A Human Coronavirus Responsible for Common Cold Massively Kills Dendritic Cells but not Monocytes

Authors: Mariana Mesel-Lemoine¹, Jean Millet², Pierre-Olivier Vidalain¹, Helen Law³, Astrid Vabret⁴, Valérie Lorin¹, Nicolas Escriou¹, Matthew L. Albert³, Béatrice Na², Frédéric Tangy¹*

¶ The two first authors equally contributed to this work

Affiliations: ¹Unité de Génomique Virale et Vaccination, Department of Virology, Institut Pasteur, CNRS URA-3015, Paris, France. ²Hong Kong University-Pasteur Research Centre and Department of Anatomy, The University of Hong Kong, Hong Kong, China. ³Unité d’Immunobiologie des Cellules Dendritiques and Centre d’Immunologie Humaine (CIH), Department of Immunology, Institut Pasteur, INSERM U818, Paris, France. ⁴Université de Caen-Basse-Normandie, EA 4655-U2RM, Laboratoire de Virologie, CHU de Caen, France.

Running Title: Dendritic cells infection by HCoV-229E

* Corresponding author:
Frédéric Tangy
Email: ftangy@pasteur.fr
Human coronaviruses are associated with upper respiratory tract infections that occasionally spread to the lungs and other organs. Although airway epithelial cells represent an important target for infection, the respiratory epithelium is also composed of an elaborate network of dendritic cells (DCs) that are essential sentinels of the immune system, sensing pathogens and presenting foreign antigens to T lymphocytes. In this report, we show that in vitro infection by human coronavirus 229E (HCoV-229E) induces massive cytopathic effects in DCs, including large syncytia formation and cell death in only few hours. In contrast, monocytes are much more resistant to infection and cytopathic effects despite similar expression levels of CD13, the membrane receptor for HCoV-229E. While the differentiation of monocytes into DCs in the presence of GM-CSF and IL-4 requires 5 days, only 24 hours are sufficient for these cytokines to sensitize monocytes to cell death and cytopathic effects when infected by HCoV-229E. Cell death induced by HCoV-229E is independent of TRAIL, FasL, TNF-α and caspase activity, indicating that viral replication is directly responsible for the observed cytopathic effects. The consequence of DC death at the early stage of HCoV-229E infection may have a potential impact on the early control of viral dissemination and on the establishment of long-lasting immune memory, explaining that people can be re-infected multiple times by HCoV-229E.
INTRODUCTION

Coronaviruses (CoV) are enveloped positive-strand RNA viruses from Coronaviridae family. Five members have been reported to infect humans including 229E, OC43, newly discovered NL63 and HKU1, and the emerging SARS-CoV. Human coronavirus (HCoV) 229E and NL63 are closely related and belong to the alpha-coronavirus genus, whereas OC43, HKU1 and SARS-CoV belong to beta-coronavirus genus. HCoVs infect airways and are responsible for different respiratory diseases (19, 44). Although the SARS-CoV was associated with a severe acute respiratory disease during the 2002-2003 pandemic, most HCoVs cause only a mild respiratory infection (49). Epidemiological studies suggest that HCoVs account for 15-30% of common colds, with only occasional spreading to the lower respiratory tract. Airway epithelial cells represent the primary target of infection (19, 44). Nevertheless, in vitro experiments demonstrate that other cell types can be infected. For example, HCoV-229E was reported to infect and replicate in neural cells, hepatocytes, monocytes and macrophages (3, 11, 14). The neurotropism of HCoV-229E and OC43 has also been documented in vivo, and a possible association with multiple sclerosis has been suggested (4). Because peripheral blood cells from the myeloid lineage can be infected by HCoVs, these cells have been proposed to serve as a vector for viral spread to neural tissues (15).

In addition to epithelial cells, the human airway epithelium possesses an elaborate network of dendritic cells (DCs). DCs serve as sentinel in the respiratory tract where they detect inhaled pathogens through the recognition of pathogen-associated molecular patterns (PAMPs) e.g., bacterial LPS or viral nucleic acids. In order to mediate this function, DCs express Pattern Recognition Receptors (PRRs), e.g., Toll-like receptors, NOD-like receptors, RIG-like receptors and C-type lectin receptors. Upon engagement, these receptors induce the migration of DCs to the draining lymph nodes and their maturation into antigen presenting...
cells (APCs). This maturation process determines the ability of DCs to stimulate an adaptive immune response against antigens that have been captured in the lungs. Therefore, these conventional DCs are at the nexus of innate and adaptive immunity in the lungs.

Several viruses that spread through the airways infect DCs. For example, DCs infection by influenza virus induces their maturation and migration to the lymph nodes to efficiently prime an adaptive immune response (1, 29, 38). In contrast, measles virus uses DCs as a trojan horse to spread from the lungs to other tissues (12, 13), while still inducing long-term immune responses. Although MV-infected DCs migrate properly to lymph nodes, their phenotypic maturation is perturbed and infected DCs fail to stimulate interacting lymphocytes (42), indicating that cross-priming by non-infected DCs might be the route for eliciting MV adaptive immune response (43). In addition, activation signals provided by lymphocytes dramatically enhance MV replication within infected DCs, and this likely contributes to the establishment of viremia (42). Thus, DC infection has different effects in disease pathogenesis, depending on host / pathogens interactions. Indeed, DC infection supports priming of T cell, yet infection of a migrating cell also contributes to spreading the virus to distal tissues.

