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Contribution of Fcγ receptors to human respiratory syncytial virus pathogenesis and the impairment of T-cell activation by dendritic cells

Roberto S. Gómez,[1] Bruno A. Ramirez,[1] Pablo F. Céspedes,[1] Kelly M. Cautivo,[1] Sebastián A. Riquelme,[1,2] Carolina E. Prado,[1] Pablo A. González[1] and Alexis M. Kalergis[1,2,3]

[1]*Millennium Institute on Immunology and Immunotherapy, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago de Chile, Chile,* [2]*INSERM U1064, Nantes, France and* [3]*Departamento de Reumatología, Facultad de Medicina. Pontificia Universidad Católica de Chile, Santiago de Chile, Chile*

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Correspondence: Drs Alexis M. Kalergis and Pablo A. González, Millennium Institute on Immunology and Immunotherapy. Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile. Alameda #340, Santiago E-8331010, Chile. Emails: akalergis@bio.puc.cl, pagonzalez@bio.puc.cl and akalergis@icloud.com
Senior author: Dr. Alexis Kalergis

Summary

Human respiratory syncytial virus (hRSV) is the leading cause of infant hospitalization related to respiratory disease. Infection with hRSV produces abundant infiltration of immune cells into the airways, which combined with an exacerbated pro-inflammatory immune response can lead to significant damage to the lungs. Human RSV re-infection is extremely frequent, suggesting that this virus may have evolved molecular mechanisms that interfere with host adaptive immunity. Infection with hRSV can be reduced by administering a humanized neutralizing antibody against the virus fusion protein in high-risk infants. Although neutralizing antibodies against hRSV effectively block the infection of airway epithelial cells, here we show that both, bone marrow-derived dendritic cells (DCs) and lung DCs undergo infection with IgG-coated virus (hRSV-IC), albeit abortive. Yet, this is enough to negatively modulate DC function. We observed that such a process is mediated by Fcγ receptors (FcγRs) expressed on the surface of DCs. Remarkably, we also observed that in the absence of hRSV-specific antibodies FcγRIII knockout mice displayed significantly less cellular infiltration in the lungs after hRSV infection, compared with wild-type mice, suggesting a potentially harmful, IgG-independent role for this receptor in hRSV disease. Our findings support the notion that FcγRs can contribute significantly to the modulation of DC function by hRSV and hRSV-IC. Further, we provide evidence for an involvement of FcγRIII in the development of hRSV pathogenesis.

Keywords: dendritic cells; Fcγ receptors; human respiratory syncytial virus; immune complexes; neutralizing antibodies; palivizumab.

Introduction

Human respiratory syncytial virus (hRSV) is an enveloped, single-stranded and negative-sensed RNA virus belonging to the *Paramyxoviridae* family, *Pneumovirus* genus.[1] Infection with hRSV is the major cause of lower respiratory tract disease in infants and young children worldwide.[2,3] Human RSV is highly infectious, affecting > 70% of children in the first year of life and nearly 100% of children by the age of 2 years.[4] Besides being highly infectious, following disease resolution hRSV interferes with the establishment of an effective immunological memory and therefore re-infections occur with high frequency.[5,6] Indeed, these features of hRSV support the notion that this virus has developed molecular mechanisms to evade the host immune response.[5,7,8] Because hRSV represents a major health burden worldwide, development of an effective vaccine against this virus is considered a major goal since its identification as a human pathogen in 1957.[9] However, despite intensive research efforts to date there are no licensed vaccines capable of inducing protective immunity against this virus in humans.[10–14]

Abbreviations: BAL, bronchoalveolar lavage; FcRs, Fc-receptors; FcγRIIb, Fcγ receptor IIb; FcγRIII, Fcγ receptor III; hRSV human respiratory syncytial virus; hRSV-IC, IgG-coated human respiratory syncytial virus; hRSV-UV, ultraviolet-treated human respiratory syncytial virus; IC, immune complex

