Respiratory Syncytial Virus G Protein CX3C Motif Impairs Human Airway Epithelial and Immune Cell Responses

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Respiratory syncytial virus (RSV) is a major cause of severe lower respiratory infection in infants and young children and causes disease in the elderly and persons with compromised cardiac, pulmonary, or immune systems. Despite the high morbidity rates of RSV infection, no highly effective treatment or vaccine is yet available. The RSV G protein is an important contributor to the disease process. A conserved CX3C chemokine-like motif in G likely contributes to the pathogenesis of disease. Through this motif, G protein binds to CX3CR1 present on various immune cells and affects immune responses to RSV, as has been shown in the mouse model of RSV infection. However, very little is known of the role of RSV CX3C-CX3CR1 interactions in human disease. In this study, we use an in vitro model of human RSV infection comprised of human peripheral blood mononuclear cells (PBMCs) separated by a permeable membrane from human airway epithelial cells (A549) infected with RSV with either an intact CX3C motif (CX3C) or a mutated motif (CX4C). We show that the CX4C virus induces higher levels of type I/III interferon (PBMCs) separated by a permeable membrane from human airway epithelial cells (A549) infected with RSV with either an intact CX3C motif (CX3C) or a mutated motif (CX4C). We show that the CX4C virus induces higher levels of type I/III interferon (IFN-α) in A549 cells, increased IFN-α and tumor necrosis factor alpha (TNF-α) production by human plasmacytoid dendritic cells (pDCs) and monocytes, and increased IFN-γ production in effector/memory T cell subpopulations. Treatment of CX3C virus-infected cells with the F(ab')2 form of an anti-G monoclonal antibody (MAb) that blocks binding to CX3CR1 gave results similar to those with the CX4C virus. Our data suggest that the RSV G protein CX3C motif impairs innate and adaptive human immune responses and may be important to vaccine and antiviral drug development.
Some of these G-mediated effects have been associated with the G protein-CX3CR1 interaction, including impaired CD4 and CD8 T cell trafficking to the lungs and cytotoxicity of CX3CR1+ cells (40), enhanced pulmonary inflammation in RSV-challenged FI-RSV-vaccinated mice (41), and G protein-induced suppression of respiratory rates in mice (36). However, little is known of the impact of the RSV G protein CX3C motif on infections in humans.

To study the role of the CX3C motif in human RSV disease, we employed an in vitro model comprised of both human airway epithelial cells (A549 cells) and human immune cells (peripheral blood mononuclear cells [PBMCs]). Most previous in vitro studies of human RSV infection, including those focused on G, have been performed by directly exposing either airway epithelial or immune cells, but not both, to the virus. For example, human lung epithelial cells (A549) or primary normal bronchial epithelial cells have been used to study the production of proinflammatory and anti-inflammatory cytokines (interleukin-8 [IL-8], macrophage inflammatory protein-1 alpha [MIP-1α], MIP-1β, interferon-gamma inducible protein-10 [IP-10], monocyte chemoattractant protein 1 [MCP-1], and RANTES) upon infection with RSV lacking the G protein (29, 42). Human PBMCs directly stimulated with the purified RSV G protein produced significant levels of tumor necrosis factor alpha (TNF-α), IL-10, and IL-12 and became unresponsive to subsequent stimulation with the whole virus (43). Stimulation of human isolated monocytes with a peptide containing the CX3C region suppressed secretion of IL-6 and prevented activation and the production of IFN-α, IL-4, and IL-17 in PBMCs, although exposure of PBMCs to RSV-infected airway cells also accelerated apoptosis of lymphocytes. Coculture of PBMCs and airway cells, as used in the study by Qin et al., permits HLA-dependent reactions to occur, which could interfere with or confound RSV-associated responses. We chose to use a two-chamber system where A549 cells and PBMCs are separated by a permeable membrane. This system prevents contact between cells but permits exchange of viruses and viral and cellular proteins between the two chambers.

In the present study, we used the two-chamber in vitro model to characterize human innate and adaptive responses to RSV infection. We compared the responses of PBMCs exposed to A549 cells infected with RSV with either an intact CX3C motif (CX3C) or a mutated motif (CX4C) and also looked at the effect that the F(ab′)2 form of the anti-RSV G protein monoclonal antibody (MAb) 131-2G had on the infection. 131-2G binds to the conserved region of G and averts the RSV CX3C-CX3CR1 interaction (MAb) 131-2G had on the infection. In the current study, we employed an in vitro model comprised of both human airway epithelial cells (A549 cells) and human immune cells (peripheral blood mononuclear cells [PBMCs]). Most previous in vitro studies of human RSV infection, including those focused on G, have been performed by directly exposing either airway epithelial or immune cells, but not both, to the virus. For example, human lung epithelial cells (A549) or primary normal bronchial epithelial cells have been used to study the production of proinflammatory and anti-inflammatory cytokines (interleukin-8 [IL-8], macrophage inflammatory protein-1 alpha [MIP-1α], MIP-1β, interferon-gamma inducible protein-10 [IP-10], monocyte chemoattractant protein 1 [MCP-1], and RANTES) upon infection with RSV lacking the G protein (29, 42). Human PBMCs directly stimulated with the purified RSV G protein produced significant levels of tumor necrosis factor alpha (TNF-α), IL-10, and IL-12 and became unresponsive to subsequent stimulation with the whole virus (43). Stimulation of human isolated monocytes with a peptide containing the CX3C region suppressed secretion of IL-6 and prevented activation and the production of IFN-α, IL-4, and IL-17 in PBMCs, although exposure of PBMCs to RSV-infected airway cells also accelerated apoptosis of lymphocytes. Coculture of PBMCs and airway cells, as used in the study by Qin et al., permits HLA-dependent reactions to occur, which could interfere with or confound RSV-associated responses. We chose to use a two-chamber system where A549 cells and PBMCs are separated by a permeable membrane. This system prevents contact between cells but permits exchange of viruses and viral and cellular proteins between the two chambers.

