High Secretion of Interferons by Human Plasmacytoid Dendritic Cells upon Recognition of Middle East Respiratory Syndrome Coronavirus

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ABSTRACT

The Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in 2012 as the causative agent of a severe respiratory disease with a fatality rate of approximately 30%. The high virulence and mortality rate prompted us to analyze aspects of MERS-CoV pathogenesis, especially its interaction with innate immune cells such as antigen-presenting cells (APCs). Particularly, we analyzed secretion of type I and type III interferons (IFNs) by APCs, i.e., B cells, macrophages, monocyte-derived/myeloid dendritic cells (MDDCs/mDCs), and by plasmacytoid dendritic cells (pDCs) of human and murine origin after inoculation with MERS-CoV. Production of large amounts of type I and III IFNs was induced exclusively in human pDCs, which were significantly higher than IFN induction by severe acute respiratory syndrome (SARS)-CoV. Of note, IFNs were secreted in the absence of productive replication. However, receptor binding, endosomal uptake, and probably signaling via Toll-like receptor 7 (TLR7) were critical for sensing of MERS-CoV by pDCs. Furthermore, active transcription of MERS-CoV N RNA and subsequent N protein expression were evident in infected pDCs, indicating abortive infection. Taken together, our results point toward dipeptidyl peptidase 4 (DPP4)-dependent endosomal uptake and subsequent infection of human pDCs by MERS-CoV. However, the replication cycle is stopped after early gene expression. In parallel, human pDCs are potent IFN-producing cells upon MERS-CoV infection. Knowledge of such IFN responses supports our understanding of MERS-CoV pathogenesis and is critical for the choice of treatment options.

IMPORTANCE

MERS-CoV causes a severe respiratory disease with high fatality rates in human patients. Recently, confirmed human cases have increased dramatically in both number and geographic distribution. Understanding the pathogenesis of this highly pathogenic CoV is crucial for developing successful treatment strategies. This study elucidates the interaction of MERS-CoV with APCs and pDCs, particularly the induction of type I and III IFN secretion. Human pDCs are the immune cell population sensing MERS-CoV but secrete significantly larger amounts of IFNs, especially IFN- α , than in response to SARS-CoV. A model for molecular virus-host interactions is presented outlining IFN induction in pDCs. The massive IFN secretion upon contact suggests a critical role of this mechanism for the high degree of immune activation observed during MERS-CoV infection.

In 2012 a novel human betacoronavirus associated with severe respiratory disease emerged in Saudi Arabia (1). Due to its geographic distribution, this new virus was classified as Middle East respiratory syndrome coronavirus (MERS-CoV) (2). MERS-CoV is associated with high fatality rates (3, 4), and case numbers globally have increased to 909 laboratory-confirmed cases with 331 fatalities (as of 21 November 2014 [<http://www.who.int/csr/don/21-november-2014-mers/en/>]). In parallel, the geographic distribution has expanded (4). MERS-CoV is the second emerging CoV with severe pathogenicity in humans within 10 years after the severe acute respiratory syndrome coronavirus (SARS-CoV) that infected approximately 8,000 people worldwide during its spread in 2003 (5). Human-to-human transmissions have been reported for MERS-CoV, but transmissibility seems to be inefficient (6, 7). MERS-CoV persists in animal reservoirs, i.e., dromedary camels (8), and transmission events between camels and contact persons have been reported (7–10). Thus, MERS-CoV infection of men has zoonotic origins, similar to SARS-CoV, but unlike SARS-CoV, where bats have been identified as the original virus reservoir, bats have been reported to host only closely related viruses of MERS-CoV (11). However, the only small-animal model developed so far

involves type I interferon receptor (IFNAR)-deficient mice expressing human dipeptidyl peptidase 4 (huDPP4; CD26), the entry receptor of MERS-CoV (12), in the lung after intranasal administration of huDPP4-expressing adenoviral vectors (13). MERS-CoV causes symptoms in humans similar to those of SARS-CoV infection, such as severe pneumonia with acute respi-

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ratory distress syndrome, leukopenia and lymphopenia (14), septic shock, and multiorgan failure. A special feature of MERS-CoV infection is that it can cause renal complications which may end in renal failure (15). The unusual tropism of MERS-CoV has been related to the wide tissue distribution of DPP4, e.g., on renal epithelial cells or leukocytes (16).

MERS-CoV replication is sensitive to type I and type III interferons (IFN) *in vitro* (17, 18), and macaques can be protected by administration of IFN- β in combination with ribavirin (19). However, a benefit of IFN- β treatment could not be confirmed in five severely ill human patients in whom disease had presumably progressed too far (20, 21). Sensitivity of MERS-CoV to IFNs indicates that innate immunity and IFN secretion are critical parameters for the outcome of MERS-CoV infection. Type I IFNs, particularly IFN- β , can be produced by most stromal cell types upon viral infection. Indeed, MERS-CoV actively suppresses type I IFN production in a variety of infected cell types, such as primary airway epithelial cells (18, 22). Additionally, professional antigen-presenting cells (APCs) are an important source of type I IFNs upon recognition of pathogen-associated molecular patterns (PAMPs) (23). Particularly, plasmacytoid dendritic cells (pDCs) have been shown to secrete large amounts of IFN- α after contact with virus (e.g., HIV-1 [24] or SARS-CoV [25]). Type I IFNs have a significant bystander effect on uninfected neighboring cells by inducing an antiviral state, activating innate immune cells, and priming adaptive immunity. On the other hand, over shooting secretion of IFN can result in cytokine dysregulation and immune pathogenesis (26).

