Human Metapneumovirus SH and G Glycoproteins Inhibit Macropinocytosis-Mediated Entry into Human Dendritic Cells and Reduce CD4⁺ T Cell Activation

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ABSTRACT

Human metapneumovirus (HMPV) is a major etiologic agent of respiratory disease worldwide. HMPV reinfections are common in healthy adults and children, suggesting that the protective immune response to HMPV is incomplete and short-lived. We used gene-deletion viruses to evaluate the role of the attachment G and small hydrophobic SH glycoproteins on virus uptake by primary human monocyte-derived dendritic cells (MDDC) *in vitro* and on subsequent MDDC maturation and activation of autologous T cells. HMPV with deletion of G and SH (Δ SHG) exhibited increased infectivity but had little effect on MDDC maturation. However, MDDC stimulated with Δ SHG induced increased proliferation of autologous Th1-polarized CD4⁺ T cells. This effect was independent of virus replication. Increased T cell proliferation was strictly dependent on contact between virus-stimulated MDDC and CD4⁺ T cells. Confocal microscopy revealed that deletion of SH and G was associated with an increased number of immunological synapses between memory CD4⁺ T cells and virus-stimulated MDDC. Uptake of HMPV by MDDC was found to be primarily by macropinocytosis. Uptake of wild-type (WT) virus was reduced compared to that of Δ SHG, indicative of inhibition by the SH and G glycoproteins. In addition, DC-SIGN-mediated endocytosis provided a minor alternative pathway that depended on SH and/or G and thus operated only for WT. Altogether, our results show that SH and G glycoproteins reduce the ability of HMPV to be internalized by MDDC, resulting in a reduced ability of the HMPV-stimulated MDDC to activate CD4⁺ T cells. This study describes a previously unknown mechanism of virus immune evasion.

IMPORTANCE

Human metapneumovirus (HMPV) is a major etiologic agent of respiratory disease worldwide. HMPV reinfections are common in healthy adults and children, suggesting that the protective immune response to HMPV is incomplete and short-lived. We found that HMPV attachment G and small hydrophobic SH glycoproteins reduce the ability of HMPV to be internalized by macropinocytosis into human dendritic cells (DC). This results in a reduced ability of the HMPV-stimulated DC to activate Th1-polarized CD4⁺ T cells. These results contribute to a better understanding of the nature of incomplete protection against this important human respiratory virus, provide new information on the entry of HMPV into human cells, and describe a new mechanism of virus immune evasion.

Human metapneumovirus (HMPV) was first reported in 2001 (1) and is now recognized as a major etiologic agent for respiratory disease, especially in very young, elderly, and immunocompromised individuals (2–4). Five to 15% of hospitalizations of young children for respiratory tract disease are due to an HMPV infection, with children under 2 years of age being most at risk for severe HMPV disease (3, 5). HMPV reinfections are common in healthy adults and children (6–9), suggesting that the protective immune response to HMPV is incomplete and short-lived.

HMPV is a nonsegmented negative-strand RNA virus of the *Paramyxoviridae* family, genus *Metapneumovirus*, subfamily *Pneumovirinae* (10). HMPV encodes three glycoproteins, the fusion protein F, the attachment glycoprotein G, and the small hydrophobic protein SH. Recombinant HMPV with deletions of the G gene (Δ G), the SH gene (Δ SH), or both (Δ SHG) retains the ability to replicate efficiently in epithelial cell lines, indicating that these proteins are not essential for replication *in vitro* (11). Moreover, the Δ G, Δ SH, and Δ SHG deletion mutants are competent for replication in the upper and lower respiratory tract of hamsters, although replication of Δ G and Δ SHG was reduced to some extent (11). Studies in African green monkeys revealed that the

Δ G mutant was strongly restricted in the upper and lower respiratory tract, whereas the absence of SH had no effect on replication (12). The Δ G, Δ SH, and Δ SHG mutants were immunogenic and protective against wild-type (WT) HMPV challenge in hamsters (Δ G, Δ SH, and Δ SHG) or African green monkeys (Δ G and Δ SH), suggesting that these gene deletions may be useful for developing live-attenuated vaccine candidates (11, 12).

Dendritic cells (DC) are an important link between the innate and the adaptive immune response. Immature DC can reside in peripheral tissue or in lymphatic tissue, where exposure to mi-

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crobes or inflammatory molecules initiates a maturation process of phenotypic and functional changes. These include an increased expression of surface markers that are correlates of DC maturation and T cell stimulatory capability, including CD38, CD83, CD80, and CD86 (13, 14). Maturing DC also secrete an array of chemokines, cytokines, and interferons involved in innate immunity and T cell activation. They also downregulate CCR1, CCR2, and CCR5 and upregulate CCR7, resulting in migration to the T cell zone of lymphatic tissue, where the DC interact by direct contact through the immunological synapse (IS) with naive and/or antigen-specific memory T lymphocytes to initiate an adaptive immune response. Naive CD4⁺ T cells can differentiate into helper (Th) subsets with distinct functions and effects on the adaptive immune response (reviewed in references 15 to 17). During reinfections with respiratory viruses, CD4⁺ T cell proliferation originates largely from antigen-specific memory CD4⁺ Th1 cells that persist from previous infection(s) and are reactivated by antigen-presenting DC (18–20).

DC generated from primary human monocytes by treatment *in vitro* with interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF; monocyte-derived DC [MDDC]) represent an appropriate model for lung DC because monocytes give rise to myeloid DC in the resting lung (21) and mucosa (22) and because MDDC are phenotypically and functionally similar to DC located at sites of inflammation *in vivo* (23, 24). In the present study, we evaluated *in vitro* the ability of recombinant HMPV lacking SH, G, or both SH and G to infect and induce maturation of human MDDC and evaluated the ability of the virus-exposed MDDC to mediate T cell activation, differentiation, and proliferation. We found that the HMPV G and SH proteins reduce the infectivity of HMPV for MDDC but that the presence of SH and G has little effect on MDDC upregulation of activation markers and HMPV-induced cytokine release of MDDC. However, we found that MDDC stimulation with HMPV lacking both SH and G induced a significantly stronger CD4⁺ T cell proliferation and activation than did stimulation with WT HMPV. This effect was observed with both live and UV-inactivated virus, suggesting that it was independent of virus replication. This effect occurs as early as the formation of the immunological synapse (IS) between DC and T cells. Using a panel of inhibitors and time course experiments, we found that ΔSHG is internalized more efficiently than is WT virus through a macropinocytosis-like pathway. Altogether, our results showed that SH and G glycoproteins reduce the internalization of HMPV by DC by a macropinocytosis-like pathway, resulting in a reduced ability of the HMPV-stimulated DC to activate CD4⁺ T cells.

MATERIALS AND METHODS

Ethics statement. Elutriated monocytes and autologous CD4⁺ T lymphocytes were obtained from healthy donors at the Department of Transfusion Medicine of the National Institutes of Health, under a protocol (99-CC-0168) approved by the Institutional Review Board (IRB) of the Clinical Center, NIH. Written informed consent was obtained from all donors.

Virus stock preparation. We previously described the construction of recombinant WT HMPV strain CAN97-83 (WT) and of the ΔSH, ΔG, and ΔSHG derivatives (11, 25). Each of these viruses expresses enhanced green fluorescent protein (GFP) from an added gene in the promoter-proximal position. In addition, the SH gene (present only in the WT and ΔG viruses) was previously modified so that tracts of A and T residues that had been sites of spontaneous mutations during passage *in vitro* had been

removed by translationally silent modifications (26). Apart from the absence of the deleted protein(s), the virions produced by the gene-deletion mutants in Vero cells were similar in protein yield, gel electrophoretic protein profile, and genome-to-PFU ratios (as determined by reverse transcription-quantitative PCR [RT-qPCR]) to WT HMPV, suggesting comparable ratios of defective virus particles between the virus preparations (11, 26; also data not shown). All viruses were grown on Vero cells, using low multiplicity of infection (MOI) ranges of 0.001 to 0.02, in the presence of 5 μg of trypsin per ml and purified by centrifugation through sucrose step gradients as described previously (27). Sucrose-purified viruses were pelleted by centrifugation to remove sucrose. Virus pellets were resuspended in Advanced RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 2 mM L-glutamine, and aliquots were snap-frozen and stored at −80°C until use. Virus titers were determined by immunoplaque assay on Vero cells under a methylcellulose overlay containing trypsin as described previously (27). UV-inactivated viruses were prepared using a Stratalinker UV cross-linker (Agilent, Santa Clara, CA) at 0.5 J/cm². Complete inactivation was confirmed by plaque assay. We confirmed by deep sequencing that defective interfering particles were absent in pneumovirus stocks prepared on Vero cells using a low MOI range of 0.001 to 0.02.

Generation of immature MDDC and purification of autologous CD4⁺ T cells. Elutriated monocytes and autologous CD4⁺ T lymphocytes were obtained from healthy adult donors at the National Institutes of Health Clinical Center Blood Bank (clinical protocol number 99-CC-0168). As described previously, monocytes were subjected to CD14⁺ positive sorting on an AutoMACS separator (Miltenyi Biotec, Auburn, CA), and were cultured in the presence of recombinant human IL-4 (R&D Systems, Minneapolis, MN) and recombinant human GM-CSF (Bayer Healthcare, Wayne, NJ) for 7 days to generate immature MDDC (27). Elutriated T lymphocytes were further purified over Ficoll (lymphocyte separation medium; Cellgro, Manassas, VA) followed by treatments with ACK lysing buffer (Lonza, Walkersville, MD) to remove remaining erythrocytes. T lymphocytes were frozen at −80°C. Prior to use, the cells were thawed and incubated overnight at 37°C. CD4⁺ T cells were subjected to positive sorting on an AutoMACS separator using magnetic microbeads coated with a CD4-specific monoclonal antibody (MAb) (Miltenyi Biotec). The purity of the CD4⁺ T lymphocytes based on the cell surface expression of CD3 and CD4 proteins was confirmed by flow cytometry to be ≥96%.

Stimulation of immature MDDC. For the analysis of MDDC infection and maturation, and for CD4⁺ T cell proliferation assays, immature MDDC were seeded in 12-well plates at 6×10^5 cells per well. Cells were mock stimulated (medium only) or stimulated with live virus at an input MOI of 1 or 3 PFU/cell (unless otherwise stated), or with an equivalent amount of UV-inactivated virus. In parallel, immature MDDC were stimulated with 1 μg/ml of lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (Sigma, St. Louis, MO) as a positive control for MDDC maturation or incubated with 1 μg/ml of the superantigen *Staphylococcus enterotoxin B* (SEB; Sigma), a strong inducer of T cell proliferation. MDDC stimulations were performed in Advanced RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 200 U/ml penicillin, and 200 μg/ml streptomycin (complete medium) at 37°C in 5% CO₂.