CoVs associated with common cold, such as HCoV-229E, are likely to interact with lung DCs in vivo. However, the susceptibility of DCs to infection by these viruses is yet unknown. In this report, we describe the in vitro infection of monocyte-derived DCs (Mo-DC) with human HCoV-229E. Infection resulted in dramatic cytopathic effects with formation of large syncytia, and cell death occurred within 24 hours. In contrast, infected monocytes from the same donors were preserved from cytopathic effects, and acquired sensitivity to cell death only after a short stimulation with GM-CSF and IL-4. Different hypothesis were tested to explain this observation.
MATERIALS AND METHODS

Production of HCoV-229E virus stocks and in vitro infection. Virus stocks were established on MRC5 cells using HCoV-229E virus strain from ATCC (VR-740). After washing, 80-90% confluent cell cultures were infected in a minimal volume of serum-free medium for 2 hours. DMEM containing 10% Fetal Calf Serum (FCS) and antibiotics was added, and infected cultures were incubated for 4-5 days at 37°C and 5% CO₂. Cytopathic effect (CPE) was monitored by optical microscopy. Cell supernatants were harvested, centrifuged for 5 minutes at 4,000 rpm, and aliquoted into cryotubes for storage at -80°C. Virus titers were determined as 50% tissue culture infective doses (TCID₅₀). MRC5 cells were seeded in 96-well plates and inoculated with serial dilutions of virus stock ranging from 10⁻¹ to 10⁻⁸. Plates were incubated for 12 hours at 37°C before adding DMEM supplemented with 10% FCS. Plates were incubated for another 6 days, and fixed with 4% paraformaldehyde before staining with crystal violet. Infected wells were numbered for each virus dilution, enabling to calculate a TCID₅₀ (26, 45). To perform in vitro infections, cell suspensions of monocytes, Mo-DCs or CD34-DCs were incubated for 2 hours at 37°C with an appropriate volume of virus stock to match the indicated multiplicity of infection (MOI). Mock infections were performed using supernatant from non-infected MRC5 cell cultures. Finally, cells were dispensed at 10⁶ cells/ml, and harvested at indicated time points.

Detection of HCoV-229E replication. Viral replication was assessed in culture supernatants of infected cells by qRT-PCR. Viral RNA was extracted from medium using an automated QiaSymphony system (Qiagen). HCoV-229E specific primers and probe previously designed and targeting the HCoV-229E N gene were used (17). The viral
quantification was calculated by using an external standard curve constituted by serial 10-fold dilutions of viral RNA transcripts (10⁸ - 10² copies). These transcripts were in-vitro-transcribed with T7 polymerase from pCR-XL-TOPO® plasmids containing the M and N genes of HCoV-229E previously cloned from HCoV-229E (strain ATCC-VR-740) MRC5 cell culture supernatants. The RNA transcripts were quantified in a UV spectrophotometer.

Production of SARS-CoV stocks and in vitro infection. Virus stocks were established on VeroE6 cells using the FFM-1 strain of SARS-CoV (kindly provided by Pr. Dr. H.W. Doerr, Institute of Medical Virology, Frankfurt University Medical School, Germany), as previously described (7). All viral stocks were stored at −80 °C in single-use aliquots and titrated in a standard limiting dilution assay on FRhK-4 cell monolayers. Infectious titers were determined as TCID50 with optical microscopic reading of CPE as described above. All work involving infectious SARS-CoV was performed in an enhanced biosafety level 3 containment laboratory with rigorous safety procedures according to WHO guidelines. In vitro infections of monocytes, Mo-DCs or CD34-DCs with SARS-CoV were performed as described above for HCoV-229E infections. Mock infections were performed using supernatant from non-infected VeroE6 cell cultures.

Preparation of monocytes and Mo-DCs. Blood cells were obtained from leukapheresis samples freshly collected from normal donors after informed consent and according to institutional guidelines. Mononuclear cells were separated on Ficoll-Hypaque gradient (density 1.077g/mL). CD14+ cells corresponding to monocytes were purified by positive selection using immunomagnetic beads coated with anti-CD14 mAbs (Miltenyi Biotech, Kohln, Germany). Purity was assessed by anti-CD14 immunostaining and flow cytometry analysis (Pharmingen, San Diego, CA, USA). DC-SIGN+ and BDCA-1+ populations were also purified by positive selection using immunomagnetic beads. CD14+ cells were plated at 2×10⁶ cells/ml in RPMI 1640 medium containing 10% FCS in the
presence of IL-4 (20 ng/ml; Gentaur Molecule Products, Belgium), and GM-CSF (100 ng/ml; Gentaur Molecule Products, Belgium). Monocyte differentiation into Mo-DCs was obtained in 5 days as previously described (10). In some experiments, DC maturation was induced using trimeric CD40-L (250 ng/ml; R&D Systems) or TNF-α (5 ng/ml; R&D Systems) plus prostaglandin E2 (PGE2, 1 µg/ml), or polyinosinic-polycytidylic acid sodium salt (Poly-IC, 1 µg/ml; Sigma).

Preparation of CD34-DCs. Peripheral blood CD34+-cells were purified from leukapheresis products collected from patients after stem-cell mobilization with G-CSF and cyclophosphamide. Permission to obtain leukapheresis samples was obtained after informed consent of the patients and approval by the institutional review board. CD34+-cells were purified using immunomagnetic beads as described elsewhere (32). Differentiation of CD34+-cells into CD34-DCs was adapted from Movassagh & al. (32). Briefly, CD34+-cells were cultured for 10 days. Medium was changed at day 3, 5 and 8, and cells were seeded at 2×10^6 cells/ml in RPMI containing 10% FCS, 1% glutamine, 2% antibiotics, 100 ng/ml GM-CSF, 50 ng/ml SCF, 50 ng/ml IL-4, 5 ng/ml TNF-α, and 300 ng/ml Flt3-L. At day 10, CD34+-cells had achieved their differentiation into CD34-DCs.

