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Activation of human monocytes after infection by human coronavirus 229E

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Abstract

Human coronaviruses (HCoV) are recognized respiratory pathogens that may be involved in other pathologies such as central nervous system (CNS) diseases. To investigate whether leukocytes could participate in respiratory pathologies and serve as vector for viral spread towards other tissues, the susceptibility of human leukocytic cell lines and peripheral blood mononuclear cells (PBMC) to HCoV-229E and HCoV-OC43 infection was investigated. Human primary monocytes/macrophages were susceptible to HCoV-229E infection, but strongly restricted HCoV-OC43 replication. Moreover, productive HCoV-229E infection of primary monocytes and of the THP-1 monocytic cell line led to their activation, as indicated by the production of pro-inflammatory mediators, including TNF-α, CCL5, CXCL10 and CXCL11 and MMP-9. Moreover, an *in vitro* chemotaxis assay showed that motility towards chemokines of THP-1 cells and primary monocytes was increased following an acute or persistent HCoV-229E infection. Taken together, these results suggest that infected monocytes could serve as a reservoir for HCoV-229E, become activated, participate in the exacerbation of pulmonary pathologies, as well as serve as potential vectors for viral dissemination to host tissues, where it could be associated with other pathologies.
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1. Introduction

Human coronaviruses (HCoV) are ubiquitous enveloped positive-stranded RNA viruses, represented by strains 229E, OC43, NL63, HKU1, and possibly SARS-CoV. Except for SARS-CoV, the etiological agent of Severe Acute Respiratory Syndrome (SARS; Ksiazek et al., 2003), HCoV have primarily been associated with mild upper and lower respiratory tract diseases, with the common cold representing the typical HCoV-induced pathology (Myint, 1995). However, over the years, HCoV have regularly been linked to other pathologies, such as severe respiratory distress syndrome in newborns (Sizun et al., 1995; Gagneur et al., 2002), and as important triggers of exacerbations of acute asthma (Nicholson et al., 1993; El-Sahly et al., 2000). More recently, both previously (229E and OC43) and newly described (NL63 and HKU1) HCoV strains were associated with more severe acute lower respiratory tract pathologies, including pneumonia, in both infants and immunocompromised patients (Woo et al., 2005; Gerna et al., 2006). Even though it is recognized that HCoV primarily infect airway epithelial cells and cause diseases of the respiratory tract in humans, viral material was reported in other cells and tissues. For example, HCoV-229E infection of human monocytic cell lines and of primary monocytes/macrophages was reported (Collins, 2002; Desforges et al., 2006). Infection of peritoneal macrophages (Patterson and Macnaughton, 1982) and murine dendritic cells expressing human aminopeptidase N (APN; Wentworth et al., 2005) by HCoV-229E suggests that this virus may use myeloid cells to disseminate to other tissues, where it could provoke other types of pathologies.

Other viruses, such as human immunodeficiency virus type 1 (HIV-1; Nottet et al., 1996; Persidsky et al., 1997), human cytomegalovirus (HCMV) (Smith et al., 2004) and its murine counterpart (MCMV) in immunocompromised mice (Reuter et al., 2004), are well-characterized examples of viruses that reach host tissues via infected leukocytes. Indeed, as previously suggested for HCMV (Smith et al., 2004), blood-borne monocytes and their differentiated counterparts, macrophages, could be involved in hematogenous dissemination of HCoV-229E to host

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tissues, including the CNS, as we previously showed this virus to be neuroinvasive (Arbour et al., 2000). In these examples of viral spread, pro-inflammatory molecules, such as cytokines, matrix metalloproteinases (MMP) and chemokines and their receptors, were shown to play a crucial role in leukocyte activation and their dissemination from blood to various tissue.

In the current study, susceptibility of the THP-1 human monocytic cell line and of peripheral blood mononuclear cells (PBMC) to infection by HCoV-229E and HCoV-OC43 was investigated to verify the possibility that these infectious agents may on the one hand activate monocytic cells that could participate in lung injury, and on the other hand use a hematogenous route to disseminate into host tissues. We show that human monocytic cells were susceptible to infection and that cell death was induced in a portion of the cells, in correlation with the initial MOI for the 229E strain. The THP-1 cell line presented a pattern of infection very similar to that of primary monocytes. Both cell types were activated following infection, as shown by the production of pro-inflammatory mediators. Moreover, infection by HCoV-229E increased cell motility and chemokine-driven cell migration. Furthermore, the establishment of a persistent infection in monocytes/macrophages is consistent with the possibility that these cells serve as a reservoir and potential vector for virus dissemination to host tissue.

2. Materials and methods

2.1. Viruses and cell lines

HCoV strains (229E and OC43) were originally obtained from the American Type Culture Collection (ATCC). HCoV-229E was produced in the human L132 cell line (ATCC CCL5) and HCoV-OC43 was produced in human HRT-18 cells (gift from Dr. David Brian, Tennessee University, Knoxville, TN, USA). Both cell lines were grown in minimum essential medium (MEM) supplemented with 10% (v/v) FBS (Wysent) and non-essential amino acid (Invitrogen). The human monocytic cell line THP-1 (gift from Dr. Daniel Oth, INRS-Institut Armand-Frappier, Laval, Quebec, Canada) was cultured in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Wysent), and 2-mercaptoethanol 2×10^{-5} M (Invitrogen) at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2.

2.2. Antibodies

Mouse IgG1 Mabs 5-11H.6 and 1-10C.3, directed against the S protein of HCoV-229E and HCoV-OC43, respectively, and anti-HEV 4-E11.3, specific for the N protein of HCoV-OC43 (gift from the late Dr. Serge Dea, INRS-Institut Armand-Frappier) were used in immunoperoxidase assays, immunofluorescence and flow cytometry.

2.3. Peripheral blood mononuclear cells (PBMC) isolation and culture

Peripheral blood was obtained from healthy volunteers. Leukocytes were separated by gradient centrifugation through Ficoll-Hypaque (Amersham Biosciences). PBMC were washed twice and viable cells brought to a final concentration of 3×10^6 mL^{-1}. Monocytes were enriched by adsorption into 24- or 6-well plastic plates (Corning) for 2 h at 37 °C in RPMI 1640 supplemented with 10 mM HEPES, 1 mM pyruvate sodium, non-essential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen). Cultures were then washed three times with medium to remove unattached lymphocytes, while adherent monocytes were fed new medium. A portion of these monocytes were cultured with 2% (v/v) autologous serum (decomplemented and filtered) and infected the next day. The remaining cells were induced to differentiate into macrophages for 7 days, with a change of medium after 4 days, in 10% (v/v) autologous serum in RPMI, before infection. Lymphocytes were cultured in RPMI supplemented with 10% (v/v) serum and 2×10^{-5} M 2-mercaptoethanol. For the chemotaxis assay and evaluation of the cytokine/chemokine profile, monocytes were isolated from PBMC by a negative immunoselection procedure, using the Monocytes Isolation Kit II (Miltenyi Biotec) and an AutoMacs apparatus. Isolated monocytes were then resuspended in SFM Macrophage medium (Invitrogen).

2.4. Infection of cells

The THP-1 human cell line was infected in triplicate at a multiplicity of infection (MOI) of 0.01, 0.1 and 1 and incubated 4 h at 37 °C, washed twice with serum-free RPMI 1640. Infections proceeded for up to 3 days in RPMI 1640 supplemented with 5% (v/v) FBS (except for evaluation of MMP-9, where the cells were incubated in RPMI without serum). Uninfected controls were prepared by incubating cells with medium alone and 4 °C controls were obtained by incubating cells with virus on ice, to measure bound virus that presumably did not penetrate the cells. Undifferentiated non-adherent monocytic THP-1 cells (1.5×10^6 cells in each well of a 6-well plate) were differentiated by incubating the cultures for 48 h with phorbol myristate acetate (PMA) (Sigma) at a final concentration of 100 nM. After this incubation period, the cells were adherent and considered to be differentiated macrophage-like cells.

