Review Article

Study of Respiratory Tissue Engineering Model for Viral Infection and Bacterial Microbiome Interaction Under Physiological and Pathological Condition

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Abstract | Respiratory epithelium is one of the mucosal epithelial which functions as protective and adaptive barrier against continuously inhaled substances including pathogens and allergens. Current research about mechanism of disease and drug development is conducted in 2D cell culture or in animal models. The 2D cell culture models poorly imitate the condition *in vivo* and provide limited utility due to mimic tissue physiology in multicellular organisms. Although animal models can be used as pre-clinical tools for new-agent screening prior to clinical testing, there is a growing awareness of the limitations of animal research and its inability to make reliable predictions for human clinical trials. Recently, 3D cell culture produced by researchers to improve better mimic tissue physiology of better *in vitro* cell culture model. It is may enable microbiologists to create infection models that combine respiratory tissue culture engineering with the virus-relevant complexity of *in vivo* models. Mechanism of infection disease have shown that the normal microbiota in host health has remarkably improved our understanding of the interactions between microbiota and invading pathogens. Commensal bacteria as microbiome can potentially influence mechanism of disease either hindering or promoting the viral infection and sometimes aggravate the disease. In this review, we discuss the histology of respiration epithelium and mucous layers, various microbiome that contribute to the interaction between the host and microbes against infection on the mucus layer in top of the respiratory epithelium, and technology that can be developed in tissue engineering techniques for reconstruction to mimic the microenvironment in respiratory tissue engineering.

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Introduction

Acute respiratory disease by viral infections are the leading cause of respiratory disease worldwide. The severity of this disease may vary markedly from asymptomatic to mild and gradually progressed into severe wheezing, bronchitis, and pneumonia. Unlike other pathogens that restricted to tropical areas, the respiratory viruses are distributed globally and efficiently transmitted from person to person (Eugenia *et al*., 2013; Proença-Módena *et al*., 2011; Troy and Bosco, 2016). In the last twenty years, several viral epidemics such as current novel coronavirus outbreak (now called SARS-CoV-2, causing the disease Covid-19), MERS-CoV in 2012, H1N1 influenza pandemic in 2009, and SARS-CoV in 2002-2003

have been recorded (Abrahao and de Arruda, 2020). Emerging of new virus are often associated with migration, deforestation, and other anthropogenic activities. This can trigger the emergence of several new diseases due to the eviction of wild animals from nature which is a reservoir of various zoonotic viruses. Accurate condition of viral infection mechanism in a laboratory setting is needed for identification of pathogen-host interaction and potential therapeutic drug development (Guan *et al*., 2020; Zhou *et al*., 2020).

Recent studies on the mechanism of virus infection and drug development research, *in-vitro* models with 2D cell cultures and in-vivo with animal models are widely used. In 2D culture, the cells were grown in monolayer condition thus offering unnatural growth kinetics and attachments. Representation of cells microenvironment can be lacking because the cell's natural microenvironment is not fully representative (Saji *et al*., 2019). Animal models can be used as pre-clinical tools for new-agent screening and understand how viruses replicate in individuals. It is important for virologists to unravel the basic mechanisms of virus pathogenesis inside the individuals. Selection of the suitable animal models which represents similar anatomy and physiology with human is important for the goal of the study (Adachi and Miura, 2014; Han *et al*., 2018; Swearengen, 2018). Animal models have several limitations e.g failure to accurately mimic the human disease progressions, lack of best practice standards for animal testing, and physiological differences that have an impact on variations in the molecular target homology (Mak *et al*., 2014).

Nowadays, tissue engineering recently proposed for the design of reliable *in vitro* models of healthy or pathological tissues and organs (Caddeo *et al*., 2017). Tissue engineering in a form of 3D cell culture receive more attention as they exhibit protein expression patterns and intracellular junctions that are similar *in vivo* states compared to classic monolayer cultures (Jaroch *et al*., 2018). This model can more faithfully recapitulate the biology of specific tissue and can be used for longer periods. Many of these models are still relatively simple, the culture of cells at an air-liquid interface or culture of two different cell types separated by a porous membrane. However, increasingly complex and sophisticated models of tissues and organs are being engineered and modified to create unique disease models in a condition that closely imitates *in vivo* microenvironment could be elaborately constructed *in vitro* (Benam *et al*., 2015; Chaicharoenaudomrung *et al*., 2019; Ramanan *et al*., 2014).

A balanced host-microbe interaction is necessary for maintaining homeostasis (Parker *et al*., 2018). Bacterial microbiome in human demonstrates inter-individual variability, and can be influenced by the environment, genetics, and diet. Recent study shows that host's normal microbiome can potentially influence viral infections either hindering or promoting the viral infection and sometimes aggravate the disease (Lima *et al*., 2019; Wilks *et al*., 2013). It significantly contributes toward protection against pathogens by competing for shared nutrients and niches or through enhancing host defense mechanisms (Kamada *et al*., 2013). Additionally, the use of tissue engineering model to study the respiratory viral infection and microbiome interaction has not been reviewed extensively. The purpose of this review is to describe the state of art co-culture of 3D epithelial respiration in tissue engineering construction to determine microbiota dysbiosis in respiratory tract and viral infection mechanism model. This review will discuss the histology of respiration epithelium and mucous layers, various microbiome that contribute to the interaction between the host and microbes against infection on the mucus layer in top of the respiratory epithelium, and technology that can be developed in tissue engineering techniques for reconstruction to mimic the microenvironment in respiratory tissue engineering.