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**MATERIALS AND METHODS**

**Cells.** A549 cells, a human alveolar adenocarcinoma cell line (45), and HEp-2 cells were maintained in minimal essential medium (MEM) (Gibco, Invitrogen Corp., San Diego, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone; Thermo Fisher Scientific, Inc., Suwanee, GA), penicillin-streptomycin, and 1-glutamine at 37°C with 5% CO2.

**Viruses.** Two RSV A2-derived strains, the wild type (wt) and the CX3C mutant (provided by A. G. P. Oomens), were generated with a reverse-genetic system as previously described (46). The wild-type strain (designated CX3C) has an intact CX3C motif (G-CX3C) in the G protein and is the parent of the mutant strain (designated CX4C), which has an A286 insertion in the CX3C motif.

An RSV G open reading frame (ORF) with a mutated CX3C site was generated from an A2-based wt G ORF by site-directed mutagenesis. Three nucleotides (GCA) encoding an alanine residue were inserted before the second cysteine (C286) to encode CWAIC (G-CX4C) instead of the wt sequence, CWAIC (G-CX3C). To generate virus expressing G-CX4C and G-CX3C, an A2-based cDNA was constructed, which lacked the G ORF and contained instead a spacer with flanking BsmBI remote-cutting sites. Next, the G-CX4C and G-CX3C ORFs were PCR amplified to contain flanking BsmBI sites and cloned into the G-lacking RSV cDNA. The resulting cDNAs contained no artificial sequences or restriction sites other than the intended CX3C mutation and were used to recover viruses by reverse genetics. Viral genomes from passage 3 stocks were verified by harvesting of viral RNA from infected cells, amplification by real-time PCR (RT-PCR), and bulk sequence analysis.

Verified viral stocks underwent one passage in HEp-2 cells and were purified by pelleting through a 20% sucrose cushion at 16,000 × g for 2 h, resuspended in MEM without FBS, and stored at −80°C.

**F(ab′)2 fragment preparation.** MAb 131-2G F(ab′)2 fragments were generated by papain digestion (Sigma-Aldrich). Briefly, protein G column-purified MAb 131-2G (47) was digested with porcine papain overnight at 37°C, and digested MAb was passed through a protein G-Sepharose column (GE Healthcare, Alpharetta, GA) to eliminate Fc fragments and undigested antibodies. Purified F(ab′)2 fragments were dialyzed and concentrated by using a Centricon spin column (Millipore, Temecula, CA) with a 30-kDa cutoff. The purity of the F(ab′)2 fragments was determined by SDS-PAGE (Bio-Rad, Hercules, CA) under nonreducing conditions. The protein concentration was determined by a Micro BCA protein assay (Pierce Protein Research Products, Rockford, IL). Endotoxin concentrations were determined by using a Limulus amebocyte lysate chromogenic endpoint assay, in accordance with the manufacturer’s instructions (Lonza, Atlanta, GA).

**Mononuclear cell isolation.** Peripheral blood from 8 random adult donors was collected under an Emory University institutional review board (IRB)-approved protocol and provided to the laboratory delinked from personal identifiers. PBMCs were isolated by centrifugation on a gradient of Ficoll-Paque Premium (GE Healthcare, Uppsala, Sweden). Briefly, blood was diluted 1:3 with phosphate-buffered saline (PBS), placed on top of the gradient, and centrifuged at 400 × g for 30 min at 21°C. Mononuclear cells were collected from the interface, washed 2 times with PBS, frozen in FBS containing 10% dimethyl sulfoxide (DMSO), and stored in liquid nitrogen until analysis.

**In vitro model.** We used a two-chamber in vitro system (Fig. 1A) with the PBMCs in the upper chamber and A549 cells in the lower chamber to model RSV infection. A549 cells were grown in 24-well tissue culture plates and infected with CX3C or CX4C virus at a multiplicity of infection (MOI) of 0.01 or a comparable concentration of mock-infected cell culture medium (supernatants from uninfected HEp-2 cells prepared in the same manner as virus stocks). The virus inoculum was incubated for 2 h at 37°C and then replaced with fresh medium. Infected A549 cells were incubated for 3 days at 37°C under 5% CO2, with fresh culture medium replacement on day 2 postinfection (p.i.), and PBMCs were then added as follows.

