Infection of human alveolar macrophages by human coronavirus strain 229E

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Human coronavirus strain 229E (HCoV-229E) commonly causes upper respiratory tract infections. However, lower respiratory tract infections can occur in some individuals, indicating that cells in the distal lung are susceptible to HCoV-229E. This study determined the virus susceptibility of primary cultures of human alveolar epithelial cells and alveolar macrophages (AMs). Fluorescent antibody staining indicated that HCoV-229E could readily infect AMs, but no evidence was found for infection in differentiated alveolar epithelial type II cells and only a very low level of infection in type II cells transitioning to the type I-like cell phenotype. However, a human bronchial epithelial cell line (16HBE) was readily infected. The innate immune response of AMs to HCoV-229E infection was evaluated for cytokine production and interferon (IFN) gene expression. AMs secreted significant amounts of tumour necrosis factor alpha (TNF-α), regulated on activation normal T-cell expressed and secreted (RANTES/CCL5) and macrophage inflammatory protein 1β (MIP-1β/CCL4) in response to HCoV-229E infection, but these cells exhibited no detectable increase in IFN-β or interleukin-29 in mRNA levels. AMs from smokers had reduced secretion of TNF-α compared with non-smokers in response to HCoV-229E infection. Surfactant protein A (SP-A) and SP-D are part of the innate immune system in the distal lung. Both surfactant proteins bound to HCoV-229E, and pre-treatment of HCoV-229E with SP-A or SP-D inhibited infection of 16HBE cells. In contrast, there was a modest reduction in infection in AMs by SP-A, but not by SP-D. In summary, AMs are an important target for HCoV-229E, and they can mount a pro-inflammatory innate immune response to infection.

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INTRODUCTION

Coronaviruses (CoVs) are large, enveloped, positive-sense RNA viruses that infect a broad range of vertebrates and cause disease of medical and veterinary significance. Infections are generally localized to the respiratory, enteric or nervous systems, but systemic disease can also be caused by some coronaviruses (Perlman, 1998). Currently five human CoVs (HCoVs) are recognized. Two HCoVs, strains 229E (HCoV-229E) and OC43, generally cause winter outbreaks of mild, self-limited upper respiratory tract infections. Identification of a novel CoV as the aetiological agent of the severe acute respiratory syndrome (SARS) epidemic of 2002–2003 led to an extensive survey to determine the role of CoVs in human respiratory diseases. The result was the discovery of two additional respiratory HCoVs, strains NL63 and HKU1 (Fouchier *et al.*, 2004; van der Hoek *et al.*, 2004; Woo *et al.*, 2005). These newly discovered HCoVs mostly cause upper respiratory tract infections (Pyrc *et al.*, 2007). However, all four of the non-SARS HCoVs can occasionally cause pneumonia and lower respiratory tract infections (Dominguez *et al.*, 2009).

HCoV-229E is a common non-SARS HCoV, with about 90 % of adults having detectable antibodies (Severance *et al.*, 2008). As stated above, most infections produce symptoms of nasal discharge, cough and sore throat (Dijkman & van der Hoek, 2009). However, HCoV-229E infection has also occasionally been associated with lower respiratory tract infections in infants, the elderly or immunocompromised individuals (Dijkman & van der Hoek, 2009; El-Sahly *et al.*, 2000; Graat *et al.*, 2003; McIntosh *et al.*, 1974; Pene *et al.*, 2003). There is also continued interest in the role of CoV infections in causing exacerbations of patients with pre-existing lung disease, especially chronic obstructive pulmonary disease (COPD) (Beckham *et al.*, 2005).

This study was undertaken to determine whether cell types found in the lower respiratory tract, namely, alveolar epithelial type II cells, type I-like cells and alveolar macrophages (AMs), were susceptible to HCoV-229E

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Two supplementary figures are available with the online version of this paper.

infection. Previously, HCoV-229E was shown to infect bronchial epithelial cells and blood monocyte-derived macrophages (Cheung *et al.*, 2005; Desforges *et al.*, 2007; Wang *et al.*, 2000), but the responses of human alveolar epithelial cells and AMs have not been defined. We also determined whether the respiratory collectins surfactant protein A (SP-A) and SP-D could inhibit HCoV-229E infection.

RESULTS

Infection of primary cultures of differentiated human lung cells

Primary cultures of alveolar type II cells, alveolar type I-like cells and AMs were evaluated. These cells are located in the gas-exchange units of the lower respiratory tract. Differentiated type II cells were cultured as reported elsewhere (Wang *et al.*, 2011). Type I-like cells are type II cells that spread out in culture and develop into a phenotype similar to type I cells in that they cease to express the surfactant proteins and express markers of type I cells (Wang *et al.*, 2007). In the adult lung, type I cells are derived from type II cells (Fehrenbach, 2001), but isolation and culture of human type I cells have not been reported. Type II cells were refractory to HCoV-229E infection in all experiments, whereas a few type I-like cells were infected, but the overall level of infection in these cultures was very low, estimated to be $<1\,\%$. Nevertheless, some of the infected type I-like cells formed syncytia that stained for viral antigens, which are indicative of CoV infection, but no other cytopathic effects were observed (Fig. 1a). The few fibroblasts that appeared in the epithelial cultures were readily infected (data not shown). In contrast, $>50\,\%$ of the human AMs were susceptible to HCoV-229E (Fig. 1a). We also evaluated the susceptibility of several human respiratory epithelial cell lines, including the lung cancer cell line A549, the bronchial epithelial cell line 16HBE14o$^-$ (16HBE), and Calu-3, H292 and H441 cells, but only 16HBE cells (Fig. 1b) and A549 cells (data not shown) were susceptible to infection. We used plaque assays to measure the kinetics of release of infectious virus particles from AMs and 16HBE cells. Both AMs and 16HBE cells were productively infected by HCoV-229E, with a 15-fold increase in virus titre by 24 h post-inoculation. Samples taken at 48 h showed a drop in virus titre in AM cultures, but a further increase in 16HBE cultures (Fig. 2). Infection of A549 cells was not studied in further detail.

