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Mini-Review

Host-pathogen interactions during coronavirus infection of primary alveolar epithelial cells

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ABSTRACT

Viruses that infect the lung are a significant cause of morbidity and mortality in animals and humans worldwide. Coronaviruses are being associated increasingly with severe diseases in the lower respiratory tract. Alveolar epithelial cells are an important target for coronavirus infection in the lung, and infected cells can initiate innate immune responses to viral infection. In this overview, we describe in vitro models of highly differentiated alveolar epithelial cells that are currently being used to study the innate immune response to coronavirus infection. We have shown that rat coronavirus infection of rat alveolar type I epithelial cells in vitro induces expression of CXC chemokines, which may recruit and activate neutrophils. Although neutrophils are recruited early in infection in several coronavirus models including rat coronavirus. However, their role in viral clearance and/or immune-mediated tissue damage is not understood. Primary cultures of differentiated alveolar epithelial cells will be useful for identifying the interactions between coronaviruses and alveolar epithelial cells that influence the innate immune responses to infection in the lung. Understanding the molecular details of these interactions will be critical for the design of effective strategies to prevent and treat coronavirus infections in the lung. *J. Leukoc. Biol.* 86: 1145–1151; 2009.

Introduction

Coronaviruses of humans and animals are increasingly being recognized as significant pathogens in the lower respiratory tract. Animals and poultry of agricultural importance, including cows, pigs, and chickens, are infected by coronavirus strains that cause respiratory and enteric diseases of varying severity. In 2002–2003, SARS-CoV emerged from wildlife to cause an epidemic with a 10% case fatality ratio. Since then, two previously unknown HCoV, NL63 and HKU1, were discovered and found to cause respiratory disease worldwide. New molecular technologies for concurrently screening clinical specimens for a large number of viruses have allowed investigators to associate these newly identified coronaviruses with a wide range of respiratory diseases, from mild upper respiratory tract infections to severe pneumonia. Primary epithelial cell cultures derived from conducting airways have been studied as targets for several respiratory viruses including SARS-CoV. It is also important to understand the role of alveolar epithelial cells in initiating and regulating local immune responses to viral infection in the alveoli through the expression of cytokines and chemokines. Until recently, the responses of alveolar epithelial cells to virus infection were studied in continuous human or animal cell lines derived from the lung. However, these cell lines do not maintain the differentiated phenotypes of alveolar cells and thus, are not optimal models for the highly specialized cell types of the alveolar epithelium. Cell culture techniques that maintain the differentiated phenotypes of primary alveolar epithelial cells permit studies on the virus/host interactions that influence immune responses to alveolar infection. Understanding the molecular details of these interactions will be critical for designing effective strategies for the prevention and treatment of respiratory virus infections.

RESPIRATORY CORONAVIRUS INFECTIONS

The repiratory and enteric tracts are common targets for coronaviruses that infect animals and poultry, including pigs, cows, dogs, rodents, and chickens. Porcine respiratory coronavirus infects the epithelial cells of the lung, and disease ranges from subclinical infection to moderate bronchointerstitial pneumo-

Abbreviations: AT1/2 cell=alveolar type I and II cell, HCoV=human coronavirus, IL-1Ra=IL-1R antagonist, KGF=keratinocyte growth factor, MHV=mouse hepatitis virus, NIH=National Institutes of Health, RCoV=rat coronavirus, RSV=respiratory syncytial virus, SARS=severe acute respiratory syndrome, SARS-CoV=SARS-associated coronavirus, SP=surfactant protein, tAT1 cell=in vitro transdifferentiated AT1-like cell, TNFR=TNF receptor

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nia, depending on the virus strain [1, 2]. Bovine coronavirus causes disease in the enteric tract and the upper and lower respiratory tracts, which has been associated with shipping fever [3]. Infectious bronchitis virus causes a highly infectious respiratory disease in the upper respiratory tract and bronchi of chickens that is especially severe in chicks [3]. Canine respiratory coronavirus was discovered in 2003 and is prevalent worldwide in populations of kenneled dogs; however, its pathogenesis and contribution to kennel cough in dogs are incompletely understood [4, 5]. The murine coronavirus MHV-1 causes fatal interstitial pneumonitis in the A/J strain of inbred mice [6]. RCoV strains cause respiratory diseases with differing degrees of severity, depending on the viral strain and the age, strain, and immune status of the animal [7–9].