Primary lymphocytes, monocytes and macrophages were infected separately in complete RPMI 1640 supplemented with 1% (v/v) serum. Infection was then performed in triplicate with both HCoV strains at an MOI of 1.0 (HCoV-229E infections at a MOI of 0.01 and 0.1 were also performed for primary monocytes) and incubated 4 h at 37 °C. Cells were then washed twice in RPMI 1640 and infection proceeded in the same medium supplemented with 2% (v/v) for monocytes and 10% (v/v) FBS for macrophages and lymphocytes in 6-well plates (Corning) at 37 °C for up to 7 days. A kinetics of infection was performed for two donors where samples of supernatant were harvested at 6, 24, 48 and 72 hpi for viral titration and cells were fixed with PBS supplemented with 4% (w/v) paraformaldehyde (Biopharm-Sigma) for 20 min and kept in PBS at 4 °C until processed for detection of viral antigens. The metabolic activity of the infected THP-1 cells or primary cells was evaluated using the MTS/PMS tetrazolium assay (Cory et al., 1991), as this represents an evaluation of cell survival after infection. MTS

(Promega) was added to the culture medium (final concentration: 0.6 mM) with PMS (Sigma–Aldrich) (final concentration: 0.014 mM). Reduction of MTS, a measure of mitochondrial respiration, and therefore a direct indication of cell viability, was monitored by optical density reading at 540 nm. For evaluation of cytokine/chemokine and MMP-9 production, serum-free medium for monocytes/macrophages (SFM, Invitrogen) was used for the culture of primary monocytes.

2.5. *Quantitation of infectious virus titers by an immunoperoxidase assay (IPA)*

The IPA was performed on L-132 cells (HCoV-229E) or HRT-18 cells (HCoV-OC43) as described previously (Sizun et al., 1998). Primary antibody was antiviral Mab, 5-11H.6 (anti-HCoV-229E) or 1-10C.3 (anti-HCoV-OC43) and secondary antibody was horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (KPL). Bound antibodies were detected by incubation with 0.025% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (Bio-Rad) and 0.01% hydrogen peroxide in PBS and infectious virus titers were calculated by the Karber method.

2.6. *Detection of viral antigens in infected cells*

Paraformaldehyde-fixed primary monocytes and macrophages (in 24-well plates) were permeabilized for 5 min with methanol at −20 °C. THP-1 cells were allowed to adhere to poly-D-lysine-coated glass slides for 45 min and were fixed the same way. Primary antibodies, Mabs 5-11H.6, 1-10.C3 and 4-E11.3 (Section 2.2), were added to plates for 1 h at room temperature. The 5-11H.6 antibody served as isotypic control for 4-E11.3 and 1-10.C3 and the latter served as isotypic control for 5-11H.6. Following three washes with PBS, the Alexa-Fluor-488 mouse-specific goat antibody was added (Invitrogen) for 1 h at room temperature. After three washes with PBS, cells were incubated for 5 min with DAPI (Sigma–Aldrich) at 1 μg/mL in order to stain the DNA and the plates were washed once and kept in PBS at 4 °C until further analysis using a fluorescence microscope. Analysis on lymphocytes was performed by flow cytometry using the undifferentiated THP-1 cell line as positive control of infection.

2.7. *Detection of viral RNA by RT-PCR*

Total RNA was extracted from primary cells using TRIzol™ (Invitrogen) according to the manufacturer's instructions. Five micrograms of total RNA were reverse transcribed at 37 °C for 90 min using 0.5 μg of oligo dT (Invitrogen), 50 U of Expand™ MoMuLV reverse transcriptase, 60 U of RNAGuard™, 0.4 mM of dNTPs and RT buffer (Roche Diagnostics). PCR primers used for detection of the N gene of HCoV-229E were E1 (nucleotides 498–521; 5′-AGGCGCAAGAATTCAGAACCAGAG-3′) and E3 (nucleotides 806–783; 5′-AGCAGGACTCTGATTACGAGAAGG-3′) (Schreiber et al., 1989). Primers O1 (nucleotides 215–238; 5′-CCCAAGCAAACTGCTACCTCTCAG-3′) and O3 (nucleotides 497–520; 5′-GTAGACTCCGTCAATATCGGTGCC-3′) were used for detection of HCoV-OC43 (Stewart et al., 1992). Twenty percent of the RT was used in the PCR and incubated with the O1 and O3 primers or E1 and E3 primers, PCR buffer (Roche Diagnostics) and 0.4 mM dNTPs. After addition of Expand™ high-fidelity PCR system DNA polymerase (Roche Diagnostics), PCR consisted of 30 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C. Amplicons were separated by electrophoresis in 1.5% (w/v) agarose.

2.8. *Cytokine/chemokine profile of the monocytic cells following infection*

To get a more complete cytokine/chemokine profile following HCoV-229E infection of human monocytic cells, the Proteome Profiler Human Cytokine Array (R&D systems), which evaluates the production of 36 inflammation-related molecules, was used according to the manufacturer's instructions.

2.9. *Production of TNF-α by THP-1 cells and primary monocytes*

To quantitate production of the TNF-α cytokine by infected THP-1 cells and primary monocytes, ELISA was performed using Quantikine™ (R&D systems) according to the manufacturer's indications. Medium was recovered at different times post-infection and was centrifuged immediately. Supernatants were harvested and kept at −80 °C until further analysis.

2.10. *Secretion of matrix metalloproteinases (MMP)*

A gelatin-based zymography, modified from Heussen and Dowdle (1980), was used to detect the secretion and gelatinase activity of MMP-2 and MMP-9, as previously described (Edwards et al., 2000). Cells were cultured in serum-free conditions and either treated with 100 ng/mL PMA, mock-infected or infected with HCoV-229E. Supernatants were collected and concentrated 10-fold by ultrafiltration using Centricon® YM-50 centrifugal filter devices (Amicon, Millipore Corporation). Ten microliters of samples were mixed with non-reducing buffer and loaded onto 9% (w/v) polyacrylamide gels containing 0.4% (w/v) gelatin. After electrophoresis, gels were incubated twice in 2.5% (v/v) Triton X-100, rinsed with distilled water and incubated in enzyme activation buffer (100 mM Tris–HCl, pH 7.4, 15 mM $CaCl_2$) overnight at 37 °C. After staining with Coomassie blue (0.1% (w/v) in acetic acid:methanol (1:3)), and destaining (acetic acid:methanol (1:3)), MMP activity was detected as clear bands of gelatin degradation. The specificity of the observed bands was confirmed by incubating the gel in activation buffer containing 10 mM EDTA, a specific inhibitor of Ca^{2+}-dependent gelatinases/type IV collagenases (data not shown). MMP-9 activity was also measured in serum-free medium (Invitrogen) following HCoV-229E infection using the Fluorokine E human MMP-9 activity assay kit (R&D systems) according to the manufacturer's instruction.

2.11. *Chemotaxis assay*

Human recombinant chemokines CCL2, CCL5 and CXCL12 (R&D systems) were diluted in RPMI 1640 supplemented with

1% (v/v) FCS. Cell migration was evaluated using Transwell inserts with a 5-μM pore size fitted in 24-well plates (Corning Costar). Briefly, 100 μL of RPMI 1640 medium supplemented with 1% (v/v) FCS, containing 5×10^5 THP-1 cells or primary monocytes were loaded into the insert above the well containing 600 μL of the same medium containing either no chemokine or either one of the CCL2, CCL5 and CXCL12 chemokines, each at 100 ng/mL. After 4 h at 37 °C, cells that migrated across the insert towards the lower chamber were harvested and counted using a hemacytometer. An aliquot of these cells was allowed to adhere for 45 min to poly-D-lysine-coated glass slides and were processed as in Section 2.6, to detect viral antigens by immunofluorescence.