Histology of respiratory tract epithelium

The respiratory system subdivides into a conducting and respiratory portion. The conducting portion deliver air to the respiratory tissue and is characterized by rigid walls that consist keep the airways open. This portion starts from the nasal cavity, pharynx, larynx, tracheal and bronchial tree. Respiratory portion located in the pulmonary alveoli, that the portion of this system where the gas exchange between blood and air occur. Alveoli are the most unique part because the microenvironment is the boundary between the air-filled space and the surfactant bordering the epithelium basal membrane that fused with a very tight capillary endothelial basal membrane (Krause, 2005).

Conduction portion of respiratory tract starts from

the nasal cavity, pharynx, and larynx that is covered by ciliated pseudostratified columnar epithelium with goblet cell, forms a continuous lining of the airway lumen homeostasis (Scherzad *et al.*, 2019; Yuksel and Turkeli, 2017). Pharynx is part of the conduction zone of respiratory system and digestive system. The pharyngeal phase serves to protect the airway during swallowing, a coordinated physiological response to prevent respiration during swallowing which lasts about 0.5 to 1.5 seconds. Swallowing mechanism itself serves as a vital protector of the airway and voluntary actions of over 30 nerves and muscles produce this coordinated movement (Panara and Padalia, 2020). The upper part of the pharynx, the nasopharynx extends from the base of the skull to the upper surface of the soft palate. This includes the space between the internal nares and the soft palate and is located above the oral cavity. In areas subject to abrasion, a nonkeratinizing stratified squamous epithelium may occur, such as on the edge of the soft palate and posterior wall of the pharynx, where these surfaces make contact during swallowing (Krause, 2005).

Larynx is part of the respiratory tract in the form of a tubular segment with primarily cartilaginous held together by a series of ligament and membrane that connects the pharynx and trachea. Internally, the laryngeal muscles move components of the larynx provides a protective sphincter at the inlet of the air passages and is responsible for phonation and breathing. The upper pair constitutes vocal cords (vestibular folds), covered by the respiratory epithelium beneath which lie numerous serous glands within the lamina propria. The anterior surface and about half the posterior surface of the epiglottis are covered by nonkeratinized stratified squamous epithelium. The vocal cords also are covered by a wet stratified squamous epithelium, but elsewhere the larynx is lined by the respiratory passage type of epithelium (Krause, 2005). Larynx does contain immunological tissue in the form of epithelial follicles (larynx-associated lymphoid tissue: LALT) in an age and disease dependent manner. This immunological activity is likely to be crucial for determining the development and outcome of infectious, inflammatory disease and neoplastic disease (Barker *et al.*, 2006).

Trachea is part of respiratory tract consists of incomplete rings of hyaline cartilage and smooth muscle that embedded into a fibrous membrane of elastic connective tissue (Brand and Schafer, 2014). These cartilages give the trachea rigidity and prevent its collapse. It extends from the lower part of the larynx to its bifurcation into two main bronchi. The average adult trachea measures about 11 cm in length with a diameter that varies from 2 to 2.5 cm. The pediatric trachea is smaller, more deeply placed, and more mobile. Tracheal luminal mucosa of lined by pseudostratified columnar respiratory epithelium consisting of ciliated and clara cells. The proportion of clara cells is increase in the caudal part of the trachea, and goblet cells that rests on a thick basal lamina are only found rarely (Fernandez *et al.*, 2008; Navarro *et al.*, 2017). In addition, goblet cells secrete mucus that contain many defensive compounds into the mucosal fluid, including mucins, antibodies, immunoglobulin A, lysozymes, histatins, nitric oxide and lactoferrin to protect the host actively (Binsker *et al.*, 2020; Patel, 2017). Both goblet cells and submucosal glands produce mucus, which forms a gel layer on the epithelial surface of the respiratory tract to act as a protective barrier against the external environment by trapping particulate matter, including pathogens. Trapped matter can then be expelled from the airways by the rhythmic beating of cilia bundles on the airway epithelium by cough (Aghapour *et al.*, 2018; Zanin *et al.*, 2016).

The bronchial structure is a continuation and branching of the trachea that allow air to be transported from the surrounding atmosphere to the lung. This structure is similar histologically to the trachea that are lined with ciliated pseudostratified columnar epithelium. The term bronchial tree is the large airways in the distal trachea that form secondary or lobar bronchi, then these branches supply the individual lung lobes and are connected to segmental or tertiary bronchi, which supply individual lung segments. The cartilaginous structure decrease with decreasing bronchial diameter. The muscularis develops into the rings of smooth muscle encircling bronchi and bronchioles. As a bronchial diameter becomes smaller, the epithelium becomes flatter and the number of secretory cells decrease with no longer have submucosal glands or cartilage. The epithelium becomes simple columnar, with clara cell replacing mucous cell and basal cells disappearing. Bronchioles end in alveolar duct, which contain spiral smooth muscle and are lined by alveolar epithelium (Chanez and Bourdin, 2008; Haschek *et al.*, 2002; Reynolds *et al.*, 2015).