To analyze the role of primary innate immune cells, especially their IFN secretion levels during MERS-CoV infection, we inoculated a range of professional APCs and pDCs with MERS-CoV. No type I or type III IFNs were produced by murine myeloid DCs (mDCs), pDCs, or peritoneal exudate cells (PECs) after contact with MERS-CoV. Most interestingly, this was also the case for all human APC cell types, which did not react to MERS-CoV with IFN secretion. Human pDCs, however, produced large amounts of IFN- α and IFN- β and moderate amounts of IFN- λ upon contact with MERS-CoV without virus amplification. The observed IFN induction was dependent on the availability of the MERS-CoV receptor DPP4, endosomal maturation, or partially on PAMP recognition via Toll-like receptor 7 (TLR7) and correlated with *de novo* expression of MERS-CoV N protein. The large amounts of type I IFNs which are secreted by pDCs during MERS-CoV infection suggest that type I IFNs hold a key position in MERS-CoV infection.

MATERIALS AND METHODS

Cell lines and viruses. Vero cells (ATCC CCL-81) and BHK-21 cells (C-13) (ATCC CCL-10) were purchased from the ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Cologne, Germany) supplemented with 2 mM glutamine and 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany) at 37°C in a humidified atmosphere containing 6% CO₂ for no longer than 6 months of culture after thawing of the original stock. MERS-CoV (EMC/2012) (14) and SARS-CoV (strain Frankfurt-1) (27) were propagated in Vero cells. Titers were determined by 50% tissue culture infection dose (TCID₅₀) titration on Vero cells (28). Virus stocks were stored in aliquots at -80°C. Inactivated MERS-CoV was generated by UV inactivation (120,000 μ J/cm² UV light [254 nm] for 90 min) (Stratalinker UV Cross-linker; Stratagene, La Jolla, CA) of 0.1 ml of virus suspension in 48-well plates on ice. Thogoto virus, an influenza-like orthomyxovirus inducing type I IFNs in

murine mDCs, lacking an elongated matrix protein [THOV(Δ ML)] (29), and vesicular stomatitis virus M2 (VSV-M2) (30), a variant of VSV with defects in M protein functionality that induces high IFN responses in cells (31), were propagated on BHK-21 cells and titrated via plaque assay on Vero cells as described previously (30).

Isolation and generation of human professional antigen-presenting cells and pDCs. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Biochrom) density gradient centrifugation from buffy coats (Blutspendedienst, Frankfurt am Main, Germany) or citrate-blood samples of anonymized healthy human volunteers. Human B cells were purified by negative selection using a B-Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured as described before (32). Monocytes were purified by positive selection using CD14 MicroBeads (Miltenyi Biotec). For generation of monocyte-derived DCs (MDDCs), 2×10^5 CD14⁺ monocytes were cultured in 96-well flat-bottom tissue culture plates using X-VIVO 15 medium (Lonza) in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF; 1,000 U/ml) (CellGenix, Freiburg, Germany) and interleukin-4 (IL-4; 1,000 U/ml) (CellGenix) for 5 days (33). For generation of GM-CSF-derived (M1) macrophages, monocytes were cultured in X-VIVO 15 medium supplemented with 10 ng/ml GM-CSF. For macrophage colony-stimulating factor (M-CSF)-derived (M2) macrophage generation, monocytes were cultured in RPMI 1640 medium containing 10% FBS, 10 mM L-glutamine, 0.5 mM penicillin-streptomycin (PAA Laboratories, Egelsbach, Germany), 0.1 mM nonessential amino acids (Biochrom), and 30 ng/ml M-CSF (R&D Systems, Wiesbaden-Nordendstadt, Germany) (34). Untouched human plasmacytoid dendritic cells (pDCs) were isolated by negative selection from PBMCs using a plasmacytoid dendritic cell isolation kit (Miltenyi Biotec) and cultured in RPMI 1640 medium (Biowest, Nuaille, France) containing 10% fetal calf serum (FCS; Lonza), 10 mM L-glutamine, and 100 ng/ml recombinant IL-3 (R&D Systems). For subsequent experiments, all APCs were seeded at a density of 2.5×10^5 APCs/well, and pDCs were seeded at a density of 2×10^4 pDCs/well in 96-well plates in 200 μ l of medium.

Generation of murine professional antigen-presenting cells. Murine bone marrow-derived myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) were generated from bone marrow cells isolated from femurs and tibias of sacrificed 6- to 10-week-old C57BL/6N mice by differentiation with GM-CSF (R&D Systems) or Flt-3L (R&D Systems) for 8 days, as described before (35). Peritoneal exudate cells (PECs) were isolated from sacrificed 6- to 10-week-old C57BL/6N mice by flushing out cells from the abdominal cavity with 5 ml of phosphate-buffered saline (PBS) and seeding at 2×10^5 cells/ml in 200 μ l of RPMI 1640 medium (Biowest).

Virus growth kinetics. Vero cells, APCs, or pDCs were infected at a multiplicity of infection (MOI) of 0.01 or 5. Cells were washed once at 1 h postinfection (hpi) and incubated in the respective cell culture medium. At the time points indicated in the figures, cell-free supernatants were sampled and stored at -80°C. Titers were determined by TCID₅₀ titration on Vero cells as described above.