The susceptibility of the primary alveolar epithelial cells and macrophages could be related to the level of expression of the HCoV-229E receptor, aminopeptidase N (CD13/APN) (Yeager *et al.*, 1992). The cellular expression level of CD13/APN was assessed by flow cytometry (Fig. 3). AMs, 16HBE cells, A549 cells and the fetal lung fibroblast line MRC-5 all expressed significant levels of CD13/APN. The MRC-5 cell line used to propagate HCoV-229E is known to express CD13/APN and served as a positive control in Fig. 3. Alveolar type I-like cells and Calu-3 cells expressed a low but detectable level of CD13/APN, whereas the level of CD13/APN was below the level of detection for alveolar type II cells and H292 and H441 cells. Hence, the expression of CD13 was related directly to their susceptibility to infection by HCoV-229E.

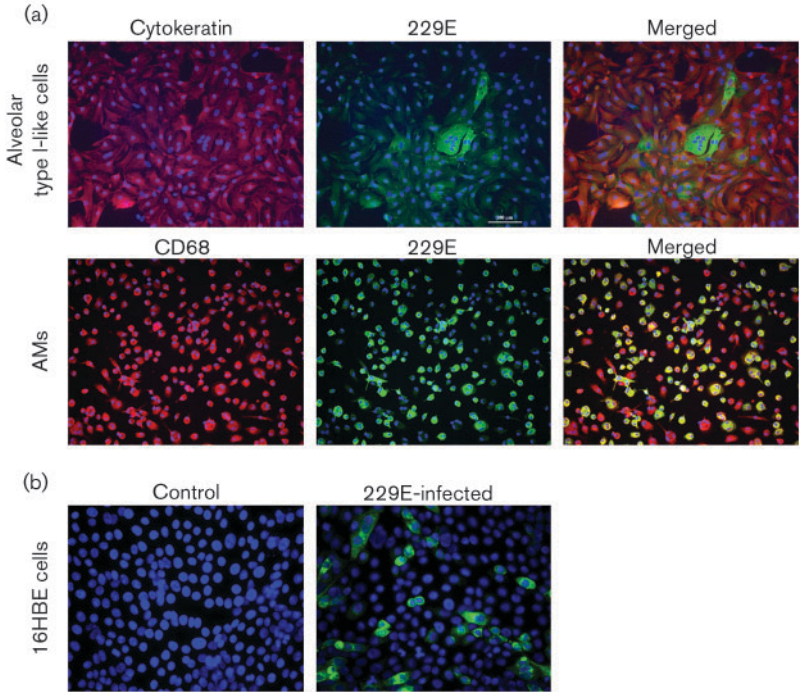

Fig. 1. Detection of cells infected with HCoV-229E. AMs, alveolar type I-like cells and 16HBE cells were cultured as described in Methods and infected with HCoV-229E. (a) Top row: alveolar epithelial type I-like cells were inoculated with HCoV-229E at an m.o.i. of 1, fixed at 24 h post-inoculation and immunostained for the epithelial cell marker cytokeratin (clone MNF116; red) and HCoV-229E proteins (green), and with DAPI to stain the nuclei (blue). Bottom row: AMs were inoculated with HCoV-229E at an m.o.i. of 1, fixed at 24 h post-inoculation and immunostained for CD68 (red) and HCoV-229E (green), and with DAPI (blue). (b) 16HBE cells were inoculated with HCoV-229E at an m.o.i. of 0.1 or control medium, fixed at 24 h post-inoculation and immunostained for HCoV-229E proteins (green) or with DAPI for the nuclei (blue).

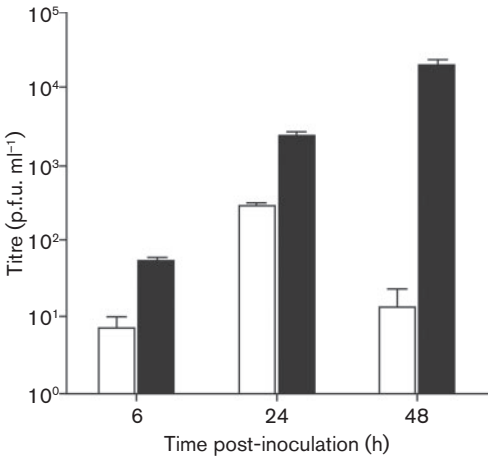

Fig. 2. Virus production by HCoV-229E-infected AMs and 16HBE cells. Supernatant from infected cells was collected at the indicated times post-inoculation (m.o.i. of 1) and virus production was measured by plaque assay. Empty bars, AMs; filled bars, 16HBE cells. The results are representative of three independent experiments (means ± SEM).