Pulmonary alveoli are the most specialized part

of the respiratory system and is responsible for its most important function, gas exchange. The microenvironment of the structure is the boundary between the air-filled space and the surfactant which is bordered by a thin layer of epithelium (pneumocytes/ type I alveolar epithelial cells) and cuboid-shaped epithelium (pneumocytes/ type II alveolar epithelial cells). The basal epithelial membrane of this structure fuses into the capillary endothelial basal membrane with high tight junctions. The design of the inter-alveolar septum that contain of smooth muscle cells, connective tissue/ fibers and elastin with complex structures providing a large surface area, a thin diffusion barrier, requirements of stability to preventing over-distension or collapse of alveoli, and also flexibility. The mechanism of O_2 and CO_2 gas exchange occurs by diffusion in alveolar membranes. Oxygen from the alveoli diffuses freely through the cells to capillaries for eventual distribution by the systemic blood flow (Alvarado and Arce, 2016; Crystal *et al*., 2008).

Mechanism of microbes infection in respiratory tract

In the respiratory tract mechanism of infection, complex interactions occur between pathogens (microorganisms) and epithelial cells that lining the respiratory tract. The airways epithelium is the primary site for viral infection and replication. The entry of viruses into respiratory epithelium cells must first penetrate the mucous layer, which provides the first line of defense against invading pathogens (Scherzad *et al*., 2019; Yuksel and Turkeli, 2017). In addition, respiratory tract epithelium consist goblet and clara cells that secrete many defensive compounds into the mucosal fluid, including mucins, antibodies, defensins, protegrins, collectins, cathlecidins, lysozyme, histatins, and nitric oxide. Oxidants, such as nitric oxide that is produced following the induction of nitric oxide synthase-2 (NOS-2), have also been shown to inhibit viral infections (Aghapour *et al*., 2018; Zanin *et al*., 2016).

High viral replication is generally known in the proximal trachea and nasopharyngeal epithelium. Increased immune cell infiltration, CD8 + cytotoxic T cells in tracheal epithelial cells are a form of immune response occurring during infection. Virus-specific cytotoxic (cell killing) T cells are the first line of adaptive immunity responsible for elimination of infected cells. Pathological abnormalities of infected trachea are often marked with reddened and swollen mucosal surfaces. Hemorrhagic tracheitis and bronchitis were observed in 50% of influenza cases (Deist *et al*., 2017; Lambert *et al*., 2016; Taubenberger and Mores, 2008). Generally, viral infection will cause innate and adaptive immune response to facilitates viral clearance. At the same time, it causes excessive inflammation and results in tissue damage (Yoo *et al*., 2013).

The entry of the virus in the host epithelial cell is strongly influenced by the receptors contained in the cell. For example, seasonal Influenza A virus binds to ASα2-6Gal receptor, which are found primarily on the surface of epithelial cells in the nasal mucosa, tracheal and bronchi. In contrast, highly pathogenic avian H5N1 virus binds to ASα2-3Gal receptor, which is found on alveolar epithelial cells (Troy and Bosca, 2016). The interaction between influenza virus and mucus was first identified in studies conducted in the 1940s and 1950s through observing receptor-destroying enzymes from Vibrio cholerae that can reduce the inhibitory action of mucoproteins over time in a manner similar to influenza viruses. The enzyme has the ability as a hydrolytic cleavage of glycosidic bonds of neuraminic acid groups (sialic acid) to D-galactose or D-galactosamine, so it is called neuraminidase or sialidase. This proves that neuraminidase plays an important role in determining whether the virus can penetrate the mucous barrier to infect the underlying cells. Neuraminidase enzyme may facilitate infection by cleaving sialic acid on mucin layer to prevent viruses from becoming trapped before they infect the epithelium (Zanin *et al*., 2016).

Underneath the mucus layer of the cells there are glycocalyx, dense forest of highly diverse glycoproteins and glycolipids. The glycocalyx, which is atop the epithelial cells, is a fuzzy and filamentous coat that is weakly acidic and consists of sulfated mucopolysaccharides. The glycocalyx has numerous functions, including structural roles in proteins, protein folding and mediation of self-recognition by the immune system (Linden *et al*., 2008). Therefore, the interaction of pathogens with the glycocalyx is very important in the viral lifecycle. However, respiratory mucus also contains sialic acids and glycoproteins, and the various virus interaction with these is also important. High sialic acid in mucosal surface results in a strongly negative surface charge, increasing the rigidity of the polymer via charge repulsion (Zanin *et al*., 2016). Sialic acid naturally occurs in prokaryotes as nine-carbon keto sugar acids derived

from N-acetylneuraminic acid (Neu5Ac). Sialidase (sialoglycosidase) that found in viruses, bacteria, protozoa, fungi, and metazoans is major enzyme that facilitate interaction between the host and pathogen by cleaving the sialic acid from sialoglycoproteins (Figure 1). This could expose that receptors for enzymatic interactions and ligand binding by contributing to biological functions, such as cellular interaction and conformational stabilization of glycoproteins in the cell membranes (Sudhakara *et al.*, 2019).