Analysis of MDDC infection rate and maturation marker expression by flow cytometry. MDDC were stimulated with virus as described above and 40 h later were carefully harvested and pelleted by centrifugation at 300 × g for 5 min. After centrifugation, MDDC were resuspended in cold washing solution (phosphate-buffered saline [PBS] [Life Technologies] with 2% heat-inactivated FBS and 2 mM EDTA [Quality Biological, Gaithersburg, MD]). Cells were stained with phycoerythrin (PE)- or allophycocyanin (APC)-labeled anti-human CD38, CD54, CD80, CD83, and CD86 monoclonal antibodies (MAbs; BD Biosciences, Palo Alto, CA) (27) on ice for 20 min in the dark. Isotype-matched MAbs served as negative controls and were included in all experiments. After incubation, cells were washed 3 times with cold washing solution and resuspended in 200 μl of

cold washing solution. Ten microliters of 200- μ g/ml propidium iodide (PI) solution (Sigma) was added to discriminate between live (PI-negative) and dead (PI-positive) cells. Data were acquired using a FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA). Cell debris and dead cells were excluded, and the expression of each maturation marker was analyzed individually (CD38, CD83, CD40, and CD54) or in combination (CD80 and CD86) using FlowJo software (Tree Star, Ashland, OR).

Measurement of cytokine production by MDDC. MDDC culture supernatants were collected at various time points (3, 14, 22, and/or 40 h) poststimulation and clarified by centrifugation at $300 \times g$ for 5 min. Clarified supernatants were stored at -80°C in the presence of a protease inhibitor cocktail (Roche Applied Science, Indianapolis, MN) until analyzed. Enzyme-linked immunosorbent assay (ELISA) kits were used to assess the concentration of alpha interferon (IFN- α) (human alpha interferon multisubtype ELISA kit; PBL Biomedical Laboratories, Piscataway, NJ) and IFN- β (Life Technologies) according to the manufacturer's instructions. All other cytokines—IL-1 β , IL-2, IL-1 α , IL-4, IL-5, epidermal growth factor (EGF), IL-6, IL-7, transforming growth factor α (TGF- α), CX3CL1 (Fractalkine), CXCL8 (IL-8), IL-10, IL-12(p70), IL-13, IL-15, IL-17, IL-1 α , IFN- γ , granulocyte colony-stimulating factor (G-CSF), GM-CSF, tumor necrosis factor alpha (TNF- α), CCL11 (Eotaxin), CCL2 (MCP1), CCL3 (MIP1 α), CCL4 (MIP1 β), CCL5 (RANTES), and VEGF—were detected with a Luminex multiplex bead assay (Linco Research, St. Charles, MO) according to the manufacturer's instructions.

Coculture of stimulated MDDC with autologous CD4⁺ T cells, CD4⁺ T cell proliferation assay, and CD4⁺ T cell intracellular cytokine staining. Immature MDDC were labeled with the far-red cell tracer 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO; Life Technologies) according to the manufacturer's instructions prior to virus or SEB stimulation. Four hours after stimulation, MDDC were extensively washed with medium and the absence of remaining infectious virus particles in the suspensions of virus-stimulated MDDC was confirmed by plaque assay as described above. In some experiments, immature DDAO-labeled MDDC were incubated for 1 h at 37°C with 5% CO_2 with 5 μM macropinocytosis inhibitor rottlerin or 20 $\mu\text{g}/\text{ml}$ anti-DC-SIGN antibody before being stimulated with 1 $\mu\text{g}/\text{ml}$ SEB or the indicated virus at an MOI of 1 or 3 PFU/cell (as indicated in the figure legends).

Autologous purified CD4⁺ T cells were labeled with the cell tracer carboxyfluorescein succinimidyl ester (CFSE, 1 μM ; Life Technologies) as described previously (28). Labeled stimulated or mock-treated MDDC were cocultured with CFSE-labeled CD4⁺ T lymphocytes at a ratio of 1 MDDC per 10 T lymphocytes for 1 to 7 days at 37°C in 5% CO_2 . In some experiments, MDDC-T cell cocultures were stimulated 4 h prior to harvesting with phorbol myristate acetate (PMA), ionomycin, and brefeldin A for intracellular cytokine staining as described previously (28). Cells were harvested and stained with blue fixable live/dead stain (Life Technologies) for 30 min at 4°C . Cells were fixed and permeabilized using the BD Perm/Wash buffer kit (BD Biosciences) and stained for 30 min at 4°C using a mix of anti-human MABs including, depending on the experiment, MABs for CD3, IFN- γ , IL-2, IL-4, IL-10, and TNF- α (labeled with APC-Cy7, PE-Cy7, PE, and APC [BD Biosciences] or QDot605 [Life Technologies]). Optimal concentrations of MABs were previously determined by titration. T cells were analyzed by flow cytometry as described previously (28). One hundred thousand events gated on live CD3⁺ cells were acquired using an LSRII flow cytometer (BD Biosciences). Single-color antibody capture beads for each antibody (BD Biosciences) were used to determine compensation, and fluorescence minus one controls were included to aid gating. Using FlowJo software, gating was performed to discriminate live, single, CD3⁺ cells; T cell proliferation was quantified by measuring the dilution of CFSE that occurs with cell division (29–31); and IFN- γ , IL-2, IL-4, IL-10, and TNF- α expression was quantified as described previously (28).

Transwell cocultures. Contributions of cell contact and soluble factors to CD4⁺ T cell proliferation were evaluated using transwell culture dishes with an 0.4- μm pore size (Costar, Cambridge, MA) to allow diffu-

sion of soluble factors but to prevent physical contact between the cells in the top and the bottom chambers. DDAO-labeled immature MDDC were seeded in 12-well plates at 6×10^5 cells per well and were mock stimulated or stimulated for 4 h with UV-WT or UV- Δ SHG at an equivalent input MOI of 3 PFU/cell or incubated with 1 $\mu\text{g}/\text{ml}$ of the superantigen SEB. After stimulation, MDDC were extensively washed with complete medium. Transwell cultures were then set up in which CFSE-labeled autologous CD4⁺ T cells (1×10^6 cells; ratio, 1 MDDC to 10 CD4⁺ T cells) were placed in the bottom chamber and stimulated MDDC (1×10^5 cells) were placed in the top and/or bottom chambers, as indicated. After 7 days, the top chambers were discarded, cells in the bottom chambers were collected, and CD4⁺ T cell proliferation was analyzed by flow cytometry.

Confocal analysis of the immunological synapse. Polarization of CD3, a marker for the immunological synapse (IS) present at the contact zone between virus-stimulated MDDC and autologous CD4⁺ T cells, was evaluated by confocal microscopy. Immature MDDC were labeled with CFSE for 10 min at 37°C , quenched, extensively washed, and stimulated for 4 h with UV-WT HMPV or UV- Δ SHG at an equivalent MOI of 3 PFU/cell. Autologous CD4⁺ T cells were subjected to negative selection to deplete naive cells using a primary cocktail of antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, T cell receptor γ/δ (TCR γ/δ), and glycophorin A conjugated to biotin and secondary anti-biotin antibody-conjugated magnetic microbeads. This was done to increase the concentration of memory T cells and thus increase the likelihood of detecting rare HMPV-specific MDDC-T cell contacts. The purity of the memory CD4⁺ T cells, based on surface expression of CD3, CD4, CD45RA, and/or CD45RO, was confirmed by flow cytometry to be $\geq 96\%$. CFSE-stained virus-stimulated MDDC were seeded onto poly-L-lysine-treated coverslips for 15 min at 37°C with 5% CO_2 . Then, memory CD4⁺ T cells were added to the adherent MDDC (ratio, 1 MDDC to 10 memory CD4⁺ T cells). After 1 h of incubation, cocultures were fixed with 4% paraformaldehyde. Fixed cells were stained for CD3 (UCHT1; Millipore, Bedford, MA) under nonpermeabilizing conditions, followed by Alexa Fluor 568-conjugated goat anti-mouse secondary antibodies (Life Technologies). Nuclei were labeled by 4',6-diamidino-2-phenylindole (DAPI).

Samples were analyzed in a blinded fashion. DC-T cell conjugates from 100 different fields were scored by confocal microscopy using a Leica DMI6000b microscope (63 $\times$ objective). For each potential synapse detected (central clustering associated with a compensatory decrease in CD3 fluorescence in closely adjacent CD3-depleted membrane areas), the microscope was adjusted to give an additional $\times 7$ magnification and a Z series of optical sections were performed at 0.3 μm . Three-dimensional reconstruction of each potential synapse was performed using Imaris 7.3.0 (Bitplane, South Windsor, CT) to quantify the mean CD3 fluorescence intensity at the contact zone and on the remaining surface of the cell. An IS was defined as the clustering of labeled CD3 at the contact zone at a mean fluorescence intensity greater than 2-fold above the CD3 mean fluorescence intensity on the remaining surface of the cell (32, 33). The contact zones that fulfilled these criteria were considered to represent ISs.

Infection study of HMPV and Δ SHG in the presence of inhibitors. The mechanism of entry of HMPV WT and Δ SHG into MDDC was investigated using a panel of inhibitors and antibodies. All inhibitors were purchased from Sigma-Aldrich and used at concentrations previously described for such studies (34–39). Anti-DC-SIGN monoclonal antibody (AZN-D1) and its corresponding mouse isotype control were purchased from Beckman Coulter (Indianapolis, IN) and used at concentrations previously described for such studies (40). Immature MDDC were seeded in 24-well plates at 1.5×10^5 cells per well. Cells were pretreated with inhibitor or antibody at the indicated concentration for 1 h at 37°C with 5% CO_2 before being infected in the presence of inhibitor or antibody at an MOI of 3 PFU/cell with GFP expressing HMPV or Δ SHG. Diluent of the inhibitors was used in some experiments to confirm the absence of side effects. Twenty-four hours postinfection (p.i.), MDDC were harvested and pelleted by centrifugation at $300 \times g$ for 5 min. Data were

acquired using a FACSCalibur flow cytometer. Cell debris and dead cells were excluded using PI staining as described above. Each of the treated MDDC samples had a viability rate of at least 70%, which compares favorably with the viability rate of 70 to 80% that was typical for MDDC cultured over a similar period of time in the absence of drugs and antibodies. This showed that the various drugs and antibodies used in this study had little or no effect on viability under these conditions. The percentage of GFP-positive MDDC was evaluated, and data were normalized as a percentage of the untreated control.

In some experiments, we used one or more entry-tracking molecules to confirm the specificity of the inhibitors EGTA, cytochalasin D (Cyt D), mannan, and rottlerin. The tracking molecules were lucifer yellow (LY; Sigma-Aldrich), which is internalized by fluid-phase endocytosis; transferrin (Tf; Alexa Fluor 647 labeled; Molecular Probes), which is internalized by receptor-mediated endocytosis; and dextran (Dex; fluorescein isothiocyanate labeled; Sigma-Aldrich), which is mainly internalized by the mannose receptor-dependent pathway. MDDC were pretreated with the indicated concentration of inhibitor as described above. Then, cells were incubated in medium containing 200 μ g/ml LY, 5 μ g/ml Tf, or 1 mg/ml Dex. After 15 min (LY and Tf) or 30 min (Dex) of incubation at 37°C, MDDC were placed on ice to halt endocytosis, washed three times with ice-cold PBS, and analyzed by flow cytometry. Data were acquired using an LSRII flow cytometer. Cell debris and dead cells were excluded using PI staining as described above. The median fluorescence intensity (MFI) of the sample minus the background MFI (mock cells) was expressed as a percentage of the no-drug control MFI.