Analysis of type I and III interferon secretion. Innate immune cells were inoculated with MERS-CoV, SARS-CoV, or UV-inactivated MERS-CoV. VSV-M2 (MOI of 0.1), THOV (MOI of 0.1), CpG 2216 (5 μ g/ml), or CpG 2006 (5 μ g/ml) (Invitrogen Life Technologies) (36) was used as a control. Cell-free supernatant was collected at 24 hpi and stored at -80°C. Supernatants of human cells were analyzed for secreted IFNs using a human IFN- α enzyme-linked immunosorbent assay (ELISA) (Mabtech AB, Nacka Strand, Sweden), human IFN- β ELISA (R&D Systems), human IL-29 (IFN- λ 1) ELISA (eBioscience, Frankfurt, Germany), or a human IL-6 DuoSet ELISA development system (R&D Systems) according to the manufacturers' instructions. Supernatants of murine cells were analyzed using a mouse IFN- α or mouse IFN- β ELISA (PBL Biomedical Laboratories, Piscataway, NJ) kit. To inhibit endosomal maturation or TLR7 signaling, pDCs were preincubated for 30 min at 37°C with 5 μ M chloroquine (Sigma) or 5.6 μ M inhibitory oligonucleotide (ODN) IRS661

FIG 1 Inoculation of murine cells with MERS-CoV. (A) Type I IFN secretion by murine immune cells. Cells were inoculated with MERS-CoV (MOIs as indicated), SARS-CoV, or the indicated positive controls. Single dots, individual experiments; horizontal lines, means. IFNs were measured at 24 hpi. b.d., below detection. Limits of detection were 12.5 pg/ml for IFN- α and 15.6 pg/ml for IFN- β . (B) Growth kinetics of MERS-CoV on Vero cells or murine APCs (MOI of 0.01). Indicated cell types were inoculated with virus, and sampled supernatants were titrated. Filled symbols, MERS-CoV; open symbols, SARS-CoV; $\diamond$, PECs; $\circ$, mDCs; $\square$, pDCs. Values are means of three independent experiments; error bars indicate standard deviations. conc, concentration.

(Invitrogen Life Technologies), respectively, and infected with MERS-CoV (MOI of 1) in the presence of inhibitors. To inhibit receptor binding of MERS-CoV, pDCs were preincubated for 30 min at 37°C with the recombinant receptor binding domain (RBD) consisting of residues 358 to 588 of the MERS-CoV spike protein (S) or with IgG1-Fc control protein (40 ng/ml) (37) before infection (MOI of 1).

qRT-PCR. A total of 2×10^4 pDCs were infected with MERS-CoV (MOI of 3) and washed once with medium at 1 hpi. Total RNA of infected cells was isolated using an RNeasy Plus minikit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Isolated RNA (10 μ l) was subjected to quantitative reverse-transcription PCR (qRT-PCR) using a SuperScript III Platinum OneStep qRT-PCR System (Invitrogen Life Technologies) with primers N2-Forward and N2-Reverse and probe N2-Probe (labeled 5' with 6-carboxyfluorescein and 3' with Black Hole Quencher 1) as described previously (38) utilizing an ABI7900 HT Fast Real-Time PCR System (Invitrogen Life Technologies). The amplification protocol was as follows: RT at 50°C for 30 min, initial denaturation at 95°C for 2 min, and PCR consisting of 40 cycles at 95°C for 15 s and 55°C for 1 min, with a final elongation at 55°C for 5 min. Data were normalized to cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, which was quantified using a SuperScript III Platinum SYBR green OneStep qRT-PCR System (Invitrogen Life Technologies) with primers GapDH fwd (5'-GGCGATGCTGGCGCTGAGTAC-3') and GapDH rev (5'-TGGTCCACACCCATGACGA-3') for human GAPDH and mGAPDH fwd (5'-CACCACTGCTTAGCCCC-3') and mGAPDH rev (5'-TCTTCTGGGTGGCAGTGATG-3') for murine GAPDH. The amplification protocol was as follows: 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 56°C for 1 min, 72°C for 30 s, and 95°C for 15 min. The normalized change in the cycle threshold (C_T) value [$\Delta C_T = C_{T(\text{MERS vRNA})} - C_{T(\text{GAPDH mRNA})}$], where vRNA is viral RNA] thus describes the difference between threshold cycle numbers for qRT-PCR signals of viral RNA and cellular mRNA for a given sample. Therefore, the lower the ΔC_T , the higher is the relative amount of vRNA in the sample. Due to exponential amplification of DNA during PCR, differences (n) between ΔC_T values were converted to x -fold ratios using the formula $x = 2^{-n}$, assuming optimal amplification for all samples.