Bacterial colonization of the respiratory tract also has a similar mechanism to be able to reach the surface of the epithelium, the bacteria must penetrate the mucous layer to gain access to epithelial cell. There are several pathogenic opportunistic factors in the airway including transient association with mucus, weak association with host carbohydrates, and strong association with host surface proteins (Siegel and Weiser, 2015). Bacterial virulence factors are molecules that influence between bacteria and host cell and/or substance, including processes such as adherence to cell membrane, colonization, and invasion. Several virulence factors mechanism is likely used to reach epithelial cell, including motility and digestion of the mucus layer by bacterial enzyme (sialogycosidase). Bacterial virulence factors act as protease, lipase, toxins, adhesion factors, biofilm, capsules made of carbohydrate and antiphagocytic factors. Bacterial capsule is known for their role in limiting optimization with complement and antibodies, also contributing to avoid mucus-mediated clearance. These factors allow bacteria to "escape" and interact with epithelial cell. For example, bacteria that sialoglycoidase enzyme can penetrate the mucus layer and reach the target cell by digest the mucus layer with their enzyme and consume oligosaccharides such in the mucus layer as a carbon source. The balance between host and bacterial colonization can be tipped in either direction by a range of factors that modify each stage of attachment, growth, and immune evasion (Zachary and McGavin, 2017).

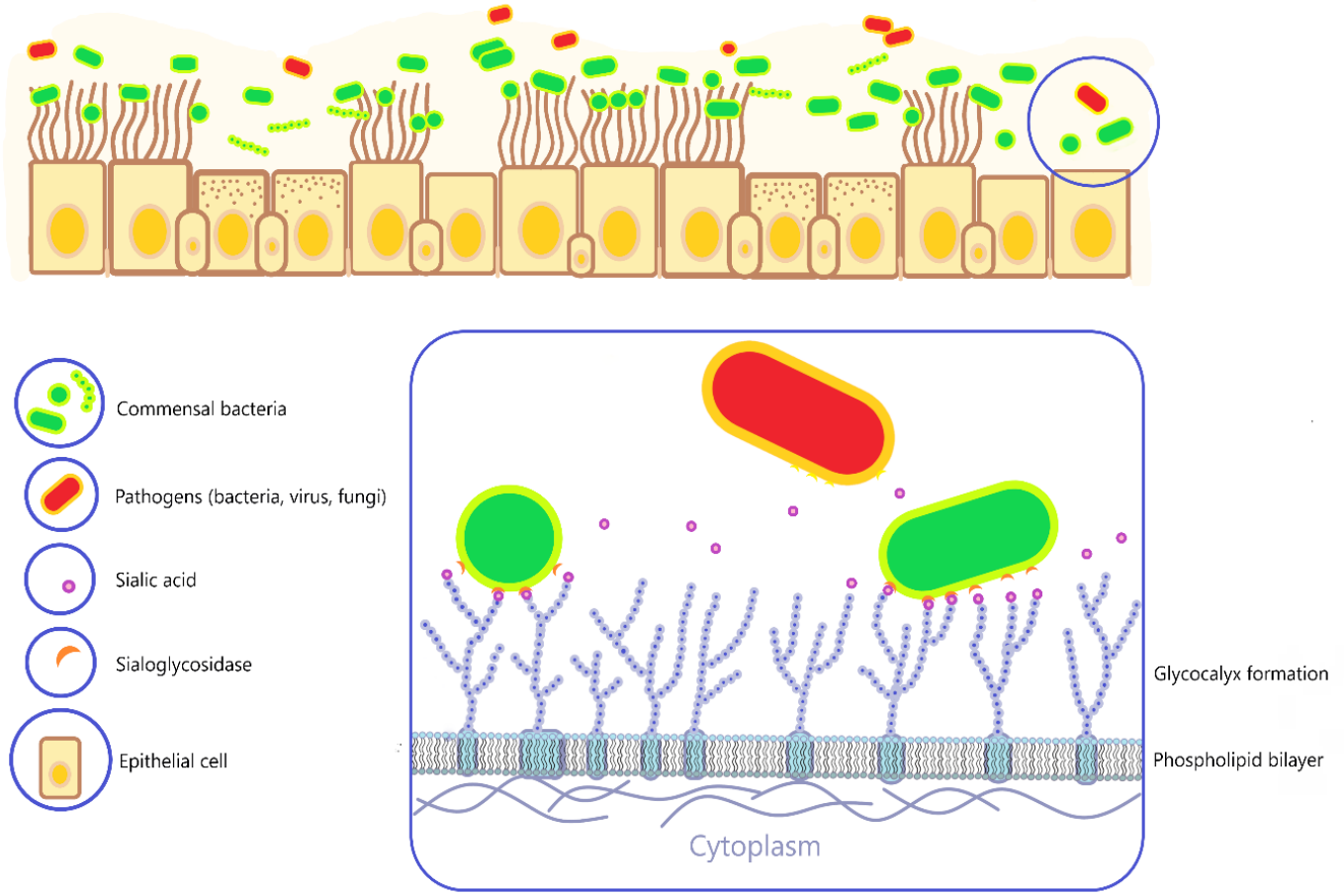

Figure 1: *Physiological condition of respiratory tract microbiome illustration. Diverse commensal bacteria in the respiratory tract live with a balanced composition without pathological conditions. Various pathogens (bacteria, viral and fungal) with relatively limited numbers are less likely to infect the host if there is no drastic changes in conditions. Glycocalyx is glycoprotein and glycolipid that cover the surface of the host epithelial cell membrane. Sialic acid which is in the structure has a place to attach to several normal microorganisms and pathogens which are assisted by sialoglycosidase.*