3. Results

3.1. Subsets of human PBMC are susceptible only to HCoV-229E infection

Infection of primary monocytes, macrophages and lymphocytes was performed at an MOI of 1 and virus replication was monitored for up to 7 days post-infection (dpi). HCoV-OC43 infectious viral particles were never detected in any of the PBMC cell components of 10 healthy donors. Similarly, HCoV-229E infectious virus could never be detected in lymphocytes. However, infectious HCoV-229E was detected at 1 dpi in monocytes and macrophages for most donors and production was low, with a maximum of 10^5 $TCID_{50}$/mL for macrophages and 10^4 $TCID_{50}$/mL for monocytes, and was below the detection level as early as 3 dpi (data not shown).

A kinetics of infectious virus production over the first 48 h post-infection (hpi) confirmed that primary monocytes and macrophages were susceptible to a weak productive HCoV-229E infection at an MOI of 1, which reached a maximum titer of 10^5 $TCID_{50}$/mL at 18 hpi, followed by a decrease at 24 and 48 hpi (Fig. 1A). In comparison, infectious HCoV-OC43 particles were below detection level as early as 24 hpi (Fig. 1A) and no HCoV-OC43 antigens could ever be detected (data not shown), indicating restriction of viral replication. Nevertheless, RT-PCR showed that HCoV-OC43 RNA remained present up to 48 hpi, even when the infection was performed at 4 °C, suggesting that it came from the inoculum that remained attached to cells (data not shown). A kinetics of infectious virus production confirmed that lymphocytes were not susceptible to infection, since neither

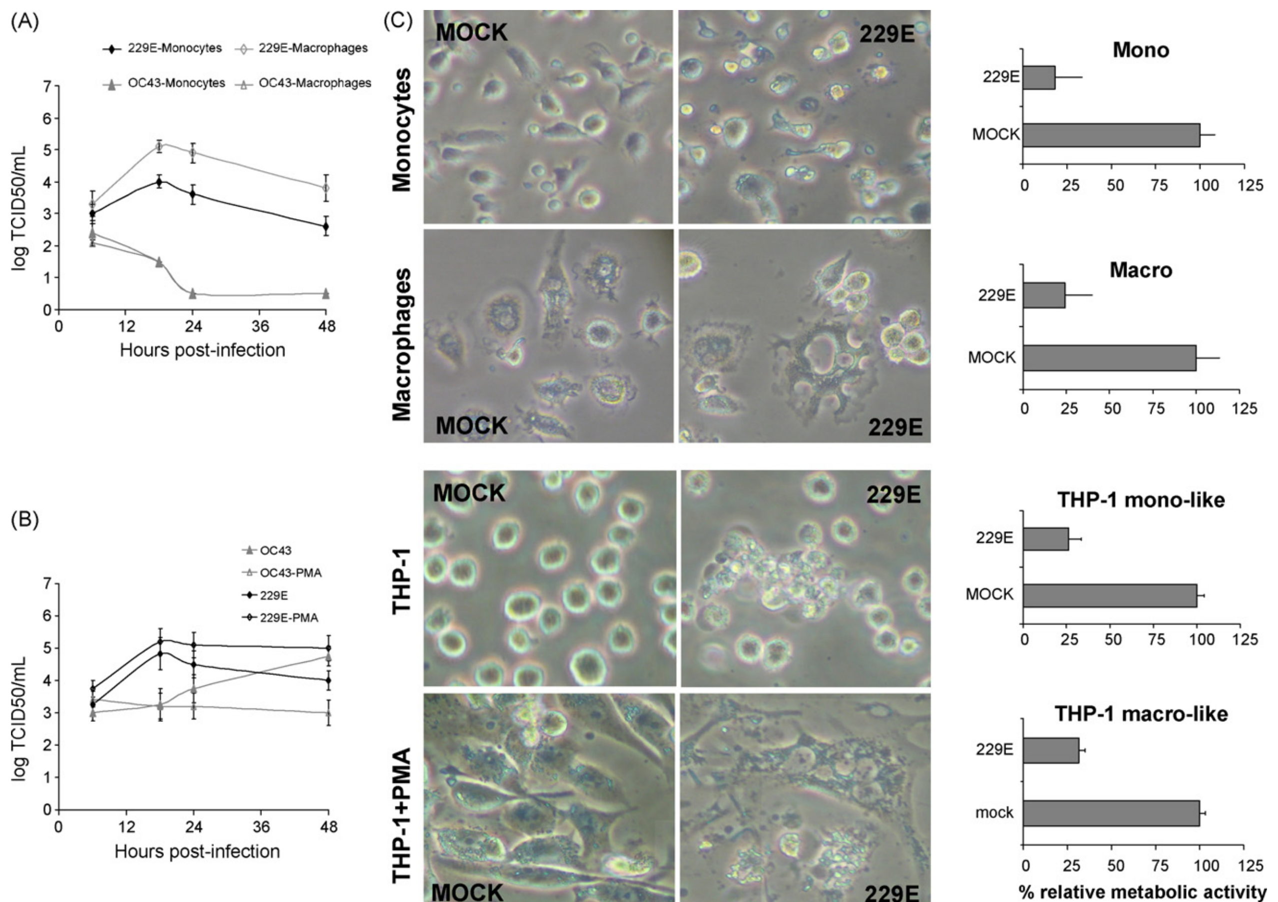

Fig. 1. Human primary monocytes and macrophages, as well as THP-1 cells, are susceptible to a productive HCoV-229E infection and to virus-induced cell death at high MOI. (A) Kinetics of infectious HCoV production in culture medium of human primary monocytes and macrophages. (B) Kinetics of infectious HCoV production in undifferentiated and PMA-differentiated THP-1 cells. In panels A and B, the limit of detection is 0.5 $TCID_{50}$/mL. (C) Evaluation of cell morphology and of the relative metabolic mitochondrial activity, indicative of cell viability, at 3 dpi in primary monocytes and macrophages, as well as in the undifferentiated and PMA-differentiated THP-1 cell line. All infections were performed at an MOI of 1, which induced cell death in a significant portion of the cell culture. Syncytia-like structures are easily observed in primary macrophages and PMA-differentiated THP-1 cells.

infectious virus nor viral antigens were ever detected (data not shown). Nevertheless, small amounts of viral RNA from both HCoV strains were detected in primary lymphocytes (data not shown).

3.2. The THP-1 human monocytic cell line: a model to study the interaction between human monocytes and HCoV-229E

Human monocytic THP-1 cells are widely used as an *in vitro* model to study interactions between viruses and monocytes (Nordoy et al., 2003; Ponzetto et al., 2004; Cooper et al., 2005; Yokota et al., 2005; Yen et al., 2006), because they display several characteristics of monocytes, and they can be induced to differentiate with PMA to more mature macrophage-like cells (Tsuchiya et al., 1982).