Five HCoV cause respiratory infections with various degrees of severity. HCoV-229E and HCoV-OC43, which were discovered in the 1960s, are a significant cause of common colds and can cause severe lower respiratory tract disease in elderly, infant, and immunocompromised patients [10–13]. In 2003, SARS-CoV was identified as the causative agent of the epidemic of SARS [14, 15]. Subsequently, two additional HCoV, HCoV-NL63 and HCoV-HKU1, were discovered to cause respiratory disease in patients worldwide [16–19]. HCoV-NL63 is associated with mild upper respiratory tract infections, laryngotracheitis (croup), and bronchiolitis and pneumonia in children [17–19]. HCoV-HKU1 is also associated with upper respiratory tract infections in children and pneumonia in elderly patients with underlying diseases [10, 20]. Because of these findings, it is important to understand the mechanisms of coronaviral pathogenesis in the lung.

Most coronaviruses, with the notable exception of bovine coronavirus, infect and cause disease in one species or a limited number of related species [21]. Although there are several animal models for SARS-CoV infection [22], there are no animal models for respiratory diseases caused by the other four HCoV [23, 24]. Therefore, it is important to study these coronaviruses in differentiated human alveolar epithelial cells in vitro. However, it is desirable to study respiratory coronaviruses for which in vitro studies in differentiated alveolar cells can be correlated with pulmonary infection in vivo. Therefore, we are studying RCoV infection in its natural host as a model for pathogenesis of respiratory coronaviruses. The study of RCoV infection in rats provides an excellent model for understanding the innate immune responses of the alveoli to infection by a respiratory coronavirus of its natural host.

VIRAL INFECTION OF THE ALVEOLAR EPITHELIUM

The alveolar epithelium consists of two morphologically and functionally distinct cell types [25]. Ninety-eight percent of the surface area of the alveolar epithelium is made up of AT1 cells, which are large, flattened, nondividing cells that function in gas exchange and fluid homeostasis [26, 27]. AT1 cells are identified in lung tissue by their morphology, specific binding to *Ricinus communis 1* lectin, and expression of T1α and aquaporin-5 [26]. AT2 cells are cuboidal, dividing cells and are progenitors for replacement of damaged AT1 cells [28]. AT2 cells produce surfactant lipids and proteins that keep the alveoli open and contribute to innate defense of the lung [29]. AT2 cells are distinguished in situ by binding to *Maclura pomifera* lectin, the presence of lamellar bodies, and expression of SP-A, SP-B, and SP-C [26, 29]. Infection of alveolar epithelial cells in vivo by respiratory viruses, including respiratory syncytial virus, influenza A virus, and SARS-CoV, can have significant effects on respiratory functions in the alveoli. Infection of AT1 cells can impair gas exchange and removal of fluid from the lung. In addition, infection of AT2 cells can compromise repair of the damaged alveolar epithelium and innate defense of the alveoli.

In autopsy specimens from SARS patients, immunohistochemistry detected SARS-CoV antigens in AT1 or AT2 cells, or both cell types, as well as in alveolar macrophages and bronchial and bronchiolar epithelial cells [30–33]. Differences in the cell types that contain viral antigen in different patients may reflect the age of the patient and/or the time after infection when the patient died. Studies on SARS-CoV infection in primate, murine, feline, and ferret models have also demonstrated infection of alveolar epithelial cells. SARS-CoV antigens were detected in AT1 cells of cynomolgus macaques 4 days after inoculation with SARS-CoV, at which time, there was diffuse alveolar damage and neutrophil infiltration in the lung [34]. van den Brand et al. [35] found SARS-CoV antigen predominantly in AT1 and AT2 cells of cats and AT2 cells of ferrets 4 days after inoculation with SARS-CoV, when all animals had diffuse alveolar damage with infiltrating neutrophils and macrophages. Although cats had no clinical signs of infection with SARS-CoV, ferrets inoculated with SARS-CoV were lethargic, and one of four ferrets died 4 days after inoculation. In aged mouse and mouse-adapted models of SARS, viral antigens were detected in alveolar epithelial cells without distinguishing AT1 from AT2 cells [36–38]. In contrast to inoculation of young mice, inoculation of aged mice with SARS-CoV causes clinical signs of disease, lymphocyte infiltration, and alveolar damage 3–9 days after inoculation [37]. Despite these clinical and histopathological signs of disease, aged mice recover from infection. The mouse-adapted SARS-CoV (MA15) isolated by Roberts et al. [36] causes lethal infection in BALB/c mice, characterized by viral antigens in bronchial and alveolar epithelial cells with cellular necrosis and infiltration of mononuclear cells. A second mouse-adapted SARS isolate (FmusX-VeroE6) causes clinical signs of disease in BALB/c mice, with a 30% mortality rate [38]. Inoculation of adult mice with this virus results in viral antigen in alveolar epithelial cells, diffuse alveolar damage, and infiltration of macrophages, lymphocytes, and neutrophils into the alveoli. Porcine respiratory coronavirus antigen has been identified by immunofluorescence in epithelial cells of the alveoli, bronchi, and bronchioli, as well as alveolar macrophages 2–6 days after inoculation of infant pigs, resulting in subclinical interstitial pneumonia [1]. RCoV infection of adult rats results in an influx of neutrophils, followed by lymphocytes and monocytes, into the respiratory tract [7, 39]. The interactions between respiratory vi-

