Apical-Out Airway Organoids and Airway-Immune Co-Cultures Are Suitable Models for Antiviral Compound Screenings and to Study Infectious Diseases

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INTRODUCTION

There has been a recent shift away from both in vivo and transformed cell line models and towards advanced primary-derived human in vitro culture systems. Advanced cell models, such as airway organoids and epithelial-immune cell co-cultures, could serve as powerful platforms to study viral infection and pathogenesis. Despite the advancements in human primary-derived in vitro models, limitations still remain, including the challenge of accessing the apical surface of epithelia in traditional organoids with basal side-out polarity, as well as the lack of heterogeneity and paracrine signaling compared to complex tissues. Towards this goal, we describe the development of two new human lung culture systems and their use in host-pathogen interaction studies and in drug screening applications (Figure 1).

1. Co-culture model with macrophages.
We developed a workflow to generate complex co-cultures of human bronchial epithelial cells (HBECs) and blood-derived macrophages. HBECs may be seeded into a semi-permeable cell culture insert and placed at the air-liquid interface. Exposure to air better recapitulates some aspects of airway physiology, and these cultures are amenable to viral infection. Since this system has the potential to more accurately recapitulate the in vivo microenvironment, we assessed the effect of M0-like, M1-like, and M2-like macrophages following respiratory syncytial virus infection.[1]

2. Apical-out airway organoids for drug screening.
The establishment of an air-liquid interface culture relies on the use of permeable cell culture inserts, which has limited scalability. In traditional extracellular matrix (ECM)-embedded airway organoids, the apical side faces inward. To address these limitations, we developed a novel ECM-free, apical-out airway organoid (AOAO) model that enables easy access to the apical surface.[2] These organoids are amenable to viral infection and were used in an antiviral drug assay.

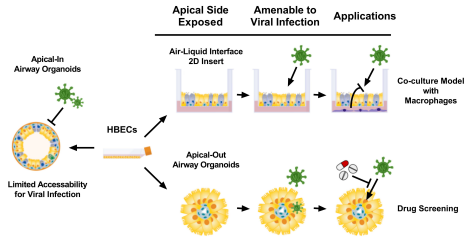

FIGURE 1. Two Human Airway Model Systems for Viral Infection.

Diagram illustrating two apical-out models for viral infection described in this work: A 2D monolayer model on cell culture inserts for co-culture systems (top), and airway organoids for drug screening (bottom).

METHODS: Co-culture and Infection

Macrophage Culture: Human blood-derived monocytes were isolated using EasySep™ Monocyte Isolation Kit and cultured in ImmunoCult™-SF Macrophage Medium (IMM), before differentiation to naive (M0-like) macrophages using M0 medium (IMM + 50 ng/mL macrophage colony-stimulating factor) for 3 - 4 days. M0-like macrophages were further differentiated for 2 - 3 days into M1- or M2-like macrophages using M1 medium (M0 medium + 50 ng/mL IFNγ + 10 ng/mL lipopolysaccharides) or M2 medium (M0 medium + 10 ng/mL IL-4), respectively.

Co-culture and RSV infection: Differentiated air-liquid interface cultures were generated from primary-tissue derived HBECs, which were first expanded in PneumaCult™-Ex Plus and subsequently differentiated in PneumaCult™-ALI (ALI) medium. After 4 weeks of differentiation at the air-liquid interface, HBECs in Transwell® inserts were placed on top of differentiated macrophages at the bottom of the lower compartment in either PneumaCult™-ALI medium or M0, M1, or M2 media. The epithelia were then infected apically with a recombinant strain of green fluorescent protein (GFP)-expressing RSV-A2 (rgRSV224, subsequently referred to as RSV, obtained from Dr. Mark Peeples Laboratory, The Research Institute at Nationwide Children's Hospital, Columbus, USA). 0.15 Multiplicity of Infection (MOI) of RSV was added and incubated for 2 hours, after which the inoculum was removed and the epithelia were airlifted. Co-cultures were maintained in ALI, M0-, M1-, or M2 medium, and collected after 72 hours for analysis, including flow cytometry for macrophage markers, ELISA for cytokine secretion, and trans-epithelial electrical resistance (TER) measurement of HBECs for barrier function.

RESULTS: Co-culture and Infection

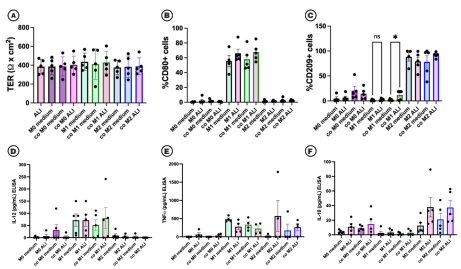

FIGURE 2. HBECs and Macrophages in Co-culture Were Not Significantly Affected by the Cell Culture Medium or Presence of the Other Cell Type.

