Developing a Nasal Organotypic Model to Investigate the Effects of the Nasal Microbiome on Susceptibility to Pathogens

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Background

The microbiota is essential to the functioning of the immune system. The nasal milieu secretes immune molecules that can be influenced by diverse bacteria. Hence commensals that enhance anti-viral responses may confer resistance to respiratory viral infection.

Our collaborators have identified 7 microbial state types (CST) defined by indicator species in the nose and recently, through analyses of nasal immune molecules, we have categorized the nasal immune profile types into 8 groups (IPT). Although the IPTs correlated with certain CSTs, the influence of the nasal microbiome on susceptibility to respiratory pathogens is still unknown.

Defining this complex relationship requires a relevant *in vitro* model which recapitulates key aspects of the *in vivo* nasal epithelium (pseudostratification, mucociliary differentiation), can sustain stable bacterial communities in a relevant environment (air-interfaced), and can support infection with respiratory pathogens (e.g., *Staphylococcus aureus,* influenza, SARS-CoV-2). Conventionally cultured cells lack innate protective features such as mucus and cilia and do not express physiological levels of innate immune mediators or pathogen entry receptors. These epithelial characteristics are crucial to reconstruct the complexity of microbiome-host-pathogen interactions in a controlled *in vitro* model. We have previously developed a nasal model capable of being infected by SARS-CoV-2, however due to variability in the source of cells, maintenance of culture consistency was difficult to achieve. Some obstacles included nonviable cells at isolation, fibroblast contaminations, and early death of differentiated cells.

Hypothesis: In this study, we aim to optimize our current nasal model to provide consistent cell cultures to support bacterial co-cultures and SARS-CoV-2 infection studies.

Methods

Primary nasal epithelial cells were isolated from nasal mucosa removed during sinus and base skull surgery for non-inflammatory indications.

Connective tissues were excised, followed by trypsin (0.25% Trypsin/EDTA) incubation to isolate nasal epithelial cells. Cells were plated into bovine type I collagen coated flasks and grown in keratinocyte serum free media (KSFM) supplemented with calcium chloride ($CaCl_2$). Epithelial differentiation into pseudostratified columnar cells was induced by removing the apical media and culturing at the air-liquid interface (ALI) in KSFM supplemented with $CaCl_2$. Calcium supplementation conditions were separated into no calcium, 0.1 mM (low), and 1.0 mM (high) during the cell growth phase followed by cell seeding into transwells for the ALI differentiation phase consisting of no calcium, 0.1 mM, and 1.0 mM supplementations creating 9 conditions (see figure 1).

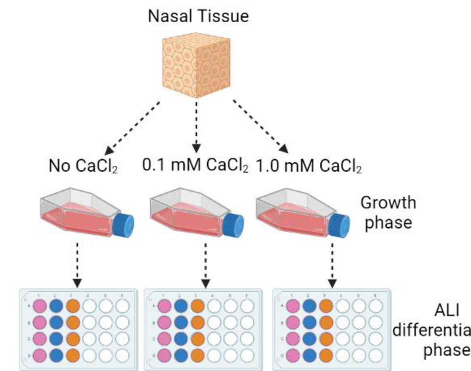

Figure 1. Conditions for supplementing calcium chloride into the growth phase and differentiating air-liquid interface (ALI) phase. Isolated tissues were cultured in no calcium, 0.1 mM, or 1.0 mM calcium in the growth phase. Then, cells from each flask were seeded into transwells and differentiated in no calcium (pink) , 0.1 mM (blue), or 1.0 mM calcium (orange) in the ALI differentiation state.

Results

Optimal isolation is defined by viable nasal epithelial cells and minimal fibroblast contamination

Isolation: Previous methods incubated tissues in Dispase overnight prior to trypsin incubation to collect isolated cells through cell strainers. However, we were successful in isolating the cells with trypsin only with 80% cell viability via Tryphan blue exclusion.

Results Cont.