ruses and alveolar epithelial cells can mediate the innate immune response to virus infection in the lung. Primary cultures of differentiated alveolar epithelial cells are a valuable model for studying these interactions.

CORONAVIRUS ISOLATION OFTEN REQUIRES DIFFERENTIATED HOST CELLS

Human respiratory coronaviruses could not be isolated from patients with colds in continuous human cell lines, but instead, virus isolation required serial blind passage in human diploid fibroblasts or human fetal tracheal organ cultures [40–44]. HCoV-229E and HCoV-OC43 caused only mild cytopathic effects in these cells, but these viruses could be used to infect human volunteers to study the pathogenesis of coronavirus infection of the upper respiratory tract [41, 45]. HCoV-NL63 is also difficult to isolate from clinical specimens [46] and can be isolated most readily in primary, differentiated human airway epithelial cells [47]. Infectious HCoV-HKU1 has not yet been propagated in any cell culture, although its entire genome sequence has been determined [16]. In contrast, SARS-CoV could be readily isolated from patients in the Vero E6 line of monkey kidney cells or in fetal rhesus kidney cells [14, 48, 49]. The reasons for the fastidious requirements of most human respiratory coronaviruses for differentiated human respiratory epithelial cells are not yet understood.

CORONAVIRUS INFECTION IN PRIMARY DIFFERENTIATED RESPIRATORY EPITHELIAL CELLS

The respiratory tract is lined with epithelial cells that have different functions in the upper respiratory tract (nasal and sinusoidal epithelium), conducting airways (tracheal and bronchial epithelium), and alveoli (alveolar epithelial cells), and all of these are susceptible to infection with a variety of respiratory viruses. Winther et al. [50] demonstrated the susceptibility of primary cultures of ciliated nasal epithelial cells to infection by HCoV-229E without cytopathic effects. Polarized cultures of differentiated, ciliated human conducting airway epithelia have also been used to study infection and the polarity of entry and release by HCoV-229E, HCoV-NL63, and SARS-CoV [47, 51–55]. These studies emphasize the importance of the differentiation state of ciliated cells for susceptibility to coronavirus infection [52, 53]. Recent advances in the cultivation of differentiated alveolar epithelial cells now allow analysis of these important cell types in virus infection.

CULTIVATION AND CORONAVIRUS INFECTION OF PRIMARY DIFFERENTIATED ALVEOLAR EPITHELIAL CELLS

As continuous cell lines derived from alveolar epithelium do not maintain their differentiated characteristics, primary cultures must be used to study differentiated AT1 and AT2 cells in vitro. Primary AT1 cells are difficult to isolate to a high yield and purity and propagate [26]. However, AT2 cells can be readily isolated from lung tissue, and under special culture conditions, they maintain their AT2 phenotype. Under different culture conditions, AT2 cells transdifferentiate into an AT1 cell phenotype (**Fig. 1**). This process of transdifferentiation occurs during repair of the alveolar epithelium in vivo and can be replicated in vitro [56–61]. Primary rat AT2 cells maintain their differentiated phenotype when they are cultured on collagen/matrigel in medium containing 5% rat serum and KGF [62, 63]. The AT2 phenotype is characterized by a cuboidal shape, lipogenesis (evident by the presence of lamellar bodies) and expression of surfactant proteins. When rat AT2 cells are cultured for 3–5 days in 10% FBS without KGF, they lose properties of AT2 cells and transdifferentiate into an AT1 cell phenotype (tAT1 cell, also called AT1-like cell) [56, 58, 59]. These cells are flattened, express markers characteristic of AT1 cells in vivo (T1α, aquaporin-5, and caveolin-1), and react with AT1 cell-specific antibodies and lectins [26, 59, 64–68]. As the markers used to distinguish AT1 cells in situ are present in tAT1 cells in vitro, tAT1 cells are a practical alternative to AT1 cell isolation for the study of AT1 cells in vitro. As with any in vitro model, the biological relevance of such studies must ultimately be confirmed in vivo.