The kinetics of HCoV-229E infection of THP-1 cells (PMA-differentiated or not) at an MOI of 1 showed a similar pattern to primary monocytes and macrophages (Fig. 1B). Moreover, like primary monocytes and macrophages, the THP-1 cells were highly susceptible to HCoV-229E-induced cell death, regardless of the state of differentiation, as mitochondrial metabolic activity dropped significantly at 72 hpi (Fig. 1C), suggesting that the decrease in infectious titers may in part be due to a lower number of viable cells. Indeed, microscopic observation of infected cells revealed an important cytopathic effect (CPE) and the formation of syncytia-like structures in primary macrophages and in PMA-differentiated THP-1 cells (Fig. 1C). Also, similarly to primary monocytes and macrophages, the PMA-differentiated THP-1 cells restricted HCoV-OC43 replication, with no detection of production of infectious virus (Fig. 1B) or viral antigens (data not shown). However, the non-differentiated THP-1 cells were susceptible to a productive HCoV-OC43 infection (Fig. 1B), with no CPE, infected cells remaining as viable as mock-infected cells at 72 hpi (data not shown).

To correlate infectious virus production and cell viability, infections were performed at a MOI of 0.01, 0.1 and 1. As shown in Fig. 2, infectious HCoV-229E production (Fig. 2A) and cell survival (Fig. 2B) were very similar and dependent on the initial MOI for both primary monocytes and THP-1 cells. Indeed, infectious virus production was delayed in infections at lower MOI (0.01 and 0.1), but reached the same level at 48 hpi as with an MOI of 1 (Fig. 2A). As expected, cell viability was higher when infection was performed at these lower MOI (Fig. 2B).

When HCoV-229E infection of human primary monocytes/macrophages was performed at a MOI of 1, infectious virus was under the detection limit at 3 dpi but viral antigens were still easily detected within the infected cells until at least 5 dpi (data not shown). Even though primary macrophages may be kept in culture for a long period of time, primary monocytes are very difficult to keep in culture for more than

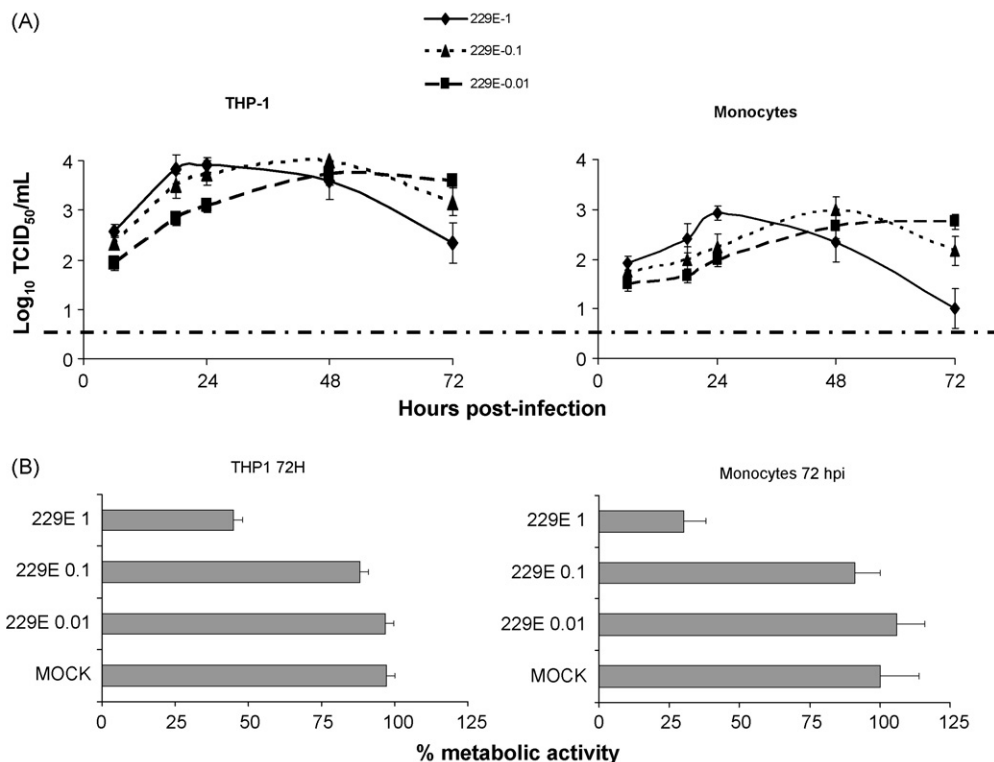

Fig. 2. Infectious HCoV-229E production and cell viability depends on the starting MOI in the human THP-1 monocytic cell line and in primary human monocytes. (A) Kinetics of infectious HCoV-229E production in culture medium after infection at different MOI (0.01, 0.1, and 1) over a 72-h period. (B) Evaluation of the relative cellular metabolic mitochondrial activity, indicative of cell viability, after infection by HCoV-229E (229E 1: infection at MOI of 1; 229E 0.1: infection at MOI of 0.1; 229E 0.01: infection at MOI of 0.01). Cell survival was in direct correlation with the starting MOI. Dotted lines represent the limit of detection of 0.5 $TCID_{50}$/mL. Results are representative of two independent experiments.