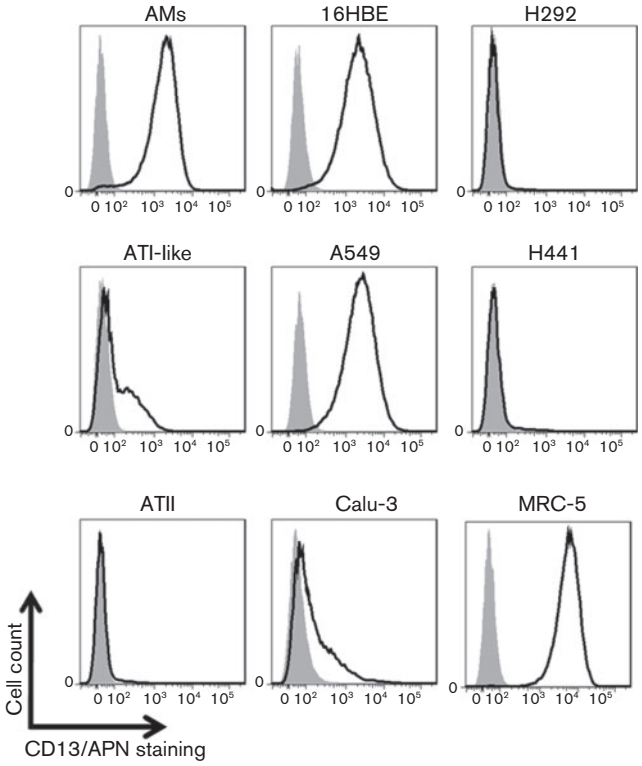

Fig. 3. Expression of APN/CD13 in human lung cells. AMs, type I-like alveolar epithelial cells (ATI-like), type II alveolar epithelial cells (ATII) and 16HBE, H292, A549, H441, Calu-3 and MRC-5 cells were cultured as for infection and then detached with trypsin. Cells were stained with a murine anti-human CD13 or isotype control antibody and PE-labelled anti-mouse IgG secondary antibody. Samples were analysed on an LSRII flow cytometer. Shaded peaks, isotype control; open peaks, CD13/APN.

Cytokine and interferon (IFN) response by AMs following HCoV-229E infection

As AMs were much more susceptible to HCoV-229E infection than alveolar epithelial cells, AMs were evaluated for their production of cytokines in response to HCoV-229E infection. To define the broad innate immune response to HCoV-229E infection, we initially used a multiplex assay. AMs from three different non-smoking donors were cultured and inoculated with HCoV-229E. The supernatant was collected at 24 h post-inoculation and the samples were analysed for 25 cytokines using an antibody bead-based Luminex assay. There was considerable variation in the levels of cytokines secreted by AMs from the three donors, but 11 of the 25 cytokines were detectable by this assay and increased in all three of the infected samples compared with the controls (Table 1). Of the cytokines tested, tumour necrosis factor alpha (TNF-α) showed the greatest increase following infection, but substantial increases in IFN-induced protein 10 (IP-10/CXCL10), macrophage inflammatory protein 1α (MIP-1α/CCL3), MIP-1β (CCL4), interleukin-7 (IL-7) and regulated on activation normal T-cell expressed and secreted (RANTES/CCL5) were also observed. In order to confirm the cytokine response in the multiplex study, we measured these proteins in culture supernatants in three additional experiments by ELISA. HCoV-229E infection of AMs increased the secretion of RANTES (CCL5), TNF-α and MIP-1β (CCL4) (Fig. 4a–c).

Because of the interest in CoV infections in smokers and in patients with COPD, we compared the secretion of cytokines in response to HCoV-229E infection in AMs isolated from smokers, ex-smokers and non-smokers (Beckham *et al.*, 2005; El-Sahly *et al.*, 2000). Unfortunately, the magnitude of the increase of TNF-α secretion after HCoV-229E infection was quite variable and there was not a consistent difference between macrophages from smokers, non-smokers or ex-smokers. However, there was an increase in TNF-α secretion following HCoV-229E infection in all groups except the ex-smokers, where the number of subjects was only four (see Supplementary Fig. S1, available in JGV Online). There was no increase in TNF-α secretion when AMs were inoculated with UV-inactivated HCoV-229E (data not shown).

One of the most important responses of many cell types to virus infection is secretion of type I IFNs, which induce an antiviral state in surrounding cells. A Luminex assay detected a small increase in IFN-α from infected AMs (Table 1), but this assay did not measure the levels of other type I or type III IFNs. Therefore, we used quantitative PCR (qPCR) assays to measure mRNA levels of IFN-β (type I IFN) and IL-29/IFN-λ1 (type III IFN) and found no significant increase in either IFN after HCoV-229E infection (Fig. 4d). There was also no increase in IL-29 protein secretion during infection (data not shown).

Table 1. HCoV-229E infection increases secretion of cytokines by AMs

Culture supernatants from HCoV-229E-infected human AMs collected at 24 h post-inoculation were assayed by Luminex multiplex technology. The data represent means ± SEM of three independent donors.