(A) TER measurements for HBECs were not significantly affected by culture in different macrophage media, nor by co-culture with macrophages. **(B)** Flow cytometry analysis revealed that M1 macrophages expressed M1-like marker CD80, and that **(C)** M2 macrophages expressed the M2 marker CD209 with no significant changes in these markers in ALI medium or after co-culture. Macrophage phenotype was confirmed by ELISA through release of **(D)** IL-12 and **(E)** TNFα for M1-like macrophages, or **(F)** IL-10 for M2-like macrophages. No significant changes were observed after co-culture. Data represent mean ± SEM (n = 4 - 5 donors).

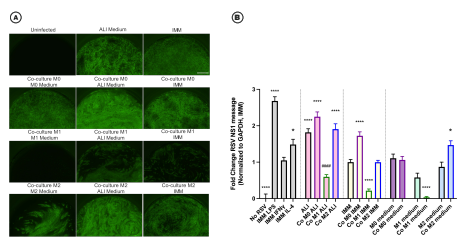

FIGURE 3. Co-culturing HBECs with M1 Macrophages Produced a Decrease in RSV Infection in HBECs.

(A) Representative images of infected co-cultures. The resulting fluorescence indicates M1 macrophages reduced the RSV infection in HBECs, whereas potent infection was observed in co-cultures containing M0 or M2 macrophages. Infection was potentiated in M0 and M2 co-cultures maintained in ALI medium compared to M0 or M2 media, indicating that the choice of medium for co-culture has the potential to affect the outcome of infection (n = 3 - 5 donors). Scale bar = 1 mm. **(B)** Quantification of NS1 by qPCR revealed co-culture with M1 macrophages in IMM or M1 medium significantly reduced RSV infection compared to infection in IMM without co-culture. Increased RSV infection was observed in co-cultures of M0 in IMM and co-cultures of M2 in M2 medium, compared to IMM alone. ALI medium reduced the ability of M1 macrophages to reduce infection. * = compared to IMM, # = compared to ALI (mean ± SEM; n = 3 - 5 donors). For statistical analysis, ANOVA was performed with Tukey's or Dunnett's post hoc tests as appropriate. * or # = p < 0.05, ** or ## = p < 0.01, *** or ### = p < 0.001, **** or #### = p < 0.0001.

METHODS: AOAO Culture and Infection

Generation of AOAOs: HBECs were cultured in PneumaCult™-Ex Plus Medium in 2D, dissociated into single cells, resuspended in PneumaCult™ AOAO Medium, and seeded at 100 cells/microwell of an AggreWell™400 24-well plate pre-treated with Anti-Adherence Rinsing Solution. Seeded cells generated aggregates after 1 - 6 days in the AggreWell™ plate. Aggregates were resuspended in the same culture medium and transferred to a new 24-well non-adherent tissue culture plate for aggregate suspension culture. After 9 - 14 days of suspension culture, functional cilia were observed at the outer side of the organoids.

Viral Infection: Day 15 AOAOs were infected with influenza A, influenza B, and rhinovirus A16 at 0.2 MOI and enterovirus D68 at 0.1 MOI. To quantify viral RNA load, 100 µL of spent medium was sampled at 24-hour intervals for 96 hours and assessed by qPCR. Cultures were topped up with fresh medium after sampling.

Antiviral Assay: To determine the effect of antiviral compounds on the viral load of enterovirus D68-infected AOAOs, 2.5 µM rupintrivir or 5 µM itraconazole was added to the cultures at 0 hours post-infection. 100 µL of medium was collected to detect viral RNA load at 24, 48, and 72 hours post-infection.

RESULTS: AOAO Culture and Infection

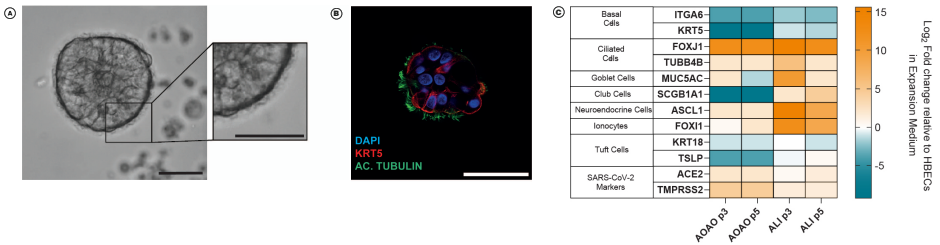

FIGURE 4. Apical-out Organoids have Outward Facing Actively Beating Cilia and Express Expected Airway Ciliated Cell Markers Similar to Air-Liquid Interface Cultures.