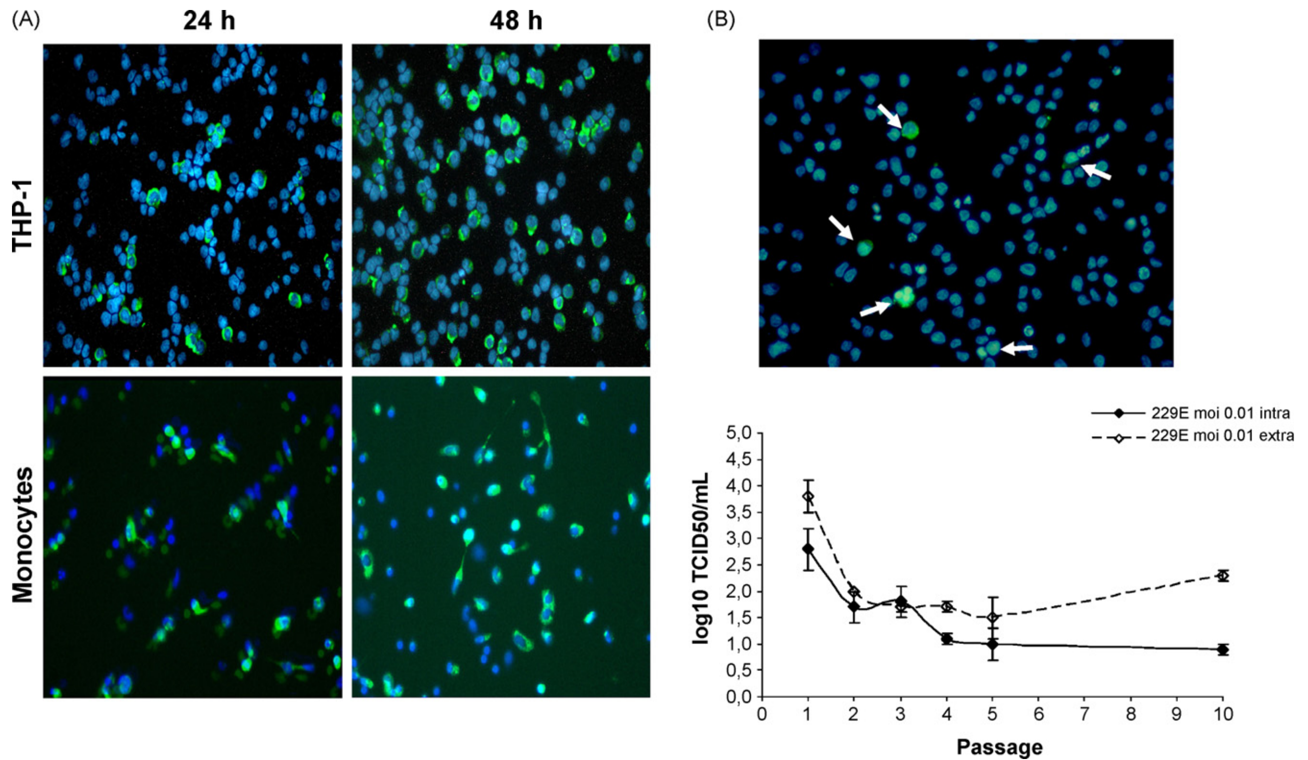

Fig. 3. Primary human monocytes and THP-1 cells are susceptible to HCoV-229E infection and THP-1 cells sustain a long-term productive infection by HCoV-229E. Infections were performed at an MOI of 0.1. Detection of HCoV-229E S protein (green), using HCoV-229E Mab 5-11H.6 in: (A) HCoV-229E-infected THP-1 cells and primary monocytes at 24 and 48 hpi; (B) THP-1 cells after 10 passages representing 2 months in culture (persistently infected cells). Cell nuclei were stained with DAPI (blue). White arrows indicate antigen-positive cells. Infectious virus titers in medium (extracellular) and within cells (intracellular) are indicated as log $TCID_{50}$/mL, showing that the long-term infection of THP-1 cells was weakly productive. Magnification 200×.

a few days. Therefore, primary cultures of monocytes did not survive long enough to characterize the establishment of a long-term infection. The THP-1 cells allowed us to investigate this possibility. When HCoV-229E infection of both primary monocytes and THP-1 cells was performed at a MOI of 0.1, the kinetics of infection was similar, as shown by infectious virus production (Fig. 2A) and detection of viral antigens (Fig. 3A). A long-term HCoV-229E infection of THP-1 cells was established for at least 10 cell passages, over a 2-month period. Indeed, a carrier-state type of persistent infection was observed: only a small proportion (1–2%) of the culture appeared to produce viral antigens, yielding a low infectious virus titer (Fig. 3B).

3.3. The THP-1 monocytic cell line and primary monocytes are activated following HCoV-229E infection

To investigate consequences of HCoV infection of THP-1 cells and of primary monocytes on their state of activation, we investigated the profile of cytokine/chemokine production following infection at a MOI of 0.1, the highest MOI that allowed survival of the majority of cells (Fig. 2B) and the establishment of a persistent infection of THP-1 cells, at 48 hpi, which corresponded to the peak of infectious virus production for all MOI tested for both THP-1 cells and primary monocytes. Results are shown in Fig. 4.

Even though the use of the cytokine array is not meant to be a precise quantitative measure of the amount of molecules produced, a reproducible increase in production of some cytokines/chemokines was observed following HCoV-229E infection in two independent experiments. Indeed, even though there was a constitutive production of low amounts of the pro-inflammatory cytokine TNF-α and of the chemokine CXCL10 in primary monocytes, of the chemokine CXCL11 in THP-1 cells and of the chemokine CCL5 (RANTES) by both types of cells, the level of production of these molecules was clearly increased after infection (Fig. 4). A slight but reproducible increase in IL-16 and CXCL12 production was also observed following infection of the THP-1 cells and primary monocytes, respectively (Fig. 4). Several other inflammatory factors (see complete list in Fig. 4) were produced by both cell types, without significant modulation after infection, including high levels of IL-1β, IL-1ra, CXCL1, MIF, CCL3, IL-6 and CXCL8. Strikingly, even though the steady-state profile of cytokine/chemokine was not exactly similar in THP-1 and primary monocytes, the production of the same four cytokines/chemokines, TNF-α, CCL5, CXCL10, and CXCL11, was reproducibly modulated the same way in either type of cells following HCoV-229E infection.

Since the production of the pro-inflammatory cytokine TNF-α was increased after HCoV-229E infection (Fig. 4), the kinetics of its production was quantitated after infection. HCoV-229E-

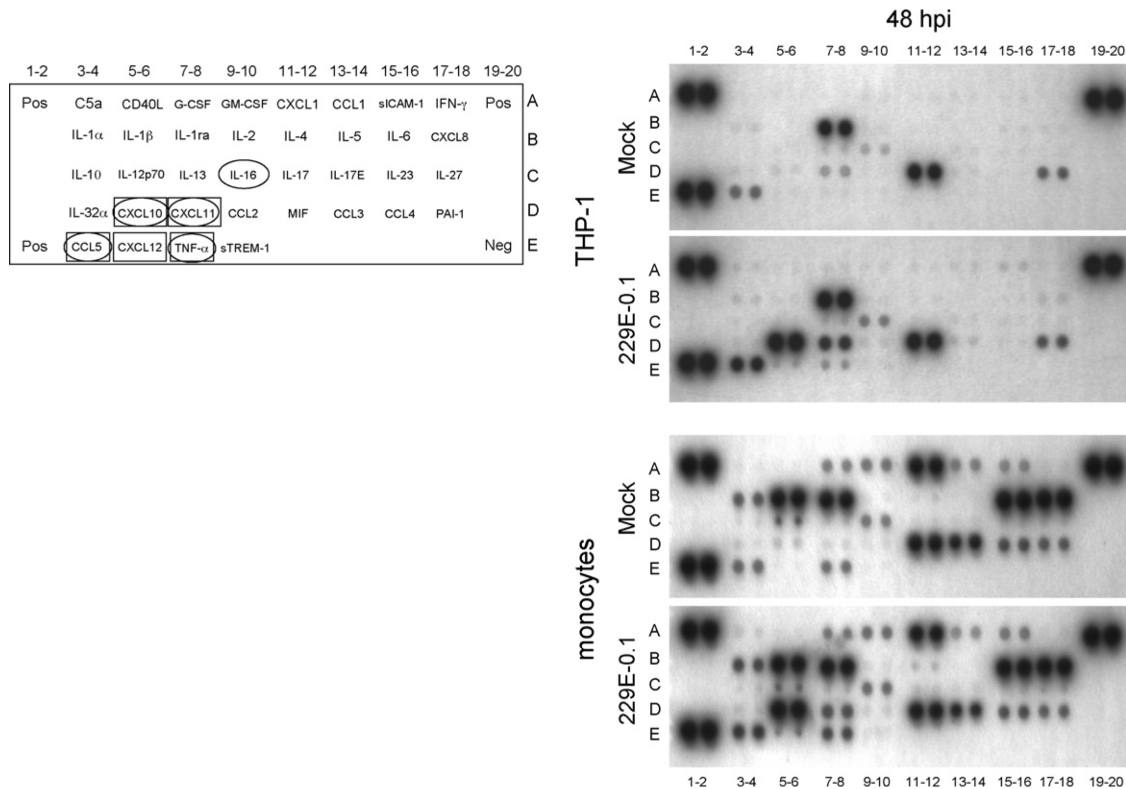

Fig. 4. Primary human monocytes and THP-1 cells showed a similar cytokine/chemokine profile after HCoV-229E infection. Evaluation of the profile of cytokines and chemokines secreted in mock-infected and HCoV-229E-infected THP-1 cells and primary monocytes using the Cytokine Array Proteome Profiler at 48 hpi. Cytokines/chemokines that were detected in the supernatant of HCoV-229E-infected THP-1 cells are circled and those that were in the supernatant of HCoV-229E-infected primary monocytes are boxed. Results are representative of two independent experiments.

infected THP-1 cells and primary monocytes produced an amount of TNF-α in direct correlation with the MOI, over a period of 72 h (Fig. 5A), confirming the result obtained with the cytokine/chemokine profiles (Fig. 4). A *t*-test analysis revealed that the production of TNF-α began to be significantly modulated at 48 hpi. Furthermore, the production of TNF-α was increased even more after HCoV-229E infection of PMA-differentiated macrophage-like THP-1 cells and UV-inactivated HCoV-229E also induced the production of a small amount of TNF-α that remained stable up until 96 hpi when compared to mock-infected THP-1 cells (data not shown).

