Severe acute respiratory syndrome (SARS)-coronavirus (CoV) produces a devastating primary viral pneumonia with diffuse alveolar damage and a marked increase in circulating cytokines. One of the major cell types to be infected is the alveolar type II cell. However, the innate immune response of primary human alveolar epithelial cells infected with SARS-CoV has not been defined. Our objectives included developing a culture system permissive for SARS-CoV infection in primary human type II cells and defining their innate immune response. Culturing primary human alveolar type II cells at an air–liquid interface (A/L) improved their differentiation and greatly increased their susceptibility to infection, allowing us to define their primary interferon and chemokine responses. Viral antigens were detected in the cytoplasm of infected type II cells, electron micrographs demonstrated secretory vesicles filled with virions, virus RNA concentrations increased with time, and infectious virions were released by exocytosis from the apical surface of polarized type II cells. A marked increase was evident in the mRNA concentrations of interferon-β and interferon-α (IFN-α) (Fig. 5) and in a large number of proinflammatory cytokines and chemokines. A surprising finding involved the variability of expression of angiotensin-converting enzyme–2, the SARS-CoV receptor, in type II cells from different donors. In conclusion, the cultivation of alveolar type II cells at an air–liquid interface provides primary cultures in which to study the pulmonary innate immune responses to infection with SARS-CoV, and to explore possible therapeutic approaches to modulating these innate immune responses.

Keywords: lung innate immune response; cytokine responses to SARS coronavirus; lung cell differentiation; air–liquid interface cultures

CLINICAL RELEVANCE

Severe acute respiratory syndrome–coronavirus (SARS-CoV) produces devastating viral pneumonia (1, 2). Pathologic changes are most prominent in the lungs, with disruptions of the epithelium in gas exchange areas and conducting airways (2–4). The epithelial cells of the alveoli and the conducting airways are the primary targets of SARS-CoV in the human lung, and they express the SARS receptor, angiotension-converting enzyme–2 (ACE2) (5–8). In autopsies of patients with SARS, coronavirus RNA and proteins have been detected in type II cells via immunocytochemistry and in situ hybridization (7, 9–14). In the aged macaque model of SARS, in which the initial site of infection in the lung can be studied, virus infection was detected in both alveolar type I and type II cells (11, 15). In human SARS autopsy specimens, the infection of alveolar macrophages was also suggested because they contained SARS antigens and formed multinucleated giant cells (2, 11). However, in vitro human alveolar macrophages, monocyte-derived dendritic cells, and monocytes are not readily susceptible to SARS-CoV, and these cell types show only very modest cytokine and interferon responses upon exposure to SARS-CoV (16–21).

One of the major clinical issues in this type of devastating viral pneumonia involves whether SARS is principally a severe primary viral pneumonia, or a systemic disease caused by a cytokine storm out of proportion to the extent of viral pneumonia (22). During the course of SARS-CoV infection, a marked elevation was evident in serum concentrations of proinflammatory cytokines, including CXCL10 (IP-10), CCL2 (MCP-1), IL-6, and CXCL8 (IL-8) (1, 22–26). However, it remains unknown whether these serum cytokines were produced by epithelial cells...
of the alveoli and conducting airways (where the virus replicates in vivo) or by inflammatory cells recruited to the site of virus infection in the lung. Based on in situ hybridization and immunocytochemistry, He and colleagues suggested that ACE2-positive infected cells in lungs of patients with SARS are the source of proinflammatory cytokines (27).

The primary goal of this research was to define the innate immune response of human alveolar type II cells to SARS-CoV. In our earlier study with submerged human alveolar type II cells on inserts, we detected a very low percentage of infected cells and could not evaluate their innate immune response (18). However, culturing and infecting the type II cell at an air–liquid interface (A/L) allowed for significant infection and characterization of their innate immune response. The robust innate immune response of these cells to SARS-CoV infection consisted of marked elevations in mRNA concentrations of interferon-β, interferon-λ, CXCL10, CXCL11, and IL-6, and these results mirror findings reported in the lungs and sera of patients with SARS (22, 23, 25).