Alternatively, host infection can be prevented by passive immunotherapy using palivizumab (Synagis™), an hRSV-specific monoclonal antibody directed to the virion surface fusion protein (F), which was approved in the USA for human use in 1998.[15,16] The protective effect of palivizumab has been demonstrated in two animal models for RSV infection, as well as in humans by decreasing hRSV-associated hospitalization rates by up to 55%, compared with placebo.[17–19] Because protection conferred by palivizumab consists of passive immunity, periodic injections of the antibody are required for effectiveness.[20,21] However, it is currently unknown whether treatment with this neutralizing antibody can block hRSV infection of immune cells, such as dendritic cells (DCs). Further, research is required to define whether systemic administration of this antibody can elicit protective immunity in the host during a simultaneous exposure to hRSV. A previous study suggests that palivizumab-coated hRSV can enhance hRSV-specific T-cell responses during hRSV infection, whereas another proposes that antibody-coated hRSV impair $CD8^+$ T-cell activation *in vitro*.[22–24] Athough antibodies against several microbes have been shown to promote the establishment of antimicrobial T-cell responses in animal models,[25–28] the opposite has also been observed.[29–31] Hence, we sought to determine whether neutralizing antibodies specific for the hRSV F protein decrease or enhance T-cell activation by antigen-presenting cells, such as DCs *in vitro* and *in vivo*.

Receptors binding to the Fc portion of immunoglobulin G (FcγRs) are expressed on the surface of immune cells, such as phagocytic antigen-presenting cells and granulocytes. These receptors can also be expressed by non-immune cells, such as mesangial cells.[32] Whereas the high-affinity FcγRI receptor binds mainly monomeric IgGs, the low-affinity FcγRIIb and FcγRIII receptors bind IgG in the form of immune complexes (ICs). These IgG–antigen conjugates are capable of inducing activating signals in immune cells when binding to FcγRIII, which promote the activation of inflammatory responses. In contrast, IC binding to inhibitory FcγRIIb leads to down-modulation of cellular responses and reduced inflammation. Furthermore, whereas IC binding to FcγRIII enhances antigen processing and T-cell activation,[25,27,33,34] binding of ICs to FcγRIIB counteracts and down-modulates this process.[26,33–36] Hence, Fcγ expression on antigen-presenting cells links humoral immunity with the modulation of T-cell immune responses.[33,34]

Dendritic cells are professional antigen-presenting cells that reside in peripheral tissues and lymphoid organs to sense, capture, process and present pathogen-derived antigens to T cells as peptides bound to either MHC class I or class II molecules.[37,38] After binding to ICs, FcγRs can modulate the capacity of DCs to uptake and present antigens to T cells.[25,26] For this reason, pathogens such as viruses have developed molecular mechanisms that exploit FcγRs to impair DC function and avoid clearance by the adaptive immune response.[29–31]

Notably, in many cases pathogen capture and degradation, antigen presentation and T-cell activation can be significantly enhanced by targeting antigens to specific FcγRs on the surface of DCs. For instance, it has been previously shown that *Salmonella enterica* serovar Typhimurium can no longer escape from degradation within DCs if delivered as ICs to FcγRs.[25,27] Furthermore, opsonized *Salmonella* promotes T-cell priming by DCs, which ultimately leads to bacterial degradation and clearance.[25,27] Similarly, FcγRs have been shown to play an important role in the context of respiratory viral infections, such as that mediated by influenza A virus.[28]

Here, we assessed the role of FcγRs in the capture and processing of IgG–hRSV (hRSV-IC) by DCs using two neutralizing monoclonal antibodies against the viral fusion protein: RS-348[39–41] and palivizumab.[17–19] We observed that FcγRs expressed on the surface of DCs mediated viral entry of hRSV-IC both, *in vitro* and *in vivo*, which hampered efficient $CD4^+$ and $CD8^+$ T-cell priming. Although treatment with palivizumab reduced viral loads in the lungs of hRSV-infected animals, administration of this antibody did not enhance the *in vivo* priming of T cells upon viral challenge. Notably, hRSV-inoculated FcγRIII$^{-/-}$ mice displayed significantly less airway inflammation than did wild-type mice, as well as reduced viral replication in the lungs. These findings suggest that FcγRIII probably plays a pro-inflammatory role during hRSV infection. Taken together, our data support the notion that FcγRs contribute to DC entry with antibody-coated hRSV, which ultimately dampens T-cell activation.