Monoclonal antibodies and flow cytometry analysis. FITC-conjugated anti-CD14, PE-conjugated anti-CCR7, APC-conjugated anti-CD83, FITC-conjugated anti-CD14, APC-conjugated anti-CD209 (DC-SIGN), PE-conjugated anti-TRAIL, FITC-conjugated anti-active caspase-3, PE-conjugated anti-caspase-3 and APC-conjugated anti-CD13 mAbs were from Pharmingen (San Diego, CA, USA). HCoV-229E spike glycoprotein was detected using mAb 5-11H.6 (2, 19). The SARS-CoV anti-M serum recognizes the C-terminal domain of SARS-CoV Membrane (M) protein (Proscience, Poway, CA, USA). TRITC-conjugated goat anti-mouse secondary antibody was from Zymed Laboratories (San Francisco, CA, USA) and...
Alexa Fluor 488 goat anti-rabbit from Invitrogen Molecular Probes (Eugene, Oregon, USA).

Negative controls were appropriate irrelevant isotype-matched mAbs.

For immunostaining, 2x10^5 cells were washed twice with PBS and 2% FCS, and then fixed in PBS containing 4% paraformaldehyde (PFA). Cells were permeabilized or not with PermWash buffer (BD Bioscience), incubated with the appropriate mAb at 4°C for 20 minutes, and washed in PBS before analysis by flow cytometry using a FACScalibur (Becton Dickinson). At least 20,000 gated events were collected and analyzed with Flowjo software (Tree Star). Results are expressed as a percentage of positive cells and/or as mean fluorescence intensity (MFI) by comparison to negative controls. Dead cells were stained according to manufacturer’s instructions with the DEAD/LIVE cell kit (Invitrogen Corporation, Carlsbad, California, USA).

**Cell culture imaging.** Monocytes or Mo-DCs were infected with HCoV-229E at a MOI of 0.05 and with SARS-CoV at MOI of 1 and cultured in the culture chambers of microscopy slides (Ibidi, München, Germany). At 24 hours post-infection, cells were fixed for 15 minutes with 4% PFA for HCoV-229E and 1 hour for SARS-CoV, for safety reasons. Free aldehyde groups were quenched in 50 mM NH4Cl, and cells were permeabilized for 5 minutes at 4°C in PBS containing 0.1% Triton X-100. Cells were incubated for 1 hour in PBS supplemented with 5% goat serum, and incubated again in the same solution containing the respective viral antibody: anti-spike glycoprotein mAb 5-11H.6 for HCoV-229E infected cells and anti-M antibody (Proscience, Poway, CA, USA) for SARS-CoV experiments. HCoV-229E and SARS-CoV infected-cells were washed twice with PBS and incubated for 1 hour with a respective secondary antibody. Finally, cells were washed with PBS and stained for 5 minutes with a PBS solution containing 4',6-diamidino-2-phenylindeole (DAPI). After PBS washing, 100 µl of Fluoromount-G was added in the wells (SouthernBiotech, Birmingham, AL, USA).
The slides were analyzed and image acquisition was performed using fluorescence microscope using a 40×-oil immersion objective.

Human primary Mo-DCs were infected with HCoV-229E at a MOI of 0.05, and incubated for 11 hours at 37°C in the culture chamber of a microscopy slide (Ibidi, München, Germany). Then, the microscopy slide was placed under an optical microscope for live cell imaging. Snapshots were captured every 30 seconds over a period of 9 hours using a 10× objective and a 1.5× magnifying lens. Finally, images were compiled into a movie, and played back at 24 frames per second.

Cytokines detection and neutralization. Cytokines produced from monocytes and Mo-DCs were measured from cell culture supernatants by multiplex bead-based LUMINEX assay (Biosource, Invitrogen Corporation, Carlsbad, California, USA) according to manufacturer’s instructions. IFN-α/β production was determined by ELISA following manufacturer’s instructions (PBL InterferonSource, Piscataway, USA). For neutralization of type I IFNs, monocytes were treated with sheep polyclonal antibody against human IFN-α (2000 IU/ml, PBL Biomedical Laboratories, Piscataway, USA) and with sheep polyclonal antibody against human IFN-β (500 IU/ml, PBL Biomedical Laboratories, Piscataway, USA). For neutralization of human IL-6 bioactivity, monocyte cultures were treated with anti-human IL-6 antibody (10 µg/ml, R&D system).

Apoptosis and infection inhibition. To block apoptosis, Mo-DCs were incubated prior to infection with caspase inhibitor Z-VAD-FMK (BD, biosciences) for 30 minutes at room temperature, and then maintained in cell culture medium. Similar experiments were performed to block apoptosis using hIL-6 (100 ng/ml; Miltenyi Biotech, Kohln, Germany) and hIFN-β (PBL Biomedical Laboratories, Piscataway, USA). To inhibit death ligand signaling, Mo-DCs were treated during and after infection with either an anti-hTRAIL mAb
(100 ng/ml; R&D Systems), or a recombinant human Fas-Fc chimera (1 µg/ml; R&D Systems), or a recombinant human TNFR-Fc chimera (1 µg/ml; R&D Systems). To block HCoV-229E infection, Mo-DCs were treated 1 hour at room temperature with 15 µg of anti-CD13 mAb (Biolegend, San Diego, CA, USA) prior to infection as described elsewhere (39). Anti-CD13 was maintained in cell culture medium.