Freshly thawed PBMCs were resuspended in MEM–10% FBS, and live cells were counted by using trypan blue exclusion staining. PBMCs were diluted to 10 × 106 live cells/ml, and 100 μl (106 cells) was added to permeable polyester Transwell inserts (0.33 cm with a 0.4-μm pore size;
Corning, Inc., Corning, NY) in 24-well plates with 3-day-infected A549 cells and incubated for 24 h at 37°C under 5% CO₂. Wells without inserts were used to assess A549-produced cytokine responses without PBMCs. In experiments with F(ab’2) 131-2G, A549 cells were pretreated for 2 h with the MAb and then infected with CX3C or CX4C virus, incubated for 3 days, and coincubated with PBMCs for another 24 h, as stated above. F(ab’2) 131-2G was added to the medium of A549 cells, the virus inoculum, and medium for PBMCs at a final concentration of 25 μg/ml. Mock-infected control A549 cells were treated in a similar fashion.

For the direct stimulation of PBMCs, freshly thawed cells were resuspended in RPMI–10% FBS and placed into a 96-well plate at 1 × 10⁶ cells/well. CX3C virus (wild type) was added at a dose of 1 MOI, and the same amount of uninfected HEp-2 cell supernatant prepared in the same manner as the virus stock (mock) was added to control wells. PBMCs were incubated for 24 h at 37°C under 5% CO₂ and for an additional 2 h in the presence of brefeldin A (GolgiPlug; BD, San Jose, CA) and then processed in the same manner as the in vitro-coincubated PBMCs for the flow cytometric analysis (see below). As a positive control for IFN-α production, CpG oligonucleotide 2336 (human TLR9 ligand) was added at a final concentration of 2.5 μg/ml. As a positive control for T cell cytokine production, staphylococcal enterotoxin B subunit (SEB) was added at a final concentration of 1 μg/ml.

**RSV replication.** The titer of infectious virus (50% tissue culture infectious dose [TCID₅₀]) was determined by a microtiter infectivity assay.

**FIG 1** In vitro model of human RSV infection. (A) Human airway epithelial cells (A549) were cultured in 24-well plates and infected with RSV strains or mock infected and cultured for 3 days, with culture medium replacement on day 2 p.i. At day 3 p.i., PBMCs from random human adult donors were added in permeable-membrane Transwell inserts and coincubated for an additional 24 h. (B to D) Responses of PBMCs after exposure to purified CX3C RSV (direct stimulation) or RSV-infected A549 cells (in vitro model). Shown are the percentages of CD69⁺ IFN-γ-producing CD4 and CD8 effector memory T cells (B), mean fluorescence intensity (MFI) of CD86 expression (C), and percentages of IFN-α-producing pDCs, mDCs, and monocytes (D). SEB or CpG was used as a positive control for T cell or innate cell responses. The data are represented as means ± SEM of independent experiments with PBMCs from 3 random donors. *, P < 0.05 versus mock infection in direct stimulation; Δ, P < 0.05 versus mock infection in the in vitro model; †, P < 0.05 versus direct stimulation (determined by a paired t test).
using an enzyme-linked immunosorbent assay (ELISA) to detect virus replication, as previously described (48). Briefly, HEP-2 cells were grown in a 96-well microtiter plate, infected with 20 μl of 10-fold serial dilutions of the virus, and incubated for 2 h at room temperature (RT) with gentle rocking; 180 μl of medium was then added to each well; and the plate was incubated at 37°C under 5% CO2 for 5 days. After the 5-day incubation, the wells were washed 3 times with PBS, fixed by incubation for 10 min at RT with 70% ethanol, dried, and stored at 4°C until testing. For the ELISA, cells were washed 4 times with PBS–0.5% Tween 20 (PBS-Tw), blocked for 1 h with PBS-Tw containing 5% bovine serum albumin (BSA), and then incubated for 2 h at 37°C with goat anti-RSV antibody (Millipore, Billerica, MA) diluted at 1:5,000. Next, wells were incubated for 1 h at 37°C with horseradish peroxidase (HRP)-conjugated donkey anti-goat antibody diluted at 1:5,000 (Jackson Immunoresearch Laboratories, Inc., West Grove, PA), washed, and developed by using o-phenylenediamine (OPD) substrate. Absorbance was read at 490/640 nm.

The replication of viruses in A549 cells was measured similarly: Transwell inserts were removed, and supernatants in the lower chamber were collected and stored for testing. The cells remaining in the 24-well plates were then washed 3 times with PBS and fixed with 500 μl/well of 70% ethanol, and the plates were dried and stored at 4°C. The ELISA was performed directly in the 24-well plates as noted above, except with 3-fold greater volumes of reagents. After the color was developed and the reaction was stopped, supernatants were transferred onto a 96-well plate to read the absorbance.

The amount of viral RNA in the basolateral supernatants of RSV-infected and mock-infected A549 cells was determined by an RT-PCR assay. Total RNA was extracted from supernatants by using the Qiagen assay. Total RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions and stored at −80°C. RT-PCR was performed by using an AgPath-ID OneStep RT-PCR kit (Applied Biosystems, Foster City, CA) and an ABI 7700 sequence detector system (Applied Biosystems, Foster City, CA). The thermal cycling conditions included 45°C for 2 min and 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 sec and 55°C for 2 min for denaturing and annealing, respectively. The primers and probes for the RSV matrix (M) gene (forward primer 5′-GGC AAA TAT GGA AAC ATA GCT GAA-3′; reverse primer 5′-TCT TTT TCT AGG ACA TTG TAY TGA ACA G-3′, and probe 5′-6-carboxyfluoro-

rescin [FAM]-TGT CCG TCT TCG CCC TCC TG-3′-black hole quencher 1 [BHQ]-1-3′), as previously described (49), were obtained from Integrated DNA Technologies. The cycle threshold (Cp) values, the number of cycles required to exceed the background level, were calculated.