Respiratory mucosal tissue microbiome

Over the last decade, human microbiome has become a hot topic since the launch of National Institutes of Health (NIH) Human Microbiome Project and the International Human Microbiome Consortium. This is seen as a new insight to understanding human health and disease by determining human microbiome (Neto *et al*., 2017). Microbiome is comprised of the collective genome of microorganisms (bacteria, archaea, viruses, and fungi) that symbiotic and pathogenic, living in and on all vertebrates (Taneja, 2017). It also revealed that the lungs have a diverse microbial community, which were previously considered sterile in health. This new concept has challenged numerous long-held assumptions regarding respiratory health and the pathogenesis of respiratory disease (Dickson *et al*., 2016). The entire surface of the respiratory tract is inhabited by niche-specific bacterial communities. Along with the anatomical development of the respiratory tract, the formation of respiratory microorganisms is considered to have an influence on the process of morphogenesis and regulation of the immune system (Man *et al*., 2017; Wilks *et al*., 2013).

Human upper respiratory structure is a combination between the outer skin surface and the mucosa that is in direct and continuous contact with the outside world. Exposure to these external environmental conditions causes the formation of ecological conditions in various microbial communities (de Steenhuijsen *et al*., 2015). The upper respiratory tract breathes at least 7000 l of air with an average number of microorganisms 10^4-10^6 bacterial cells per cubic meter of air inhaled per day. There are five of the most common genera of bacteria found in nasopharynx, including *Moraxella*, *Staphylococcus*, *Corynebacterium*, *Streptococcus*, and *Dolosigranulum* (Figure 2). Commensal bacteria can positively interact with the host and each other by colonizing the upper airways. Interaction of competition usually occurs between commensal and opportunistic pathogens to fight over nutrients in the airways of freely glucose and iron in limited quantities (Wang *et al*., 2018; Kumpitsch *et al*., 2019).

In the pharynx, the most common microbiome found include *Prevotella*, *Capnocytophaga*, *Campylobacter*, *Veillonella*, *Streptococcus*, *Neisseria* and *Haemophilus*. Some species of bacteria such as *Streptococcus pneumonia*, *Staphylococcus aureus*, *Haemophilus influenza* and *Mycoplasma pneumonia* are known to be pathogenic, however they can adapt to the pharyngeal environment, rendering the host asymptomatic (Gao *et al*., 2014). Whereas in the laryngeal area several other bacteria that cause respiratory infection are found including *Chlamydia*, *Bordetella*, *Aggregatibacter*, *Pseudomonas*, and *Bacteroides* (Gong *et al*., 2013). Some of these variation in microbiota diversity and bacterial composition are often associated with a risk of laryngeal carcinoma and vocal cord polyps (Gong *et al*., 2017; Wang *et al*., 2019).

Microbiome that lives in the lower respiratory tract plays an important role against micro-environmental homeostasis that serves as a defense against potential invading viral infection. Generally, some bacteria that are often found in lower respiratory tract microbiome in healthy individuals include *Prevotella*, *Veillonella*, *Streptococcus*, *Fusobacterium*, and *Hemophilus* (Gu *et al*., 2019). In the trachea microbiome bacteria reflects membership from both the oral cavity and upper airways. The anatomic location of the trachea that connects the upper and lower respiratory tract is the reason it can occur. Some of the microbiome that

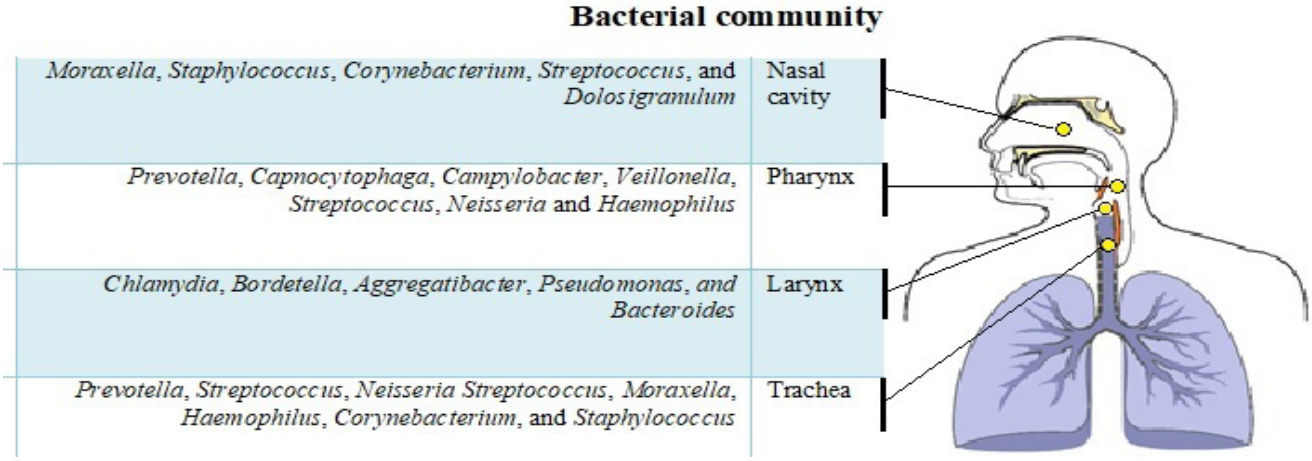

Figure 2: *Bacterial community along the nasal cavity, pharynx, larynx, and trachea.*

found in trachea are *Prevotella*, *Streptococcus*, *Neisseria*, *Streptococcus*, *Moraxella*, *Hemophilus*, *Corynebacterium*, and *Staphylococcus* (Perez-Losada *et al*., 2017, 2018).