Immunoblotting. For detection of CoV N protein expression, 5×10^4 pDCs were incubated with MERS-CoV (MOI of 3) and washed once with medium at 1 hpi or 8 hpi. For blocking experiments, cells were preincubated with the respective blocking agents as described above or with human DPP4/CD26 affinity-purified polyclonal antibody (R&D Systems) or goat IgG control (R&D Systems) (40 μ g/ml) (12) for 30 min at 37°C

before infection. Subsequently, washed pDCs were lysed and subjected to immunoblot analysis as described previously (39). MERS-CoV N protein was detected using polyclonal rabbit anti-MERS-CoV serum (1:1,000) with donkey horseradish peroxidase (HRP)-conjugated anti-rabbit IgG(H+L) (1:10,000) (Rockland, Gilbertsville, PA); β -actin was detected by mouse monoclonal anti- β -actin antibody (AC-15; 1:5,000) (ab6276; Abcam, Cambridge, United Kingdom) with HRP-conjugated rabbit anti-mouse secondary antibody (Invitrogen Life Technologies). Pierce ECL 2 Western blotting substrate (Thermo Scientific) on Amersham Hyperfilm ECL (GE Healthcare) was used for detection of specific bands.

Flow cytometry analysis. Flow cytometry was performed on an LS-RII-SORP fluorescence-activated cell sorter (FACS; BD, Heidelberg, Germany), and data were analyzed using FACSDiva, version 6.1.3, or FCS Express, version 3 (De Novo Software, Los Angeles, CA). Cells were stained and analyzed as described previously (39) using the following antibodies: murine anti-human CD26-phycoerythrin (PE) (BA5b; Biolegend, San Diego, CA), murine anti-human CD14-fluorescein isothiocyanate (FITC) (M5E2; BD), murine anti-human CD19-PE (HIB19; BD Bioscience), murine anti-human CD123-PE (9F5; BD Bioscience), or murine anti-human DC303-allophycocyanin (APC) (BD Bioscience) according to the manufacturers' instructions. Fc block was performed with Gammagard (1.25 mg/ml; Baxter, Deerfield, IL). Viability was checked by Fixable Viability Dye eFluor 780 (eBioscience).

RESULTS

Analysis of type I IFN secretion in murine immune cells. Due to the sensitivity of MERS-CoV to IFNs and the important role of innate immune cells in pathogen recognition and IFN secretion, we were interested in which innate immune cell subsets produce type I or type III IFNs upon contact with MERS-CoV. Therefore, type I IFN secretion by murine APCs and pDCs inoculated with MERS-CoV was analyzed first. Murine PECs (mainly macrophages), mDCs, or pDCs were inoculated with MERS-CoV or SARS-CoV. For murine mDCs and PECs, THOV(ΔML) served as a positive control for IFN secretion (29). Murine pDCs were inoculated with CpG 2216 oligonucleotide to test the cells' reactivity. All murine immune cells revealed robust IFN- α and IFN- β responses to the adequate positive controls but no induction of type I IFN after contact with MERS-CoV or with SARS-CoV (Fig. 1A). Next, viral replication of MERS-CoV in murine APCs was

FIG 2 Inoculation of human immune cells with MERS-CoV. (A) Type I and III IFN secretion by human immune cells. Human cells were inoculated with MERS-CoV (MOIs indicated), SARS-CoV (MOI of 1), or the indicated positive control (CpG 2006, VSV-M2, or CpG 2216). Supernatants were sampled at 24 hpi, and secreted IFNs were determined by specific ELISAs. (B) Isolated RNA was used for qRT-PCR. MERS RNA signals were normalized to cellular GAPDH mRNA [$\Delta C_T = C_{T(\text{MERS RNA})} - C_{T(\text{GAPDH mRNA})}$]. ΔC_T values and respectively calculated x -fold amounts of RNA normalized to an MOI of 0.1 were determined. (C) Titers of MERS-CoV or SARS-CoV (control) in human immune cells (APCs) infected at an MOI of 0.01 or on pDCs infected at an MOI of 5. (D) IL-6 secretion by human B cells upon inoculation with stimulating CpG 2006. Supernatants were sampled at 24 h after inoculation with CpG 2006, and secreted IFNs were determined by ELISA. Individual donors are displayed as single dots, and horizontal lines indicate means. Limits of detection were 7 pg/ml for IFN- α , 50 pg/ml for IFN- β , and 8 pg/ml for IFN- λ . b.d., below detection; filled symbols, MERS-CoV; open symbols, SARS-CoV; $\diamond$, B cells; $\triangle$, M1 macrophages; ∇ , M2 macrophages; $\square$, MDDCs; $\square$, pDCs. Growth in human APCs (MOI of 0.01) (i, left), or in human pDCs (MOI of 5) (ii, right) was determined. Values are means of three independent experiments; error bars indicated standard deviation. *, $P < 0.05$.

controlled since inhibition of type I IFN production in MERS-CoV-infected cells has been described previously (17), potentially decoupling replication from IFN secretion. Productive viral replication in immune cells was quantified by titration of the supernatant of inoculated cells to detect released infectious progeny virus (Fig. 1B). Two days postinfection, permissive Vero cells produced high peak titers of 5×10^6 TCID $_{50}$ /ml and 1×10^7 TCID $_{50}$ /ml of MERS- and SARS-CoV, respectively (Fig. 1B, panel i). In contrast, no infectious virus considerably above the limit of detection (1×10^2 TCID $_{50}$ /ml) was detected in the supernatants of any murine cell population for both MERS- and SARS-CoV (Fig. 1B, panel ii).