RESULTS

HCoV-229E induces massive cytopathic effects in Mo-DC cultures but not in monocytes. Although CoVs associated with common cold, such as HCoV-229E, are likely to interact with DCs in the upper respiratory tract, the in vitro susceptibility of human DCs to infection by these viruses is yet unknown. To address this question, we infected human primary monocytes and monocyte-derived DCs (Mo-DC) with HCoV-229E. We first assessed the expression on the surface of monocytes and Mo-DCs of aminopeptidase N (CD13), a membrane bound metalloprotease previously identified as a receptor for human HCoV-229E (52). CD13 is expressed on epithelial cells from the lungs and the intestine but also on monocytes, granulocytes, and neuronal cells. Both monocytes and Mo-DCs expressed high levels of CD13 (Figure 1D). Human primary monocytes and Mo-DCs were then infected with HCoV-229E at a multiplicity of infection (MOI) of 0.05 and cultivated for 24 hours. Bright field observation of cells at 24 hours post-infection revealed a massive cytopathic effect in Mo-DCs, while monocyte cultures remained largely unaffected (Figure 1A). The cytopathic effect in Mo-DCs was assessed by the presence of cellular aggregates, large syncytia and numerous cellular debris. Infectious virus was required for killing cells since no cytopathic effect was observed when HCoV-229E virus was UV inactivated (Figure 1A; right panel). To evaluate the kinetic of cell death after infection, time-lapse microscopy was recorded on
infected Mo-DC cultures (supplementary Movie 1). This recording started 11 hours after
infection, and snapshots were captured every 30 seconds for 9 hours. Small syncytia
connected to each other by branched projections were visible as early as 11 hours post-
infection. These structures progressively fused with neighbouring cells to form large syncytial
structures. Finally, a rapid and near complete lysis of both syncytia and isolated cells was
observed at 20 hours post-infection.

To quantify cell death induced by HCoV-229E in Mo-DC cultures, we determined the
number of living cells in infected cultures by trypan blue exclusion at different time points
and different MOI, including 0.05, 0.1 and 0.5 (Figure 1B). Cell death was massive in Mo-DC
cultures, even at the lowest MOI 0.05, with virtually almost no viable cells detected at 24
hours post infection. In contrast, monocytes were resistant to cell death with limited loss of
viable cells at 48 hours after inoculation, even with the highest MOI of 0.5. In the same
experiment, we also quantified the status of caspase-3 activation since this protease plays a
central role in the execution-phase of programmed cell death. In agreement with our
observation showing that only Mo-DCs were killed by HCoV-229E infection, caspase-3 was
activated in Mo-DCs but not in monocytes (Figure 1C). Thus, death of Mo-DC induced after
HCoV-229E infection is associated to caspase-3 activation. We then compared the effects of
SARS-CoV, which is much more pathogenic in human than HCoV-229E and spreads to
lower respiratory tract possibly through dendritic cells transfer (51). Although viral infection
was detected by anti-spike and anti-M immunostainings, no cytopathic effect was induced by
SARS-CoV infection in Mo-DCs (Figure 1E), in agreement with previous reports (28, 46).
The same result was obtained when using 20 times higher SARS-CoV MOI (data not shown).
Altogether, these observations show that HCoV-229E (but not SARS-CoV) induces massive
cytopathic effects in Mo-DC cultures, whereas monocytes do not exhibit this unusual
susceptibility.
Mo-DCs, but not monocytes, are highly susceptible to HCoV-229E infection. To determine whether cytopathic effects of HCoV-229E correlate with a higher susceptibility to infection, Mo-DCs and monocytes were infected, cultured, and then immunostained for the expression of viral spike glycoprotein. As expected, large syncytia of Mo-DCs were positive for HCoV-229E spike protein expression thus demonstrating viral infection (Figure 2A). In contrast, only a small fraction of monocytes expressed the viral spike glycoprotein after exposure to a similar inoculum of HCoV-229E and no cell fusion occurred. Immunostaining and flow cytometry analysis of infected cells revealed that a large fraction of Mo-DCs (>85%) expressed the spike glycoprotein of HCoV-229E at 10 hours post infection, while only few monocytes were positively stained whatever the MOI used (Figure 2B). These results suggest that Mo-DCs are much more susceptible to infection by HCoV-229E than monocytes. This was confirmed when viral RNA load was measured by qRT-PCR in cell cultures supernatants (Figure 2C). Viral RNA load increased during the first 8 hours post-infection in both Mo-DC and monocyte, thus demonstrating viral replication, but was much higher in Mo-DC cultures. Interestingly, viral RNA load detected in monocyte cultures increased when cells were infected with a higher MOI, thus demonstrating that monocytes are permissive to HCoV-229E infection, but Mo-DC are clearly much more susceptible. Furthermore, HCoV-229E never induced massive cytopathic effects in monocyte cultures, even when performing infection with higher MOI (Figure 1B; right panel). In conclusion, Mo-DC are not only more susceptible to viral replication but also to HCoV-229E-induced cytopathic effects.