Materials and methods

Mice

Wild-type (WT) C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice (C57BL/6 background) were generously provided by Dr. T. Takai (Tohoku University, Tohoku, Japan) and Dr. K. Smith (University of Cambridge, Cambridge, UK), respectively. The OT-I and OT-II transgenic mice strains[42] expressing specific T-cell receptors for I-A^b/$OVA_{323–337}$ and H-$2K^b$/$OVA_{257–264}$, respectively, were kindly provided by Dr. R. Steinman (The Rockefeller University, New York, NY). All animal procedures used in this study are based on both the *Handbook for Standard Biosafety*, Conicyt 2008, Chile; and the *Guide for the Care and Use of Laboratory Animals* (NRC 2011). All procedures were performed under the supervision of a veterinarian and approved by the institutional bioethical committee.

Virus preparation and titration

Monolayers of confluent HEp-2 cells (CCL-2, American Type Culture Collection, Manassas, VA, USA) were infected with 3×10^7 plaque-forming units (PFU) of hRSV serogroup A strain 13018-8 (clinical isolate obtained from the Instituto de Salud Pública de Chile) or a recombinant hRSV encoding the green fluorescent protein (GFP) kindly provided by Dr Mark E. Peeples (The Research Institute at Nationwide Children's Hospital) and maintained in OptiMEM-I (Gibco, São Paulo, Brazil) media at 37° and 5% CO_2. After 12 hr of incubation, culture medium was replaced with fresh OptiMEM-I and incubated for 48 hr. Then, infected-HEp-2 cell supernatants were harvested and stored in small aliquots (1 ml) at −80°. Virus was titrated over HEp-2 cells in 96-well plates and screened for syncytia formation after crystal violet staining. Viral titres in supernatants were estimated in HEp-2 cells (25 000 cells/well) monolayers. Cells were infected with serial dilution of 20 µl of hRSV per well on 96-well plates and incubated at 37° for 48 hr. Screening for detection and quantification of syncytia was performed after cell fixation with 1% paraformaldehyde–PBS. Also titration was performed by immunochemistry; cells were permeabilized with Saponin 0·2%–PBS for 20 min and then blocked with BSA 0·1%–PBS for 10 min. Next, cells were incubated with mouse monoclonal anti-N antibody developed in our laboratory (clone 1E9D1[43]) for 1 hr (dilution 1 : 750 in PBS–BSA), washed twice and then incubated with horseradish-peroxidase-conjugated anti-mouse IgG (dilution 1 : 2000) for 45 min. Next, cells were washed twice and the substrate TRUE BLUE Peroxidase (KPL, Gaithersburg, Maryland, USA) was added and incubated for 10 min. Lysis plaques were visualized with a microscope and quantified. Ultraviolet inactivated virus (hRSV-UV) was generated by exposing 2 ml virus preparations covered with an ice-pack for 45 min over a 302 nm, 15 W lamp transilluminator as previously described.[7,44] 'Mock' consist of supernatants from uninfected HEp-2.