Statistical analysis. Data sets were assessed for significance using parametric one-way repeated-measure analysis of variance (ANOVA) with the Tukey or Bonferroni *post hoc* tests or *t* test for normally distributed data sets or the nonparametric Friedman test with Dunn's *post hoc* test for nonnormal data sets. A $\log_{10}$ transformation was applied to data sets when necessary to obtain equal standard deviations among groups, a necessary requirement of both tests. Statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, CA). Data were considered significant only at a *P* value of <0.05.

RESULTS

Effects of HMPV G and SH proteins on the infection rate and maturation of human MDDC. We investigated the effects of HMPV SH and G on human MDDC infection, maturation, and autologous T cell activation *in vitro*. To do so, we used recombinant WT HMPV or Δ G, Δ SH, or Δ SHG gene-deletion mutants that expressed enhanced GFP from a separate, added gene placed in the promoter-proximal position. All of the viruses had been completely sequenced and confirmed to be identical apart from the gene deletions. Previous studies showed that the HMPV G protein inhibits innate immune responses in epithelial cells by inhibiting RIG-I-dependent gene expression and in MDDC by inhibiting Toll-like receptor 4 (TLR4)-dependent signaling (41, 42). In addition, the HMPV SH protein has been implicated in reducing the activation of NF- κ B in epithelial cells (43). As a benchmark, we evaluated the effects of the gene deletions on the type I IFN response in the human airway epithelial A549 cell line infected at a high MOI (data not shown). This showed that deletion of G resulted in a 2- to 3-fold increase in the secretion of IFN- α and - β , whereas deletion of SH did not increase IFN secretion. None of the deletions individually or in combination had a noticeable effect on the efficiency of infection of A549 cells, which ranged from 70 to 90%, depending on the experiment (not shown).

Next, we evaluated infection of MDDC with WT HMPV and Δ SHG at an MOI of 3 PFU/cell. Twenty-four or 40 h later, flow cytometry was used to evaluate the percentage of cells with robust

FIG 1 Deletion of HMPV G and SH proteins increases infectivity of human MDDC. Immature human MDDC were mock stimulated or infected at an MOI of 3 with GFP-expressing WT or Δ SHG HMPV. Twenty-four or 40 h poststimulation, the cultures were harvested for analysis. (A) Dot plot of data from a typical donor, showing GFP expression versus forward scatter (FSC; a measure of cell size) for live MDDC. The population of GFP-positive cells is boxed, and its percentage is indicated. (B and C) The percentage of GFP⁺ MDDC (B) and the median fluorescent intensity of the GFP⁺ subpopulation of MDDC (C) were analyzed by flow cytometry (*n* = 9 and 7 different donors at 24 and 40 h poststimulation, respectively). The box plots show the median (horizontal line), flanked by the second and third quartiles; the outer bars show the ranges. Individual donors are represented by different symbols. Statistical differences are indicated by asterisks (see Materials and Methods): *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

GFP expression as a marker for infected cells with substantial viral gene expression (Fig. 1). For WT virus, the median percentages of GFP⁺ MDDC were 4% and 8.6% at 24 and 40 h p.i., respectively (Fig. 1A and B), consistent with our previous results (27). Deletion of either the G or SH protein alone did not significantly affect the percentage of GFP⁺ MDDC (median % GFP⁺ cells = 8.9 and 9.0%, respectively, at 40 h p.i.; data not shown), while deletion of both SH and G resulted in a significant increase in the percentage of GFP⁺ MDDC at 24 h p.i. (median of 12% GFP⁺ cells, *P* < 0.001) compared with WT HMPV.

We also evaluated, as an indicator of viral gene expression, the intensity of GFP expression by the GFP⁺ subpopulation (Fig. 1C). The deletion of the G gene, alone (data not shown) or together with the SH gene (but not the deletion of SH alone), significantly reduced the level of GFP expression, showing that the presence of HMPV G increased the level of viral gene expression. This would be consistent with the observation in epithelial cells noted above that deletion of the HMPV G gene results in an increase in the

expression of type 1 IFN, since an increase in the IFN-induced antiviral state would be anticipated to reduce viral gene expression. However, in MDDC, deletion of the G gene was associated with only a slight and nonsignificant increase in IFN production compared to WT virus ($n = 4$ different donors; Fig. 2A). It also is formally possible that Δ SHG is less efficient at gene expression for some other reason (although a previous examination by Northern blotting assays of RNA from epithelial cells did not suggest that [11]).

We next analyzed the level of expression of the maturation markers CD38, CD54, CD80, CD83, and CD86 in MDDC at 40 h poststimulation (Fig. 2B). As previously described (27), stimulation with UV-inactivated WT (UV-WT) HMPV did not induce appreciable changes in the expression of MDDC maturation markers compared to mock-treated cells. Stimulation with live WT HMPV resulted in a low level of induction and cell surface expression of maturation markers, as previously described (28, 44), and this was not significantly changed by deletion of the G and/or SH gene, showing that the G and SH proteins did not detectably influence MDDC maturation (Fig. 2B). Expression of TNF- α , CCL4, and CCL5 by HMPV-stimulated MDDC was not affected by deletion of G and/or SH (Fig. 2D). We also compared the effects of HMPV, UV-WT, Δ SHG, and UV- Δ SHG on MDDC maturation by qRT-PCR using a gene card containing 62 human genes related to DC maturation. As shown previously (27), HMPV and UV-WT had very little effect on expression of maturation-related genes and were very poorly stimulatory for DC maturation; we also found that UV-WT and UV- Δ SHG were indistinguishable ($n = 4$ different donors; data not shown). Thus, the HMPV SH and G proteins appeared to have a limited impact on MDDC maturation and cytokine release in response to stimulation with live or inactivated virus.

The HMPV G and SH proteins reduce CD4⁺ T cell proliferation. We next evaluated the effect of the G and SH proteins on the ability of HMPV to induce proliferation of autologous CD4⁺ T cells. The CD4⁺ T cells were prelabeled with CFSE, and cell division was monitored by dilution of the CFSE dye visualized by flow cytometry.

Figure 3A presents results from a single representative donor, and Fig. 3B summarizes results from multiple donors ($n = 8$ donors). As expected, mock-stimulated MDDC did not induce detectable proliferation (median, 0.7% of proliferated cells). MDDC stimulated at an MOI of 1 PFU/cell with live WT HMPV induced stronger proliferation than did MDDC stimulated with UV-inactivated HMPV (median, 6.6% and 1.5%, respectively, although this difference was not statistically significant; $P > 0.05$).

Surprisingly, live Δ SHG induced a significant increase in CD4⁺ T cell proliferation (median of 22.8%) compared to WT HMPV (6.6%; $P < 0.01$). UV- Δ SHG also induced a substantial and significant increase in CD4⁺ T cell proliferation (median of 27.2%) compared to UV-WT HMPV (median of 1.5%; $P < 0.001$) (Fig. 3B). Comparable results were obtained using an MOI of 3 PFU/cell ($n = 9$ different donors; data not shown). We note that the individual donors appeared to segregate into high-response and low-response groups based on the magnitude of T cell proliferation, as we also have noted previously (28).

We also tested HMPV with deletions of SH and G alone. In experiments using cells from five to six different donors stimulated with virus at an MOI of 3 PFU/cell, live Δ SH or Δ G induced a similar level of CD4⁺ T cell proliferation as did live WT HMPV.

In the context of UV-inactivated viruses, UV- Δ SH or UV- Δ G induced a slightly stronger CD4⁺ T cell proliferation than did UV-WT HMPV, although the difference was not significant (data not shown). These results show that the deletion of both SH and G proteins is required to allow for increased proliferation in response to HMPV. This establishes a role for these proteins in inhibiting CD4⁺ T cell proliferation. Increased proliferation in response to Δ SHG was observed not only with live virus but also with UV-inactivated virus.

We investigated whether this inhibitory effect of SH and G was evident throughout the MDDC/CD4⁺ T cell coculture period of 7 days. We used UV-inactivated virus because of the absence of confounding effects of the cytokine response induced by virus replication. In coculture experiments with cells from three different donors (two donors using an MOI of 1 PFU/cell and one donor using an MOI of 3 PFU/cell), we observed that the greater level of T cell proliferation associated with UV- Δ SHG than with UV-WT HMPV was evident on day 4, the earliest time of reliable detection of proliferation, and on all subsequent days (results not shown). Using cells from 5 additional donors and using an MOI of 3 PFU/cell, we confirmed that the difference on day 4 was statistically significant ($P < 0.05$; results not shown). This suggests that the inhibitory effects of G and SH on CD4⁺ T cell proliferation occur early in the coculture.

The SH and G proteins reduce the number of Th1 polarized CD4⁺ T cells. We also measured the expression of IFN- γ (Th1 cytokine), IL-4 (Th2 cytokine), IL-10 (immunosuppressive cytokine), TNF- α (proinflammatory cytokine), and IL-2 (a marker of T cell proliferation) by proliferated CD4⁺ T cells in MDDC-CD4⁺ T cell cocultures. These experiments were done with live and UV-inactivated WT HMPV and Δ SHG, and cytokine expression was measured by polychromatic flow cytometry. The strategy for gating and Boolean analysis was performed as described previously (28). In experiments employing cells from nine different donors, we found only minimal numbers of IL-4-producing cells at any time point (not shown), and in a pilot experiment with cells from one donor, we detected only minimal numbers of IL-10-producing cells. This indicated that HMPV induced a Th1 response and no significant Th2 response, consistent with our previous observations (28). Therefore, we focused on the Th1 response, and on the production of the two Th1 cytokines IFN- γ and TNF- α , as well as IL-2 from six donors.

We first analyzed the kinetics of cytokine expression by proliferated CD4⁺ T cells from days 1 to 7, using cells from a single donor (Fig. 4A). In cocultures where MDDC were stimulated with live or UV-inactivated WT or Δ SHG viruses, the number of cells expressing IFN- γ , TNF- α , and/or IL-2 increased over time; by day 7, MDDC stimulated with live or UV Δ SHG induced at least 2-fold more CD4⁺ T cells that were single positive, double positive, or triple positive for T cell cytokines than did MDDC stimulated with live or UV-WT HMPV (Fig. 4A). Comparable results were obtained in an additional experiment using cells from another donor.