Blocking cell entry or replication of HCoV-229E into Mo-DCs prevents cytopathic effects. Once inside Mo-DCs, HCoV-229E replicates actively as demonstrated by cell surface expression of the viral spike glycoprotein and detection of viral RNA load (Figure 2). Interestingly, UV-inactivated virus was ineffective at inducing cytopathic effect in Mo-DCs, suggesting that viral replication in infected cells is linked to the CPE (Figure 1A; right panel).
To demonstrate that HCoV-229E-induced cytopathic effects and cell death are dependent on viral entry, Mo-DC infection was performed in the presence of anti-CD13 mAb to block virus binding to its receptor. Pre-treating Mo-DC cultures with anti-CD13 mAb protected cells from virus-induced cytopathic effects (Figure 3A), confirming that virus entry into Mo-DCs is essential. In addition, we tested whether type I interferon (IFN-β) could rescue Mo-DCs from HCoV-229E-induced cytopathic effects. Indeed, IFN-α/β are essential antiviral cytokines that can control viral replication by inducing a large cluster of immune factors. Mo-DCs were pretreated with increasing doses of IFN-β for 24 h before HCoV-229E infection. As shown in Figure 3B, even a low dose of IFN-β (100 IU/ml) was sufficient to prevent HCoV-229E replication, cytopathic effects, and massive cell death in Mo-DC cultures. Altogether, these results demonstrate that HCoV-229E-induced cytopathic effects require viral entry in Mo-DCs and sustained viral replication.

**Different populations of conventional DCs show a similar susceptibility to HCoV-229E-induced cytopathic effects.** Several subsets of conventional DCs have been described (20, 30, 35, 36). To determine if other DC populations also show a high susceptibility to HCoV-229E, we tested DCs derived from CD34+ precursor cells (9, 40). Mo-DCs and CD34-derived DCs (CD34+-DCs) show similar morphologies and antigen uptake/presentation capacities (18, 24). We first demonstrated that CD34+-DCs express significant amounts of CD13 on their surface (Figure 4B). Then, we investigated whether this DC population was as susceptible as Mo-DCs to HCoV-229E infection. We observed massive cell death at 24 hours post-infection in both DC populations as determined by trypan blue exclusion (Figure 4A). Furthermore, infection of CD34+-DCs with HCoV-229E was assessed by viral spike glycoprotein expression on their surface (Figure 4C), thus demonstrating viral infection. Caspase-3 activation was also observed in HCoV-229E-infected CD34+-DCs (Figure 4D). In addition, we also tested peripheral blood DCs purified on the basis of BDCA-1 or DC-SIGN.
expression (BDCA-1⁺-DCs or DC-SIGN⁺-DCs). Both BDCA-1⁺-DCs or DC-SIGN⁺-DCs rapidly died upon HCoV-229E infection similarly as for Mo-DCs (Figure 4A). Altogether, these results show that all tested populations of conventional DCs are highly susceptible to HCoV-229E-induced cell death.

Together, GM-CSF and IL-4 induce monocyte differentiation into immature DCs. However, DCs ability to stimulate T cells still requires their maturation into professional APCs. This maturation is induced by PAMPs or by cellular factors such as CD40L, TNF-α or PGE2. To determine whether Mo-DCs susceptibility to HCoV-229E infection depends on their maturation state, we pre-incubated cells in the presence of poly-IC, CD40L or PGE2+TNF-α for 16 hours before infection with HCoV-229E and the expression of CD80 and HLA-DR maturation markers was analyzed. Mo-DCs cultured 16 hours in presence of poly-IC, CD40L or PGE2+TNF-α up-regulated the expression of CD80 and HLA-DR (Figure 4E). However, whatever the maturating agent used, Mo-DC maturation did not confer resistance to infection and did not protect cells from HCoV-229E-induced cell death (Figure 4F).

**Cell death induced by HCoV-229E infection is independent of FasL, TRAIL and TNF-α.** Death receptors are transmembrane proteins that transmit apoptotic signals initiated by specific ligands such as FasL, TNF-α and TRAIL. They recruit and activate cysteine proteases of the caspase family that are essential mediators of programmed cell death or apoptosis, and were found activated in HCoV-229E-infected Mo-DCs (Figure 1C). To evaluate the role of FasL, TNF-α and TRAIL receptors on the death induced by HCoV-229E infection of Mo-DCs, we neutralized the binding of these ligands during infection by using different inhibitors: an anti-TRAIL mAb, a recombinant human TNFR-Fc chimera, and a recombinant human Fas-Fc chimera (Figure 5A). We observed that blocking Fas, TNF-α or
TRAIL pathways did not rescue Mo-DCs from HCoV-229E-induced death. We controlled in Jurkat cells that anti-TRAIL, TNF-R/Fc and FAS/Fc were efficient to block apoptosis induced by their cognate ligands, i.e. TRAIL, TNF-α and FasL, respectively (data not shown). Caspases can be activated through pathways that are independent of membrane death receptors. To determine whether cell death induced by HCoV-229E relies on caspases, we pretreated cells with Z-VAD-FMK, a general caspase inhibitor that irreversibly blocks their catalytic site (48). As shown in Figure 5B, Z-VAD-FMK did not prevent the death of HCoV-229E-infected Mo-DC. We controlled in Jurkat cells that Z-VAD-FMK efficiently blocked the apoptosis induced by TNF-α (data not shown). Altogether, these observations suggest that even if HCoV-229E infection activates caspases in Mo-DC (Figure 1C), cell death does not require caspase activation, but rather results from cytolysis and/or necrosis of multinucleated syncytia induced by replicative infection.