The composition and alteration of the microbiome community in the bronchial and lung are often associated with asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). In healthy subject, microbiome composed of oropharynx bacteria which migrate to bronchial tree through aspiration. Some of these bacteria are *Streptococcus* and *Veillonella* that are found in the bronchial mucosa at low loads. Changes in the microenvironmental conditions cause modification of bacterial composition by overgrowth of specific bacteria. In pathological conditions such as asthma, COPD, and CF, high load of several bacteria such as *Pseudomonas*, *Hemophilus* and *Legionella* are found (Garcia *et al*., 2014; Millares *et al*., 2017). Local pH, blood flow, temperature, epithelial cells, oxygen levels, and disposition of inflammatory cells are some conditions that affect the proliferation and dynamic change of the microbial diversity of the respiratory tract (O'Dwyer *et al*., 2016).

3D tissue engineered upper respiratory tract epithelium to investigate early infection

The development of *in vitro* models to determine the interaction of microbes and host cells begins with observation using a host cell that is grown in a monolayer 2-D flat. This approach advances our understanding of the mechanisms underlying infection and disease in host cells. However, it is experiencing problems due to differences between in-vivo and in-vitro conditions which cause changes in the original behavior of host cells and microbes. Some of these differences are more dominant in microenvironment conditions including oxygen tension, extracellular matrix (ECM), homotypic or heterotypic cells surrounding the single cell, cytokines, hormone, microbiota composition/localization, and biomechanical forces (e.g., fluid shear, stretch, compression) (Barrila *et al*., 2018; Barthes *et al*., 2014). Therefore, natural microenvironments of monolayer 2-D flat cells are not fully represented. Recently, 3-D cell cultures in tissue engineering are used to improve better *in vitro* cell culture models that resemble *in vivo* conditions in multicellular organisms (Saji *et al*., 2019). This model can mimic the nature of tissues and show changes in viability, morphology, differentiation status, proliferation capacity, and gene expression profiles. One of the benefits is to find out the mechanism of viral infection and the impact that occurs *in vitro* on tissue-specific hosts, innate immune function, cell cycle status, and polarity resembling *in vivo*. In order to get the appropriate tissue engineering model, several things to consider include the selection of scaffold and extracellular matrix (ECM), cell type, medium, biomechanical forces to form microenvironment (Barrila *et al*., 2018; Ramanan *et al*., 2014).

Construction of 3D microenvironment of upper respiratory tract

Construction models that can be used to mimic the upper airway epithelium begin with two-dimensional (2D) monolayers, differentiated airway pseudostratified cell layers either submerged or at the air-liquid interface (ALI) (between 2D and 3D) and 3D cell culture models. In general, there are several main components for tissue engineering construction, including scaffold to support cell attachment and growth, cells with which to populate the scaffold, and bioreactors that provide the nutrients needed by developed tissue, waste removal, and other physical and biochemical stimuli to generate organized and functional tissue (Calle *et al*., 2017). The choice of scaffold is most often derived from collagen, to mimic the natural extracellular matrix or, a decellularized biomimetic structure or tissue can be used as a substrate for cell culture. The choice of substrate is important because it can affect cell adhesion, polarity, cell differentiation and barrier formation (De Rudder *et al*., 2018).

Scaffold for respiratory tract tissue engineering

The complex nature of respiratory organs requires the development of hybrid scaffolding that has more than one material to be able to resemble in-vivo. The mechanism and interaction between pathogens and hosts in the development of tissue engineering is focused on respiratory epithelium based on three basic approaches: in situ homing (adhesion, proliferation, and support migration to cells); cell sheet technology (release intact cell sheets with deposited ECM); and fluid interface techniques (promote epithelial differentiation into multi-lineage phenotypes) (Kumar *et al*., 2017). Scaffold material commonly applied in the development of respiratory tissue engineering consists of natural polymers (collagen), synthetic polymeric materials (Poly-cap-caprolactone), and composites of natural synthetic combination materials (Dekorin-gelatin-PCL). Most research on tissue engineering to date has used primary

tracheobronchial or alveolar epithelial cells which are mixed cell populations containing endogenous progenitors that may contribute to proliferation in 3D construction (O'Leary *et al*., 2015).

Natural polymers are known to originate from the decellularization process of organs or tissues, so they can form ECM with low antigenicity and minimal risk of allograft rejection in regenerative medicine. Collagen, chitosan, and gelatin are natural scaffolds that can provide bioactive properties to interact with cells. The limitation of this scaffold is the lack of tissue and organs for decellularization and low mechanical stability, while synthetic biomaterials can be produced at low cost, in large quantities and have a longer shelf life and provide more mechanical stability (Eltom *et al*., 2019; Khazraee *et al*., 2018). In addition to determining the material, the design and fabrication of scaffolding is also very important to mimic the shape of in-vivo respiratory tissue so that it has good biocompatibility, biodegradability and better cell viability (Dhasmana *et al*., 2020). Various fabrication techniques have been used in the past to produce different materials into scaffolding for tissue engineering purposes. These techniques include solvent casting and particle washing, gas foam, fiber meshes and fiber bonding, phase separation, melt molding, emulsion freeze drying, solution casting, freeze drying and bioprinting (Henkel and Hutmacher, 2013). Scaffold design research for artificial trachea with cylindrical structure with coverage with ciliated respiratory mucosa, and adequate cartilage has been carried out by combining the fabrication techniques of electrospun polycaprolactone (PCL) nanofibers (inner) and 3D-printed PCL microfibers (outer) (Kim *et al*., 2020).