We also analyzed cytokine expression in CD4⁺ T cells on day 7, the day of maximum proliferation, in cocultures using cells from six different donors with stimulation of MDDC by live or UV-inactivated WT HMPV or Δ SHG (proliferation data at day 7 for these cultures are shown in Fig. 3B). The percentages of cytokine-producing cells were only significantly different between UV-WT and UV- Δ SHG for the expression of IFN- γ /TNF- α ⁺ cells ($P <$

FIG 2 Effect of HMPV G and SH proteins on MDDC maturation and cytokine release. Immature MDDC derived from 7 different donors were mock stimulated, stimulated with 1 μ g/ml LPS, or infected at an MOI of 3 PFU/cell with recombinant GFP expressing WT HMPV, Δ G, Δ SH, or Δ SHG or with an equivalent amount of UV-WT HMPV. (A) The expression of IFN- α and IFN- β was analyzed by ELISA of cell culture supernatants ($n = 4$ different donors). Individual donors are represented by different symbols. (B) The level of expression of the maturation markers CD38, CD54, CD80, CD83, and CD86 was analyzed by flow cytometry. (C) Forty hours poststimulation, we analyzed the cell surface expression of maturation markers CD38, CD54, CD80, CD83, and CD86 on GFP⁺ versus GFP⁻ MDDC by flow cytometry. Data were expressed as a ratio of the MFI of GFP⁺ to that of GFP⁻ MDDC. (D) Medium supernatants at 40 h postinfection collected from duplicate wells for each treatment were tested for cytokine production using a multiplex bead assay ($n = 5$ different donors). Only representative cytokines are shown. The box plots show the median (horizontal line), flanked by the second and third quartiles. The outer bars show the range of values. Statistically significant differences from live WT HMPV are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

FIG 3 Effect of the HMPV G and SH proteins on CD4⁺ T cell proliferation. Immature DDAO-labeled MDDC were mock stimulated or stimulated with an MOI of 1 PFU/cell with the indicated live virus or with an equivalent amount of UV-inactivated virus. Four hours later, MDDC were washed to remove noninternalized virus and cocultured with CFSE-labeled autologous CD4⁺ T lymphocytes at a ratio of 1 MDDC for 10 CD4⁺ T cells (a ratio used in all experiments in this study). CD4⁺ T cell proliferation was measured after 7 days of coculture by flow cytometry to detect CFSE dilution indicative of cell division. (A) CD4⁺ T cell proliferation from a typical donor presented as a dot plot, showing CD3 staining versus CFSE. The population of T cells with CFSE dilution is gated in each diagram, and the number as a percentage of live cells is indicated. (B) Percentage of CD4⁺ T cells that have proliferated at day 7 for multiple donors. Each donor is represented by a different symbol. The box plots show the median (horizontal line), flanked by the second and third quartiles; the outer bars show the ranges. The statistical significance of the difference between UV-WT and UV-ΔSHG and live WT and live ΔSHG is indicated (**, $P < 0.01$; ***, $P < 0.001$; $n = 8$ different donors).

0.01), IL-2/TNF- α ⁺ cells ($P < 0.01$), and TNF- α ⁺ cells ($P < 0.05$; data not shown).

However, when we analyzed the number of cytokine-expressing cells, both live and UV-ΔSHG induced significantly higher numbers of single-positive, double-positive, and triple-positive CD4⁺ T cells than did live and UV-WT HMPV, respectively (Fig. 4B and C). These results show that the increased CD4⁺ T cell proliferation induced by HMPV with deletion of the G and SH proteins results in an increased number of Th1-polarized CD4⁺ T cells. This suggests that HMPV G and SH have the effect of limiting the proliferative Th1 T cell response to HMPV.

The lower level of CD4⁺ T cell proliferation associated with G and SH depends on contact between virus-stimulated MDDC and CD4⁺ T cells. The absence of differences in the expression of maturation markers and cytokine genes by MDDC following stimulation with UV-WT or UV-ΔSHG suggested that the restricted CD4⁺ T cell proliferation associated with UV-ΔSHG might depend on the contact between the virus-stimulated MDDC and CD4⁺ T cells. We tested this hypothesis using a transwell system. Immature MDDC were labeled with DDAO and mock stimulated or stimulated for 4 h with UV-WT or UV-ΔSHG at an equivalent input MOI of 3 PFU/cell or incubated with the superantigen SEB. Four hours after stimulation, MDDC were extensively washed with complete medium. Transwell cultures were set up in which CFSE-labeled autologous CD4⁺ T cells were placed in the bottom compartment of each culture, and MDDC stimulated as described above were placed in the top and/or bottom compartments to evaluate possible effects of direct contact versus soluble factors. The top and bottom compartments were separated by a membrane with a pore size of 0.4 μ m, which would prevent physical contact between cells in each compartment but allow passage of soluble factors. Seven days after incubation, the top chambers were removed and cells in the bottom chambers were collected and analyzed by flow cytometry to evaluate CD4⁺ T cell proliferation.

Figure 5 shows results of one representative experiment, using cells from one donor (the experiment was performed two more times, each with cells from additional donors, and each with comparable results). As expected, CD4⁺ T cells alone (Fig. 5, culture 1) or cocultured with mock-stimulated MDDC (culture 2) did not proliferate, whereas SEB-treated MDDC placed with the T cells in the bottom compartment induced strong CD4⁺ T cell proliferation (culture 3). In addition, MDDC stimulated with UV-ΔSHG, placed in the bottom compartment with the T cells (culture 5), induced a stronger proliferation of CD4⁺ T cells than did MDDC stimulated with UV-WT HMPV (culture 4), as observed in previous experiments in this study. Addition of SEB to the top chamber induced a strong proliferative response by CD4⁺ T cells cocultured in the bottom well with mock-stimulated MDDC (culture 6). This showed that the transwell membrane was permeable to SEB superantigen. In contrast, addition to the top chamber of MDDC stimulated with UV-WT (culture 7) or UV-ΔSHG (culture 8) did not induce any proliferative response of CD4⁺ T cells cocultured with mock-stimulated MDDC in the bottom chamber. This confirmed that direct contact between virus-stimulated MDDC and T cells rather than soluble factors was required to induce CD4⁺ T cell proliferation and that there was no significant antigen diffusion from the virus-stimulated MDDC in the top chambers to the bottom chambers. The addition of mock-stimulated MDDC to the top chamber did not affect proliferation of CD4⁺ T cells cocultured in the bottom chamber with MDDC stimulated with UV-WT (culture 9) or UV-ΔSHG (culture 10). The presence in the top chamber of MDDC stimulated with UV-ΔSHG did not increase the proliferation of CD4⁺ T cells cocultured in the bottom chamber with MDDC stimulated with UV-WT HMPV (culture 11). This showed that UV-ΔSHG-stimulated MDDC did not release soluble factors capable of increasing T cell proliferation in response to MDDC stimulated with UV-WT HMPV. Conversely, the presence in the top chamber of MDDC stimulated with UV-WT HMPV did not reduce the proliferation of CD4⁺ T cells cocultured in the bottom chamber with MDDC

FIG 4 Effect of the HMPV G and SH proteins on CD4⁺ T cell cytokine expression. Expression of IFN- γ , TNF- α , and IL-2 individually and in combination was determined in live, CD4⁺ T cells from cocultures of MDDC and autologous CD4⁺ T cells. Immature MDDC were stimulated with live WT HMPV or Δ SHG at an MOI of 1 PFU/cell or with an equivalent amount of UV-WT HMPV or UV- Δ SHG. (A) Number of proliferated CD4⁺ T cells expressing cytokines in all possible combinations from days 1 to 7 ($n = 1$ donor). (B) Total number of proliferating CD4⁺ T cells expressing cytokines in all possible combinations at day 7 using cells from six different donors. The box plots show the median (horizontal line), flanked by the second and third quartiles; the outer bars show the ranges. Statistical differences between live or UV-inactivated viruses are indicated by asterisks (see Materials and Methods; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (C) Summary of data from panel B expressed as number of positive cells. In the stacked bar graphs, the overall height of the bar indicates the total median number of CD4⁺ proliferating T cells expressing cytokines in all possible combinations, and the height of each color (coded as shown in the inset) indicates the median number of an individual cytokine or cytokine combination.

FIG 5 The increased $CD4^+$ T cell proliferation induced by UV- Δ SHG depends on MDDC- $CD4^+$ T cell contact. Immature DDAO-labeled MDDC were mock stimulated (illustrated as black cells), stimulated with UV-WT (blue) or UV- Δ SHG HMPV at an equivalent input MOI of 3 PFU/cell, or treated with SEB (gray). Four hours after stimulation, MDDC were extensively washed. Twelve different cultures (numbered 1 to 12) were prepared using transwells with top and bottom compartments separated by a membrane with an $0.4\text{-}\mu\text{m}$ pore size, permeable to soluble factors but not to cells. CFSE-labeled autologous $CD4^+$ T cells (green) were placed in the bottom chamber of each culture. Various combinations of stimulated MDDC were placed in the top and/or bottom compartments as shown and as described in the text, and one culture (culture 6) was prepared with soluble SEB in the top compartment. Seven days after incubation, the top chambers were discarded and cells in the bottom chambers were collected and analyzed by flow cytometry to evaluate $CD4^+$ T cell proliferation, expressed as percentage of live cells (percentage is noted below the gated proliferated population). The results are for one representative donor of three.

stimulated with UV- Δ SHG (culture 12). This showed that MDDC stimulated with UV-WT HMPV did not release soluble factors capable of inhibiting T cell proliferation in response to MDDC stimulated with UV- Δ SHG. Taken together, these results show that the lower level of $CD4^+$ T cell proliferation associated with UV-WT HMPV than with UV- Δ SHG depends strictly on contact between the virus-stimulated MDDC and $CD4^+$ T cells, consistent with a direct inhibitory effect of the SH and G proteins present on virus-exposed MDDC.

MDDC stimulated with UV- Δ SHG establish more conjugates with $CD4^+$ T cells than do MDDC stimulated with UV-WT HMPV. We next compared the abilities of MDDC stimulated with UV-WT or with UV- Δ SHG to establish conjugates with $CD4^+$ T cells, as characterized by the formation of an IS, which is the primary step to initiate T cell proliferation. MDDC were labeled with CFSE, stimulated with UV-WT or UV- Δ SHG at an equivalent MOI of 3 PFU/cell for 4 h, and cocultured on coverslips with autologous $CD4^+$ T cells for 1 h. The $CD4^+$ T cells had been depleted of naive T cells in order to increase the abundance of HMPV-specific memory T cells (Materials and Methods). After 1 h of incubation, the cocultures were fixed and stained for CD3, and the nuclei were stained with DAPI. The slides were examined by fluorescence confocal microscopy in a blinded fashion in order

to detect ISs. For this analysis, an IS was defined as the clustering of labeled CD3 at a point of contact between an MDDC (labeled with CFSE) and a $CD4^+$ T cell, with a mean fluorescence intensity greater than 2-fold above the CD3 mean fluorescence intensity on the remaining surface of the cell. In an activated $CD4^+$ T cell, the clustering of CD3 at the contact zone is associated with a compensatory decrease in CD3 fluorescence in surrounding areas. The contact zones that fulfilled these criteria were considered to represent ISs. Cells from two donors were used, and 100 fields were analyzed for each virus per donor.