Cytokine analysis. The supernatants from the lower chamber were harvested on day 3 p.i. (6 h after addition of inserts) and on day 4 p.i. (24 h after addition of inserts), stored at −80°C, and then tested for cytokine levels. For cytokine testing, the freshly thawed supernatants were centrifuged at 450 × g for 3 min to eliminate cell debris and analyzed by Luminex magnetic bead kits for IFN-α2, IFN-α1 (IL-29), and IFN-λ2 (IL-28a) (Millipore). The assay was performed according to the manufacturer’s instructions but with half the recommended quantity of beads, biotinylated antibody, and fluorescent dye-labeled streptavidin. Serial dilutions of standards for each analyte were used in each test to calculate the concentration of the analyte according to the manufacturer’s instructions.

Flow cytometric analysis of PBMCs. PBMCs were harvested from the Transwell inserts after 24 h of coincubation with RSV or mock-infected A549 cells (as noted above). Cells were transferred from inserts into separate wells in a 96-well plate. Residual cells on the insert were acquired by washing the inserts 2 times with Versene (0.48 mM EDTA) solution (Gibco, Invitrogen), with 5 min of incubation at 37°C during each washing step. The cells were centrifuged at 450 × g for 3 min and resuspended in 200 μl of the original supernatant, to which brefeldin A (BD GolgiPlug) was added and incubated for 2 h at 37°C under 5% CO2. The cells were then washed 2 times with staining buffer (BD Pharmingen), stained with live/dead fixable red dye (Invitrogen), incubated for 1 h at 4°C with TruStain (BioLegend, Inc., San Diego, CA) to block Fc receptors to decrease nonspecific staining, and stained for 30 min at 4°C with two panels of fluorochrome-labeled antibodies, one to test for innate (monocytes/dendritic cells) responses and one to test for memory/effector T (TEM) cell responses.

The in vitro response panel consisted of antibodies against CD3, CD19, and CD56 (Brilliant Violet 570; BioLegend); CD14 (allophycocyanin [APC]-Cy7; BioLegend); HLA-DR (V500; BD Horizon); CD11c (Alexa Fluor 700; eBioscience, Inc., San Diego, CA); CD303a (fluorescein iso-thiocyanate [FITC]; eBioscience); and CD86 (phycoerythrin [PE]-Cy7; BioLegend). Monocytes and dendritic cells were analyzed after excluding dead cells within the CD3+CD19+CD56- subpopulation as CD14+ HLA-DR+ monocytes, CD14+ HLA-DR+CD11c+CD303a- myeloid dendritic cells (mDCs), and CD14+ HLA-DR+CD11c+CD303a+ plasmacytoid dendritic cells (pDCs). The activation and maturation status of the cells was determined by staining for surface expression of CD86 and HLA-DR. Cytokine production was assessed by intracellular staining as previously described (50). Briefly, after surface staining, the cells were washed 2 times with staining buffer, fixed for 10 min at RT with BD fluorescence-activated cell sorter (FACS) lysing solution, washed, permeabilized for 10 min at RT with BD FACS permeabilization solution 2, and incubated for 30 min at 4°C with fluorochrome-labeled antibodies against IFN-α2b (V450; BD Horizon), TNF-α (PE; eBioscience), and MCP-1 (APC; eBioscience).

The memory/effector T cell panel consisted of antibodies against CD3 (eFluor 605NC; eBioscience), CD4 (APC-H7; BD Biosciences), CD8 (V500; BD Horizon), CD45RA (Alexa Fluor 700; BioLegend), CCR7 (PE-Cy7; BD Pharmingen), and CD69 (APC; BioLegend). Memory/effector T cells were analyzed after excluding dead cells within CD3+CD4/CD8 subsets as CD45RA+CCR7+ central memory T (Tcm) cells and CD45RA+CCR7+ TEM cells and only within the CD8+ subpopulation as CD45RA+CCR7+ effector T cells expressing CD45RA+ TcmRA cells. The activation and functional status of T cells were determined by surface expression of CD69 and production of cytokines. Intracellular cytokine staining was performed in the same manner as for monocytes and dendritic cells i.e., cells were stained with fluorochrome-labeled antibodies against IFN-γ (FITC; BioLegend) and IL-4 (PE; BioLegend).

Flow cytometry analysis was performed by using a 4-laser BD LSR-II instrument (BD Biosciences) and FlowJo software (Tree Star, Inc., Ashland, OR).

Data analysis. Statistical analysis and graphs were performed by using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Data are expressed as means ± standard errors of means (SEM) of independent experiments with PBMCs from 5 random donors [3 donors for P(ab’)_2 131-2G experiments] and 3 replicates for each experimental condition. The Wilcoxon matched-pairs test was employed to determine the significance of differences between CX3C RSV, CX4C RSV, and mock infec-
tions. The Mann-Whitney test was used for comparison of the different viruses and mock-infected A549 cells without PBMCs. A P value of <0.05 was considered to be statistically significant.