Cytokine	Control (pg ml^{-1})	HCoV-229E-inoculated (pg ml^{-1})
IL-1Ra	323 ± 119	555 ± 183
IL-2R	1530 ± 640	2650 ± 690
IL-6	289 ± 214	511 ± 307
IL-7	72 ± 51	168 ± 58
IL-12p40/p70	95 ± 41	150 ± 43
TNF-α	155 ± 71	3190 ± 1410
IFN-α	33 ± 12	49 ± 6
MIP-1α/CCL3	6290 ± 2830	22000 ± 10400
MIP-1β/CCL4	3950 ± 1580	6930 ± 2750
IP-10/CXCL10	162 ± 143	874 ± 455
RANTES/CCL5	269 ± 203	544 ± 195

Influenza infection markedly increases the mRNA levels of IFN-β and IL-29 in human AMs (Wang *et al.*, 2009), so these cells are able to produce a type I and type III IFN response.

Effects of SP-A and SP-D on HCoV-229E infection

Surfactant proteins play a critical role in protecting the alveolus from inhaled pathogens (Haagsman *et al.*, 2008; Hartshorn, 2010). We determined the binding of SP-A and SP-D to HCoV-229E virions using a solid-phase assay (Fig. 5a, b). Both surfactant proteins bound similarly to HCoV-229E virions in a dose-dependent manner. Virus binding was nearly saturated at 10 μg, indicating that 1 μg surfactant protein can bind about 10 μg virus. These results demonstrated that SP-A and SP-D have the capacity to bind HCoV-229E and suggested that surfactant proteins might modulate infection within the lower respiratory tract.

To assess the ability of SP-A and SP-D to block infection, we chose the cell line 16HBE, which does not contain endogenous SP-A or SP-D and is readily infected by HCoV-229E. Both SP-A and SP-D inhibited virus infection in a concentration-dependent manner (Fig. 5c). However, SP-D caused a greater reduction in HCoV-229E infection at both 3 and 10 μg ml^{-1} than the same concentrations of SP-A. Treatment of the virus inoculum with 10 μg SP-D ml^{-1} resulted in a >60 % drop in the number of infected cells compared with wells without SP-D. However, the level of inhibition of virus infection was modest compared with the inhibition of influenza infection (H3N2) in Madin–Derby canine kidney (MDCK) cells assayed under the same

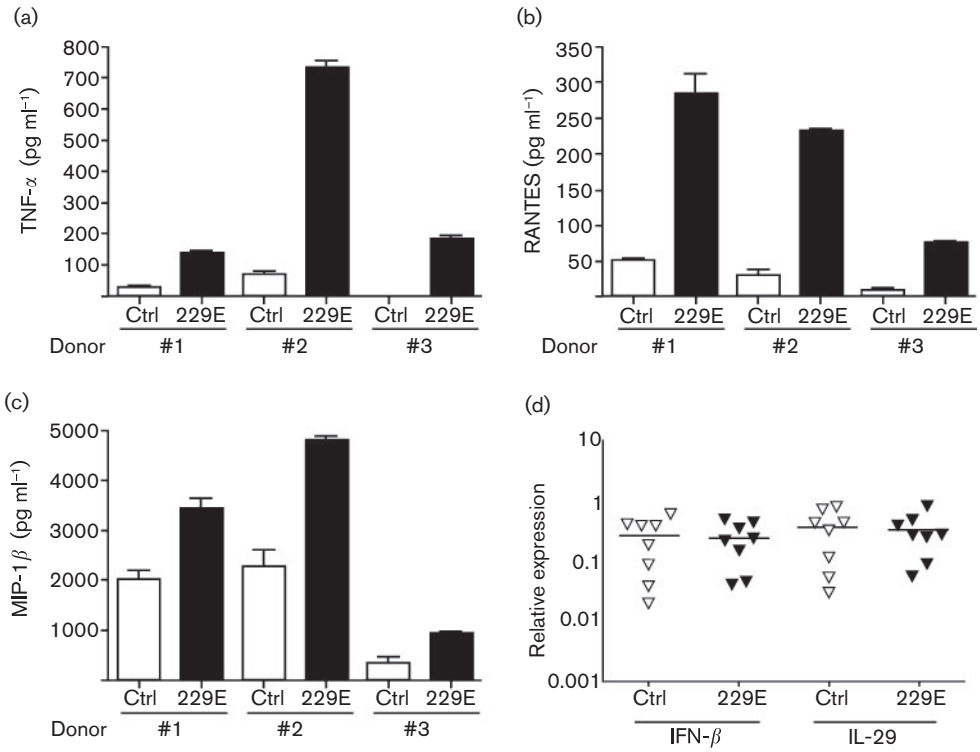

Fig. 4. Cytokine secretion and IFN response by AMs in response to HCoV-229E infection. AMs were cultured and infected with HCoV-229E at an m.o.i. of 1 and the culture supernatants were collected 24 h later. (a–c) Levels of the cytokines TNF-α (a), RANTES (CCL5) (b) and MIP-1β (CCL4) (c) were measured by ELISA and compared with levels in the control medium (Ctrl). (d) mRNA levels of IFN-β and IL-29 in AMs from eight donors 24 h after inoculation with HCoV-229E or control medium.