Neutralization assays

Human RSV-IC was produced by incubating 5×10^5 PFU with different dilutions of a neutralizing anti-RSV Fusion protein monoclonal antibody (anti-F RS-348, kindly provided by Dr Pierre Pothier, Université de Bourgogne, France) (stock concentration 2·8 mg/ml) for 2 hr at 4° or incubating 5×10^5 to 5×10^7 PFU of hRSV with 50 µg/ml of palivizumab for 15 min at 37°. HEp-2 cells were grown in 24-well plates at 2×10^5 cells per plate and 24 hr later were inoculated either with hRSV or hRSV-IC at a multiplicity of infection (MOI) equal to 1. Untreated cells were included as a control. The neutralizing capacity of different antibody dilutions was evaluated by flow cytometry. Forty-eight hours after inoculation, cells were gently washed with PBS to remove debris and then mechanically detached by pipetting. For assessing hRSV-F protein expression, cells were stained with RS-348 in PBS/BSA 2% for 1 hr at 4°. Then, cells were washed and stained with an anti-mouse IgG-FITC antibody (BD Pharmingen, San Jose, CA, USA) for 1 hr. For hRSV-N protein expression cells were first fixed with PBS/paraformaldehyde (PFA) 2% for 30 min. Then, cells were permeabilized with Saponin 0·2%–PBS for 20 min. Later, cells were stained with an anti-N antibody (clone 1E9D1) in 0·2% Saponin–2% BSA-PBS, washed and finally stained with a goat anti-mouse IgG-FITC antibody (BD Pharmingen). Samples were evaluated in a FACS-Canto II flow cytometer. Dead cells were excluded by FSC/SSC analysis and by viability staining.

Real-time PCR for detection of RSV RNA

Total RNA was obtained from lungs and DCs by using TriZol Reagent (Invitrogen, Waltham, MA, USA), as suggested by the manufacturer and reverse transcribed to cDNA by the use of Improm-II Reverse transcription system (Promega, Madison, WI, USA) with random primers. Then, hRSV N protein and β-actin RNA were detected by real-time PCR using Brilliant QPCR Master Mix (Stratagene, La Jolla, CA, USA) on an Mx3000P thermal cycler (Stratagene, La Jolla, CA, USA). Data were expressed as copy numbers of hRSV N RNA per 5000 copies of β-actin. Primers used for hRSV N gene detection were forward: 5′-GAG ACA GCA GCA TTG ACA CTC CT-3' and reverse: 5′-CGA TGT GTT GTT ACA TCC ACT-3′. Detection of β-actin was used as a housekeeping reference gene with primers forward: 5′-AGG CAT CCT GAC CCT GAA GTA C-3′ and reverse: 5′-TCT TCA TGA GGT AGT CTG TCA G-3′. For the infection kinetic experiments, data were expressed as copy numbers of hRSV N RNA per ng of total cDNA.

Cytokine ELISA

Release of interleukin-6 (IL-6), IL-10 and IL-12 by DCs was measured 24 hr after challenge with hRSV, hRSV-IC or hRSV-UV. Briefly, ELISA plates (Maxisorb; Nunc, Rochester, NY, USA) were coated with 50 ng/well of purified anti-IL-6 (clone MP5-20F3), anti-IL-10 (clone JES5-2A5) or anti-IL-12 (clone 9A5) antibodies in 50 µl PBS. Then, plates were blocked with PBS-BSA 3% and 200 µl of supernatant from cultures was added to each well and incubated overnight at 4°. After this time, wells were washed twice with PBS and 25 ng/well of anti-IL-6-biotin (clone MP5-32C11), anti-IL-10-biotin (clone SXC-1) or anti-IL-12-biotin (clone C17.8) was added in BSA 1%–PBS. Finally, plates were washed and incubated with streptavidin-horseradish peroxidase. All antibodies used

for ELISA were purchased from BD Pharmingen. 3-3′-5-5′-Tetramethyl-benzidine, final concentration 100 μg/ml (Sigma-Aldrich, St Louis, MO, USA) was used as a colorimetric substrate. Enzymatic reaction was stopped with 2 M H_2SO_4 and absorbance was recorded at 450 nm. Recombinant IL-2, IL-6, IL-10 and IL-12 (BD Pharmingen, San Jose, CA, USA) were used as standards for cytokine quantification.