RESULTS

In vitro model of human RSV infection. To study human airway and immune cell responses to RSV, we developed a two-chamber in vitro model comprised of human A549 cells and PBMCs separated by a permeable membrane (Fig. 1A). The system prevents direct cell-to-cell interaction but allows exchange of virus, viral proteins, and cellular mediators between the two chambers, i.e., cross talk between immune and airway epithelial cells.

To characterize this in vitro model, we examined the kinetics of RSV replication and cytokine responses in A549 cells and compared the response of PBMCs exposed to RSV-infected A549 cells to that of PBMCs exposed to purified RSV. The kinetics of RSV infection in A549 cells (data not shown) showed that day 3 p.i. was
the first day with consistent, detectable replication of virus and RSV-associated increases in levels of cytokines and chemokines. We chose day 3 p.i. to add PBMCs to the infected A549 cells. By day 4 p.i., the titers of infectious virus (TCID₅₀) in supernatants were ≈1 × 10⁶ (range, 8.0 × 10⁵ to 1.3 × 10⁶). To compare direct stimulation of PBMCs with RSV to stimulation with RSV-infected A549 cells, we used the same donor PBMCs, the same time of incubation (24 h), and the same inoculum (1 × 10⁶ TCID₅₀) of the purified wild-type CX3C strain of RSV. We determined the intracellular production of IFN-γ (Fig. 1B) and IL-4 (data not shown) to assess responses in CD4 and CD8 TEM cells and the expression of the maturation marker CD86 (Fig. 1C) and intracellular IFN-α (Fig. 1D) and MCP-1 (data not shown) to assess responses in dendritic cells and monocytes.
Stimulation of PBMCs with purified RSV or RSV-infected A549 cells gave a similar pattern of responses, although exposure to RSV-infected A549 cells induced higher levels of innate immune responses. The percentages of IFN-α-producing pDCs and MCP-1-producing mDCs were significantly higher in PBMCs exposed to RSV-infected A549 cells than in PBMCs stimulated with purified virus. Although the average percentages of IFN-α-producing monocytes, IFN-γ-producing CD8 TEM cells, and IL-4-producing CD4 TEM cells were higher for PBMCs exposed to infected A549 cells, the difference compared to purified virus stimulation did not reach statistical significance. The control responses of the PBMCs exposed to the mock-infected cells were similar to the responses of directly mock-stimulated PBMCs, showing that uninfected A549 cells do not induce PBMC responses.

These findings indicate that our in vitro system of exposing PBMCs to RSV-infected A549 cells effectively detects responses and may be especially suited to studies of innate immune responses.

**Altering the CX3C site does not affect virus replication in A549 cells.** A549 cells were infected with two RSV strains: the wild type (CX3C), containing an intact CX3C motif in the G protein, or a mutant virus (CX4C), with an additional alanine preceding the second cysteine. Both RSV strains had similar replication rates in human A549 cells, as indicated by the amount of RSV antigens detected by ELISA (Fig. 2A) and the relative amount of viral RNA detected by real-time PCR (Fig. 2B). The addition of PBMCs to A549 cells on day 3 p.i. was associated with a minor decrease in viral replication for both viruses. Thus, mutating the CX3C site did not impact RSV replication in A549 cells. We also noted that the virus did slowly cross the membrane, as evident by the lack of infectious virus in the upper chamber at 6 h and the presence of low levels at 24 h after the addition of the PBMCs (Fig. 2C), as would be expected with a 0.4-μm pore diameter. The addition of F(ab’)_2 fragments of 131-2G to the cells (Fig. 2D) slightly decreased the levels of replication for both viruses. It appeared that the MAb affected the CX3C virus more than the CX4C strain, reducing the difference in replication between the two viruses; these effects, though, were not statistically significant.

To minimize the effect that differences in virus replication might have on responses, we used an inoculum of the virus that gave similar levels of replication for the two viruses at the study

<table>
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<th>PBMC donor</th>
<th>Virus replication (RSV ELISA absorbance)</th>
<th>Difference in RSV ELISA absorbance</th>
<th>Estimated difference in response values associated with difference in RSV ELISA absorbance</th>
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<td>IFN-γ-producing CD8 TEM cells (%) 1.17 0.288 11,738 1.17 1.49 1.24</td>
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**Fig 3** Antiviral responses of A549 cells infected with wild-type RSV (CX3C) and mutant RSV with an altered CX3C motif of the G protein (CX4C). A549 cells were mock infected or infected with the CX3C or CX4C RSV strain and incubated for 4 days, with culture medium changed on day 2 postinfection. Supernatants from the bottom chamber were collected on days 3 and 4 postinfection (6 h and 24 h after addition of inserts, respectively). The Luminescence assay was performed with collected supernatants to measure production of IFN-α2, IFN-λ1, and IFN-λ2. The data are represented as means ± SEM of 3 repeated independent experiments. *, P < 0.05 versus mock infection; ∆, P < 0.05 versus CX3C (determined by the Mann-Whitney test).
time points. However, experimental variation left some differences in levels of virus replication. To determine the effect of these differences on our results, we calculated the estimated values of responses by interpolating from a standard curve of virus replication (RSV antigen-specific ELISA absorbance) versus the response measure values for both viruses. One set of PBMCs was used to generate the standard curve. Table 1 shows the individual replication results for each PBMC set, the difference in levels of replication between the two viruses observed for each experiment, and examples of the estimated change in response values associated with the difference in replication.

