Pivoting to Work on Covid-19

Getting Started in Pulmonary *in vitro* Research with the Right Oxygen

There has been a flood of publications from researchers all over the world racing to get findings out to the clinicians, researchers, and policy makers battling the COVID-19 pandemic. Lost in the torrent is critical information from earlier studies about oxygen and ROS for cell-based science. This mini-review is designed to connect old and new for the researchers pivoting to join the COVID-19 fight and interested in physiologically relevant conditions for *in vitro* cell-based studies.

Artifact is introduced into cell-based assays when they are cultured in room air. This is true even for airway cells, and even when the cell line has been cultured in room air for decades. [1] Room air cell handling adds ROS and oxidative stress to cells in culture. [2] So it is essential to have the physiologically relevant oxygen (physioxic) conditions for your cell-based assays for translatable results.

Be aware that some of the reports here have not been subjected to proper peer-review yet. Despite the newness of the field, these early findings have supported a rapid and critically important evolution in COVID-19 treatments.

SARS-CoV-2 Infection Causes COVID-19 Disease

Unless you've been under a rock for the last few months, you know that patients with COVID-19, the disease caused by the SARS-CoV-2 coronavirus, can suffer an atypical pneumonia and the sickest patients can develop life-threatening Acute Respiratory Distress Syndrome (ARDS) [3]. These patients require ICU beds, ventilator support, and high-intensity staffing levels. While deficiencies in surveillance testing throw hard statistics into doubt, the COVID-19 disease is far too often fatal, particularly for older people with co-morbidities such as COPD and cardiovascular diseases. Sepsis is a systemic inflammatory response COVID complication that adds to the mortality rates. Unfortunately, new COVID-related syndromes are still being identified as testing becomes more widespread and confirmed case numbers grow.

Even patients that recover from fierce COVID-19 immunological responses are often left with long-term decreased lung capacity from scarring. Long after each wave of infections pass, there will be a new set of patients that will need continuing treatment to recover lung function.

We have a long journey ahead with these patients and new treatments will still be needed for years.

Key Targets in the Lungs

Type I pneumocytes are the thin cells that cover 95% of the alveolus and allow gas to cross from the air-side to the blood in capillaries that wrap around the outside of the alveolus. Type II pneumocytes produce surfactant which reduces the surface tension of the liquid on the surface of the alveolus and keeps it from collapsing. [4] These cells are responsible for not only replacing themselves, but also dividing and differentiating to become new squamous Type I pneumocytes when they are destroyed.

SARS-CoV-2 and ACE2 in the Lungs

SARS-CoV-2 coronavirus binds to Angiotensin Converting Enzyme 2 (ACE2) receptors in the lung and many other tissues before entering and infecting cells. Antibodies from patients that have recovered from SARS (2003 version) blocks SARS-CoV-2 (2020 version) from infecting ACE2-expressing cells *in vitro*. [5] ACE2 fusion proteins also blocked infection by SARS-CoV-2. [6] These findings, among other evidence, point to ACE2 being the key receptor for this virus.

Type II pneumocytes are a major target of SARS-CoV, propagating new virus and suffering widespread damage. Type II pneumocytes express ACE2 and associated enzymes transmembrane protease serine 2 (TMPRSS2) and Cathepsin L (CTSL). [7] [8]Lung progenitor cells (CD34+Oct4+) can be preferentially infected by SARS-CoV (2003 version) as compared to more mature pneumocytes. [9]

ACE2 can help protect lungs from damage, so loss of ACE2 in SARS infection may contribute to ARDS in infected patients. [10] But, ACE Inhibitors, which are commonly prescribed for high blood pressure, may help prevent pneumonia in hypertensive patients and in patients with ARDS. [11] The effect of targeting ACE2 with well-known ACE2 inhibitors may be a mixed bag and doctors so far have avoided it.

Taken together, these findings mean that as the COVID-19 infected lungs try to replace damaged and dead lung cells, the replacement stem and progenitor cells are also being targeted by the virus in a 1-2 punch. This may be behind the long periods of time it takes for patients to recover, as well as the lung scarring that may reduce lung capacity, perhaps permanently, for some patients.

ACE2 Receptors are Regulated by Oxygen Levels

Lung oxygen levels vary greatly from the highest in upper airways to much lower levels in alveoli (Reviewed in [12]). With incoming gases immediately mixing with outgoing gases in the airways, even in the trachea, oxygen levels are normally lower than room air.