(A) Representative brightfield image of a day 15 differentiated AOAOs with cilia on the outer surface. **(B)** Fluorescent immunocytochemistry analysis of day 15 differentiated AOAOs express ciliated cell marker Acetylated Tubulin (green) and basal cell marker Keratin 5 (KRT5, red). Cilia localize on the outer surface confirming the apical-out polarity. **(C)** Heatmap showing fold changes in the RNA levels of common differentiation markers in AOAOs cultured in PneumaCult™ AOAO Medium (n = 3 donors). Ciliated cell markers *FOXJ1* and *TUBB4B* are upregulated in AOAOs compared to HBECs in Expansion Medium (PneumaCult™-Ex Plus), and at a similar level as 2D differentiated cultures on inserts in ALI medium. Low expression of secretory lineages (i.e. *MUC5AC*) was detected in AOAOs compared to ALI medium. Scale bars = 50 µm.

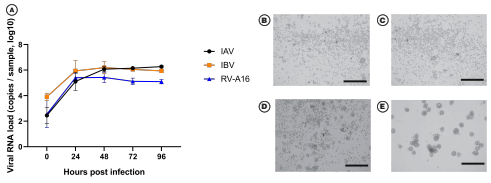

FIGURE 5. AOAOs are Susceptible to Infection with Influenza A, Influenza B, and Rhinovirus A16.

(A) Successful viral infection and replication were observed in AOAOs using influenza A (IAV), influenza B (IBV), and rhinovirus A16 (RV-A16). Data are represented as copies/sample, log10 of the mean ± SD (n = 3). Cytopathogenic effect (CPE) following infection with **(B)** IAV, **(C)** IBV, and **(D)** RV-A16, compared to **(E)** mock-infected AOAOs at 48 hours post-infection. AOAOs infected with IAV or IBV displayed high levels of CPE. In contrast, AOAOs infected with RV-A16 displayed only moderate levels of CPE, indicating that differences in the pathogenicity of these viruses could be detected using AOAOs (n = 3 donors). Scale bars = 300 µm.

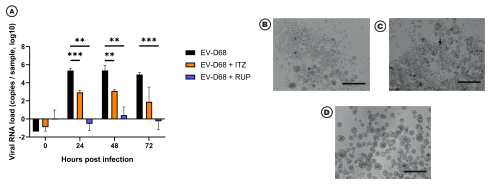

FIGURE 6. AOAOs are a Suitable Model for Antiviral Drug Screenings.

(A) Quantification of the viral RNA load in enterovirus (EV-D68)-infected AOAOs with no addition of drug (black bars), with 5 µM itraconazole (ITZ, orange bars) or 2.5 µM rupintrivir (RUP, blue bars). ITZ treatment significantly inhibited in vitro EV-D68 replication primarily during the first 48 hours, whereas RUP completely abolished replication (mean ± SD, n = 3 donors). ** = p < 0.01 and *** = p < 0.001. **(B)** CPE after 72 hours following infection of AOAOs with EV-D68. Infected AOAOs treated with antiviral drugs showed partial to complete CPE rescue when treated with **(C)** ITZ and **(D)** RUP, respectively. Scale bars = 300 µm.

Summary

- Our work showcases two different models to study viral infection with different characteristics.
  - A co-culture system that can accommodate assays requiring increased complexity with its capacity to model epithelial and immune cell responses and interplay.
  - A cost-effective, completely defined, and scalable workflow for AOAO generation that overcomes the limitations of current primary system models used to study viral and antiviral responses.
- Co-culturing HBECs with M1 macrophages considerably decreased the initial RSV infection, while co-culture with M0 or M2 macrophages increased the severity of infection,
  - These results demonstrate that different macrophage subsets play different roles during RSV infection, and these interactions could be modeled using our co-culture system.
- AOAOs generated with this ECM-free, defined, and scalable workflow:
  - Are susceptible to infection with influenza A, influenza B, rhinovirus A16, and enterovirus D68.
  - Successfully modeled the antiviral effect of rupintrivir and itraconazole in viral RNA load and CPE following enterovirus D68 infection, indicating their potential in antiviral drug screening applications.

References:
1 Ronaghan N. et al., PLoS One 2022; 17(10):e0276013.
2 Stroulios G. et al., Sci Rep 2022; 12(1):7673.