Interferon production by human APCs upon contact with MERS-CoV. Although MERS-CoV did not induce any reactivity in murine immune cells, reactivity of human immune cells seemed not too unlikely as SARS-CoV exhibited such a pattern of IFN induction (25). Therefore, we analyzed next if and which human innate immune cell subset(s) produces type I or type III IFNs upon inoculation with MERS-CoV. Human B cells, M1 and M2 type macrophages,

MDDCs, and pDCs were inoculated with MERS-CoV or SARS-CoV. As positive controls for IFN secretion, M1 and M2 macrophages and MDDCs were inoculated with VSV-M2 (30), B cells were inoculated with the B cell-stimulating CpG oligonucleotide CpG2006 (40), and pDCs were inoculated with pDC-stimulating CpG oligonucleotide CpG2216 (41). Untreated cells served as mock controls. Human B cells, M1 macrophages, M2 macrophages, and MDDCs did not secrete type I or type III IFNs upon inoculation with MERS-CoV, despite being responsive to appropriate stimuli (Fig. 2A). General responsiveness of B cells was confirmed by IL-6 secretion after stimulation with CpG 2006 (Fig. 2D) (40). In contrast, human pDCs secreted large amounts of IFN- α , IFN- β , or IFN- λ (up to 40, 0.3, or 0.1 ng/ml, respectively) upon contact with MERS-CoV (Fig. 2A), with the highest secretion at an intermediate MOI of 1. Interestingly, this did not correlate with rates of infection. pDCs inoculated with increasing MOIs of MERS-CoV revealed an approximately linear correlation of viral RNA detectable in infected pDCs, as determined by calculating the normalized ΔC_T values of qRT-PCR analysis and converting the detected differences into fold changes (Fig. 2B). The

FIG 3 Dissecting type I and III IFN induction in human pDCs. Impact of different parameters on IFN induction in pDCs after inoculation of MERS-CoV (MOI of 1). (A to C) Live virus. pDCs were incubated with UV-inactivated virus (UV) or live virus (MERS-CoV). (D to F) Entry receptor. Infection was performed in the presence or absence of the MERS-CoV S receptor binding domain (RBD) or IgG1-Fc control protein (Ctrl). (G to I) Endosomal maturation. Infection was performed in the presence of chloroquine. (J to L) TLR recognition. Infection was performed in the presence of TLR7 inhibitor (IRS661). IFNs were sampled at 24 hpi. Mock and MERS-CoV data for live virus experiments are the same as displayed in Fig. 2. Mock, uninfected cells; sham, infected but untreated cells. Single dots, individual donors; horizontal lines, means. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

largest amounts of secreted IFNs at an MOI of 1 were about 8-, 4-, or 1.5-fold higher, respectively, than IFN levels measured after inoculation with SARS-CoV (5 ng/ml IFN- α , 0.07 ng/ml IFN- β , and 0.07 ng/ml IFN- λ). In addition, responses of human pDCs to CpG 2216 (8.6 ng/ml IFN- α , 600 pg/ml IFN- β , and 80 pg/ml IFN- λ) were remarkably less strong than to MERS-CoV but clearly detectable (41).

MERS-CoV replication in human innate immune cells. To analyze whether production of IFNs corresponds to productive replication of MERS-CoV in the respective human innate immune cell subsets, we inoculated cells with MERS-CoV and SARS-CoV at a low MOI of 0.01. Productive viral replication was quantified by titration of the supernatant of inoculated cells to detect released infectious progeny virus (Fig. 2C). Similar to findings with murine APCs (Fig. 1B, panel ii), no infectious virus considerably above the limit of detection (1×10^2 TCID₅₀/ml) was detected in the supernatants of any human cell population for both MERS- and SARS-CoV (Fig. 2C). Thus, no productive replication of MERS-CoV in APCs and pDCs became evident after infection at a low MOI. Since replication of MERS-CoV in M1 macrophages or MDDCs after infection at a high MOI has been reported (42, 43), human pDCs were additionally infected at an MOI of 5 to test, if, e.g., putative antiviral cellular restriction factors may be overcome by infection at high MOIs, and infectious virus in supernatants was titrated. A slowly decreasing titer with an initial set point (1 hpi) of 2×10^4 TCID₅₀/ml was detected in the supernatant (Fig. 2C, panel ii). This indicates only inefficient replication of MERS-CoV in pDCs in our hands even after inoculation at a high MOI. Thus, IFN secretion by pDCs is not linked to virus amplification.

Inhibition of IFN production by pDCs upon MERS-CoV contact. Next, we aimed to study the recognition of MERS-CoV in human pDCs. Therefore, human pDCs were inoculated with UV-inactivated MERS-CoV particles (corresponding to an MOI of 1 before inactivation).

UV-inactivated MERS-CoV induced secretion of similar amounts of IFN- α (50 ng/ml) and IFN- λ (0.06 ng/ml) but significantly reduced amounts of IFN- β (0.08 ng/ml) compared to values with untreated MERS-CoV (Fig. 3A to C). These results indicate the requirement for replication-competent virus particles to induce IFN- β secretion, whereas IFN- α and IFN- λ are induced by replication-defective virus particles as well, as evident by similar differences in induction between UV-inactivated and untreated MERS-CoV compared to mock controls.