ACE2 receptors are regulated by oxygen levels and Hypoxia Induced Factor 1 alpha (HIF-1a). [13] The Nobel Prize in Medicine was awarded in 2019 to the first researchers to characterize HIF-1a. HIF-1a, which is rapidly destabilized by room air oxygen levels in traditional room air cell culture conditions [14], is intimately tied to signaling pathways key to cytokine responses.[15]

Notch, also an oxygen-sensitive signaling molecule, has also been suggested as a clinical target in COVID-19 associated cardiopulmonary disease. [16]

These findings mean that having the appropriate oxygen level for studying ACE2 *in vitro* is essential for translatability of results from *in vitro* to *in vivo.*

COVID-19, Reactive Oxygen Species, and Lung Inflammatory Cytokines

High serum levels of pro-inflammatory cytokines such as IL-1, TNF, and IL-6 have been found in the sickest of COVID-19 patients, prompting usage of available anti-IL-6 and anti-IL-6R treatments. [17] Soluble ACE2, as a treatment, also lowers serum IL-6 in patients with ARDS. (reviewed in [11]). CO2 hypercapnia may also protect against ventilator-induced lung injury and IL-6 related inflammation. [18]

Reactive oxygen species (ROS) and nitric oxide (NO) at the cellular level are generated by immune cells during infections, but can also be quite damaging to local tissues. ROS can regulate local physiologic reactions [19]. They can enhance lung infection with viruses such as influenza. NO can also shift the type of T helper cells that develop during an immune response. (Reviewed in [20])

Room air cell handling adds extra reactive oxygen species (ROS) and oxidative stress to cells in culture. [2] This means that culturing cells in traditional room air conditions is the wrong setting for physiologically relevant COVID-19 studies.

MSC, ARDS and Oxygen

Treatment with mesenchymal stromal cells (MSC) has been shown to be beneficial in rat models of ARDS [21] and MSC-derived extracellular vesicles can help alleviate damage from ischemia in lungs. [22] Immunomodulatory function in MSC themselves depends upon HIF-1 alpha, an oxygen-sensing transcription factor. [23] Room air oxygen during large-scale expansion can shift MSC metabolic processes, altering MSC function and reducing therapeutic potency [24].

So getting the oxygen levels right for MSC in culture is critical to their subsequent therapeutic effect. MSC have been approved for clinical use on an expanded access compassionate use basis for human COVID-19 clinical trials.

Lung Co-Morbidities

SARS-CoV-2 has had its worst effects on patients with underlying illnesses. Increased oxidative stress in the lungs can also contribute to disease processes in Chronic Obstructive Pulmonary Disease (COPD), Asthma, and Lung Cancer [25]

While data on COVID-19 patients with Asthma and lung cancer is still evolving, COPD has been shown to significantly increase COVID-19 mortality [26]. Smoking tobacco may also change ACE2 expression in the lungs, increasing ACE2 expression levels and changing the cell types expressing it. [27] Each of these lung co-morbidities may induce hypoxemia, a lowering of blood oxygen levels, which can then contribute to COVID-related damage in other body systems.

This means that even if you are studying SARS-CoV-2 in other systems than Lung, proper culture pathophysioxic levels are necessary for mimicking the COVID-related hypoxic state of the patient.

What is the Right Oxygen Level to Use *in vitro* for My COVID-19 Cell-Based Assays?

The answer here comes down to the three L's of physioxia; location, location, and location. To get started looking for the proper oxygen conditions for your cell type of interest, start with these excellent reviews [12] [28]

The Concern about Room Air Oxygen for COVID-19 Research is Real

Room air oxygen is supraphysioxic and stressful for cells *in vitro*. Room air incubators are not at room air oxygen levels, neither are they at physioxic levels. [28] [12] HIF-1a levels are modulated within single minutes of oxygen changes. [14] Viruses have altered propagation in cells cultured at the wrong oxygen levels. [29]

It is essential that researchers use the proper equipment to maintain physioxic or pathophysioxic levels for cell-based research

The Right Stuff

Explore equipment for carrying out physiologically relevant *in vitro* COVID-19 assays at: https://www.biospherix.com/covid-19

Discover accessories that upgrade standard, peoplecentric cell culture equipment with new cell-focused capabilities. These include incubator subchambers with hypoxic or physioxic control that insert within existing laboratory incubators. These also protect COVID-19 samples from room-air disturbances that happen when the incubator door opens to uncontrolled room air.

Even brief disturbances in hypoxic cell conditions alter HIF levels and can skew important *in vitro* data. To enhance translatability of cell-based COVID-19 research, learn about the fully Cytocentric platform which combines modular incubators with interconnecting hoods. These systems provide continuously controlled O2, CO2, pH, and temperature levels which mimic those measured in any tissue, including human lung.

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