MATERIALS AND METHODS

Lungs were obtained from deidentified patients whose lungs had been deemed unsuitable for transplantation and were donated for medical research. The Committee for the Protection of Human Subjects at National Jewish Health has deemed the use of human lung cells from deceased lung donors does not constitute human subject research and is therefore exempt from Institutional Review Board approval. (Correspondence to this effect from the Committee for the Protection of Human Subjects at National Jewish Health is available upon request).

Isolation and Culture of Human Alveolar Type II Cells at an Air–Liquid Interface

Cells were isolated as previously described (28, 29). Isolated cells were resuspended in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS, and plated at a density of 1 × 10^5 per cm^2 on six-well or 24-well Millicell inserts (Millipore Corp., Bedford, MA) precoated with a mixture of 50% Matrigel (BD Biosciences, San Jose, CA) and 50% rat-tail collagen. After cell adherence for 48 hours, the medium was switched for 2 days to 5% heat-inactivated human serum plus 10 ng/ml IL-4, 10 ng/ml IL-13, and 10 ng/ml KGF, 0.1 mM isobutylmethyl xanthine was switched for 2 days to 5% heat-inactivated human serum plus 10% FBS, and plated at a density of 1 × 10^5 per cm^2 on six-well or 24-well Millicell inserts (Millipore Corp., Bedford, MA) precoated with a mixture of 50% Matrigel (BD Biosciences, San Jose, CA) and 50% rat-tail collagen. After cell adherence for 48 hours, the medium was switched for 2 days to 5% heat-inactivated human serum plus 10 ng/ml keratinocyte growth factor (KGF), 0.1 mM 8-bromo-cyclic adenosine monophosphate (cAMP), 10 ng/ml IL-4, and 10 ng/ml IL-13. At this time, cultures were converted to A/L conditions. Cells were incubated for an additional 4 days with medium containing KIA, IL-4, IL-13, and 10 mM dexamethasone (D). After 8 days of culture on the inserts and 6 days under A/L conditions, cells were used for virus inoculation or harvested.

SARS-CoV Infection

The Urbani strain of SARS-CoV was kindly provided by Dr. Bellini at the Centers for Disease Control (Atlanta, GA), and was propagated in Vero E6 cells. All work with infectious SARS-CoV was performed in the Biosafety Level 3 suite at the School of Medicine, University of Colorado (Aurora, CO). Type II cells were inoculated on the apical surface with 250 µl per six-well plate insert (or 50 µl per 24-well plate insert) of KIAD with IL-4-supplemented and IL-13-supplemented medium containing SARS-CoV (1 × 10^5 plaque-forming units (pfu)/ml). After 4 hours of incubation at 37°C, the virus inocula and control media were aspirated from the apical cell surface, and cultures were incubated for a total of 24 or 48 hours after virus inoculation under A/L conditions, after which they were extracted to analyze viral and cellular RNA, or fixed and assayed via electron microscopy or immunofluorescent labeling with antibodies to viral or cell proteins. The yield of infectious virus was titered in washes of apical cell surfaces and basal medium by plaque assays on Vero E6 cells.

The methods for immunocytochemistry, the quantification of infected cells, virus production, plaque assays, the isolation of RNA, quantitative PCR, flow cytometry, surface biotinylation, and statistical analysis are described in the online supplement.

RESULTS

Culturing Primary Human Alveolar Type II Cells at an Air–Liquid Interface Maintains Their Cellular Differentiation