Virus replication had unequal effects on the responses measured. For example, virus replication had a greater impact on the percentage of TNF-α than IFN-α-producing pDCs or IFN-γ-producing CD8 TEM cells, as the estimated values of responses were higher for TNF-α-producing dendritic cells (Table 1). To estimate this influence on differences in responses between CX3C and CX4C viruses, we normalized the original data to replication of both viruses using the estimated response values from the standard curve.

The CX3C motif alters antiviral responses in A549 human airway epithelial cells. To study innate antiviral responses in A549 cells, we measured the production of IFN types I and III in response to infection with CX3C and CX4C viruses (Fig. 3). Newly
identified type III IFNs have antiviral activity like type I IFNs (51), and although type I and type III IFN receptors are unrelated, they trigger similar Jak-STAT signaling pathways (52, 53).

RSV infection of A549 cells induced both IFN-I/H9251 and IFN type III (IFN-I/H9261/2) on day 3 p.i. and day 4 p.i. Both viruses induced approximately 400- to 1,000-times-higher levels of IFN type III (11,430 to 78,940 pg/ml of IFN-I/H9261 and 26,550 to 42,350 pg/ml of IFN-I/H9261) than of IFN type I (27 to 72 pg/ml of IFN-I/H9251). Interestingly, the CX4C virus induced even higher levels of both IFN types I and III. The levels of IFN-I/H9261 and -H9261 were significantly higher for the CX4C virus than for the CX3C virus on both day 3 and day 4 p.i., while the IFN-I/H9251 levels were significantly higher only on day 4 p.i. Normalization of responses to virus replication did not change the pattern or significance of differences seen with the original data. Thus, RSV with an intact G protein CX3C motif appears to suppress antiviral responses in A549 cells.

**Altering the CX3C site does not decrease activation/matura-
tion of dendritic cells and monocytes.** After 24 h of exposure to RSV-infected A549 cells, PBMCs were harvested from the inserts and stained with fluorochrome-labeled antibodies to distinguish mDCs, pDCs, and monocytes. The activation/maturation state of dendritic cells and monocytes was assessed by the expression of the costimulatory molecule CD86 and the increased expression of major histocompatibility complex class II (HLA-DR) (Fig. 4). CD86 (B lymphocyte/accessory cell activation antigen B7-2) is a ligand for T cell-costimulatory CD28/cytotoxic-T lymphocyte-associated antigen 4 (CTLA-4) molecules, provides costimulatory signals necessary for T cell activation, and appears

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**FIG 5** Innate immune responses of human PBMCs coincubated with A549 cells infected with CX3C or CX4C virus. A549 cells were mock infected or infected with the CX3C or CX4C RSV strain and cultured for 3 days, with culture medium replacement on day 2 p.i. and addition of PBMCs in permeable-membrane inserts on day 3 p.i. After coincubation for an additional 24 h, PBMCs were harvested and analyzed. The counterplot shows the distribution of plasmacytoid dendritic cells expressing CD86 and intracellular IFN-α (data for PBMCs from a single donor). CpG (ODN 2336), a human TLR9 ligand, was used as a positive control for IFN-α production. Graphs show the percentages of myeloid (mDC) and plasmacytoid (pDC) dendritic cells and monocytes producing IFN-α, TNF-α, and MCP-1. The data are represented as means ± SEM of independent experiments with PBMCs from 5 random donors and individual paired results for each donor. †, P < 0.05 versus mock infection; Δ, P < 0.05 versus CX3C (determined by the Wilcoxon matched-pairs test).
on the surface of dendritic cells and monocytes/macrophages after their transformation into fully efficient antigen-presenting cells (54, 55).

Exposure of PBMCs to RSV-infected A549 cells increased the surface density of CD86 and HLA-DR on both dendritic cells and monocytes for all donors. The wild-type and CX4C viruses had similar effects, suggesting that altering the CX3C motif in the RSV G protein does not change innate immune cell activation and maturation.

The CX3C motif impairs innate antiviral responses in pDCs. Plasmacytoid DCs are a major source of type I IFN and, as well as mDCs and monocytes, exhibit strong proinflammatory response to the antigens (56–58). In order to evaluate the impact of the RSV CX3C motif on the functionality of innate immune cells, we assessed cytokine production in dendritic cells and monocytes within PBMCs exposed to RSV-infected A549 cells.

In our in vitro model, both viruses induced significant increases in percentages of IFN-α-, TNF-α-, and MCP-1-producing monocytes and mDCs and percentages of IFN-α- and TNF-α-producing pDCs for most donor PBMCs (Fig. 5). We also found that the CX4C virus induced significantly higher percentages of IFN-α- and TNF-α-producing pDCs and TNF-α-producing monocytes. There were, however, no significant differences between the two viruses in percentages of cytokine-producing mDCs. Normalization of the data to virus replication had a slight effect on the differences in responses between the two viruses that did not change the statistical significance obtained with the original percentages of IFN-α-producing pDCs and TNF-α-producing monocytes. The normalized percentages of TNF-α-producing pDCs had a pattern similar to the original data, but the difference between CX3C and CX4C virus-exposed PBMCs was no longer significant.