Cells for scaffold repopulation

Development of human disease models *in vitro* often used cell lines and primary cells such as tracheal epithelial, alveolar epithelial and bronchial epithelial cell lines to better represent respiratory tissue because specific cell types can accurately recapitulate the phenotype of the disease (Estermann *et al*., 2020; Thiebes *et al*., 2015). Immortal cell lines that are often used to mimic the respiratory tract are 16HBE (human bronchial epithelial cells) and Calu-3 and A549 (cells from human lung carcinoma). These cells have the advantages of easy acquisition, source reliability, and stable for repeat experiments. But these cells are dependent on the availability of tissue and are difficult to obtain, reproduce slowly, have a limited life span with the altered genotype compared to *in vivo* tissue, and the inability to obtain a desired disease phenotype. The development of tissue engineering using induced pluripotent stem cells (iPSC) provides an extraordinary opportunity to overcome this limitation. that is because of its ability to express several transcription factors and the resulting pluripotent cells can renew themselves indefinitely. The cell can also differentiate into several types of cells depending on the induction and the conditions given. The combination of different cell types within a model is a strategy accurately represent the host microenvironment and the interactions therein (Benam *et al*., 2015).

Basically the cells are seeded directly on the surface of the initial scaffold until a monolayer cell culture is obtained and then it can be removed, combined, and shaped in such a way as to obtain a 3D formation where the microenvironment conditions of the artificial tissue can be regulated (Mandrycky *et al*., 2017). Cells that seeded on the scaffold aim to deposit extracellular matrix components such as fibronectin and collagen. Research with the aim of modeling the interaction between host and pathogen with epithelial cells as physical barriers to pathogens that colonize the human respiratory tract its integrity is a key requirement. Parameters to consider in the formation of monolayer cells such as physiological epithelial function usually depend on the formation of tight junctions (TJs) that unite the epithelial cells that form a barrier. Expression of the Zonula occludens 1 (ZO-1) protein which is an important component of TJ can accurately depict inter-cellular contours on the apical side of the epithelial cell layer (Marrazzo *et al*., 2016).

Biomechanical forces and bioreactor

The architecture of respiratory tissue has very high complexity to be emulated in the form of 3D tissue engineering and has the same biomechanical properties of breathing. The complex structure consists of compartments throughout the network in a flexible and dynamic matrix that continues to move at the two cell layer interfaces, with a balanced combination of structural and functional conditions (Mandrycky *et al*., 2017). Mechanical properties and forces affect the modulation of cell fate, especially on stem cell differentiation. In 3-D scaffold, stem cells through the addition of growth factors and mechanical strength

have been shown to induce desired cell differentiation and tissue formation (Roomans, 2010). In bronchial epithelium, mechanical forces in the form of bronchoconstriction as well as the cyclical stretching of the airway can have an impact to the luminal level by the presence of air flow and transduction of the cilia resulting in additional mucus. However, in addition, it can influence activation promoting airway remodeling that is influenced by ECM, increased epithelial properties such as solute transport, viral gene delivery, and secretion of ECM modifying proteins (Prakash *et al.*, 2015).

Current bioreactors usually contain an integrated 3D cell construction, e.g., epithelium and endothelium; or epithelial and fibroblastic, or several types of cells that aim to mimic conditions *in vivo*. Conditions inside the bioreactor such as downward or cross flow forces, microgravity environments, rotating wall cell culture systems, fluid shear, compression, stretched and pressure loads are used to achieve dynamic conditions. This is done to stimulate the differentiation and proliferation of cells into tissue, especially in stem cells, known as mechano-differentiation (Selden and Fuller, 2018). Some research on the development of respiratory tissue techniques shows that mechanical stimulation in the form of air flow most often occurs in bioreactors. Controlled air flow in the bioreactor is known to contribute to the formation of tight junctions in confluent monolayers of lung epithelial cells. Additionally, the incorporation of perfusion and ventilation in the bioreactor of the airway allows maintenance of the survival of epithelial and endothelial cells (Panoskaltsis, 2015; Petersen *et al.*, 2011; Poon *et al.*, 2012). Mechanical stimuli such as water-liquid interfaces and ventilation produced by negative pressure are needed especially for the development of lung tissue. Measurement microfluidic parameters in real-time are also needed to provide complex bioprocesses that mimic the microenvironment of the lungs (Tebyanian *et al.*, 2019).