Figure 6A shows a typical MDDC- $CD4^+$ T cell contact without polarization of CD3, representing a contact that did not result in a detectable IS, as a negative example. Figure 6B shows two examples of MDDC- $CD4^+$ T cell contacts in which CD3 polarization was evident (arrows), indicative of the formation of ISs (MDDC are stained green; CD3 is red; nuclei are dark blue). Movies S1 and S2 in the supplemental material show the three-dimensional (3D) reconstruction and modeling of typical MDDC- $CD4^+$ T cell contacts without and with CD3 polarization, respectively. The data are summarized in Fig. 6C. As expected, due to the low frequency of HMPV-specific $CD4^+$ T cells, the number of ISs detected in cells from the two different donors was low. With MDDC stimulated with UV-WT HMPV, a single IS involving contact between

FIG 6 Visualization of immunological synapses between UV-ΔSHG or UV-WT HMPV-stimulated MDDC and autologous CD4⁺ T cells. Immature CFSE-labeled MDDC (green) were stimulated with UV-WT or UV-ΔSHG at an MOI of 3 PFU/cell for 4 h and were placed on coverslips with autologous CD4⁺ T cells. After 1 h of incubation, the cells were fixed and immunostained for CD3 (red) and nuclei (DAPI, blue). Using cells from two different donors, 100 fields per virus were inspected by confocal microscopy in a blinded fashion in order to detect ISs, which were identified by polarization of CD3 at a contact zone between an MDDC and a T cell. (A and B) Confocal photomicrographs of contacts between MDDC and T cells visualized under white light (left panels, with the DC and T cells identified) and by fluorescence (right panels). (A) MDDC-T cell contact without polarization of CD3, as an example negative for an IS. (B) Two examples of MDDC-T cell contact with CD3 polarization (white arrows) indicative of IS; MDDC were previously stimulated with UV-ΔSHG. (C) Summary of analysis of 100 fields for each treatment for two donors (numbered 1 and 2). Each symbol represents a T cell with CD3 polarization, indicative of an IS. The y axis plots the MFIs associated with the IS and with the T cell membrane outside the contact zone (Mb).

one CD4⁺ T cell and one MDDC was detected for each donor. With MDDC stimulated with UV-ΔSHG, five ISs were observed for each donor: three each involved contact between one CD4⁺ T cell and one MDDC, and two involved separate contacts between one CD4⁺ T cell and two MDDC (open circles in Fig. 6C for each donor). The MFI for CD3 was measured for the contact zone and the surrounding membrane region (y axis in Fig. 6C), showing the increase in fluorescence associated with the contact zone. The intensities of the ISs were substantially greater with UV-ΔSHG than with UV-WT HMPV. Taken together, these data show that the ISs formed with MDDC stimulated with UV-ΔSHG compared to those formed with MDDC stimulated with UV-WT HMPV were more frequent and had a greater clustering of CD3.

HMPV is internalized into DC through an endocytic pathway that is more efficient when SH and G are deleted. As noted, deletion of SH and G was associated with an increase in the ability of HMPV to infect MDDC, as measured by GFP expression as a surrogate for viral gene expression (Fig. 1A), and also was associated with an increase in CD4⁺ T cell activation mediated by

MDDC stimulated with either live or UV-inactivated virus (Fig. 3). Thus, SH and G had comparable inhibitory effects on the pathway leading to robust infection and on the pathway leading to antigen presentation. This suggested that SH and G inhibited virus uptake and internalization, since this step is common to the two pathways.

To compare uptake in the presence and in the absence of SH and G, immature MDDC were inoculated with UV-WT or UV-ΔSHG and, at different times during the next 4 h, aliquots of cells were taken, extensively washed to remove unbound virus particles, and cocultured with CD4⁺ T cells. T cell proliferation was evaluated at day 7. Figure 7A shows results of one experiment, using cells from one donor (the experiment was performed one additional time, with cells from another donor, and with comparable results). We found that for UV-ΔSHG, an exposure time of MDDC to virus of 2 h was sufficient for MDDC to induce maximum proliferation, whereas for UV-WT and MDDC, 4 h of virus exposure did not stimulate the MDDC sufficiently to induce maximum T cell proliferation. When the MDDC were not washed to

FIG 7 HMPV WT and Δ SHG enter MDDC by endocytosis. (A) Effect of contact time between virus and MDDC on CD4⁺ T cell proliferation. DDAO-labeled immature MDDC derived from one donor were inoculated with the equivalent of 3 PFU/cell of UV-WT or UV- Δ SHG. Aliquots of MDDC were removed at 5, 30, 120, or 240 min postinoculation; extensively washed to remove unbound virus particles; cocultured with autologous CD4⁺ T cells for 7 days; and analyzed for T cell proliferation. (B to F) Effect of calcium depletion (B, C, and F) and inhibition of actin polymerization (D, E, and F) on the internalization of live HMPV WT and Δ SHG or the entry-tracking dye LY (Materials and Methods). LY was used as a control to confirm the effectiveness of the actin polymerase inhibitors in blocking LY uptake and the lack of nonspecific inhibition of calcium depletion on LY uptake. Immature MDDC were pretreated with the indicated concentration of EGTA (B, C, and F), CytD (D and F), or LatA (E) for 1 h at 37°C and then infected with 3 PFU/cell of HMPV WT or Δ SHG expressing GFP or 200 μ g/ml of LY. Twenty-four hours postinfection, the percentage of GFP-positive MDDC was quantified by flow cytometry. LY uptake was performed for 15 min (see Materials and Methods for details). Panel B is a dot plot of data from a typical donor, showing GFP expression versus forward scatter (FSC; a measure of cell size) for live MDDC. The population of GFP-positive cells is boxed, and its percentage is indicated. In panels C to F, data are normalized as a percentage of the untreated control from $n = 6$ and 3 different donors for the viruses and the LY, respectively. Each histogram shows the mean with the standard error. The statistical significance of the difference between nontreated (NT) WT or Δ SHG and its corresponding treated samples is indicated by an asterisk (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

remove virus prior to cocultivation, the WT-stimulated MDDC became as efficient as those stimulated with Δ SHG (not shown). This indicated that the presence of SH and G was associated with a reduced rate of uptake of HMPV.

Next, we investigated whether HMPV uptake by MDDC involved endocytosis (which can include receptor-mediated endocytosis, phagocytosis, or macropinocytosis) as opposed to cell surface fusion mediated by the HMPV F protein. First, we investigated calcium dependency, which is a hallmark of endocytosis in general, and also is required for binding by C-type lectin receptors (CLRs). Immature MDDC representing 6 different donors were preincubated for 1 h with different concentrations of the calcium chelator EGTA, infected with GFP-expressing HMPV WT or Δ SHG, and analyzed for GFP expression at 24 h p.i. (Fig. 7B and C). GFP expression of MDDC infected with HMPV WT or Δ SHG was almost completely abrogated in the presence of 10 mM EGTA (88.3% $\pm$ 5.5% and 87.6% $\pm$ 4.2% reduction of GFP expression, respectively, compared to untreated cells), indicating that calcium-dependent en-

docytosis was essential for WT and Δ SHG entry into MDDC. As an additional control, we showed that treatment of MDDC with EGTA did not inhibit the uptake of the tracking molecule LY (Materials and Methods; also Fig. 7F), which has been previously shown to be calcium independent (45).

We also evaluated the dependency for actin polymerization, another hallmark of endocytosis, using the inhibitor cytochalasin D (Cyt D) or latrunculin A (Lat A) (Fig. 7D and E; $n = 6$ donors). As a control, we confirmed that the concentrations of Cyt D that we used inhibited the uptake of LY, confirming the effect of the drug (Fig. 7F). Each inhibitor significantly inhibited GFP expression. For example, addition of 20 μ M Cyt D resulted in a reduction of GFP expression by 67.7% $\pm$ 7.1% or 96.9% $\pm$ 3.1% in MDDC infected with WT or Δ SHG, respectively. Addition of 10 μ M Lat A resulted in a reduction of GFP expression of 75.8% $\pm$ 2.9% or 88.5% $\pm$ 3.8% in MDDC infected with WT or Δ SHG, respectively. Interestingly, MDDC infection with Δ SHG appeared to be more sensitive to the higher concentrations of Cyt D (2 μ M and 20

FIG 8 Effect of HMPV G and SH proteins on C-type lectin receptors and dynamin-dependent endocytosis pathways. The effect of C-type lectin receptor blocking or dynamin-dependent endocytosis inhibition on the entry of HMPV and Δ SHG was evaluated by flow cytometry. Immature MDDC were pretreated with the indicated concentrations of mannan, an anti-DC-SIGN MAb (AZN-D1), or the dynamin inhibitor Dynasore for 1 h at 37°C and then infected at an MOI of 3 PFU/cell with GFP-expressing HMPV WT or Δ SHG. Twenty-four hours postinfection, the percentage of GFP-positive MDDC under each condition was evaluated. In parallel, the effectiveness and lack of nonspecific inhibition by mannan were confirmed by its ability to inhibit the uptake of the tracker molecule Dex, which depends on the mannose receptor, and its lack of effect on uptake of LY and Tr, which are taken up by other pathways (see Materials and Methods for details). (A to D) Data were normalized as a percentage of the untreated control from $n = 6$ (A), 5 (B), 7 (C), or 3 (D) different donors. Each histogram shows the mean with the standard error. The statistical significance of the difference between nontreated (NT) WT or Δ SHG and its corresponding treated samples is indicated by an asterisk (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

μ M, $P < 0.01$ and $P < 0.001$, respectively) or Lat A (10 μ M, $P < 0.01$) than did infection with WT, suggesting a greater dependency on actin polymerization for Δ SHG entry. Altogether, these results provided evidence that HMPV WT and Δ SHG enter MDDC via an active endocytic mechanism and that this is more efficient with Δ SHG than with WT.

G and SH proteins mediate internalization of HMPV WT through DC-SIGN and dynamin-dependent pathways. The marked calcium dependence observed for entry, as noted above, raised the possibility of CLR involvement. CLRs are pattern recognition molecules that bind carbohydrate ligands highly conserved recognition domains. For example, DC-SIGN is a CLR that is highly expressed on MDDC (46). We therefore evaluated the effects of the pan-CLR inhibitor mannan as well as the well-characterized DC-SIGN-specific blocking monoclonal antibody (MAb) AZN-D1 (Fig. 8A and B) on HMPV infection using live WT and Δ SHG viruses expressing GFP. As a control, 20 mg/ml of mannan did not affect LY and Tf uptake whereas Dex uptake was efficiently inhibited (60% inhibition, Fig. 8D; Materials and Methods), consistent with expectations. These results showed that the concentrations of mannan that we used specifically inhibit the mannose receptor-dependent pathway.

The percentage of MDDC infected with HMPV WT was partially reduced in a dose-dependent manner by mannan ($55.5 \pm 8.2\%$ reduction at the 20-mg/ml concentration, $n = 6$ donors, $P \leq 0.001$) or AZN-D1 ($47.7 \pm 6.1\%$ reduction at the 20- μ g/ml concentration, $n = 5$ donors, $P \leq 0.001$), suggesting that a CLR,

and specifically DC-SIGN, is involved in HMPV uptake into MDDC. Interestingly, the Δ SHG virus was insensitive to these CLR-specific agents (no statistical difference with untreated cells and $P < 0.05$ compared to WT at all tested concentrations). This suggests that SH and/or G is necessary for uptake of HMPV by DC-SIGN.