The addition of F(ab')2 fragments of anti-G protein MAb during CX3C infection increased the production of IFN-α and TNF-α in pDCs and monocytes (Fig. 6) to levels similar to those with the CX4C virus. The response of the CX4C virus was not significantly changed by addition of the MAb. These findings suggest that the intact CX3C motif downregulates the human innate immune response associated with IFN-α and TNF-α production in pDCs and monocytes.

The CX3C motif diminishes adaptive antiviral responses of CD4/CD8 T cells. In addition to innate immune responses, we tested effector/memory responses in CD4/CD8 T cell subpopulations. Three effector/memory T cell subsets were characterized by the expression of the homing receptor CCR7 and the CD45 naive isofrom CD45RA, i.e., central memory (T_{CM}), effector memory (T_{EM}) and CD45RA+ effector memory (T_{EMRA}) T cells (59). The T_{EMRA} cells typically belong to the CD8 subset (59). The few CCR7-CD45RA+ (T_{EMRA} phenotype) CD4 T cells that could be distinguished (60) were, in our gating strategy, included in the CD4 T_{EM} subpopulation.

CD4 and CD8 effector and memory T cells from PBMCs from the 5 donors exposed to RSV-infected A549 cells responded with significant increases in percentages of activated CD69-positive IFN-γ- and IL-4-producing cells (Fig. 7). The CX4C virus induced a significantly higher percentage of IFN-γ-producing cells in CD4 T_{CM}, CD8 T_{EM}, and T_{EMRA} subpopulations than the CX3C virus. The percentage of IFN-γ-producing CD4 T_{CM} and CD8 T_{EM} cells after CX4C exposure was also higher, but the difference did not reach statistical significance. Notably, normalization of the data to virus replication did not affect the pattern or statistical significance observed with the original percentages of IFN-γ-producing memory T cells.

In contrast to IFN-γ responses, the numbers of IL-4-produc-
ing cells were similar between PBMCs exposed to wild-type-infected and those exposed to CX4C virus-infected A549 cells, except for the CD4 TCM subpopulation, where 3 of 5 tested PBMC samples had a significantly higher percentage of IL-4-expressing cells after exposure to the CX4C virus than after exposure to the wild-type strain. After normalization to virus replication, the difference in the percentages of IL-4-producing CD4 TCM cells between the two viruses no longer reached statistical significance.

The addition of F(ab’)2 anti-G MAb during CX3C infection gave results similar to those with CX4C virus with or without the MAb, i.e., higher percentages of IFN-γ-producing CD4 T CM, CD8 TEM, and TEMRA subpopulations (Fig. 8), and support our findings with the CX3C and CX4C viruses. Overall, these data indicate that the RSV G protein CX3C motif downregulates adaptive immune responses, particularly the production of IFN-γ in effector/memory T cell subsets.

**DISCUSSION**

The G protein is recognized as being a substantial contributor to the RSV-induced host response to infection (61). It also appears that the G protein CX3C chemokine motif in particular contributes to some of these effects in mice (36, 40, 41). Our data obtained in an *in vitro* model of the human immune responses to RSV show that the CX3C motif is also likely relevant to both innate and adaptive immune responses during human RSV infection.

Three studies directly link the CX3C motif to RSV disease. In one study, FI-RSV-vaccinated mice which were challenged with RSV that had a deletion of the G gene or that had a Cys-to-Arg mutation at the CX3C motif (CX3R) or were pretreated with anti-
CX3CR1 antibody had marked decreases in the enhanced pulmonary inflammation otherwise seen in FI-RSV-vaccinated mice (41). In another study, mice challenged with the RSV CX3R mutant had significantly higher levels of effector T cell trafficking into inflamed lungs and T cell production of IFN-γ/IL-4 than mice challenged with wild-type RSV (40). In the third study, mice given the RSV G protein intravenously had a decreased respiratory rate, but mice given the CX3R mutant G or anti-CX3CR1 antibody before administration of the G protein did not experience a decrease in the respiratory rate (36).

All three studies showing a role of the CX3C-CX3CR1 interaction in host immune responses were performed with the murine system. Our data using the two-chamber in vitro RSV infection model identified CX3C effects on both the innate and adaptive responses in human cells. The differences between intact (CWAIC) and mutated (CWAIAC) CX3C motifs suggest a significant effect on both antiviral and inflammatory innate responses. To assess antiviral responses in human airway epithelial cells, we tested the production of both type I and type III IFNs in RSV-infected A549 cells. The newly characterized IFN type III includes IFN-λ1, -λ2, and -λ3 (52, 62), and its production occurs primarily in epithelial cells (63–65). Okabayashi et al. suggested that production of IFN-λ, but not IFN type I, is the primary antiviral defense against RSV infection in normal bronchial epithelial cells (66). Similarly, we found that RSV infection of A549 cells induced much higher levels of IFN-λ than of IFN-α (Fig. 3). Low-level or absent production of IFN-α by RSV-infected airway epithelial cells (primary cultures) has been well documented (67, 68). RSV appears to effectively suppress the host IFN-α/β responses by the expression of the nonstructural proteins NS1 and NS2 (69–71). In addition, the RSV attachment G protein has also been suggested to suppress type I IFN responses by reducing the expression levels of the cytokine-signaling SOCS3 protein and the interferon-stimulated gene ISG15 (72). Our data show significant increases in levels of IFN-λ1 and -λ2 in A549 cells infected with the CX4C virus (Fig. 3B and C), suggesting that the CX3C chemokine motif contributes to the suppression of IFN production. We found that A549 cells express fairly low levels of CX3CR1 (less than 1% of total cells [data not shown]). This low expression level of CX3CR1 in A549 cells appears to be sufficient to affect IFN type I/III production but not high enough to significantly increase infection (Fig. 2).