Tissue engineering for microbiome model

Respiratory tissue engineering modeling to observe host and microbiome interaction in co-culture epithelial cells model *in vitro* offer the possibility to investigate if in a controlled environment with reduced complexity compared to *in-vivo*. It is therefore hoped that the respiratory tissue engineering model will not only focus on colonization with single species of microbe or probiotic but also can be used to monitor short-term host-microbe interactions, such as innate immune responses of the epithelial cells and physiological changes of bacterial and epithelial cells such as cytokine production and tight junction functionality. The first respiratory tissue engineering study *in vitro* model was done for bacterial biofilm formation on differentiated Calu-3 epithelial cells at ALI in co-culture with *Haemophilus* influenzae bacteria (Starner *et al.*, 2006). Similar modeling mechanisms are also used to study *Staphylococcus aureus* colonization of airway epithelial cells and observations of physiological changes that occur during the colonization process. However, this causes the epithelial layer completely disrupted within 1 day after inoculation (Kiedrowski *et al.*, 2016). The model demonstrates limitations in the time frame of host microbial culture and the ability to replicate the respiratory tract microenvironment in an appropriate manner due to cell toxicity, duration of microbial culture by interaction between epithelial culture cells and bacteria that are limited to hours or days. Based on these studies several things that need to be considered that will affect the success of the model, namely the number of microbes that are infected in cells ranging from 0.01-10 bacteria / host cell. Flow washing in the bioreactor is also a matter to be considered to prevent over colonization from occurring, however, the failure of the host compartment due to inadequacy in mimicking the host microenvironment as the most frequent host cell due to undifferentiation or differentiated epithelial cell culture (De Rudder *et al.*, 2018).

The most recent work on respiratory tissue engineering in 3D is a comprehensive human tracheobronchial mucosa from the pseudostratified epithelium of primary cells of human origin and the underlying stromal tissue. In this research, a model was developed that could mimic native tissue such as mucociliary differentiation from epithelial sheets and basement membrane formation. Furthermore, the addition of *Haemophilus influenzae* bacteria results in bacterial associations and crossing the mucous layer. these results can potentially be adjusted to study medium / long term host-pathogenic processes (Marrazzo *et al.*, 2016). Other development of respiratory engineering is ALI model of human bronchioles with epithelial and endothelial tissue known as the lung-in-a-chip, developed based on microfluidic devices that mimic the microarchitecture and dynamic microenvironment

of alveolar-capillary units of living human lungs. Soft lithography-based microfabrication is used for building a three-dimensional microchannel system in such a way as to obtain the formation of alveolar-capillary interfaces, human alveolar epithelial cells and pulmonary microvascular endothelial cells favored into the upper and lower chambers. This model is claimed to be used to observe inflammatory mechanisms, such as cytokines and proinflammatory bacteria, to the upper alveolar compartment which induces activation of endothelial cells on opposite sides of the membrane and increases the expression of their adhesion molecules (Huh *et al*., 2012; Huh, 2015). As in previous studies of microbiome in tissue engineering models were carried out in the intestinal tract using the intestine on a chip model, which at present is at an early stage of development microbial loads in the lumen. The model is used to observe the interaction of microbial and host-cell viability, gene-expression profiles during culture, mucus-producing human intestinal epithelial and complex community of living commensal gut microorganisms (Jalili *et al*., 2019; Poceviciute and Ismagilvo, 2019). In the 3D structure model, two popular transformed intestinal epithelial cells (IECs), Caco-2 and HT29 cells propagated in ECM such as Matrigel, an analog basement membrane that helps direct the polarity of the epithelium. Recently, intestinal pluripotent stem cells are also used *in vitro* and are known as human intestinal organoids (HIOs). This complex spheroid consists of an epithelial layer surrounded by a mesenchymal layer containing fibroblasts and smooth muscle cells. Bioreactors in the form of chambers are designed to allow shear stresses to be applied to the apical and basolateral sides of the membrane and permeability is measured over time. Commensal bacteria can be added to the apical tract to simulate the effects of intestinal microbiome and to be observed (Hewes *et al*., 2020; Pearce *et al*., 2018; Zhou *et al*., 2018). This concept seems to be applicable to respiratory tissue engineering although it seems that there are some complexities that need to be adjusted, such as cell type and properties, ECM scaffold used, and dynamic microenvironment systems for microbiome in bioreactors.

Conclusions and Recommendations

Respiratory tissue has a high complexity based on the anatomical structure of diverse epithelium and has various functions from nasal cavity to alveolar. In addition, knowledge about the role of microbiomes physiologically and pathologically to the mechanism of viral infection is still limited to the identification of microbes found based on metagenomics. The development of *in vitro* tissue engineering models in the respiratory tract seems to be very necessary given the lack of knowledge about various aspects of the pathophysiology of the respiratory tract. Challenges in this model include achieving a balanced condition between the microenvironment to support the host epithelial tissue and normal microbiome to obtain suitable tissue engineering conditions that are similar *in vivo*. Research related to modeling that occurs *in vitro* can represent events that occur *in vivo* and help determine physiological conditions, the mechanism of the emergence of pathogenic infections, and the development of effective treatment strategies for respiratory diseases in the future.

Novelty Statement

This review article aims to provide an overview of the various complex mechanisms that occur when infection occurs in the host. Some of them occur due to the role of the microbiome in the respiratory system which has so far been neglected, especially the in vitro infection model using tissue engineering. Thus, the development of an in vitro infection model in respiratory tissue engineering would be more suitable for mimicking the infectious mechanism of the host in the future.

Author's Contribution

RSK conducts articles with appropriate topics based on recent literature review study, designs and compiles images, also selects included literature. RDA adds to the study literature, revises the image so that it fits, and adds discussion to this article. The final version was approved by all authors.

Conflict of interest

The authors have declared no conflict of interest.

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