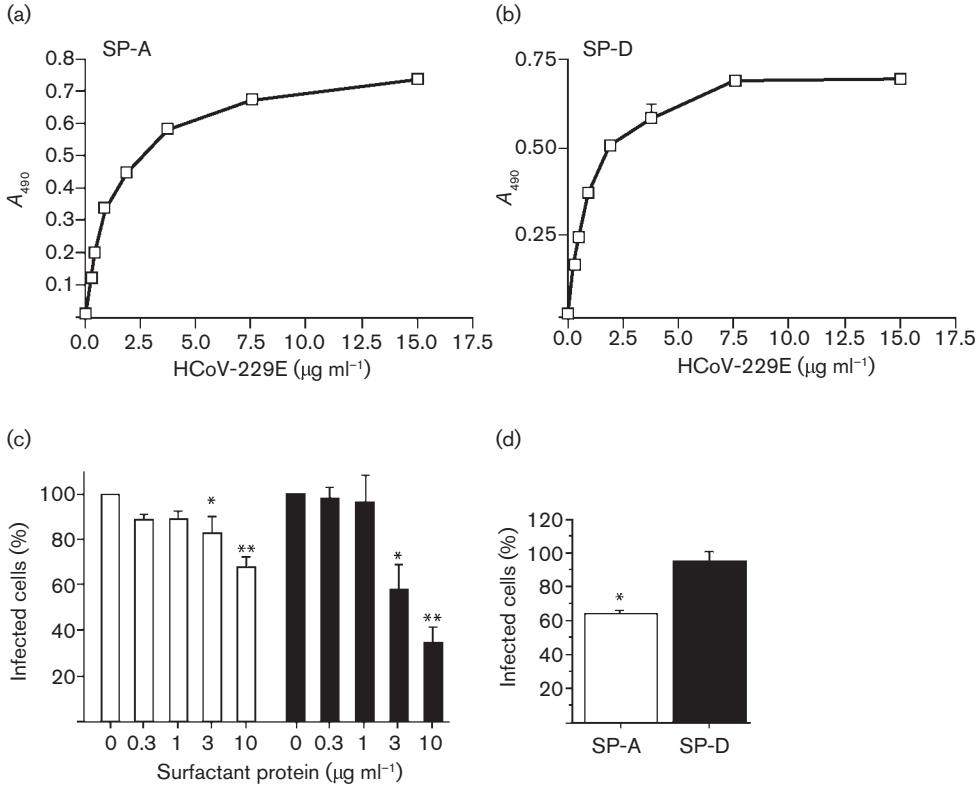

Fig. 5. Binding and inhibition of infection of HCoV-229E by surfactant proteins SP-A and SP-D. The surfactant proteins were bound to an ELISA plate, followed sequentially by binding of the HCoV-229E virus, goat anti-HCoV-229E antibody and an HRP-conjugated anti-goat IgG antibody. (a, b) Binding curves for HCoV-229E bound to 1 μg SP-A (a) or SP-D (b) per well. (c) Inhibition of HCoV-229E infection of 16HBE cells after pre-incubation of the virus with SP-A or SP-D. The number of cells infected with virus alone was set at 100 %, and this control was compared with cells that were infected with virus plus increasing concentrations of surfactant proteins. The results are shown as means ± SEM for three independent experiments. Open bars, SP-A; filled bars, SP-D. *$P<0.05$; **$P<0.01$. (d) Effect of SP-A and SP-D on HCoV-229E infectivity in human AMs. The number of cells infected with virus alone was set at 100 %, and the effect of 10 μg SP-A ml^{-1} (open bar) or 10 μg SP-D ml^{-1} (filled bar) on HCoV-229E infection of human AMs (m.o.i. of 1) at 7 h post-inoculation is shown ($n=3$). *$P<0.05$.

conditions with the same proteins (see Supplementary Fig. S2, available in JGV Online). Similar experiments were conducted with human AMs and pre-treatment of HCoV-229E with SP-A or SP-D prior to infection. Only pre-treatment with SP-A reduced infection of AMs significantly (Fig. 5d). Hence, the effects of SP-A and SP-D depend on the virus and the cell type being studied.

DISCUSSION

Of the three human lung cell types that were evaluated, AMs were the most readily infected by HCoV-229E. AMs were not only susceptible to infection, but also released a significant amount of infectious virus. AMs have a critical role in preventing dust, toxins and pathogens from damaging the lung surface. The response of AMs to virus infection includes a central role in initiating the innate immune response, including secretion of cytokines. AMs produced a variety of cytokines in response to HCoV-229E infection, and these cytokines are known to activate or be chemotactic for monocytes, neutrophils and lymphocytes. The cytokines produced by AMs in our study were similar to the cytokines detected following HCoV-229E infection of human monocytes and the monocytic cell line THP-1 (Desforges *et al.*, 2007).

Although HCoV-229E produced a cytokine response in AMs, it did not result in a significant increase in IFN expression. As AMs are considered to be an important source of type I IFNs following infection by RNA viruses (Kumagai *et al.*, 2007), our results suggest that HCoV-229E may have a mechanism to block the IFN pathway in AMs. Several SARS-CoV gene products may downregulate or inhibit the IFN-signalling pathway during SARS-CoV infection (de Lang *et al.*, 2009; Kopecky-Bromberg *et al.*, 2006, 2007; Minakshi *et al.*, 2009; Narayanan *et al.*, 2008). Similar gene products of HCoV-229E may be able to inhibit IFN during infection of AMs and should be the subject of additional studies. However, it should be noted

that blood monocyte-derived macrophages were found to develop a type I and type III mRNA IFN response to HCoV-229E (Cheung *et al.*, 2005). Hence, the differences in response might be due to functional differences between AMs and blood monocyte-derived macrophages. Additional studies are indicated to define further the IFN response to HCoV-229E.