To determine the route of MERS-CoV cell entry necessary for viral replication, we first analyzed the role of the virus receptor DPP4. Analysis of DPP4 surface expression by flow cytometry indicated surface expression of DPP4 on human pDCs (Fig. 4A). Indeed, preincubation of human pDCs with the receptor binding domain (RBD) of MERS-CoV (37) to block active MERS-CoV entry reduced secretion of IFNs by pDCs. Secretion of IFN- α was reduced 10-fold (3 ng/ml versus 30 ng/ml), that of IFN- β was reduced 26-fold (0.03 ng/ml versus 0.8 ng/ml), but that of IFN- λ was reduced only slightly (37 pg/ml versus 43 pg/ml) compared to levels in control-treated cells (Fig. 3D to F). For IFN- λ the impact of one outlier data point with high IFN- λ secretion within this experiment influenced the data. In an additional data set, RBD

FIG 4 CD26 expression and functionality of human pDCs. (A) Expression of MERS-CoV receptor DPP4 on human pDCs. pDCs were stained with anti-DPP4 antibody and analyzed via flow cytometry. ITC, isotype control antibody. (B) Viability of pDCs of three different donors (D1 to D3) treated with inhibitors. pDCs were treated as indicated. At 24 hpi, cells were stained for viability and analyzed via flow cytometry. Dead, cells killed by osmotic shock. (C) Block of CpG 2216-induced IFN secretion by MERS-CoV RBD. Secretion of the indicated IFNs was determined in the presence or absence of the MERS-CoV S receptor binding domain (RBD) or IgG1-Fc control protein (Ctrl) upon MERS-CoV infection or the DPP4-independent stimulus CpG 2216. IFNs were sampled at 24 hpi. Single dots, individual donors; horizontal lines, means. b.d., below detection.

blocked IFN- λ , again, for each of the three studied donors (Fig. 4C). However, also after stimulation by CpG2216 the secretion of all IFNs was strongly reduced in the presence of RBD protein (1.5 ng/ml versus 150 ng/ml IFN- α , <50 pg/ml versus 0.16 ng/ml IFN- β , 4 pg/ml versus 40 pg/ml IFN- λ), indicating general immune-suppressive properties of the RBD protein (Fig. 4C).

To evaluate if MERS-CoV particles are endocytosed and if MERS-CoV is recognized in the endosome, endosomal maturation and, thus, the endosomal route of entry and IFN induction (44) were inhibited by chloroquine. Of note, 24 h after cotreatment with chloroquine or RBD and MERS-CoV, viability of pDCs was not impaired (Fig. 4B). When pDCs were infected with MERS-CoV (MOI of 1) in the presence of chloroquine, the secretion of IFNs was reduced by factors of 11 for IFN- α (5 ng/ml versus 55 ng/ml), 35 for IFN- β (0.6 ng/ml versus 0.016 ng/ml), and 2.3 for IFN- λ (60 pg/ml versus 140 pg/ml) (Fig. 3G to I). These data indicate that the endosomal route is critical for sensing of MERS-CoV infection by human pDCs. Since viral RNA can be recognized as a PAMP in the endosomes of pDCs by TLR7, we inhibited TLR7 via the inhibitory ODN IRS661. IFN- α production was 1.5-fold decreased upon TLR7 inhibition (15 ng/ml versus 25 ng/ml) compared to infection in the presence of a noninhibiting control oligonucleotide. IFN- β production was 3-fold decreased (0.25 ng/ml versus 0.73 ng/ml), and IFN- λ production was 2-fold decreased upon TLR7 inhibition (36 pg/ml versus 77 pg/ml)

FIG 5 Infection of human pDCs by MERS-CoV. (A) Quantification of viral N RNA in human or murine pDCs by qRT-PCR in the presence or absence of chloroquine, normalized to cellular GAPDH mRNA [$\Delta C_T = C_{T(MERS\ vRNA)} - C_{T(GAPDH\ mRNA)}$] at indicated time points after inoculation. (B to D) Immunoblot analysis of N protein expression in murine pDCs (B) or human pDCs (C and D) after inoculation with MERS-CoV (MOI of 3). pDCs of three different donors (D1 to D3 and D4 to D6) were infected in the presence of blocking anti-DPP4 serum (DPP4), the receptor binding domain of MERS-CoV S protein (RBD), or respective controls (Ctrl) or with UV-inactivated (UV) or live MERS-CoV in the presence (Chl) or absence (sham) of chloroquine. Cells were lysed at the indicated time points and subjected to analyses.

(Fig. 3J to L). Thus, secretion of all IFNs analyzed was reduced upon inhibition of TLR7. These data indicate involvement of TLR7 in sensing MERS-CoV RNA and in IFN induction upon MERS-CoV infection of pDCs.

Transcription of MERS-CoV N RNA in infected pDCs. Even though no significant productive viral replication was observed, initial steps of viral infection and replication may take place in pDCs and could be responsible for triggering cytosolic pattern recognition receptors (PRRs). To analyze MERS-CoV infection of pDCs, onset of viral transcription was monitored by qRT-PCR of N protein RNA in infected pDCs. For this purpose, total RNA of human pDCs infected with MERS-CoV (MOI of 3) was isolated, and amounts of MERS-CoV N RNA was quantified at 1 hpi and 6 hpi and normalized to cellular housekeeping gene mRNA (GAPDH) (Fig. 5A). The relative amount of N RNA increased from 1 hpi to 6 hpi by 14-fold, indicating onset of viral gene transcription. In line with IFN-blocking experiments, only a minimal increase in relative N RNA levels was detected when human pDCs were pretreated with chloroquine (1.2-fold) or when murine pDCs were used as the substrate (no increase) (Fig. 5A).