Antigen that is bound by DC-SIGN is internalized by clathrin-dependent endocytosis (47). The large GTPase dynamin II is required for clathrin-dependent endocytosis (as well as caveolin-dependent endocytosis and phagocytosis), where it functions to pinch off endocytic vesicles from the plasma membrane. We therefore investigated the effects of the dynamin inhibitor Dynasore on live HMPV infection. GFP expression in MDDC infected with WT was partially inhibited in a dose-dependent manner by Dynasore ($33.3 \pm 5.9\%$ reduction at 50 μ M, $n = 7$ donors, $P \leq 0.001$), whereas at this concentration, Δ SHG was insensitive to this inhibitor (no statistical difference with untreated cells but $P < 0.001$ and $P < 0.01$ compared to WT at the 100 μ M and 50 μ M concentrations, respectively) (Fig. 8C).

The conclusion that WT HMPV, but not Δ SHG, interacted with DC-SIGN raised the formal possibility that this interaction might inhibit DC function and thus might account for the reduced T cell activation observed with DC stimulated with WT HMPV compared to that stimulated with Δ SHG. To evaluate this, we blocked DC-SIGN by incubation of DC with MAb AZN-D1 prior to exposure to WT HMPV or Δ SHG ($n = 1$; data not shown). However, this treatment did not increase the ability of

FIG 9 Effect of HMPV G and SH proteins on macropinocytosis. The effect of inhibitors of macropinocytosis on the entry of HMPV and Δ SHG was evaluated by flow cytometry. Immature MDDC were pretreated with the indicated concentrations of macropinocytosis inhibitors rottlerin and amiloride EIPA or DMA, the PI3K inhibitor wortmannin, or the protein kinase C (PKC) inhibitor GF109203X for 1 h at 37°C and then infected at an MOI of 3 PFU/cell with GFP-expressing HMPV WT or Δ SHG. Twenty-four hours postinfection, the percentage of GFP-positive MDDC under each condition was evaluated. In parallel, the effectiveness and lack of nonspecific effects of rottlerin were confirmed by demonstrating its ability to inhibit the uptake of LY, which is dependent on macropinocytosis, and its marginal effects on the uptake of Tf and Dex, which are taken up by other pathways (see Materials and Methods for details). (A to E and G) Data were normalized as a percentage of the untreated control from $n = 6$ (A to E) or 3 (G) different donors. Each histogram shows the mean with the standard error. The statistical significance of the difference between nontreated (NT) WT or Δ SHG and its corresponding treated samples is indicated by an asterisk (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (F) Immature DDAO-labeled MDDC were incubated for 1 h with 5 μ M rottlerin before being stimulated with an MOI of 1 PFU/cell with the indicated live virus or with an equivalent amount of UV-inactivated virus or 1 μ g/ml SEB. SEB treatment was used as a control to show that rottlerin did not have nonspecific inhibitory effects on the coculture system. Four hours later, MDDC were washed to remove noninternalized virus and cocultured with CFSE-labeled autologous CD4⁺ T lymphocytes at a ratio of 1 MDDC for 10 CD4⁺ T cells. CD4⁺ T cell proliferation was measured after 7 days of coculture by flow cytometry to detect CFSE dilution indicative of cell division. Data were normalized as a percentage of the untreated control from $n = 3$ different donors. Each histogram shows the mean with the standard error.

HMPV WT-stimulated DC to stimulate T cell proliferation, and thus, there was no evidence of DC-SIGN-mediated inhibition.

Taken together, these results indicate that WT HMPV can be taken up by MDDC by receptor-mediated endocytosis involving DC-SIGN. However, this was dependent on the presence of SH and/or G and was not operative for Δ SHG. The efficient uptake of Δ SHG, together with the incomplete effects of the inhibitors on WT HMPV, indicated that another entry mechanism must exist.

Macropinocytosis is the main entry pathway for HMPV WT and Δ SHG. The sensitivity of Δ SHG to EGTA, Cyt D, and Lat A noted above indicated that this alternative entry mechanism presumably involves endocytosis. However, the insensitivity of Δ SHG to Dynasore noted above indicated that caveolin-mediated

endocytosis and phagocytosis are not involved, since these pathways depend on dynamin (48). Macropinocytosis has been found to be involved in the uptake of an increasingly wide array of viruses. Therefore, we investigated its possible involvement using three well-known inhibitors of macropinocytosis: rottlerin, 5-(N-ethyl-N-isopropyl)amiloride (EIPA), and dimethyl amiloride (DMA) (Fig. 9). Amilorides inhibit the Na⁺/H⁺ ion exchange pump in the plasma membrane affecting the intracellular pH, resulting in the cessation of macropinocytosis. The precise mechanism of macropinocytosis inhibition by rottlerin is still unknown, but rottlerin was recently shown to be more specific than EIPA or DMA in blocking macropinocytosis in DC (34). We confirmed the specificity of rottlerin in inhibiting macropinocytosis under

our experimental conditions by showing that it inhibits the uptake of LY but not Tr or Dex (Fig. 9G).

GFP expression of MDDC infected with WT was significantly reduced by $90.4\% \pm 5.0\%$ with 5 μM rottlerin and by $35.5\% \pm 10.3\%$ with 20 μM DMA. GFP expression of MDDC infected with ΔSHG was significantly reduced by $88.0\% \pm 2.4\%$ with 5 μM rottlerin and by $49.3\% \pm 22.3\%$ with 50 μM EIPA (Fig. 9A, B, and C).

In many cell types, macropinocytosis is also dependent on several kinases, including phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC), which are involved in signaling pathways that promote membrane ruffling and macropinosome formation. However, PKC is not critical for macropinocytosis in DC (34). We found that the percentage of GFP-expressing MDDC infected with WT or ΔSHG was significantly reduced in the presence of 10 μM wortmannin, an inhibitor of PI3K ($99.1\% \pm 0.2\%$ and $99.8\% \pm 0.2\%$, respectively), but not in the presence of GF109203X, an inhibitor of PKC (Fig. 9D and E). Interestingly, ΔSHG infection of MDDC was more sensitive to PI3K inhibition than was infection by WT, as the percentage of GFP-expressing MDDC infected with ΔSHG was significantly reduced at the concentration of 1 μM ($P \leq 0.05$), whereas GFP expression of MDDC infected with WT HMPV was not.

Finally, we investigated whether inhibition of MDDC macropinocytosis by treatment with rottlerin, and the resulting inhibition of uptake of WT and ΔSHG HMPV, indeed resulted in reduced CD4^+ T cell proliferation. To do so, MDDC were stimulated with UV-inactivated or live WT or ΔSHG HMPV, or SEB, in the presence of 5 μM rottlerin as described above, before being cocultured with autologous CD4^+ T lymphocytes. CD4^+ T cell proliferation and cytokine production were evaluated at day 7, as described above. This showed that CD4^+ T cell proliferation indeed was strongly inhibited by rottlerin treatment for both WT and ΔSHG (reductions of $89.6\% \pm 0.4\%$ and $73.1\% \pm 17.1\%$, respectively; $n = 3$ donors). As a consequence of the reduction of the proliferation, the number of cytokine-expressing T cells also was reduced (data not shown). This confirmed that uptake of HMPV via macropinocytosis is necessary for MDDC to be able to efficiently stimulate memory CD4^+ T cells and that reduced uptake resulted in reduced proliferation. In contrast, rottlerin did not affect CD4^+ proliferation mediated by SEB-treated MDDC (Fig. 9F), showing that the drug did not have nonspecific inhibitory effects on the coculture system.

DISCUSSION

A decade after it was first described, HMPV is widely recognized as an important etiologic agent of respiratory disease. There is no vaccine or specific antiviral therapy for HMPV. Similar to respiratory syncytial virus, HMPV can reinfect symptomatically throughout life without significant antigenic change (6–9). This may reflect virus-mediated inhibition of host immune responses. DC maturation and T cell activation are critical steps in the adaptive immune response. In the present study, we evaluated the effects of the HMPV attachment G and small hydrophobic SH glycoproteins on infection and maturation of primary human MDDC and autologous CD4^+ T cell activation.

We found that the G and SH proteins are not essential for HMPV to infect human MDDC, and indeed, the absence of both proteins resulted in a significant, 3-fold increase in infectivity measured 24 h postinfection. However, even in the absence of SH

and G, the infectivity of HMPV for MDDC was nearly 10-fold less than that for epithelial cells. We previously noted this low infectivity and suggested that this may be one means by which HMPV reduces its immunologic impact, since efficient infection of professional antigen-presenting cells would provide for greater immune stimulation. In epithelial cell-like cell lines, deletion of SH and/or G has little effect on HMPV replication *in vitro* (11). *In vivo*, deletion of G results in a substantial restriction in replication in hamsters and an even greater restriction in African green monkeys, whereas deletion of SH had little or no effect (11, 12).

We previously reported that HMPV induces a low level of maturation of immature MDDC compared to the positive-control LPS, which induces strong DC maturation (27). This low level of maturation was not significantly changed by the deletion of the SH or G proteins, suggesting that SH and G do not have a regulatory or inhibitory effect on maturation or cytokine production by MDDC. There were two marginal exceptions to this general observation. First, HMPV lacking G, SH, or both G and SH proteins induced a slight (but not significant) increase in type I IFN production by MDDC compared to WT HMPV. These results are consistent with previously published data showing that HMPV G inhibits cytokine release in human MDDC (41) and that both G and SH proteins inhibit the activation of transcription factors belonging to NF- κB in a human lung epithelial cell line (A549 cell line) (42, 43). Our results suggest that the inhibition of type I IFN release by the HMPV G protein is more modest in human MDDC than in epithelial cells, possibly due to the low infectivity of HMPV for MDDC. In addition, deletion of G or both SH and G induced a significant increase in the expression of CD38 on the subset of virus-exposed MDDC that were GFP $^+$ (e.g., those cells that were robustly infected [Fig. 2C]). CD38 is involved in DC migration, survival, and Th1 polarization of interacting T cells (13), which suggests an inhibitory effect of G on these factors. The increase in CD38 expression associated with deletion of G might be directly related to the slight increase in type I IFN expression, since type I IFN induces expression of CD38 but not of CD40, CD54, CD80, CD83, and CD86 (49). In any event, deletion of SH and G had little effect on MDDC maturation.

We next evaluated the effect of deleting the HMPV G and/or SH protein on the activation of autologous CD4^+ T cells. Surprisingly, MDDC stimulated with HMPV lacking both G and SH proteins significantly increased proliferation of autologous CD4^+ T cells compared to MDDC stimulated with WT HMPV. This increase was observed with both live and UV-inactivated $\Delta\text{SH}/\text{G}$ virus, showing that this effect was independent of virus replication.

In the absence of virus replication (i.e., with UV-inactivated preparations), the deletion of either the G or the SH protein alone resulted in a modest increase in the ability of stimulated MDDC to direct CD4^+ T cell proliferation (data not shown), while the deletion of both had an effect that was greater than the sum of the individual effects. Thus, both proteins contribute to limit the CD4^+ T cell proliferation. This is somewhat comparable to the effects observed with measles virus (MeV) glycoproteins F and HA, which were shown to inhibit T cell proliferation. Similar to the HMPV glycoproteins, both MeV glycoproteins appear to be involved in this inhibition, as the removal of either one of these proteins abolished inhibition (50–55). However, stimulation of MDDC with increasing concentrations of HMPV, containing increased amounts of HMPV SH and G proteins, resulted in increas-

ing CD4⁺ T cell proliferation (data not shown), whereas for MeV, increasing concentrations of F and HA proteins resulted in an increased inhibition of T cell proliferation (50, 53). This suggests that the HMPV G and SH proteins are not as effective in inhibiting the CD4⁺ T cell proliferation as are the measles virus F and HA proteins.