The cellular tropism for AMs that we observed for HCoV-229E differed from the cellular tropism that we found for sialodacryoadenitis virus (SDAV), a rat CoV. SDAV readily infected bronchial and alveolar epithelial cells, especially type I cells, but not AMs (Funk *et al.*, 2009). For SARS-CoV, we also found infection of alveolar type II cells, but not AMs (Mossel *et al.*, 2008). The cellular tropism of HCoV-NL-63 and HCoV-HKU-1 needs to be defined more precisely, but both are reported to infect bronchial epithelial cells (Banach *et al.*, 2009; Pyrc *et al.*, 2010). Hence, some respiratory CoVs infect epithelial cells, whereas others infect AMs.

The ability of AMs to respond to virus infection is probably mediated in part by prior exposure to toxins and micro-organisms that are present in air inhaled into the lung. One of the most common environmental exposures of human lungs is cigarette smoke. However, because of variability in the TNF-α response among donors, we were not able to demonstrate a difference in the TNF-α response to HCoV-229E based on smoking history. In a recent study on the cytokine response of monocyte-derived macrophages to influenza, there was also a significant variation in the cytokine response among individual donors (Sakabe *et al.*, 2011). There is a well-known large variation in the response of human neutrophils to lipopolysaccharide (Wurfel *et al.*, 2005). This individual variability poses a problem for experimental design, but highlights the potential importance of studying primary human cells. The biological basis for this variability is probably of clinical significance. In some other models of innate immunity, the levels of cytokine expression by AMs isolated from smokers are reduced compared with those from non-smokers (Chen *et al.*, 2007). In a mouse-model system, when AMs from mice of a single genetic background were exposed to cigarette smoke and to poly(I:C), a mimic of viral dsRNA, there was a significant reduction in the levels of TNF-α, IL-6 and RANTES compared with AMs from control animals (Gaschler *et al.*, 2008). Cigarette-smoke exposure probably also has additional effects beyond cytokine expression and alters the host response in other ways that were not the subject of our investigation.

Alveolar type II primary epithelial cells that were cultured in a system highly permissive for influenza infection were found to be refractory to infection with HCoV-229E (Wang *et al.*, 2009, 2011). However, a few type I-like cells could be infected. Because of the very low level of infection of type I-like cells, we did not evaluate their cytokine or IFN response. The very low level of infection of type I-like cells and the absence of infection in type II cells was probably due to the very low level of expression of the receptor CD13/APN (Fig. 3). It was interesting to note the large variation in expression of CD13/APN in different epithelial lung cell lines. This indicates the importance of verifying the results in cell lines with primary cultures. 16HBE, H292 and Calu-3 cells are all used as models of bronchial epithelial cells, but have marked differences in expression of CD13/APN. Although A549 cells do not express surfactant proteins, they are sometimes used as a model of alveolar type II cells. In this study, we found another difference: A549 cells expressed CD13 and were susceptible to HCoV-229E, whereas human type II cells did not express CD13 and did not support HCoV-229E infection.

SP-A and SP-D have been shown to be important components of innate immunity in the lung and are potentially very important for influenza infections, including the 2009 pandemic H1N1 infections (Hartshorn *et al.*, 2000; Job *et al.*, 2010). SP-D binds to carbohydrates on the influenza haemagglutinin and neuraminidase by its carbohydrate-recognition domain (Job *et al.*, 2010; Reading *et al.*, 1997). SP-D binding to haemagglutinin inhibits both virus infection and haemagglutination activity. SP-A interacts with influenza primarily through sialic acid residues on *N*-linked carbohydrates of SP-A and not through its carbohydrate-recognition domain, but it can also inhibit infection. SP-D binds the SARS-CoV surface spike protein, although the effects of SP-D in SARS infection have not been determined (Leth-Larsen *et al.*, 2007). Our study indicated that both SP-A and SP-D bound HCoV-229E. SP-D was more effective at inhibiting infection of 16HBE cells and SP-A was more effective at inhibiting infection of AMs. As we tested both the SP-A and SP-D preparations for inhibition of infection of MDCK cells by influenza virus (Supplementary Fig. S2), we knew that the proteins were biologically active. In addition, the level of the surfactant proteins in alveolar lavage fluid may increase during the course of infection and impede propagation of infection into the distal lung. For example, in SDAV infection of rats, SP-D but not SP-A levels increase in lavage fluid during the course of the infection as it resolves (Funk *et al.*, 2009).

In conclusion, the cellular environment of the alveolus is not very conducive to HCoV-229E production. Neither differentiated alveolar type II nor type I-like epithelial cells were infected easily *in vitro*, and SP-A and SP-D might provide some level of protection. Although AMs could be infected, they did not appear to produce large amounts of virus. When AMs became infected, they secreted cytokines and chemokines that can attract other immune cells capable of aiding in the destruction of infected cells. Although no single factor was identified, lower respiratory tract infections of HCoV-229E probably require some compromise of the normal innate immune system. For example, many cases of lower respiratory tract infections involve patients that are diagnosed as immunocompromised or have been co-infected with another respiratory

virus (Dominguez *et al.*, 2009; Gaunt *et al.*, 2010; Kuypers *et al.*, 2007). Understanding how HCoV-229E is still able to initiate infections in the lower respiratory tract under these circumstances will require further investigation.