Matrix metalloproteinases (MMP) can also be linked to monocyte activation. Therefore, the modulation of MMP-2 and -9 production and activity (type IV collagenases) was investigated. Two bands of gelatinolysis were identified as pro-MMP-9 (92 kDa) and pro-MMP-2 (72 kDa), based on their molecular weight in polyacrylamide gels and their inhibition in presence of EDTA. While pro-MMP-9 appeared to be constitutively expressed in the THP-1 cell line and primary monocytes, an increased expression was observed after 72 hpi with HCoV-229E, when compared to mock-infected cells (Fig. 5B). PMA was used as a positive control (data not shown). Even though PMA induced an increase in the expression of pro-MMP-2 and MMP-9 in THP-1 cells, no modulation of MMP-2 was detected upon infection of either cell type (data not shown). Quantitation of active MMP-9 by fluorescence ELISA revealed that the amount of MMP-9 secreted in the medium started to increase at 48 hpi and that the differences in MMP-9 secretion were statistically significant (*t*-test) between mock- and infected-cells (Fig. 5C).

3.4. The THP-1 monocytic cell line and primary monocytes show an increase in motility and chemokine-driven migration following HCoV-229E infection

Since monocytic cells were activated by HCoV-229E infection, their potential to respond to chemokines was evaluated by a chemotaxis assay. Our results indicate that an acute or persistent infection induced an increase in motility of the THP-1 cells and primary monocytes, and in the capacity of these cells to respond to CCL5 and CXCL12-driven migration *in vitro* (Fig. 6). Even though HCoV-229E-infected THP-1 cells were more responsive to the CCL2-driven migration at 24 hpi compared to mock-infected THP-1 cells, this increase was only transient, since no difference in migration was observed at 48 hpi (Fig. 6A and B). Persistently infected THP-1 cells were also slightly more responsive to the CCL2-driven migration. On the other hand, HCoV-229E and mock-infected primary monocytes were equally responsive to the CCL2-driven migration (Fig. 6A and B). Immunofluorescence analysis revealed that only a portion of the migrating cells were producing viral antigens (Fig. 6C).

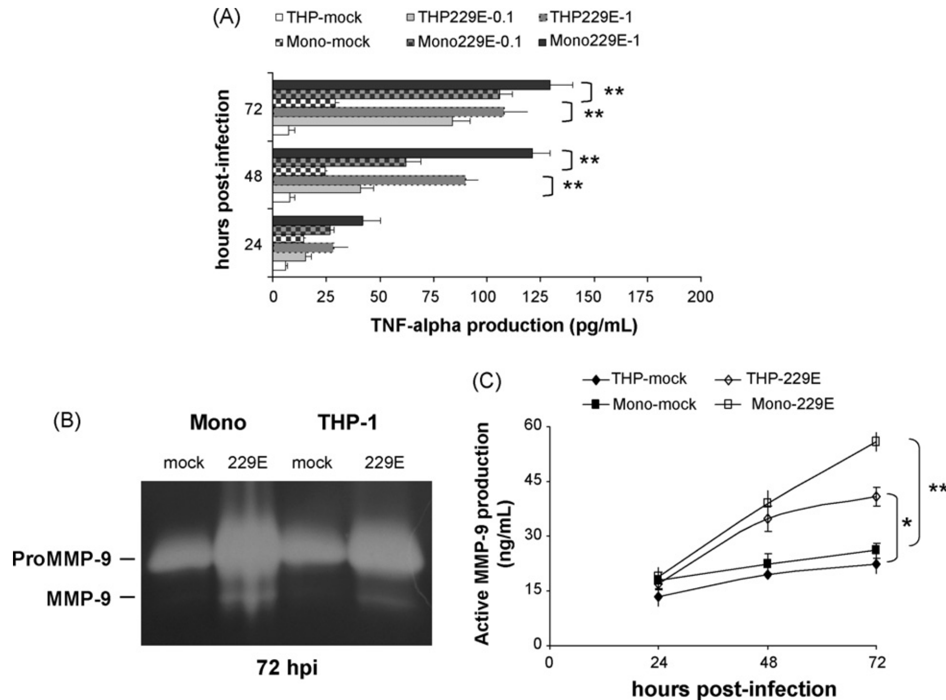

Fig. 5. Production and secretion of TNF-α and MMP-9 was significantly increased in THP-1 cells and primary monocytes after HCoV-229E infection. (A) TNF-α secretion in mock-infected THP-1 cells and primary monocytes, and in HCoV-229E-infected cells (MOI of 0.1 and 1) for 24, 48, and 72 h. (B) MMP-9 secretion in mock-infected or HCoV-229E-infected cells (MOI of 0.1) at 72 hpi. Cell supernatants were collected and assayed for MMP-9 content by gelatin-based zymography on polyacrylamide gels. (C) Active MMP-9 secretion in mock-infected and HCoV-229E-infected THP-1 cells and primary monocytes (MOI of 0.1) over a period of 72 h. Results are representative of two independent experiments. The levels of statistical significance between mock- and HCoV-infected cells were evaluated for each condition. The p values were calculated by a t-test and are represented by * where the difference between mock and infected cells was significant. $^{*}p < 0.05$; $^{**}p < 0.01$.

4. Discussion

HCoV are recognized respiratory pathogens, infecting mostly airway epithelial cells (Wang et al., 2000), but viral material was detected in host tissue other than the respiratory tract, including the CNS (Arbour et al., 2000). Since they have access to lung tissue and express CD13 (APN), the HCoV-229E receptor (Yeager et al., 1992), on their cell surface (Ashmun and Look, 1990), infected activated leukocytes could participate in initiating an overwhelming immune response that could lead to more serious respiratory pathologies, as well as represent vehicles for viral spread to other tissues, like it was shown for HIV-1 (Nottet et al., 1996; Persidsky et al., 1997) and human cytomegalovirus (HCMV) (Smith et al., 2004). Therefore, the present study was designed to evaluate this possible mechanism for HCoV-related pulmonary diseases, such as asthma exacerbations and respiratory distress syndrome in newborns, and in dissemination to host tissues such as the CNS.

The OC43 strain of HCoV (HCoV-OC43) was reported to be capable of productively infecting primary monocytes and macrophages (Collins, 1998, 2002). However, in our study, only viral RNA was detected (data not shown). This could be explained by virus attachment to cell surface lectin-like molecules, as shown for SARS-CoV (Lau and Peiris, 2005) and other enveloped viruses such as HIV, Dengue and hepatitis C virus (Altmeyer, 2004). Alternatively, it may be related to the differentiation state of monocytes and its correlation with susceptibility to HCoV-OC43 infection. Indeed, promonocytic THP-1 cells, which were susceptible to a weak productive HCoV-OC43 infection, represent less differentiated cells than primary monocytes/macrophages. Upon PMA-induced differentiation, they lost susceptibility to infection, which is consistent with monocytes being only susceptible to infection during a brief period during their differentiation. This was also suggested for HIV-1 (Schuitemaker et al., 1992; Pauza et al., 1988), HCMV (Weinshenker et al., 1988) and measles virus (Helin et al., 1999).

The detection of HCoV-OC43 RNA in primary monocytes/macrophages (data not shown) is consistent with these cells participating in virus dissemination, as suggested for SARS-CoV (Yilla et al., 2005). However, infectious HCoV-OC43 particles were not detected as early as 24 hpi post-infection, suggesting that viral RNA detected at 48 hpi represents non-infectious virus, still cell-associated.