In our initial studies on SARS-CoV infection with human alveolar type II cells in traditional submerged cultures, we detected only a very low level of infection. Subsequently, we evaluated a variety of changes in the culture system to improve the rate of infection. We found that susceptibility to SARS-CoV infection and the maintenance of differentiation were achieved by culturing cells at an A/L on Millicell inserts coated with a matrix of rat-tail collagen and Matrigel, and by providing a medium that contained IL-13 and IL-4 as well as KGF, isobutylmethyl xanthine, 8 Br-cAMP, dexamethasone, and 5% heat-inactivated human serum (30). Cells contained readily identifiable lamellar bodies according to phase microscopy (Figure 1A). In general, increased type II cell differentiation was observed, as evidenced by the expression of the surfactant proteins (SPs) (Figure 1B). As shown in Figure 1B, the air–liquid interface condition increased the expression of ACE2, SP-A, proSP-B, proSP-C, and ABCA3, but not fatty-acid synthase, compared with the submerged condition in this experiment. However, the magnitude of effect of the A/L condition was more variable with human type II cells than with our experiments on primary rat type II cells (31) (Figure E1 in the online supplement). In addition, we found an increase in ACE2 expression in our cultures after adding IL-4 and IL-13, a finding that differs from results with Vero E6 cells regarding the effect of IL-13 on ACE2 expression (32) (Figure 1B). In general, the level of ACE2 expression, as measured by immunoblotting of whole-cell extracts, was slightly increased in type II cells cultured under A/L conditions, but ACE2 concentrations varied considerably between cells from individual donors (Figure E1). However, according to flow cytometry and surface biotinylation, we were unable to detect any increase in the surface expression of ACE2 in cells maintained under A/L conditions, compared with cells maintained in submerged cultures (data not shown).

SARS-CoV Readily Infects Primary Human Alveolar Type II Cells Cultured at the Air–Liquid Interface

To test whether type II cells cultured at the A/L were more susceptible to SARS-CoV infection than type II cells in submerged cultures (18), human type II cells cultured at an A/L on six-well inserts were inoculated on the apical surface with 250 µl of medium containing SARS-CoV at an estimated multiplicity of 2 pfu/cell, and allowed to adsorb for 4 hours at 37°C. Then the apical fluid and inoculum were aspirated, and cells were maintained under A/L conditions. SARS-CoV reproducibly caused productive infection of the primary human alveolar type II cells cultured at the A/L. We found little evidence of infection at 4 hours after virus inoculation, but by 24 hours after virus inoculation, infected cells containing viral antigens in the cytoplasm were readily detected. SARS-CoV–infected cells were readily observed in all primary alveolar type II cultures on Millicell inserts at the A/L, although the percentages of infected cells varied considerably in different areas of the same insert and between different human donors. In four donors, the percentage of cells infected ranged from 3.4–34.2% at 24 hours, and from 2.4–27.2% at 48 hours.

To demonstrate that the SARS-CoV–infected cells were type II cells, we used antibodies to several type II cell differentiation markers, namely, thyroid transcription factor-1 (TTF-1), SP-A, and E-cadherin. Nearly 100% of the cells expressed SP-A. Dual labeling showed that the infected cells were epithelial and expressed TTF-1 and SP-A (Figure 2). These markers were chosen because they could be readily detected in the methanol-fixed cells required for identifying the viral antigens.
Coronavirus RNA is synthesized in virus-induced double-membrane vesicles that comprise the sites of viral RNA replication. The characteristic double-membrane vesicles that comprise the sites of viral RNA replication are depicted in Figure 3B (34, 35), and virions were observed budding into vesicles (Figure 3A) and adsorbed to the apical membrane (Figure 3B).

Infected Alveolar Type II Cells Cultured at the Air–Liquid Interface Produce Infectious SARS-CoV

To analyze SARS-CoV infection in type II cells at an A/L, we used both real-time quantitative PCR and plaque assays (Figure 4). Quantitative real-time PCR revealed a consistent 1.5 log increase in viral mRNA at 24 hours after inoculation, as shown for six donors (Figure 4A). Each donor was deidentified and received a number. Most donors were used for only one type of experiment because of the limited number of cells isolated from each donor. Cells were isolated from more than 20 donors in the course of these experiments.

Because no medium was recoverable from the cells at the A/L, we washed the apical surface at designated times after virus inoculation, and titrated the released virus in the apical wash as well as in the basolateral medium by a plaque assay. Equally high yields of released virus from the apical wash and cell-associated virus from the frozen and thawed samples were detected at 24 hours, and the virus titers in specimens harvested at 48 hours were lower but similar. Very little virus was detected in the basolateral medium (Figure 4B).