METHODS

Isolation and culture of primary cells from human lung. Human AMs and type II cells were isolated as described previously (Wang *et al.*, 2007, 2009). Briefly, de-identified donor lungs that were not suitable for transplant were obtained through the National Disease Research Interchange (Philadelphia, PA, USA) and the International Institute for the Advancement of Medicine (Edison, NJ, USA). The Committee for the Protection of Human Subjects at National Jewish Health approved this research. The middle lobe was perfused and lavaged with HEPES-buffered saline containing 2 mM EDTA and then with HEPES-buffered saline alone. The lavage fluid was retained for isolation of AMs. Briefly, the lavage fluid was centrifuged at 4 °C for 10 min and washed in Dulbecco's modified Eagle's medium (DMEM). The red blood cells were lysed with Pharm Lyse (BD Biosciences) and macrophages were either plated immediately or resuspended in freezing medium (10 % DMSO and 90 % FBS) and stored in aliquots in liquid nitrogen for use at a later time.

Further treatment of the lung for isolation of type II epithelial cells involved digestion with elastase and isolation of the cells by differential centrifugation and gradient purification (Wang *et al.*, 2011). Macrophages were removed from the cell suspension using negative selection with CD14-coated Dynal magnetic beads (Invitrogen) and IgG-coated Petri dishes. Assessment of cell purity was made by staining for cytokeratin (mAb clone CAM 5.2; DakoCytomation). Epithelial cells were either plated immediately or resuspended in freezing medium and stored in aliquots in liquid nitrogen.

Cell culture. AMs were resuspended in DMEM supplemented with 10 % FBS, 2 mM glutamine and antibiotics (2.5 g amphotericin B ml^{-1}, 100 g streptomycin ml^{-1}, 100 U penicillin G ml^{-1} and 10 g gentamicin ml^{-1}) and plated on tissue-culture plates or glass coverslips. After a 2 h or overnight incubation to allow adherence, the cells were washed with DMEM to remove the non-adherent cells. The purity of isolated AMs was typically 90 % before plating and close to 100 % after adherence in culture, as measured by CD68 staining. AMs were plated at a density of 2×10^5 or 5×10^5 cm^{-2} depending on the experiment. AMs were cultured for at least 2 days before infection. Most infections were carried out on AMs cultured for 2 days.

Alveolar epithelial type II cells were cultured in DMEM supplemented with 10 % FBS, 2 mM glutamine and antibiotics as above. Cells were plated on Millicell inserts (Millipore) that had previously been coated with a mixture of rat-tail collagen and Matrigel (BD Biosciences) in 10 % FBS. The cells were cultured in DMEM with 1 % charcoal-stripped FBS along with keratinocyte growth factor, isobutylmethyl-xanthine, 8-bromo-cAMP, dexamethasone and antibiotics to achieve their differentiated phenotype, as described previously (Wang *et al.*, 2007, 2011).

Transdifferentiation of type II cells into type I-like cells was accomplished by plating cells on rat-tail collagen-coated dishes or glass coverslips at a density of 0.5×10^5–1×10^5 cm^{-2} in DMEM with 10 % FBS and antibiotics (Wang *et al.*, 2007). After 24–48 h for adherence, the medium was changed to DMEM with 5 % FBS and cultured for an additional 6 days. These cells differentiate towards the type I cell phenotype but retain some type II cell features, such as remnant lamellar bodies.

16HBE cells were originally isolated from human bronchial epithelial cells and transformed with the origin-defective simian virus 40 genome (Cozens *et al.*, 1994). The 16HBE cells used in this study grow readily in monolayers and were obtained from Marc Hershenson (University of Michigan, MI, USA; Page *et al.*, 2003). This cell line was grown on tissue-culture plates or glass coverslips using DMEM with 10 % FBS and antibiotics.

Virus. HCoV-229E was originally obtained from the ATCC and was propagated in human diploid lung MRC-5 fibroblasts (ATCC) at 34 °C in high-glucose DMEM supplemented with 1 mM sodium pyruvate, 1 mM HEPES, 1 mM non-essential amino acids and antibiotics. The virions were purified by sucrose density-gradient ultracentrifugation and stored in TMS buffer [25 mM Tris/HCl (pH 6.5), 25 mM maleic acid, 100 mM NaCl] with 5 % glycerol (Wentworth & Holmes, 2001).

Virus infections. Cells were inoculated with sucrose gradient-purified HCoV-229E using an m.o.i. of 1 or as noted in the text. After a 1 h adsorption period, the cells were washed twice with DMEM before replacing with DMEM plus FBS and antibiotics, and incubated at 37 °C. Control inoculated cells were incubated for 1 h with TMS buffer diluted in DMEM at the same ratio as the virus inoculum and served as controls in the data in the figures. At 24 h after inoculation, cells grown on coverslips were fixed in methanol at −20 °C. At the indicated time intervals after inoculation, medium from inoculated cultures was collected for virus titration, ELISAs and cytokine analysis. Viral antigen and cell markers were detected in cells grown on coverslips by immunostaining. HCoV-229E was detected in infected cells using a goat polyclonal antibody and visualized using an anti-goat secondary antibody conjugated to Alexa Fluor 488 (Invitrogen) (Wentworth & Holmes, 2001). The primary antibodies for cell markers were mAbs for cytokeratin for type I epithelial cells (clone MNF116; DakoCytomation) and CD68 for AMs (clone KP1; DakoCytomation). The secondary antibody was conjugated to Alexa Fluor 594 (Invitrogen).