On the other hand, primary monocytes and macrophages were susceptible to HCoV-229E infection (Fig. 1A), in contrast with a report of an abortive HCoV-229E infection of peritoneal macrophages (Patterson and Macnaughton, 1982). Our results with primary monocyte-derived human macrophages not only confirm susceptibility to HCoV-229E, but demonstrate their production and release of small amounts of infectious virus into the culture medium. Moreover, detection of HCoV-229E antigens up to 5 dpi (data not shown) suggests that virions still remain in cells for a while even after a short burst of infectious virus produc-

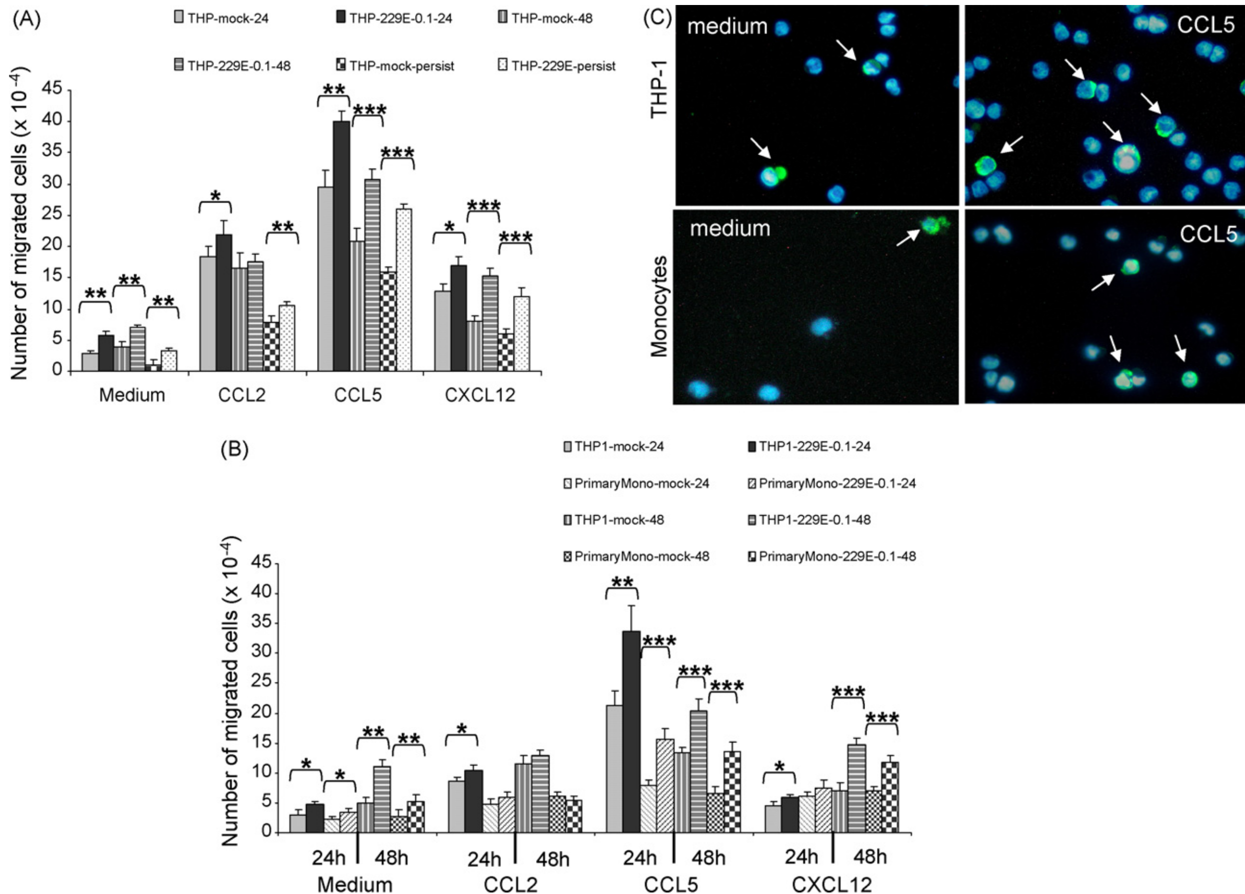

Fig. 6. THP-1 cell and primary monocyte motility and chemokine-driven migration are increased after HCoV-229E infection. Infections were performed at a MOI of 0.1. (A) Chemotaxis assay showed that HCoV-229E infection of THP-1 cells by itself induced a significant increase in cell motility and migration across Transwell inserts. CCL5 and CXCL12-driven migration was also significantly increased following infection but CCL2-driven migration was only significantly modified at 24 hpi and in persistently infected cells. (B) A comparative chemotaxis assay showed that both THP-1 cell and primary human monocyte motility and chemokine-driven migration across Transwell inserts was significantly increased. CCL5-driven migration was already significantly increased at 24 hpi and remained at that level until 48 hpi and CXCL12-driven migration was significantly increased at 48 hpi. CCL2-driven migration was only significantly increased at 24 hpi for the THP-1 cells but not significantly modified for primary monocytes. (C) A representative field of THP-1 cells and primary monocytes that have migrated across Transwell inserts and were allowed to adhere to poly-D-lysine-coated glass slides. Detection of HCoV-229E S protein (green cells with white arrows) was performed using the Mab 5-11H.6. Cell nuclei were stained with DAPI (blue). Results are representative of three independent experiments. Mock-infected THP-1 cells (THP-mock) at 24, 48 hpi and at 60 dpi (persist: persistently infected kept in culture for 2 months, representing 10 passages) were compared to HCoV-229E-infected THP-1 cells (THP-229E-0.1) at 24, 48 hpi and 60 dpi and mock-infected primary monocytes (PrimaryMono-mock) at 24 and 48 hpi were compared to HCoV-229E-infected primary monocytes (PrimaryMono-229E-0.1) at 24 and 48 hpi. The levels of statistical significance between mock- and HCoV-infected cells were evaluated for each condition. The p values were calculated by a t-test and are represented by * where the difference between mock and infected cells was significant. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

tion. Kinetics of monocyte infection (Fig. 1A) suggests that it is productive, as previously reported (Collins, 2002). However, in contrast to the latter report, our results show a transient productive infection, with titers starting to drop between 24 and 48 hpi when an initial MOI of 1 is used. We also show that infection led to cell death in a portion of the cell culture in a MOI-dependent manner (Fig. 1C), which may involve apoptosis (Collins, 2002).

Our results clearly indicate that susceptibility of THP-1 cells to HCoV-229E infection correlates very well with results on primary cells. A weakly productive long-term HCoV-229E infection of THP-1 cells at lower initial MOI was also previously reported for HIV-1 (Mikovits et al., 1990) and cells remained responsive to PMA-induced differentiation into macrophage-like cells and produced more infectious virus (Konopka et al., 1993). Moreover, even though monocytes have a life span of only a few days in circulation (Cline et al., 1978), a long-term infection in THP-1 monocytic cells is highly relevant, since during their migration towards tissue, monocytes differentiate into macrophages, which are long-lived cells that can harbor virus for a long period of time. Persistently infected THP-1 cells had an increased motility and were still responsive to chemokines (Fig. 6A), which strongly suggests that monocytes/macrophages could be a reservoir and potential vector for HCoV-229E spread into host tissue, as suggested for HCMV (Söderberg-Naucler et al., 1994; Sinclair and Sisson, 1996). Moreover, we show that HCoV-229E infection of THP-1 cells and primary monocytes leads to their activation, as indicated by the production of several inflammation-related mediators and responsiveness to chemokine gradients.