Our in vitro model allows us to also look at the effect of RSV-infected airway cells on immune cell responses. We found that the presence of PBMCs did not significantly affect virus replication, while the membrane allowed virus to pass through (Fig. 2). The microenvironment of RSV-infected A549 cells induced a variety of responses in PBMCs, including significant increases in levels of activation markers and intracellular cytokines for both innate and adaptive immune cells.

We found that PBMCs exposed to RSV-infected A549 cells had increased maturation and percentages of IFN-α-, TNF-α-, and MCP-1-producing dendritic cells and monocytes (Fig. 4 and 5). Similar to our findings, previous studies showed the ability of RSV to induce production of these cytokines in isolated human pDCs and mDCs (73, 74). However, our data show that these RSV-induced responses are impaired by the CX3C motif. The intact CX3C motif was associated with decreases in the percentages of IFN-α- and TNF-α-producing pDCs and TNF-α-producing monocytes, while the mutation of the CX3C site to CX4C and the presence of MAb 131-2G restored the production of these cytokines in innate immune cells (Fig. 5 and 6). The G protein was previously shown to affect both TLR-dependent and -independent (RIG-I/MDA5) pathways involved in the expression of IFN type I and other proinflammatory cytokines. For example, pre-

**FIG 8** Memory T cell responses of human PBMCs coincubated with A549 cells infected with CX3C or CX4C virus in the presence of F(ab’2), fragments of anti-G protein antibody. A549 cells were mock infected or infected with the CX3C or CX4C RSV strain and cultured for 3 days, with culture medium replaced on day 2 p.i. and PBMCs added to the permeable-membrane inserts on day 3 p.i. For the F(ab’2)-treated wells, A549 cells were pretreated with F(ab’2), 131-2G MAb for 2 h before infection, and MAb was included in medium changes and medium for the PBMCs. The PBMCs were harvested 24 h after exposure to A549 cells and studied by flow cytometry. The graph shows the percentages of CD69+ central memory (TCM), effector memory (TEM), and CD45RA+ central memory (TCM) CD69+ effector memory (TEM), and CD45RA+ effector memory (TEM) T cells producing IFN-γ. The data are represented as means ± SEM of independent experiments with PBMCs from 3 random donors. *, P < 0.05 (determined by the Wilcoxon matched-pairs test).
treatment of human monocytcs with a G protein peptide containing the CX3C region prevented LPS-induced cytokine production (32), and both membrane and soluble forms of G protein inhibit either or both TLR3/4- and RIG-I/MDA5-mediated IFN type I induction in human monocyte-derived DCs (26). Our data suggest that the RSV G protein can affect TLR-dependent/independent signaling pathways, probably through CX3C binding to the fractalkine receptor CX3CR1. The majority of effector immune cells, including dendritic cells and monocytes, express CX3CR1 (19–21). Expression of this receptor allows them to penetrate inflamed tissues through the chemotactic function of the CX3CR1 ligand fractalkine. A substantial role for the fractalkine-CX3CR1 interaction has been well documented for chronic inflammatory cardiovascular, neuronal, and lung diseases (75–77). CX3CR1 also appears to play an important role in pattern recognition receptor (PRR)-mediated innate immune responses. For instance, CX3CR1-deficient mice have decreased expression levels of TLR4 (78) as well as impaired bacterial clearance and production of TNF-α and IFN-γ by macrophages (79). In addition, CX3CR1 signaling has been shown to increase the activation of NF-κB (79), the common transcriptional factor in the production of antiviral and inflammatory cytokines (80).

We also found that the CX3C motif in the RSV G protein affected the adaptive immune response, as indicated by diminished percentages of IFN-γ-producing effector/memory T cells compared to the CX4C virus and treatment with F(ab’)2 131-2G (Fig. 7 and 8). These data are consistent with previous studies in murine models showing a downregulation of Th1-mediated immune responses by the RSV CX3C motif (40, 41). Fraticelli et al. have shown that CX3CR1 was predominantly expressed by Th1-polarized cells and that Th1 but not Th2 cells responded to fractalkine stimulation (81). Our data suggest that the RSV G protein, which competes with fractalkine for CX3CR1 binding, may prevent this stimulation and suppress Th1-biased inflammatory responses. Interestingly, certain CX3CR1 haplotypes were found to be associated with an increased risk of severe RSV bronchiolitis in infants (82). The same single-nucleotide polymorphisms in the CX3CR1 gene were shown to confer low-affinity binding between CX3CR1 and fractalkine (83–85).

In conclusion, in this in vitro model of human RSV infection, we demonstrate that the RSV G protein CX3C motif has an impact on virus-host interactions in A549 human airway epithelial cells, immune cells, and their interplay and is likely an important determinant for the course of human RSV infection. The CX3C motif in the RSV G protein may be a target for future vaccine and antiviral drug development. An understanding of the mechanisms of CX3C-induced impairment of the host immune response should help clarify its role in treating and preventing RSV disease.

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