Alveolar Type II Cell Cultures at the A/L Display a Vigorous Innate Immune Response to SARS-CoV Infection

Concentrations of multiple proinflammatory cytokines and chemokines have been reported to be increased in the lungs and sera of patients with SARS (22, 23, 25, 26). Because alveolar type II cells at the A/L were readily infected by SARS-CoV, we were able to measure changes in expression of selected genes of the innate immune system in these cells in response to SARS-CoV infection (Figure 5). For technical and biosafety reasons, we did not attempt to measure concentrations of interferon or cytokine proteins from infected cultures. The SARS-CoV infection of type II cells induced markedly increased concentrations of the mRNAs encoding type I and type III interferons, as well as the interferon-responsive genes ISG56, OAS1, Mx1, CXCL10, and CXCL11 (Figure 5). The SARS-CoV infection of type II cultures also elicited large increases in the concentrations of chemokines that recruit inflammatory cells (CXCL8, CCL5, CXCL10, and CXCL11). Thus, human alveolar type II cells are capable of initiating robust innate immune responses that could contribute to the cytokines and chemokines measured in the lungs and sera of patients with SARS. In addition, chemoattractants released by epithelial cells would be expected to recruit inflammatory cells in vivo, and to initiate the innate immune response.
Some Alveolar Type I–Like Cells Are Susceptible to SARS-CoV Infection in A/L Cultures

The focus of these studies involved differentiated human type II cells, but we also performed a limited number of experiments to determine whether type I–like cells transdifferentiated from type II cells grown under A/L conditions were also susceptible to SARS-CoV. In our previous study (18), we detected no SARS-CoV infection of human alveolar type I–like cells under submerged conditions, and ACE2 protein expression was below the level of detection. In pilot experiments with unselected donors, we detected very little infection of type I–like cells under A/L conditions at 24 hours after inoculation. However, when we undertook screening to select a donor (i.e., patient 101) who expressed a high concentration of ACE2 in the freshly isolated cells, we observed that transdifferentiated type I–like cells could be infected with SARS-CoV (Figures E1 and E2). Hence, these alveolar type I–like cells at the A/L were also susceptible to SARS-CoV, although they were much less susceptible to infection than differentiated human type II cells. These type I–like cells derived from type II cells in vitro may differ from type I cells in vivo (36).

DISCUSSION

The goal of this study involved developing a primary culture system with human alveolar type II cells that was permissive to SARS-CoV infection, so that their innate immune response to infection could be defined. Human alveolar type II cells maintained at an A/L were well differentiated (Figures 1–3) and permissive for SARS-CoV infection, as shown by immunocytochemistry, by increases in the synthesis of viral RNA and protein, and by the production and release of infectious virions. Although the air–liquid interface culture conditions greatly increased the susceptibility of type II cells to SARS-CoV, the mechanism for this increased susceptibility to infection is not yet established. In the lung, very little fluid is present on the apical surface of the alveolar epithelium (37, 38). Hence, the A/L conditions approximate conditions in vivo. A/L culture conditions improved the differentiation state of type II alveolar...
epithelial cells in vitro, and greatly enhanced their susceptibility to SARS-CoV infection.