Cytokines produced by monocytes do not account for their resistance to HCoV-229E-induced cell death and cytopathic effects. We searched for cellular soluble factors that could account for monocyte resistance to HCoV-229E-induced cell death and cytopathic effects. Specific cytokines such as IFN-α/β and IL-6 could provide resistance to viral infection. We performed multiplex bead-based Luminex assays and ELISA to determine cytokine/chemokine expression profiles in culture supernatants of monocytes and Mo-DCs collected 6, 12 and 24 hours after infection with HCoV-229E. Results obtained at 24 hours are presented in Table I. Tested cytokines and chemokines were all induced in monocytes as soon as 6 hours after infection with HCoV-229E, reaching prominent inductions of TNF-α (x27), IFN-α (x19), MCP1 (x16), IL-6 (x11) and IFN-γ (x9) at 24 hours. In contrast, for Mo-DCs, no increased induction was observed at early time points (not shown), and a more restricted induction profile limited to IFN-α (x24), IL-8 (x16) and TNF-α (x9) at 24 hours after infection. Some of these cytokines that were differentially induced, like IL-6, could
account for the resistance of monocytes to HCoV-229E infection, cytopathic effects and cell death. To test this hypothesis, Mo-DCs were pre-treated for 24 hours with UV-inactivated supernatants from infected monocyte cultures. Then, Mo-DCs susceptibility to infection by HCoV-229E was determined. As shown in Figure 6A, monocyte culture supernatant did not confer resistance to HCoV-229E-induced cell death and cytopathic effects. Thus, resistance of monocytes to HCoV-229E-induced cell death is probably not mediated by a soluble factor, but rather relies on an intrinsic property of this cell type that is lost during differentiation into Mo-DCs. It should be noted that monocyte culture supernatants contained 113 pg/ml of IFN-\(\alpha\) and 31 pg/ml of IFN-\(\beta\) (Table 1), which correspond to 17 and 5 IU/ml, respectively. These levels are far much lower than the 100 to 1000 IU/ml doses of IFN-\(\beta\) used to inhibit HCoV-229E replication in Mo-DCs in Figure 3B.

**HCoV-229E susceptibility is early acquired during monocyte differentiation into Mo-DCs.** The differentiation of monocytes into immature DCs is usually achieved after 5 days of culture in the presence of both GM-CSF and IL-4. To determine at which stage of differentiation monocytes acquire their susceptibility to infection, we cultivated purified CD14\(^+\) monocytes either in medium alone, GM-CSF, IL-4 or both cytokines. At different time points, cells were harvested and tested for the induction of cytopathic effects by HCoV-229E infection (MOI of 0.05). After only 24 hours of culture in presence of both GM-CSF and IL-4, although cells retain a cell-surface phenotype of monocytes, expressing CD14 but no CD11c (Figure 6B), they became susceptible to HCoV-229E as assessed by the presence of large syncytia, dead cells and cellular debris (Figure 6C, upper panel). Again, this susceptibility paralleled with high levels of viral spike glycoprotein expression and activation of caspase 3 (Figure 6C, lower panel). The strong increase of viral spike protein on cell membrane, as in Mo-DCs, argues that this protein, which is responsible for membrane fusion, is the cause of syncytia formation and cell death. Interestingly, monocytes treated with IL-4...
alone also became susceptible to HCoV-229E infection and massive cytopathic effects were observed. A similar trend was observed when treating monocytes with GM-CSF alone, but cytopathic effects were much less pronounced. This suggests that signaling events predominantly induced by IL-4, but partially overlapping with GM-CSF, are responsible for monocytes sensitization.

DISCUSSION

Although infection with the emerging SARS-CoV was associated with a severe acute respiratory disease, most human CoVs are responsible for mild upper respiratory tract infections, such as common colds, with only occasional spreading to the lower respiratory tract. Most respiratory viruses interact with DCs in the upper respiratory tract, which results in initiating an antiviral immune response but may also result in the spreading of the virus as a result of DC migration to draining lymph nodes. In this study we investigated the interaction between HCoV-229E and human DCs. We observed that HCoV-229E infection causes massive cytopathic effects, resulting in rapid death of DCs. Cell-death correlates with a high surface expression of HCoV-229E spike protein, which is responsible for cell-cell fusion, and the formation of large syncytia that blow-up in a very short time (10 hours, supplemental movie). In contrast, monocytes from the same donors are less susceptible to infection and resist to cytopathic effects and cell death despite similar expression levels of CD13, the cell surface receptor for HCoV-229E. Monocytes rapidly acquire susceptibility to HCoV-299E infection upon a short differentiation in the presence of GM-CSF and/or IL-4. DC differentiation might down-regulate a restriction factor present in monocytes or induce the expression of an unknown cellular factor increasing susceptibility to HCoV-229E infection like a co-receptor on the surface of DCs. Infection and killing of DCs is dependent upon viral
entry and viral replication, as blocking virus entry with an anti-CD13 antibody or inactivating the virus with UV protected cells. Pretreating cells with blocking antibodies against TNF-α, FAS-L, TRAIL, and IFN-α/β or with the caspase inhibitor Z-VAD-FMK did not protect infected Mo-DCs from death. We therefore suggest that cell death induced by HCoV-229E is not an apoptotic process, but rather a direct consequence of virus replication and viral spike protein expression on cell surface. Consistent with this interpretation, the only situation in which we observed protection was when cells were pre-treated with IFN-β, which prevented infection by HCoV-229E. However, when Mo-DCs were infected with HCoV-229E and only then treated with IFN-β, viral replication occurred, inducing cytopathic effects and massive cell death (data not shown). This suggests that, as previously described for other respiratory viruses (5, 6), HCoV-229E encodes a virulence factor that blocks IFN-α/β signaling and prevents the induction of antiviral interferon-stimulated genes (ISG) in Mo-DCs. This might explain that endogenous type I IFNs induced after HCoV-229E infection are unable to block viral replication and cytopathic effects in Mo-DCs. Monocytes exposed to HCoV-229E also responded by producing type I IFNs, yet blockade of IFN-α/β binding did not alter susceptibility to infection. Altogether, this suggests that monocytes resistance to HCoV-229E-induced cytopathic effects is independent of endogeneous type I IFNs production, but rather relies on the expression of a yet unknown restriction factor or the absence of a cellular factor increasing susceptibility to viral infection like a co-receptor.