We showed that the differentiation status of primary alveolar epithelial cells is critical in determining susceptibility to SARS-CoV infection [69]. Human alveolar epithelial cells that were maintained with the AT2 phenotype supported infection by SARS-CoV, whereas cells from the same donor that were transdifferentiated in vitro to a tAT1 cell phenotype were resistant to infection. In contrast to SARS-CoV, HCoV-229E replicates

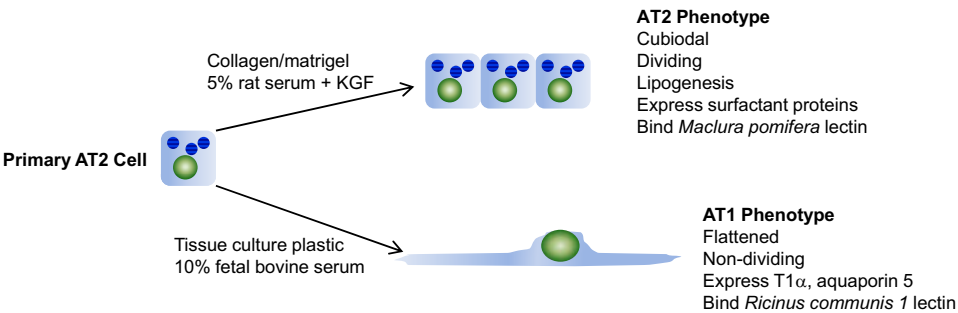

Figure 1. Schematic representation of culture conditions for primary differentiated alveolar epithelial cells. AT2 cells are isolated from rat lung and are cultured to maintain an AT2 phenotype or transdifferentiate into a tAT1 cell phenotype.

and causes cytopathic effects in cloned AT2 cells, which resemble an AT1 cell phenotype [70]. Our studies using RCoV infection of primary differentiated rat alveolar epithelial cells were the first to demonstrate coronavirus infection in tAT1 and AT2 cells in vitro [71, 72].

INNATE IMMUNE RESPONSES OF ALVEOLAR EPITHELIAL CELLS TO VIRUS INFECTION

In the lung, the roles of AT2 cells and alveolar macrophages in initiating and regulating an immune response have been studied extensively. AT2 cells produce inflammatory mediators upon exposure to inhaled microbes or particles and regulate the functions of immune cells, including macrophages, dendritic cells, and lymphocytes in the lung [29, 73–75]. In vitro, human bronchial epithelial cells and AT2 cells produce cytokines and chemokines in response to infection with viruses including RSV, influenza A virus, and SARS-CoV [76–80]. The innate immune functions of AT1 cells have only been recognized recently. Expression of chemokines by primary differentiated tAT1 cells in vitro is increased by exposure to IL-1α, IL-1β, or LPS [64, 65, 81, 82]. In primary cultures of murine tAT1 cells, influenza A virus induces expression of CCL2 and CCL5, resulting in transmigration of monocytes [83].

We have shown that rat tAT1 cells in vitro express cytokines and chemokines upon infection with RCoV [71]. This study was the first to show that virus infection of primary tAT1 cells induces a proinflammatory response. RCoV infection of tAT1 cells induces expression of cytokines, including GM-CSF, IFN-γ, and TNF-α, and chemokines, predominantly those of the CXC family [71]. The primary functions of CXC chemokines are to recruit and activate neutrophils. We hypothesize that RCoV infection in the lung induces CXC chemokine expression by AT1 cells, which in turn, recruits neutrophils to the lung. The role of neutrophils during RCoV infection of the lung is unknown, but these cells may contribute to viral clearance and immunopathology. Rat tAT1 cells are a valuable model in which to study the virus/host interactions that regulate this response. Using this model, we showed that like RCoV infection, UV-inactivated RCoV induces CXC chemokine expression in rat tAT1 cells [71], so virus replication is not required to induce the chemokine response in rat tAT1 cells. Dual immunolabeling of viral antigen and CXC chemokines in rat tAT1 cells showed that CXC chemokines are expressed predominantly from uninfected cells in the culture [71]. Therefore, expression of CXC chemokines during RCoV infection of tAT1 cells may be mediated by a paracrine mechanism. We found that RCoV-infected rat tAT1 cells treated with IL-1Ra had markedly decreased expression of CXC chemokines relative to cells without IL-1Ra [71]. Treatment with soluble TNFR protein did not affect chemokine expression by RCoV-infected tAT1 cells. Thus, signaling through the IL-1R likely mediates CXC chemokine expression by rat tAT1 cells during RCoV infection (**Fig. 2**). As IL-1α and IL-1β signal through the IL-1R, either or both of these cytokines may contribute to CXC chemokine expression during RCoV infection of tAT1 cells. Manzer et al. [65, 82] showed that rIL-1α and rIL-1β induce expression of CXC chemokines by rat tAT1 cells in vitro. Rat tAT1 cells are a valuable model for investigating the early events in innate immune responses to respiratory coronavirus infections.