Transwell experiments showed that the ability of the G and SH proteins to reduce CD4⁺ T cell proliferation strictly depended on contact between the virus-stimulated MDDC and the CD4⁺ T cells. Direct visualization of contacts between virus-stimulated MDDC and memory CD4⁺ T cells showed that the contacts involving WT HMPV-stimulated MDDC compared to those involving Δ SHG-stimulated MDDC were fewer.

Deletion of SH and G was associated with (i) increased infectivity in MDDC by live HMPV and (ii) increased CD4⁺ T cell activation in response to MDDC stimulated with live or UV-inactivated HMPV. These observations suggested that SH and G inhibited a common step in the pathway to infectivity and the pathway to MDDC-mediated activation of T cells. A likely step was virus uptake and internalization. A time course experiment indicated that, in addition to decreasing the efficiency of infectivity, SH and G were associated with a reduced rate of internalization. Studies with inhibitors indicated that uptake of WT and Δ SHG HMPV was mediated by endocytosis rather than surface fusion. WT HMPV appeared to be taken up by two different pathways. One pathway was receptor-mediated, clathrin-dependent endocytosis that involved DC-SIGN and required SH and/or G (and thus did not operate for Δ SHG). However, this appeared to account for only part of the uptake. A second pathway, which appeared to be more important, was found to involve macropinocytosis. Macropinocytosis is a constitutive property of immature DC that is primarily responsible for nonspecific uptake of fluid, solutes, membrane, ligands, and small particles attached to the plasma membrane. It is becoming increasingly recognized as a pathway for viral uptake and entry. Macropinocytosis is a ligand-induced process and requires the interaction between the virus and the cell surface, which can involve multiple different types of contacts: some providing anchoring to the membrane, others needed for activation of receptors. It has been shown that some viruses bind to heparan sulfate proteoglycans and other glycans or integrins that end up influencing actin dynamics and inducing macropinocytosis (see review in reference 56).

Although HMPV WT could be taken up by two pathways, namely, DC-SIGN-mediated endocytosis and macropinocytosis, whereas Δ SHG could be taken up only by the latter pathway, uptake of Δ SHG was significantly more efficient than that of HMPV WT. This implies that macropinocytosis was more efficient in the absence of SH and G. This is consistent with the time course experiment noted above, in which uptake of HMPV WT was much slower than that of Δ SHG. Thus, we conclude that macropinocytosis is the major uptake pathway for WT HMPV (and the only pathway for Δ SHG) and that SH and G reduce the efficiency of macropinocytosis. Our conclusions on viral uptake are offered with the caveat that we did not directly visualize uptake but rather measured the expression of GFP as a marker. However, we believe that GFP expression is a reasonable marker because HMPV is a relatively simple cytoplasmic virus that bears its own polymerase and has a relatively simple mode of gene expression. Thus, GFP expression is closely related to entry of the viral nucleocapsid across the cellular membrane.

Interestingly, macropinocytosis has also been shown recently to be the main internalization pathway used by respiratory syncytial virus in human tissue culture cells (57). Examples have been reported in which the glycoproteins of other viruses affect viral internalization. For example, the poxvirus semaphorin A39R protein was shown to inhibit phagocytosis by DC (58). Conversely, the Ebola virus glycoprotein was shown to stimulate macrophagocytosis to facilitate viral entry (36). In the present study, we suggest that the HMPV SH and G proteins inhibit macropinocytosis by DC, with the effect of reducing antigen uptake and presentation and reducing T cell activation. This represents a previously unknown mechanism of virus immune evasion. It is possible that deletion of SH and G revealed a domain in F that might, for example, result in increased binding and uptake by DC.

The increased CD4⁺ T cell proliferation induced by HMPV lacking the G and SH proteins resulted in an increased number of IFN- γ ⁺, TNF- α ⁺, and IL-2⁺ CD4⁺ T cells. Thus, by reducing the CD4⁺ T cell proliferation, the HMPV G and SH proteins restrict the Th1 polarized response. This suggests that *in vivo*, the HMPV G and SH proteins, by interfering with the internalization of HMPV into DC, would reduce the CD4⁺ T cell proliferation and Th1 polarized response and thus would reduce and alter the immunological footprint of this virus, limiting the protective immune response. Such effects of these two proteins should be considered in the development of a live-attenuated vaccine for this virus.

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REFERENCES

1. van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, Osterhaus AD. 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat. Med.* 7:719–724. <http://dx.doi.org/10.1038/89098>.
2. O'Gorman C, McHenry E, Coyle PV. 2006. Human metapneumovirus in adults: a short case series. *Eur. J. Clin. Microbiol. Infect. Dis.* 25:190–192. <http://dx.doi.org/10.1007/s10096-006-0116-0>.
3. Williams JV, Harris PA, Tollefson SJ, Halburnt-Rush LL, Pingsterhaus JM, Edwards KM, Wright PF, Crowe JE, Jr. 2004. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. *N. Engl. J. Med.* 350:443–450. <http://dx.doi.org/10.1056/NEJMoa025472>.
4. Falsey AR, Erdman D, Anderson LJ, Walsh EE. 2003. Human metapneumovirus infections in young and elderly adults. *J. Infect. Dis.* 187:785–790. <http://dx.doi.org/10.1086/367901>.
5. Hamelin ME, Abed Y, Boivin G. 2004. Human metapneumovirus: a new player among respiratory viruses. *Clin. Infect. Dis.* 38:983–990. <http://dx.doi.org/10.1086/382536>.
6. Williams JV, Wang CK, Yang CF, Tollefson SJ, House FS, Heck JM, Chu M, Brown JB, Lintao LD, Quinto JD, Chu D, Spaete RR, Edwards KM, Wright PF, Crowe JE, Jr. 2006. The role of human metapneumovirus in upper respiratory tract infections in children: a 20-year experience. *J. Infect. Dis.* 193:387–395. <http://dx.doi.org/10.1086/499274>.
7. Falsey AR. 2008. Human metapneumovirus infection in adults. *Pediatr. Infect. Dis. J.* 27:S80–S83. <http://dx.doi.org/10.1097/INF.0b013e3181684dac>.
8. Walsh EE, Peterson DR, Falsey AR. 2008. Human metapneumovirus