Because DCs are major sensors to detect viral infection and prime adaptive immunity, viruses have evolved strategies to interfere with their development, maturation, function or viability to suppress or escape immune response. In this regard, killing DCs can be an efficient viral strategy to delay or prevent the establishment of adaptive immune responses. Infections by measles virus, human immunodeficiency virus or lymphocytic choriomeningitis virus deplete DC populations in infected hosts (12, 22, 37). In vitro experiments have also
demonstrated that filoviruses, vaccinia virus, herpes simplex virus, H5N1 influenza virus, and measles virus induce DC apoptosis or cytolysis in a few days (16, 31, 33, 41, 47). As well, human echovirus is extremely cytopathic toward DCs and induces their death in less than 24 hours (27). DCs killing by HCoV-229E, if it happens in vivo in human infection, could delay the induction of an adaptive immune response, thus providing time to replicate in the infected host. Furthermore, this could affect the establishment of a long-term immunological memory to the virus, explaining that people can be re-infected multiple times by HCoV-229E (8).

Massive death of infected DCs may also act as a host defense mechanism to prevent virus spreading in the body. Because DCs are located at every possible entry site of the body, they are one of the first cell type encountered by incoming viruses. Upon stimulation by pathogen-associated molecular patterns, DCs migrate from peripheral tissues to draining lymph nodes where they elicit antigen-specific T lymphocytes. Many viruses use DCs as a vehicle to penetrate draining lymphoid organs, but also to interfere with their APC functions so that immune response is skewed towards inappropriate cytokine profiles. In the case of HCoV-229E, the extreme susceptibility of DCs to infection probably prevents this virus from using them as a “Trojan Horse”. Although this hypothesis needs to be further supported by experimental data, it was recently shown that DCs infected by *Legionella pneumophila* undergo apoptosis to restrict bacterial replication and spreading (34). Interestingly, SARS-CoV which spreads to the lower respiratory tract and is therefore associated with a much more severe respiratory disease than HCoV-229E does not induce massive cell death and cytopathic effects in DCs, arguing in favor of this hypothesis.

Our most striking observation is that, compared to DCs, monocytes from the same donors are resistant to HCoV-229E infection. However, when stimulated for only 24 hours with IL-4 alone, and to some extend with GM-CSF alone, monocyte cultures became susceptible to infection. This suggests that signaling events induced by IL-4, and also...
somewhat induced by GM-CSF, are responsible for monocyte sensitization to HCoV-229E infection. For example, both IL-4 and GM-CSF lead to the activation of PI3K and GRB2/MAPK pathways which seem to be critical in the biological functions of DCs (23, 25). In a previously published work, Collins et al. found that monocytes/macrophages undergo apoptosis when infected with HCoV-229E (11). However, they did not describe such massive cell death and cytopathic effects as we observed in Mo-DCs. This difference could be due to different cell isolation and purification methods. Indeed, monocytes obtained by adhering peripheral blood mononuclear cells quickly differentiate into macrophages, as acknowledged by the authors themselves. This could account for their susceptibility to HCoV-229E infection, whereas monocytes positively selected by magnetic beads are resistant, as shown in the present report.

What are the mechanisms allowing monocytes, but not DCs, to prevent cell death and massive cytopathic effects upon HCoV-229E infection? We observed that monocytes produced 16 times more IL-6 than Mo-DCs upon infection. This could explain the resistance of monocytes since IL-6 is an IFN-like cytokine with antiviral properties. However, neutralizing IL-6 in infected monocyte cultures did not confer susceptibility to HCoV-229E, and adding recombinant IL-6 into infected DC cultures did not confer resistance to the virus (data not shown). As well, supernatants collected from infected monocytes did not protect Mo-DCs. Thus, a soluble co-factor does not account for monocyte resistance to HCoV-229E infection. Interestingly, it has been shown that bovine viral diarrhoea virus (BVDV), a positive strand RNA virus that belongs to the Flaviviridae family, has opposite effects on these two cell types, DCs being resistant whereas monocytes are rapidly killed by the infection (21). HIV is another example demonstrating that cells from macrophages lineage have different levels of susceptibility to infection. Although macrophages and monocytes both express HIV-1 entry receptors, monocytes freshly purified from peripheral blood are
resistant to HIV-1 infection. In contrast, monocyte-derived macrophages are highly susceptible to infection. As an explanation, Wang et al. demonstrated that freshly isolated monocytes express higher levels of anti-HIV-1 microRNAs than monocyte-derived macrophages (50). This suggests that the expression of specific antiviral miRNA is a possible mechanism underlying monocyte resistance to HCoV-229E. The precise mechanism which triggers the susceptibility of monocytes to infection after a short stimulation with IL-4 or GM-CSF remains to be elucidated.

In conclusion, we demonstrate in this report that HCoV-229E, which is commonly spread among human population, infects and destroys very rapidly human Mo-DCs, whereas monocytes are resistant. The next steps of this work is to identify the molecular basis of DC susceptibility and monocyte resistance to HCoV-229E infection, but also the mechanisms that regulate this phenotype, and how this determines the severity of the disease.