NEUTROPHILS IN RESPIRATORY VIRUS INFECTIONS

Neutrophils infiltrate tissues early after viral infection and, through the expression of proinflammatory cytokines and chemokines, can direct the subsequent recruitment of monocytes and lymphocytes. For example, in infants with RSV bronchiolitis, neutrophils accounted for 93% and 76% of inflammatory cells in the upper and lower airways, respectively [84, 85]; however, the specific functions of neutrophils during RSV infection are unclear [86]. Neutrophils infiltrate the respiratory tract by 18 h after inoculation of mice with influenza A virus, and increased numbers of neutrophils have been associated with highly pathogenic influenza virus infections in mice [87, 88]. Depletion of neutrophils from mice exacerbated infection with a highly pathogenic recombinant influenza virus strain containing the hemagglutinin and neuraminidase genes of the 1918 influenza virus [87]. Thus, neutrophils can play a role in protection from virulent influenza virus infection. In infection with less virulent strains of influenza A virus, neutrophils can have a protective effect [89, 90] or no effect [91] on viral replication and pathogenesis. Thus, with different virus strains, neutrophils can have different functions in the innate immune response to respiratory infections.

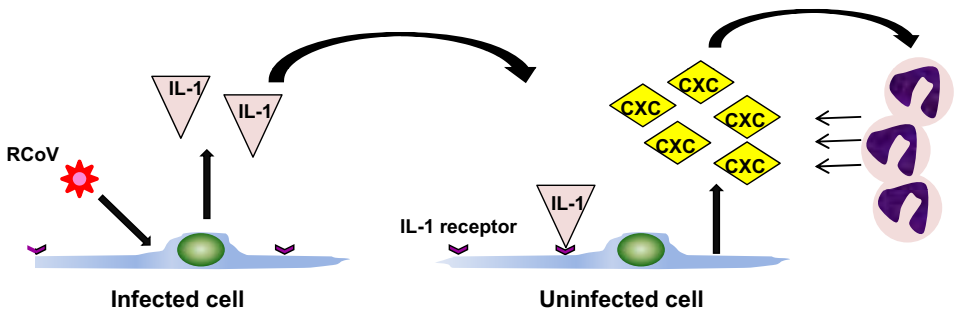

Figure 2. Model of RCoV-induced expression of CXC chemokines in primary rat tAT1 cells. RCoV infection induces expression of IL-1α and/or IL-1β, which signal through the IL-1R on uninfected cells to induce expression of CXC chemokines, likely recruiting neutrophils to the site of infection.

Neutrophils also infiltrate tissues infected by coronaviruses, including SARS-CoV, RCoV, and MHV. A high neutrophil count in the blood of SARS patients at the time of hospital admission was associated with a poor prognosis [92, 93]. A mouse-adapted isolate of SARS-CoV (F-musX-VeroE6) causes lethal infection in adult, but not young, mice [38]. The disease severity in adult mice correlates with increased pulmonary inflammation consisting predominantly of neutrophils, which are also the predominant cell type detected in the nasal exudates from chickens infected with infectious bronchitis virus and are believed to contribute to disease pathology [94]. These findings suggest that neutrophils can contribute to immune-mediated pathology in some coronavirus infections. Infection of rats with RCoV results in infiltration of neutrophils to the respiratory tract early after inoculation, followed by the recruitment of macrophages and lymphocytes [7, 8, 39, 95]. Infection of mice with a neurotropic murine coronavirus, MHV-JHM, results in infiltration of neutrophils into the brain by 1 day after inoculation, which then promotes the recruitment of other types of inflammatory cells into the brain, likely through loss of the blood brain barrier [96]. Despite the presence of neutrophils in coronavirus-infected tissues, their role in the clearance and/or immunopathology of coronavirus infections is largely unknown. Future studies on the responses of neutrophils to RCoV-infected tAT1 cells in vitro may elucidate the role of neutrophils in the pathogenesis of respiratory coronavirus infections.

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