Innate immunity serves as the first response to protect host cells from viral infection, and the characteristics of the innate immune response to a pathogen can significantly shape subsequent adaptive immune responses and affect the outcome of infection (39). When the alveolar epithelium becomes infected, infected cells alert other cells and organs to the infection by secreting cytokines and chemokines. The infected epithelial cells are likely the first responders, and initiate the innate immune response to a pathogen can significantly shape subsequent adaptive immune responses and affect the outcome of infection (29, 41, 42). The cytokine responses of human type II alveolar epithelial cells at the A/L were similar to those detected in the sera and lungs of patients and in nonhuman primates infected with SARS-CoV (15, 24, 43). He and colleagues suggested that resident ACE2-expressing lung epithelial cells, and not the subsequently recruited inflammatory cells, constitute the primary source of elevated cytokines in patients infected with SARS-CoV, and that these epithelial-derived cytokines play an important role in the acute lung injury and pathogenesis of SARS (27). Our data show that type II alveolar cells likely contribute to the high concentrations of cytokines and chemokines detected in the gas-exchange portion of the lungs in patients with SARS. In the sera of patients with SARS, the cytokine that increases most rapidly is CXCL10 (IP-10) (22, 23). Similarly, in our type II cells at the A/L, the level of CXCL10 mRNA expression increased more than 1,000 fold by 24 hours after virus inoculation (Figure 5). The other cytokine that appears to predict the clinical course and outcome of SARS is IL-6 (43). The concentration of mRNA encoding IL-6 was elevated approximately 10-fold in our SARS-CoV-infected alveolar type II cultures at the A/L (Figure 5). In these particular experiments, we did not evaluate ultraviolet light-inactivated virus, but we knew from previous studies that type II cells do not present a cytokine or interferon response to ultraviolet light-inactivated influenza (40). Additional studies will be required to define the cell–cell interactions whereby infected epithelial cells signal to nearby uninfected parenchymal cells or inflammatory cells. We found that rat alveolar type I–like cells infected with rat coronavirus stimulated noninfected cells in the culture to secrete cytokines and chemokines through an IL-1–dependent mechanism (30). Paracrine signaling by IL-1, which is released from infected type II cells during the activation of the inflammasome, may also play important roles, both in protecting type II cells from infection and in amplifying the innate immune response.

We explored the hypothesis that the increased rate of SARS-CoV infection in type II cells under A/L conditions was attributable to an increased concentration of ACE2, the SARS-CoV receptor. We observed that type II cells from donors who expressed higher concentrations of ACE2 and from culture conditions that increased ACE2 expression generally correlated with a higher susceptibility to SARS-CoV infection. However, we did not measure ACE2 concentrations in cells from enough different donors who were also inoculated with SARS-CoV, and we did not measure the extent of virus infection to prove this quantitatively. Although immunoblotting showed a modest but variable increase in ACE2 expression by type II cells when cultured at the A/L compared with submerged conditions, we did not detect a consistent increase in the surface expression of ACE2 by either flow cytometry or surface biotinylation (data not shown). Similarly, when we transdifferentiated type II cells at the A/L into type I–like cells that expressed very low levels of ACE2, we observed very little SARS-CoV infection. However, in type I–like cells transdifferentiated from a type II cell donor selected to express high concentrations of ACE2, we did observe the infection of type I–like cells (Figures E1 and E2). Because we did not demonstrate a clear increase in the surface expression of ACE2 under A/L conditions, we cannot conclude that the enhanced role of infection of type II cells at the A/L was attributable solely to the concentration of ACE2.

We did not focus on the potential of adding IL-4 and IL-13 to these cultures. De Lang and colleagues reported that IL-4 and IL-13 decreased ACE2 expression in Vero E6 cells (32). However, as shown in Figure 1, we found that IL-4/IL-13 actually increased ACE2 expression in our primary human alveolar type II cells. Hence, we added IL-4/IL-13 to increase ACE2 expression and...
and this concentrated virus might accelerate secondary rounds of infection. In pilot studies, we observed that an increased susceptibility of type II cells to SARS-CoV infection required A/L culture conditions both before and after virus inoculation.

Other investigators identified Oct-4–positive lung cells that could be infected with SARS-CoV (43, 48). In neonatal mice, these cells expressed club cell secretory protein but not alveolar epithelial cell markers (48). In human lung tissue, these cells did not express cytokeratin or surfactant proteins (43). Our cells were different and did not express Oct-4 or the club cell secretory protein, but they expressed surfactant proteins and cytokeratin, and contained characteristic lamellar bodies.

In conclusion, culturing at the A/L renders primary human alveolar type II cells susceptible to productive SARS-CoV infection, and the cells mount a robust innate immune response, similar to that observed in the lungs and sera of patients with SARS. Type II alveolar epithelial cells cultured at an A/L offer an excellent in vitro system in which to isolate and propagate emerging respiratory viruses that present with fastidious requirements for growth in highly differentiated human lung cells. These cultures also provide excellent in vitro models in which to evaluate the effects of candidate therapeutics that target the innate immune responses to respiratory virus infection.

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**References**