The effects of SP-A and SP-D on HCoV-229E infection were determined in a similar way to assays used for influenza infectivity by Hartshorn *et al.* (2000). Briefly, purified surfactant protein or a buffer control was combined with HCoV-229E at an m.o.i. of 0.1 and incubated together in PBS with calcium and magnesium (PBS^{++}) at 37 °C for 45 min. The mixture of virus and surfactant protein was then applied to a monolayer of 16HBE cells in the wells of a 96-well plate and incubated for 1 h. The concentrations of surfactant proteins tested are indicated in the text. After a further 6 h incubation, the cells were washed with PBS, fixed in methanol (10 min at −20 °C) and washed three times with PBS^{++} buffer. Cells were stained with goat anti-HCoV-229E antibody and then with a secondary antibody conjugated to Alexa Fluor 594 (Invitrogen) and DAPI. Infected cells were counted using an inverted fluorescence microscope and the mean was determined across three wells for each treatment.

Plaque assays. Stocks of purified virus or medium from HCoV-229E-infected cultures were diluted serially in DMEM and used to inoculate triplicate wells of near-confluent MRC-5 cells in a six-well plate. After a 1 h adsorption period at 37 °C, the inoculum was removed and the cells were overlaid with minimal essential medium containing 8 % FBS, antibiotics and 0.5 % SeaKem LE agarose (Cambrex). Plaques were stained after 48 h incubation at 37 °C, with an agarose-overlay medium containing 6 % neutral red (Sigma-Aldrich) (Wentworth & Holmes, 2001).

Cytokine analysis. A Luminex antibody bead kit (BioSource; Invitrogen) was used to measure human chemokines and cytokines produced by infected cells. This method allows simultaneous evaluation of 25 human cytokines, chemokines and growth factors:

IL-1α, IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IFN-α, IFN-γ, TNF-α, granulocyte–macrophage colony stimulating factor (GM-CSF), monocyte chemotactic protein 1 (MCP-1), MIP-1α, MIP-1β, IP-10, eotaxin, RANTES and monokine induced by IFN-γ (MIG). The Luminex assay was performed at the National Jewish Luminex Core Facility according to the manufacturer's instructions. To generate a standard curve, twofold serial dilutions of appropriate standards were prepared in DMEM. Standards and supernatant samples were pipetted at 50 µl per well and mixed with 50 µl of the bead mixture. After a 1 h incubation, the wells were washed three times with washing buffer using a vacuum manifold. A secondary PE-conjugated antibody was added for 45 min, the wells were washed twice and the samples were analysed using a Bio-Plex fluorescent reader (Bio-Rad). One hundred beads were counted for each analyte per well and values (pg ml^{-1}) were calculated using the Bio-Plex Manager software (Bio-Rad).

Cytokine ELISAs. Medium from virus-inoculated or control cells was harvested at 24 h after inoculation for the measurement of TNF-α, RANTES (CCL5) and MIP-1β (CCL4). ELISAs were purchased from ElisaTech and developed according to the kit instructions. Measurements were made from at least three independent human donor samples with reported values shown as means ± SEM of at least two replicate wells.

Flow cytometry. AMs, type-I like alveolar epithelial cells, type II cells and cell lines (16HBE, A549, H292, H441, Calu-3 and MRC-5) were cultured as for infection. Cells were removed from the culture surface with trypsin. After blocking with human Fc receptor blocking reagent (Miltenyi Biotec), the cells were stained with anti-human CD13/APN antibody (BD Biosciences) or isotype control (BD Pharmingen), followed by secondary PE-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch). Samples were analysed on a BD LSRII flow cytometer (National Jewish Health Flow Core). We did not systematically evaluate the effect of trypsin on CD13 expression in these studies, and the values indicate relative expression and not the precise level of expression.

Surfactant protein solid-phase assay. Purified surfactant proteins (SP-A or SP-D) were coated onto microtitre wells (1 µg per well in 0.1 M $NaHCO_3$, pH 9.6) and incubated at 4 °C overnight. SP-A was isolated from alveolar proteinosis lavage fluid and SP-D was a human recombinant protein. Non-specific binding was prevented by incubation with buffer A [20 mM Tris/HCl (pH 7.4), 150 NaCl, 5 mM $CaCl_2$] in 3 % powdered milk. Virus protein, in the amount indicated in the text, was incubated at 37 °C for 1 h in buffer A. Following the incubation period, the wells were washed with buffer A and 3 % skimmed milk. A goat anti-HCoV-229E antibody was used for detection of the virus, followed by incubation with a secondary HRP-conjugated rabbit anti-goat antibody. The primary and secondary antibodies were each incubated at 37 °C for 1 h in buffer A. The binding of HCoV-229E to surfactant protein was determined using *o*-phenylenediamine as a substrate for the peroxidase reaction and measured by absorbance at 492 nm (A_{492}). Control conditions included wells without added viral protein.

Real-time qPCR. Measurement of IFN mRNA levels was performed with qPCR. The primers and probes for IFN-β and IL-29 TaqMan assays (Applied Biosystems) have been described previously (Wang *et al.*, 2009). Expression of 36B4 (the acidic ribosomal phosphoprotein P0) was used to normalize the amount of mRNA for each sample (Wang *et al.*, 2007).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 4. A paired *t*-test was used to compare the difference between two groups. One-way analysis of variance was used to compare the difference among two or more groups. Appropriate post-hoc tests were selected for multiple comparisons. A value of $P \leqslant 0.05$ was considered significant for all methods. The effect of HCoV-229E on TNF-α secretion (legend to Supplementary Fig. S1) was carried out by a Wilcoxon non-parametric *t*-test.

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