Expression of MERS-CoV nucleocapsid protein in infected human pDCs. To back up mRNA expression data, the onset of viral protein translation was monitored by immunoblot analysis

of viral N protein expression in infected pDCs. For this purpose, human pDCs were infected with MERS-CoV (MOI of 3), and N protein expression was checked (Fig. 5C and D). As expected, no CoV N protein was detected in murine pDCs (Fig. 5B) and human pDCs at 1 hpi (Fig. 5C and D). However, at 8 hpi CoV N protein expression was clearly demonstrated in human pDCs, indicating the onset of viral gene expression in infected human pDCs. In contrast, using polyclonal anti-DPP4 antibody or the RBD, MERS-CoV N protein expression was decreased in comparison to that in the respective control, indicating infection of pDCs via DPP4 (Fig. 5C). Moreover, when human pDCs were inoculated with UV-inactivated MERS-CoV, no expression of N protein was detected, thus indicating that intact viral genomes are crucial for N protein expression (Fig. 5D). In addition, inhibition of endosomal maturation was accompanied by considerably less N protein expression in infected cells at 8 hpi (Fig. 5D). Therefore, expression of MERS-CoV N depends on receptor binding and endosomal maturation, arguing for the endosomal pathway as a primary route of MERS-CoV entry into human pDCs.

DISCUSSION

Our data reveal that primary human pDCs produce large amounts of type I and type III IFNs in response to MERS-CoV infection. Sensing depends on receptor availability, endosomal uptake, and, at least partially, functionality of TLR7. Moreover, we detected expression of MERS-CoV N mRNA and protein in the absence of progeny virus, suggesting unproductive infection of human pDCs. Similar to data obtained upon SARS-CoV infection, secretion of IFNs was exclusively found in pDCs (25), but the amounts of IFNs induced by MERS-CoV were significantly higher.

In parallel, stimulation with CpG 2216 also resulted in lower, but clearly detectable, amounts of IFNs. Thereby, integrity of pDCs can be assumed since the amounts of secreted IFNs were comparable to those in previously published data (25, 41) considering the fact that in the present study only one-third the amount of pDCs was used and that IFN-containing supernatants were harvested after 24 h.

When human B cells, macrophages, or MDDCs were inoculated with MERS-CoV, no type I or III IFNs were detected in the supernatant of infected cells. In line with these data, Zhou et al. (42) could not detect upregulation of type I IFN mRNA upon infection of human macrophages. Also in infected human MDDCs, only minor induction of IFN- α mRNA was detected, and no induction of IFN- β mRNA synthesis was detected (43).

In contrast, large amounts of IFN- α were detected when human pDCs were inoculated with MERS-CoV. IFN- α can be induced in pDCs after recognition of PAMPs in the endosome, e.g., via TLR7 (45). In our experiments, secretion of IFN- α was strongly inhibited by chloroquine treatment. Indeed, chloroquine is an inhibitor of endosomal maturation and can inhibit IFN production induced by viruses (e.g., HIV) via PRRs within the endosome (46). When the endosomal PRR of pDCs for viral RNA, TLR7, was inhibited, secretion of IFN- α also decreased. Taken together, these data argue for endosomal recognition of MERS-CoV and potentially recognition via TLR7 in mature endosomes of pDCs. The pattern of IFN- λ secretion by human pDCs after MERS-CoV inoculation followed that of IFN- α , as expected, since IFN- λ is induced by stimuli similar to those that induce IFN- α (47).

IFN- β was also secreted in significant amounts by human

pDCs upon MERS-CoV inoculation. IFN- β can be induced after recognition of PAMPs by cytosolic PRRs such as MDA-5 or RIG-I (48). Indeed, we demonstrated the onset of viral gene expression in human pDCs. In line with this, UV inactivation of MERS-CoV, which damages the viral genome and thereby inhibits transcription and amplification of viral RNA, significantly reduced IFN- β secretion, but secretion of IFN- α or IFN- λ remained on similar levels. Thus, cytoplasmic recognition of viral replication intermediates seems to be responsible for IFN- β induction. However, MERS-CoV-induced IFN- β secretion was also blocked by chloroquine, an effect that cannot be explained if we postulate direct viral entry across the plasma membrane after contact of the viral spike glycoprotein S with its receptor CD26/DPP4, as assumed for MERS-CoV entry into lung epithelial cells (49). To use this entry pathway, MERS-CoV S has to be activated by cellular exopeptidases; mainly, transmembrane protease serine 2 (TMPRSS2) has been demonstrated to be responsible for cleavage during MERS-CoV entry into Calu-3 cells (49). In contrast, we demonstrate that endosomal maturation is crucial for MERS-CoV entry into pDCs. Interestingly, uptake of MERS-CoV via the endosomal route has already been described as an alternative pathway for entry into, e.g., Vero cells (49). Here, lysosomal proteases such as cathepsin L are required to activate the MERS-CoV S protein (50), but their activity depends on endosomal maturation (51). Hence, chloroquine-mediated inhibition of IFN- β secretion by pDCs after contact with MERS-CoV argues for receptor-mediated endocytosis of MERS-CoV particles and activation of MERS-CoV S protein by endosomal proteases, such as cathepsin L, finally resulting in cytosolic entry of MERS-CoV across the endosomal membrane. Indeed, expression of mRNA and MERS-CoV N protein was considerably decreased in the presence of chloroquine, indicating chloroquine-mediated inhibition of infection.