- infections in adults: another piece of the puzzle. *Arch. Intern. Med.* 168: 2489–2496. <http://dx.doi.org/10.1001/archinte.168.22.2489>.
9. Okamoto M, Sugawara K, Takashita E, Muraki Y, Hongo S, Nishimura H, Matsuzaki Y. 2010. Longitudinal course of human metapneumovirus antibody titers and reinfection in healthy adults. *J. Med. Virol.* 82:2092–2096. <http://dx.doi.org/10.1002/jmv.21920>.
 10. Collins PL, Crowe JE. 2007. Respiratory syncytial virus and metapneumovirus, p 1601–1646. In Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (ed), *Fields virology*, 5th ed, vol 2. Lippincott Williams & Wilkins, Philadelphia, PA.
 11. Biacchesi S, Skiadopoulos MH, Yang L, Lamirande EW, Tran KC, Murphy BR, Collins PL, Buchholz UJ. 2004. Recombinant human metapneumovirus lacking the small hydrophobic SH and/or attachment G glycoprotein: deletion of G yields a promising vaccine candidate. *J. Virol.* 78:12877–12887. <http://dx.doi.org/10.1128/JVI.78.23.12877-12887.2004>.
 12. Biacchesi S, Pham QN, Skiadopoulos MH, Murphy BR, Collins PL, Buchholz UJ. 2005. Infection of nonhuman primates with recombinant human metapneumovirus lacking the SH, G, or M2-2 protein categorizes each as a nonessential accessory protein and identifies vaccine candidates. *J. Virol.* 79:12608–12613. <http://dx.doi.org/10.1128/JVI.79.19.12608-12613.2005>.
 13. Frasca L, Fedele G, Deaglio S, Capuano C, Palazzo R, Vaisitti T, Malavasi F, Ausiello CM. 2006. CD38 orchestrates migration, survival, and Th1 immune response of human mature dendritic cells. *Blood* 107: 2392–2399. <http://dx.doi.org/10.1182/blood-2005-07-2913>.
 14. Reis e Sousa C. 2006. Dendritic cells in a mature age. *Nat. Rev. Immunol.* 6:476–483. <http://dx.doi.org/10.1038/nri1845>.
 15. Zhou L, Chong MM, Littman DR. 2009. Plasticity of CD4+ T cell lineage differentiation. *Immunity* 30:646–655. <http://dx.doi.org/10.1016/j.immuni.2009.05.001>.
 16. King C. 2009. New insights into the differentiation and function of T follicular helper cells. *Nat. Rev. Immunol.* 9:757–766. <http://dx.doi.org/10.1038/nri2644>.
 17. Zhu J, Paul WE. 2010. Heterogeneity and plasticity of T helper cells. *Cell Res.* 20:4–12. <http://dx.doi.org/10.1038/cr.2009.138>.
 18. MacLeod MK, Clambey ET, Kappler JW, Marrack P. 2009. CD4 memory T cells: what are they and what can they do? *Semin. Immunol.* 21:53–61. <http://dx.doi.org/10.1016/j.smim.2009.02.006>.
 19. Löhning M, Hegazy AN, Pinschewer DD, Busse D, Lang KS, Hofer T, Radbruch A, Zinkernagel RM, Hengartner H. 2008. Long-lived virus-reactive memory T cells generated from purified cytokine-secreting T helper type 1 and type 2 effectors. *J. Exp. Med.* 205:53–61. <http://dx.doi.org/10.1084/jem.20071855>.
 20. Geiger R, Duhon T, Lanzavecchia A, Sallusto F. 2009. Human naive and memory CD4+ T cell repertoires specific for naturally processed antigens analyzed using libraries of amplified T cells. *J. Exp. Med.* 206:1525–1534. <http://dx.doi.org/10.1084/jem.20090504>.
 21. Jakubczik C, Tacke F, Ginhoux F, Wagers AJ, van Rooijen N, Mack M, Merad M, Randolph GJ. 2008. Blood monocyte subsets differentially give rise to CD103+ and CD103– pulmonary dendritic cell populations. *J. Immunol.* 180:3019–3027.
 22. Varol C, Landsman L, Fogg DK, Greenshtein L, Gildor B, Margalit R, Kalchenko V, Geissmann F, Jung S. 2007. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J. Exp. Med.* 204:171–180. <http://dx.doi.org/10.1084/jem.20061011>.
 23. Xu Y, Zhan Y, Lew AM, Naik SH, Kershaw MH. 2007. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J. Immunol.* 179:7577–7584.
 24. Cheong C, Matos I, Choi JH, Dandamudi DB, Shrestha E, Longhi MP, Jeffrey KL, Anthony RM, Kluger C, Nchinda G, Koh H, Rodriguez A, Idoyaga J, Pack M, Velinzon K, Park CG, Steinman RM. 2010. Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. *Cell* 143:416–429. <http://dx.doi.org/10.1016/j.cell.2010.09.039>.
 25. Biacchesi S, Skiadopoulos MH, Tran KC, Murphy BR, Collins PL, Buchholz UJ. 2004. Recovery of human metapneumovirus from cDNA: optimization of growth in vitro and expression of additional genes. *Virology* 321:247–259. <http://dx.doi.org/10.1016/j.virol.2003.12.020>.
 26. Biacchesi S, Murphy BR, Collins PL, Buchholz UJ. 2007. Frequent frameshift and point mutations in the SH gene of human metapneumovirus passaged in vitro. *J. Virol.* 81:6057–6067. <http://dx.doi.org/10.1128/JVI.00128-07>.
 27. Le Nouen C, Munir S, Losq S, Winter CC, McCarty T, Stephany DA, Holmes KL, Bukreyev A, Rabin RL, Collins PL, Buchholz UJ. 2009. Infection and maturation of monocyte-derived human dendritic cells by human respiratory syncytial virus, human metapneumovirus, and human parainfluenza virus type 3. *Virology* 385:169–182. <http://dx.doi.org/10.1016/j.virol.2008.11.043>.
 28. Le Nouen C, Hillyer P, Munir S, Winter CC, McCarty T, Bukreyev A, Collins PL, Rabin RL, Buchholz UJ. 2010. Effects of human respiratory syncytial virus, metapneumovirus, parainfluenza virus 3 and influenza virus on CD4+ T cell activation by dendritic cells. *PLoS One* 5:e15017. <http://dx.doi.org/10.1371/journal.pone.0015017>.
 29. Perfetto SP, Chattopadhyay PK, Roederer M. 2004. Seventeen-colour flow cytometry: unravelling the immune system. *Nat. Rev. Immunol.* 4:648–655. <http://dx.doi.org/10.1038/nri1416>.
 30. Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay P, Roederer M. 2006. Quality assurance for polychromatic flow cytometry. *Nat. Protoc.* 1:1522–1530. <http://dx.doi.org/10.1038/nprot.2006.250>.
 31. Seder RA, Darrah PA, Roederer M. 2008. T-cell quality in memory and protection: implications for vaccine design. *Nat. Rev. Immunol.* 8:247–258. <http://dx.doi.org/10.1038/nri2274>.
 32. Arhel N, Lehmann M, Clauss K, Nienhaus GU, Piguet V, Kirchhoff F. 2009. The inability to disrupt the immunological synapse between infected human T cells and APCs distinguishes HIV-1 from most other primate lentiviruses. *J. Clin. Invest.* 119:2965–2975. <http://dx.doi.org/10.1172/JCI38994>.
 33. Pryshchep S, Sato K, Goronzy JJ, Weyand CM. 2006. T cell recognition and killing of vascular smooth muscle cells in acute coronary syndrome. *Circ. Res.* 98:1168–1176. <http://dx.doi.org/10.1161/01.RES.0000220649.10013.5c>.
 34. Sarkar K, Kruhlak MJ, Erlandsen SL, Shaw S. 2005. Selective inhibition by rotterlin of macropinocytosis in monocyte-derived dendritic cells. *Immunology* 116:513–524. <http://dx.doi.org/10.1111/j.1365-2567.2005.02253.x>.
 35. Sandgren KJ, Wilkinson J, Miranda-Saksena M, McInerney GM, Byth-Wilson K, Robinson PJ, Cunningham AL. 2010. A differential role for macropinocytosis in mediating entry of the two forms of vaccinia virus into dendritic cells. *PLoS Pathog.* 6:e1000866. <http://dx.doi.org/10.1371/journal.ppat.1000866>.
 36. Nanbo A, Imai M, Watanabe S, Noda T, Takahashi K, Neumann G, Halfmann P, Kawaoka Y. 2010. Ebolavirus is internalized into host cells via macropinocytosis in a viral glycoprotein-dependent manner. *PLoS Pathog.* 6:e1001121. <http://dx.doi.org/10.1371/journal.ppat.1001121>.
 37. Laliberte JP, Weisberg AS, Moss B. 2011. The membrane fusion step of vaccinia virus entry is cooperatively mediated by multiple viral proteins and host cell components. *PLoS Pathog.* 7:e1002446. <http://dx.doi.org/10.1371/journal.ppat.1002446>.
 38. Schelhaas M, Shah B, Holzer M, Blattmann P, Kuhling L, Day PM, Schiller JT, Helenius A. 2012. Entry of human papillomavirus type 16 by actin-dependent, clathrin- and lipid raft-independent endocytosis. *PLoS Pathog.* 8:e1002657. <http://dx.doi.org/10.1371/journal.ppat.1002657>.
 39. Vanarsdall AL, Wisner TW, Lei H, Kazlauskas A, Johnson DC. 2012. PDGF receptor- α does not promote HCMV entry into epithelial and endothelial cells but increased quantities stimulate entry by an abnormal pathway. *PLoS Pathog.* 8:e1002905. <http://dx.doi.org/10.1371/journal.ppat.1002905>.
 40. Shan M, Klasse PJ, Banerjee K, Dey AK, Iyer SP, Dionisio R, Charles D, Campbell-Gardener L, Olson WC, Sanders RW, Moore JP. 2007. HIV-1 gp120 mannoses induce immunosuppressive responses from dendritic cells. *PLoS Pathog.* 3:e169. <http://dx.doi.org/10.1371/journal.ppat.0030169>.
 41. Kolli D, Bao X, Liu T, Hong C, Wang T, Garofalo RP, Casola A. 2011. Human metapneumovirus glycoprotein G inhibits TLR4-dependent signaling in monocyte-derived dendritic cells. *J. Immunol.* 187:47–54. <http://dx.doi.org/10.4049/jimmunol.1002589>.
 42. Bao X, Liu T, Shan Y, Li K, Garofalo RP, Casola A. 2008. Human metapneumovirus glycoprotein G inhibits innate immune responses. *PLoS Pathog.* 4:e1000077. <http://dx.doi.org/10.1371/journal.ppat.1000077>.
 43. Bao X, Kolli D, Liu T, Shan Y, Garofalo RP, Casola A. 2008. Human metapneumovirus small hydrophobic protein inhibits NF- κ B transcriptional activity. *J. Virol.* 82:8224–8229. <http://dx.doi.org/10.1128/JVI.02584-07>.
 44. Le Nouen C, Hillyer P, Winter CC, McCarty T, Rabin RL, Collins PL, Buchholz UJ. 2011. Low CCR7-mediated migration of human monocyte derived dendritic cells in response to human respiratory syncytial virus

- and human metapneumovirus. *PLoS Pathog.* 7:e1002105. <http://dx.doi.org/10.1371/journal.ppat.1002105>.
45. Sallusto F, Cella M, Danieli C, Lanzavecchia A. 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J. Exp. Med.* 182: 389–400. <http://dx.doi.org/10.1084/jem.182.2.389>.
 46. Figdor CG, van Kooyk Y, Adema GJ. 2002. C-type lectin receptors on dendritic cells and Langerhans cells. *Nat. Rev. Immunol.* 2:77–84. <http://dx.doi.org/10.1038/nri723>.
 47. Cambi A, Beeren I, Joosten B, Fransen JA, Figdor CG. 2009. The C-type lectin DC-SIGN internalizes soluble antigens and HIV-1 virions via a clathrin-dependent mechanism. *Eur. J. Immunol.* 39:1923–1928. <http://dx.doi.org/10.1002/eji.200939351>.
 48. Mercer J, Schelhaas M, Helenius A. 2010. Virus entry by endocytosis. *Annu. Rev. Biochem.* 79:803–833. <http://dx.doi.org/10.1146/annurev-biochem-060208-104626>.
 49. Trepiakas R, Pedersen AE, Met O, Svane IM. 2009. Addition of interferon-alpha to a standard maturation cocktail induces CD38 up-regulation and increases dendritic cell function. *Vaccine* 27:2213–2219. <http://dx.doi.org/10.1016/j.vaccine.2009.02.015>.
 50. Schlender J, Schnorr JJ, Spielhoffer P, Cathomen T, Cattaneo R, Billeter MA, ter Meulen V, Schneider-Schaulies S. 1996. Interaction of measles virus glycoproteins with the surface of uninfected peripheral blood lymphocytes induces immunosuppression in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 93:13194–13199. <http://dx.doi.org/10.1073/pnas.93.23.13194>.
 51. Niewiesk S, Eisenhuth I, Fooks A, Clegg JC, Schnorr JJ, Schneider-Schaulies S, ter Meulen V. 1997. Measles virus-induced immune suppression in the cotton rat (*Sigmodon hispidus*) model depends on viral glycoproteins. *J. Virol.* 71:7214–7219.
 52. Weidmann A, Fischer C, Ohgimoto S, Ruth C, ter Meulen V, Schneider-Schaulies S. 2000. Measles virus-induced immunosuppression in vitro is independent of complex glycosylation of viral glycoproteins and of hemifusion. *J. Virol.* 74:7548–7553. <http://dx.doi.org/10.1128/JVI.74.16.7548-7553.2000>.
 53. Klagge IM, ter Meulen V, Schneider-Schaulies S. 2000. Measles virus-induced promotion of dendritic cell maturation by soluble mediators does not overcome the immunosuppressive activity of viral glycoproteins on the cell surface. *Eur. J. Immunol.* 30:2741–2750. [http://dx.doi.org/10.1002/1521-4141\(200010\)30:10<2741::AID-IMMU2741>3.0.CO;2-N](http://dx.doi.org/10.1002/1521-4141(200010)30:10<2741::AID-IMMU2741>3.0.CO;2-N).
 54. Dubois B, Lamy PJ, Chemin K, Lachaux A, Kaiserlian D. 2001. Measles virus exploits dendritic cells to suppress CD4+ T-cell proliferation via expression of surface viral glycoproteins independently of T-cell trans-infection. *Cell. Immunol.* 214:173–183. <http://dx.doi.org/10.1006/cimm.2001.1898>.
 55. Muller N, Avota E, Schneider-Schaulies J, Harms H, Krohne G, Schneider-Schaulies S. 2006. Measles virus contact with T cells impedes cytoskeletal remodeling associated with spreading, polarization, and CD3 clustering. *Traffic* 7:849–858. <http://dx.doi.org/10.1111/j.1600-0854.2006.00426.x>.
 56. Mercer J, Helenius A. 2012. Gulping rather than sipping: macropinocytosis as a way of virus entry. *Curr. Opin. Microbiol.* 15:490–499. <http://dx.doi.org/10.1016/j.mib.2012.05.016>.
 57. Krzyzaniak MA, Zumstein MT, Gerez JA, Picotti P, Helenius A. 2013. Host cell entry of respiratory syncytial virus involves macropinocytosis followed by proteolytic activation of the F protein. *PLoS Pathog.* 9:e1003309. <http://dx.doi.org/10.1371/journal.ppat.1003309>.
 58. Walzer T, Galibert L, De Smedt T. 2005. Poxvirus semaphorin A39R inhibits phagocytosis by dendritic cells and neutrophils. *Eur. J. Immunol.* 35:391–398. <http://dx.doi.org/10.1002/eji.200425669>.