Growth: Previous growth of isolated cells resulted in fibroblast contamination as the rate of growth of fibroblasts outpaced epithelial cells. With supplementation of 0.1 mM or 1.0 mM of $CaCl_2$ in KSFM, it enhanced the growth of epithelial cells compared to no calcium added and indirectly reduced fibroblast contamination (see figure 2). Ensuring greater growth of epithelial cells to minimize fibroblasts was more effective than performing differential trypsin to remove fibroblast. Differential trypsin removed epithelial cells in the process, which reduced the competition against fibroblast growth.

ALI: In previous ALI cultures, cells began to undergo cell death at around 20 days post-ALI condition, creating gaps in the confluent layer. Non-confluent epithelial models are ineffective barriers against respiratory infections nor bacterial co-cultures. We found that ALI supplemented with high $CaCl_2$ achieved greater transepithelial electrical resistant (TEER) values up to day 28 post-ALI compared to low calcium or no calcium supplementations in the ALI differentiation phase (see table 1).

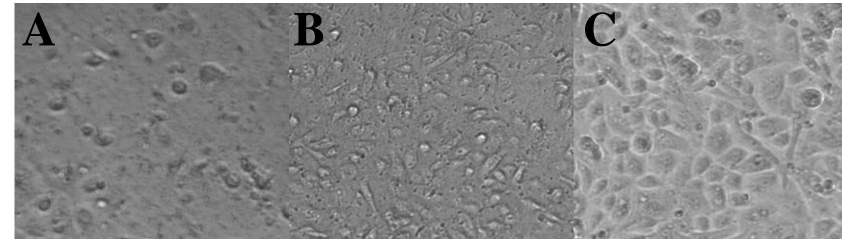

Figure 2. Day 3 post isolation of nasal epithelial cells grown in 3 different calcium concentrations. **A)** Keratinocyte serum-free media (KSFM) with no calcium. **B)** KSFM supplemented with 0.1 mM of $CaCl_2$. **C)** KSFM supplemented with 1.0 mM of $CaCl_2$. Photos taken under 50x total magnification

Table 1. TEER values measured in Ohms at different time points post-air liquid interface.

	No Calcium Plate			0.1mM Calcium plate			1.0mM Calcium plate		
Days	0-> 0	0 -> 0.1	0 -> 1.0	0.1 -> 0	0.1 -> 0.1	0.1 -> 1.0	1.0 -> 0	1.0 -> 0.1	1.0 -> 1.0
D4	141 Ω	164 Ω	141 Ω	164 Ω	154 Ω	170 Ω	82 Ω	75 Ω	150 Ω
D9	92 Ω	175 Ω	222 Ω	118 Ω	133 Ω	343 Ω	86 Ω	67 Ω	176 Ω
D14	44 Ω	109 Ω	205 Ω	38 Ω	102 Ω	169 Ω	135 Ω	151 Ω	139 Ω
D28	28 Ω	74.3 Ω	124 Ω	34 Ω	109 Ω	149 Ω	33 Ω	12.7 Ω	86 Ω

Red = calcium concentration (mM) at growth phase. Blue = calcium concentration (mM) at differentiation state.

Conclusions

We further optimized the isolation of cells from donor tissue through trypsin-only incubation and enhanced the growth of cells with supplementation of high $CaCl_2$ (1.0 mM) which indirectly minimized fibroblast contamination. Additionally, continuation of high $CaCl_2$ supplementation during ALI prolonged the epithelial layer and barrier integrity as measured by TEER.

Significance

This study provides a foundation for future viral-microbiota-host interaction models aimed at better understanding the effects of the nasal microbiome on susceptibility to respiratory pathogens using a consistent and replicable model.

Future Works

- Continue to verify mucociliary differentiation using hematoxylin and eosin (H&E) staining techniques
- Create a baseline for SARS-CoV-2 luciferase intensity at differ multiplicity of infection for our nasal model
- Mimic IPT *in vitro* by treating nasal models with cytokines to investigate susceptibilities of IPT to wild-type SARS-CoV-2 challenge

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