Furthermore, we blocked cell attachment of MERS-CoV to pDCs by blocking DPP4 with recombinant viral RBD. Block of receptor binding led to a significant reduction of IFN production following virus inoculation. However, CpG-stimulated IFN induction was blocked by recombinant RBD as well, indicating the immune suppressive properties of the MERS-CoV RBD in human pDCs. DPP4 is described as an activating receptor on T lymphocytes (52–54), but its function in DCs has thus far been linked only to T cell stimulation (55). Thus, the reasons for the eventually immune-suppressive properties of the RBD remain to be elucidated. Nevertheless, the remarkable inhibition of N protein expression by both RBD and anti-DPP4 serum indicates the necessity of DPP4 as an entry receptor for endocytotic uptake and subsequent infection on human pDCs. Receptor-dependent endocytosis as an uptake pathway for MERS-CoV may also explain the absence of IFN induction in murine pDCs after contact with the virus. In contrast to human DPP4, murine DPP4 is no suitable receptor for promoting infection with MERS-CoV (56). Thus, lack of binding of MERS-CoV to murine DPP4 should reduce endosomal uptake of virus particles, thereby reducing the amount of PAMPs which can be sensed by PRRs, resulting in strongly reduced IFN induction in murine pDCs.

To summarize the data, a model explaining the mechanism how of MERS-CoV induces type I IFN in human pDCs may be proposed (Fig. 6). MERS-CoV binds to its entry receptor DPP4 on the surface of pDCs, triggering receptor-mediated endocytosis of viral particles. In the mature endosome, MERS-CoV RNA is sensed by TLR7, inducing IFN- α secretion. Furthermore, MERS-

FIG 6 Model for MERS-CoV-induced type I IFN secretion in human pDCs. The figure schematically depicts the life cycle of MERS-CoV in human pDCs and events triggering secretion or infection of type I IFNs. Successful inhibition of IFN secretion at single steps is indicated. Inhibitors or proteins which have been analyzed are depicted in bold. Question marks point out steps during assembly or release of viral particles, the block of which could be responsible for absence of significant viral replication in pDCs. α , anti.

CoV spike proteins become cleaved by endosomal proteases during or after endosome maturation. This cleavage allows fusion of viral and endosomal membranes, causing release of the viral genome into the cytoplasm. In the cytoplasm, expression of viral proteins starts. We hypothesize that MERS-CoV RNA replication intermediates are recognized by cytosolic PRRs, resulting in full-blown induction of IFN- β . However, assembly or release of new progeny viral particles is impaired by yet unknown mechanisms in stages subsequent to viral gene expression. This absence of significant MERS-CoV replication in pDCs in contrast with the virus's replication in MDDCs (43) or macrophages (42) may be explained by the significant (biological and functional) differences between pDCs and other DC subsets (57). In line with this hypothesis, influenza A virus replication was also demonstrated in mDCs but shown to be blocked at postentry steps in pDCs (58).

Entry receptors are crucial for MERS-CoV recognition by pDCs, and lack of functional entry receptors in mice leads to lack of IFN production in murine cells. Meanwhile, it has been shown that murine DPP4 cannot be used as a MERS-CoV entry receptor; as demonstrated by expression of human DPP4 in mouse lungs via

adenoviral vectors, this is sufficient to gain (partial) susceptibility to MERS-CoV infection (13).

Moreover, type I (59) and type III (17) IFNs inhibit MERS-CoV replication *in vitro*. A characteristic feature of type I IFNs is that effects are seen at small concentrations. In previous studies replication of MERS-CoV was inhibited by type I and III IFNs in different cell types *in vitro* already starting at a ng/ml concentration range (17, 59, 60). The IFN- α levels obtained in the experiments surpassed such amounts at least 40-fold; thus, the amounts of IFNs produced by pDCs upon MERS-CoV infection can be supposed to be relevant. The release of type I and III IFNs may protect against MERS-CoV-induced pathogenicity. It is therefore counterintuitive that MERS-CoV induces significantly higher secretion of IFNs than SARS-CoV when infecting pDCs, but clinically recorded MERS patients having a worse prognosis than SARS patients (5). However, secretion of extraordinarily large amounts of type I IFNs can result in aberrant immune activation. Zhou et al. already speculated that an induced cytokine storm could be the reason for the severity of illness on the basis of large amounts of proinflammatory cytokines and chemokines such as IL-12 or

IP-10 secreted by human macrophages upon MERS-CoV infection (42). In human SARS patients, aberrant IFN-stimulated gene expression and cytokine responses, compared to those of healthy individuals, were indeed observed (61). Patients that had such kinds of hyperimmune activation were more likely to succumb to the infection (62). Furthermore, the severity of SARS correlated with large amounts of inflammatory cytokines in serum (63), and symptoms of disease became usually worse after virus clearance (64). For these reasons, immune-mediated pathogenesis has been proposed for SARS-CoV infection (62). Whether such a pathomechanism also applies to MERS and whether pDCs are really the major source of IFNs in such a setting remain to be demonstrated in further studies. However, the up to 8-fold-enhanced IFN type I secretion upon MERS-CoV infection compared to SARS-CoV infection might then hint at overshooting immune reactions being potentially one factor for the higher mortality rate observed in MERS patients.

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