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REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Cytopathic effects of HCoV-229E on Mo-DCs. (A) Bright field microscopy of monocytes and Mo-DC cultures either mock-treated or infected with HCoV-229E (MOI = 0.05), and then cultured for 24 hours. (B) Monocytes (closed circle) and Mo-DCs (closed triangle) were infected with HCoV-229E at MOIs of 0.05, 0.1 and 0.5. Viable cells were enumerated at indicated time points by trypan blue exclusion (mean of 3 donors). (C) Detection of the active form of caspase-3 in HCoV-229E-infected Mo-DCs and monocytes by cytometry (one representative experiment out of 5). (D) CD13 expression on monocytes and Mo-DCs. Cells were stained with an anti-CD13 monoclonal antibody (open line) or an isotypic control (closed line) and analyzed by flow cytometry. Data shown is representative of three different donors. (E) Mo-DCs infected with SARS CoV (MOI = 1 for 24 hours). Upper part shows bright field image of Mo-DCs either mock-treated or infected with SARS-CoV. Lower part shows immunostaining of viral spike glycoprotein and membrane envelope protein (M) (red = anti-spike; green = anti-M; blue = DAPI). Data is representative of three independent experiments.

**Figure 2.** Susceptibility of monocytes and Mo-DCs to HCoV-229E. (A) Viral spike (S) glycoprotein expression in monocytes and Mo-DC cultures mock-treated or infected with HCoV-229E (MOI = 0.05), and then cultured for 10 hours (red = anti-spike immunostaining; blue = DAPI staining). (B) Cell-surface expression of HCoV-229E spike glycoprotein on mock-treated and HCoV-229E-infected cells (one representative experiment out of three). (C) Kinetics of viral RNA produced in medium from monocytes and Mo-DCs infected with HCoV-229E at MOIs of 0.05, 0.1 and 0.5 (mean of 2 donors).

**Figure 3.** The role HCoV-229E cell entry and replication on Mo-DC death. (A) Mo-DCs were incubated or not with anti-CD13 mAb prior to infection with HCoV-229E. Cell
death was determined by the observation of cytopathic effects and formation of syncytia by bright field microscopy (one representative experiment out of 3). (B) Mo-DCs were treated or not with increasing amounts of IFN-β prior to infection with HCoV-229E, and the percentages of cells expressing spike viral protein or activated caspase-3 were determined by flow cytometry (one representative experiment out of 3).

**Figure 4: Killing of conventional DCs by HCoV-229E.** (A) CD34⁺-DCs, BDCA-1⁺-DCs and DC-SIGN⁺-DCs were either mock-treated (open symbols) or infected with HCoV-229E (closed symbols) at different MOI (0.05, 0.1 and 0.5). Viable cells were enumerated at indicated time points by trypan blue exclusion (mean of 2 donors). (B, C, D) CD34⁺-DCs were infected with HCoV-229E, cultured for 24 hours, and then immunostained for CD13, spike glycoprotein or active caspase-3 expression. Cells were analyzed by flow cytometry. (E) Mo-DC were treated with poly-IC, CD40L or PGE2+TNF-α to induce maturation, and then infected with HCoV-229E. CD80 and HLA-DR expression levels were determined by flow cytometry. (F) Cell death was determined using DEAD/LIVE staining.

**Figure 5. Death of HCoV-229E-infected Mo-DCs is independent of TRAIL, TNF-α, FasL and caspase activity.** (A) Mo-DCs were infected with HCoV-229E and incubated for 24 hours in the presence of anti-TRAIL blocking antibodies, TNFR-Fc chimera or FAS-Fc chimera. Cell death was determined by trypan blue exclusion (one representative experiment out of 3). (B) Inhibition of caspase activity does not prevent cell death in Mo-DC cultures. Mo-DCs were pre-incubated for 24 hours in the presence of Z-VAD-FMK, a potent broad-spectrum caspase inhibitor, then infected by HCoV-229E in the presence of Z-VAD-FMK. After 24 hours of culture, cell death was determined using DEAD/LIVE staining (one representative experiment out of 3).
Figure 6: Analysis of monocyte resistance to cytophatic effects and cell death. (A) Mo-DCs were infected with HCoV-229E at MOI 0.05 after pre-incubation for 24 hours with culture supernatants from mock-treated monocytes, HCoV-229E-infected monocytes, or UV-treated supernatants from HCoV-229E-infected monocytes. Viable cells were enumerated at indicated time points by trypan blue exclusion. Results correspond to the mean of 3 donors. (B) Phenotype analysis of monocytes incubated for 24 hours in presence of GM-CSF and IL-4. Cells were stained with an anti-CD14 monoclonal antibody or anti-CD11c (open lines) or an isotypic control (closed lines) and analyzed by flow cytometry. Data shown is representative of three different donors. (C) Monocytes become susceptible to HCoV-229E-induced cell death when induced to differentiate into Mo-DCs. Monocytes were treated for 24 hours with GM-CSF or IL-4 or both combined, and then infected with HCoV-229E. After 24 hours, cells were immunostained for spike glycoprotein and active caspase-3 expression.
Table I: Cytokine and chemokine levels (pg/ml) detected in culture supernatants from mock and HCoV-229E-infected monocytes and Mo-DCs (24 hours of infection). All cytokines and chemokines were measured by multiplex bead-based LUMINEX assay (mean of 3 donors), except for IFN-α and IFN-β (*) measured by ELISA (mean of 6 donors).

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Monocytes</th>
<th>Mo-DCs</th>
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<tbody>
<tr>
<td></td>
<td>Mock</td>
<td>229E</td>
</tr>
<tr>
<td>IL-6</td>
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A) Images showing Mo-DC and Monocytes under Mock and HCoV-229E conditions.

B) Graph showing Mo-DC and Monocytes cell counts over a range of spike values.

C) Graphs showing viral RNA copies/ml over hours for different MOI values (0.05, 0.1, 0.5).