The faint increase in IL-16 production by infected THP-1 cells (Fig. 4) could be caused by apoptosis of a small portion of the culture, as reported for primary monocytes (Elssner et al., 2004). However, the increase in IL-16 was not seen in primary monocytes, as these cells produced it constitutively (Fig. 4). Others have reported a faint increase in CXCL8 production and no production of TNF-α following HCoV-229E infection of THP-1 cells (Yen et al., 2006). This apparent discrepancy may be due to their use of THP-1 cells manipulated to overexpress DC-SIGN, which could have altered their capacity to produce TNF-α, or to the fact that they measured TNF-α production at 1 dpi, whereas we analyzed its production over a 3-day period. The same may apply to the apparent discrepancy in the production of CXCL8 in HCoV-229E infected THP-1 cells (Yen et al., 2006). Constitutive production of high levels of cytokines/chemokines by primary monocytes (Fig. 4) is not surprising: for instance CXCL8, IL-1β, IL-6 and TNF-α were shown to be constitutively produced by human monocytes (Naldini et al., 2006).

The pro-inflammatory cytokine TNF-α was constitutively produced at very low levels in THP-1 and primary cells (Fig. 5A), consistent with reports for THP-1 cells (Heidinger et al., 2006) and primary monocytes (Nguyen et al., 2006). Interestingly, we show that TNF-α production was modulated by viral replication in a MOI-dependent manner in both cell types (Fig. 5A). This cytokine was linked to some asthmatic complications and in acute respiratory distress syndrome (ARDS; Mukhopadhyay et al., 2006), two pathologies associated with sporadic HCoV infection (Nicholson et al., 1993; Sizun et al., 1995; El-Sahly et al., 2000; Gagneur et al., 2002). On the other hand, TNF-α was shown to increase ICAM-1 expression on endothelial cells (Weirtheimer et al., 1992) including those of the BBB (Defazio et al., 1998), and ICAM-1 is known to facilitate the passage of leukocytes (including monocytes) through the BBB (Dietrich, 2002). Therefore, considering the neuroinvasive properties of HCoV-229E, the production of TNF-α by infected monocytes could lead to an increase spread of these cells towards other tissues, including the CNS.

The strong production of the CXCL10 and CXCL11 chemokines after HCoV-229E infection is very interesting since these chemokines have a high chemotactic potential towards T cells (Qin et al., 1998) and they were upregulated and provoked an increased recruitment of T cells after HIV-1 infection of monocyte-derived macrophages (Foley et al., 2005). The increased production of CCL5 in HCoV-229E-infected cells is also of interest as this chemokine has a chemoattracting potential for memory and activated T cells (Schall et al., 1990), NK cells (Loetscher et al., 1996), dendritic cells (Lin et al., 1998), and monocytes (Fine et al., 2001). Therefore, migration of HCoV-229E-infected monocytes into the respiratory tract could lead to recruitment of additional immune cells that may eventually cause an uncontrolled upregulation of inflammatory molecules, as is the case in ARDS. Indeed, an increased level of expression of matrix metalloproteinases (MMP) was seen in patients with ARDS (Corbel et al., 2002), an interesting observation considering that HCoV-229E was associated with severe respiratory distress syndrome in newborns (Gagneur et al., 2002). Moreover, it was recently shown that the *in vitro* recruitment of human peripheral blood leukocytes exposed to supernatants of SARS-CoV and HCoV-229E-infected THP-1 DC-SIGN cells (Yen et al., 2006) was significantly higher compared to supernatant from uninfected cells.

In the particular case of the CNS, a role for CCL5 in the pathogenesis of MHV-induced neurologic disease was suggested, which correlated with macrophage infiltration and demyelination (Lane et al., 2000). Also, expression of CCL5 and its CCR5 receptor was strongly upregulated during CNS infection by West Nile virus, which was associated with an important infiltration of immune cells including macrophages (Glass et al., 2005). As HCoV-229E is also neuroinvasive (Arbour et al., 2000), migration of HCoV-229E-infected monocytes into the CNS is plausible and the increased production of MMP-9 by monocytes following HCoV-229E infection was also previously described for HIV-1-infected monocytes (Dhawan et al., 1995).

Given that MMP-9 is implicated in promoting leukocyte trafficking across the BBB (Gidday et al., 2005; Bar-Or et al., 2003), the upregulation of MMP-9 in THP-1 cells and primary monocytes following HCoV-229E infection is highly relevant. A release of cytokines, such as TNF-α, during infection may also be involved in the upregulation of MMP in monocytes/macrophages (Welgus et al., 1992). The significant induction of TNF-α production by monocytes after HCoV-229E infection (Fig. 5A) suggests a synergistic effect between MMP-9 and TNF-α to facilitate the migration of monocytes into tissues, including the CNS. The MMP-9 production of monocytes was enhanced in the presence of chemokines such as CCL5 and CXCL12 (Webster and Crowe, 2006). A role for neutrophil-derived MMP-9 (Zhou et al., 2002) was also suggested in the breakdown and integrity loss of the BBB (Zhou et al., 2003) during MHV infection of the CNS.

The increased motility of HCoV-229E-infected monocytes (Fig. 6) is consistent with the hypothesis that infected monocytes could serve as vectors for viral spread to tissues, as suggested for HCMV (Smith et al., 2004). Furthermore, the increased chemokine-driven migration of infected cells suggests that migration of HCoV-229E-infected monocytes towards tissue could be facilitated during an inflammatory response in which the production of CCL2 and CCL5 would be increased within the inflamed tissue. The production of CCL5 appear more important than that of CCL2 in facilitating migration of HCoV-229E infected cells towards tissue, since the increased migratory capacities of infected cells were more evident towards CCL5 (Fig. 6). The increased responsiveness to CXCL12 (Fig. 6) is also very interesting since this chemokine is constitutively produced in tissues such as the CNS (Cartier et al., 2005). This latter chemokine was shown to facilitate the passage of lymphocytes and monocytes through endothelial cells *in vitro* (Wu et al., 2000) and was implicated in the baseline traffic of T cells and monocytes in the CNS (Calderon et al., 2006).

Monocytes can be divided into two main subsets, a short-lived “inflammatory subset” that homes to inflamed tissue to trigger an immune response, and a “resident” subset, with a longer life span, that homes to noninflamed tissues, including the lungs where they serve to replenish either the alveolar macrophages or the dendritic cell populations (Landsman et al.,

2007), and the brain, where they serve as putative microglia precursors (Geissmann et al., 2003). Either type of monocytes may propagate a viral infection (Reuter et al., 2004). Therefore, virus-induced monocyte activation could facilitate their passage through endothelial cells towards host tissue, especially the CNS in immunocompromised individuals, as reported for murine cytomegalovirus (MCMV) (Reuter et al., 2004). The fact that HCoV-229E could only infect partially immunocompromised transgenic mice (Lassnig et al., 2005) suggests that HCoV-229E could also take advantage of immunosuppression to gain access to tissue other than the respiratory tract, including the CNS in susceptible individuals.

Altogether, our results indicate that monocytes are susceptible to HCoV-229E infection and that they become activated shortly after being infected, as shown by their increased capacity to produce pro-inflammatory mediators and chemokines. This could have important consequences on the development of more serious respiratory tract diseases and even in the capacity of the virus to invade other tissues. Even though HCoV-229E is a respiratory pathogen, our previous studies did demonstrate that it has neuroinvasive properties. Since monocytes have access to CNS, our observation that monocytes/macrophages support coronavirus replication is consistent with the possibility that monocytes may contribute to HCoV-229E spread to the CNS. Moreover, the long-term infection of the THP-1 monocytic cell line strongly suggests the possibility of a viral reservoir within monocyte subsets and is consistent with human monocytes harboring HCoV and participating in virus spread towards tissue in general and particularly the CNS.

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