DC viability and antigen-presentation assays

Bone marrow-derived DCs from C57BL/6, FcγRIII$^{-/-}$ and FcγRIIb$^{-/-}$ mice were prepared as previously described.[25] On day 5 of culture, DCs were inoculated for 24 hr with hRSV or hRSV-IC at an MOI equal to 1. The hRSV-IC was prepared with neutralizing concentration of anti-F RSV 348 antibody (0·86 mg/ml). Simultaneous to virus inoculation, for antigen presentation assays, DC cultures were treated with 10 ng/ml of $OVA_{257–264}$ peptide or 20 ng/ml of $OVA_{323–339}$ peptide. Viability of DCs was determined by trypan blue exclusion. In the respective assays, FcγRIII and FcγRIIb were blocked by incubating DCs for 2 hr with 5 μg/ml of anti-CD16/CD32 blocking monoclonal antibody (clone 2.4G2; BD Pharmingen) before DC inoculation with either hRSV or hRSV-IC. The DCs were then cultured with purified OT-II and OT-I T cells at a DC : T-cell ratio equal to 1. OT-II CD4$^+$ T cells or OT-I CD8$^+$ T cells were purified from spleens of transgenic mice and isolated by negative selection (purity > 95%, Miltenyi Biotech, Bergisch Gladbach, Germany). Release of IL-2 and interferon-γ (IFN-γ) was measured after 20 hr of DC–T-cell co-culture as previously described.[25] As a control, cell viability was determined by Trypan blue exclusion during and after the co-culture experiments.

Flow cytometry

Flow cytometry analyses were performed on FACSCanto-II flow cytometer or FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). For determination of DC infection with hRSV, DCs were inoculated as mentioned above. Forty-eight hours after inoculation, cells were double-stained with anti-CD11c-allophycocyanin (APC) (clone HL3; BD Pharmingen) and anti-hRSV Fusion protein (clone RS-348, same one used for immune complex formation).[41] After washing, cells were stained with a goat anti-mouse IgG-FITC (BD Pharmingen). Maturation of DCs was determined with fluorescence-labelled antibodies against CD40, CD80, CD86, H-2K^b and I-A^b as previously described[27] (all antibodies from BD Pharmingen). For analysis, our gating strategy first considered CD11c$^+$ cells, which were evaluated for the expression of all other markers. Acquired data were analysed using FCS Express (DeNovo Software V4) or FlowJo Software v.8·4 for Macintosh (Tree Star Inc., Ashland, OR, USA). For *in vivo* evaluation of DC infection and T-cell activation at the lungs of control and hRSV-infected mice, animals were killed either at day 7 or 17 post-infection with an overdose of ketamine/xylazine. Lungs were extracted and homogenized through a 70-μm cell-strainer in PBS containing 10 mM EDTA–10% fetal bovine serum (FBS). For T-cell activation, 3×10^6 lung cells were stained with a mixture of antibodies containing anti-CD69-FITC, anti-CD8α-phycoerythrin (PE), anti-CD3-peridinin chlorophyll protein (PerCP), anti-T-cell receptor-β-APC, anti-CD4-PE-Cy7 and anti-CD25-APC-Cy7 (all antibodies from BD Pharmingen) in PBS–FBS 2% for 30 min at room temperature. To assess conventional DC infection at the lungs, 5×10^6 lung cells were incubated with a mixture of antibodies containing anti-EpCAM-PE, anti-CD11b-PerCP, anti-Ly-6G-APC, anti-CD11c-PE-Cy7, anti-I-A I-E-APC-Cy7 and anti-N-hRSV-Alexa 488 (clone 1E9D1) in PBS-Saponin 0·2%-FBS 2% for 1 hr at room temperature. Samples were analysed by FACS, which was set to acquire at least 3×10^4 CD11c$^+$ I-A/I-E$^+$ Ly-6G$^-$ EpCAM$^-$ cells, that were then analysed for the surface expression of hRSV Nucleoprotein as recently described by our group.[44]

Passive antibody immunization and hRSV challenge

Lyophilized palivizumab (Synagis™) was reconstituted in 100 μl of sterile water to a final concentration of 12·5 μg/μl. Then, WT, FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice (C57BL/6 background) were injected intraperitoneally with 50 mg/kg (approximately 1·25 mg per mouse) of palivizumab (a humanized neutralizing monoclonal antibody (IgG1) against the hRSV fusion protein). At day 1 after immunization WT, FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice were anaesthetized with 150 μl of a 0·8% ketamine–0·1% xylazine solution in PBS 1× (NaCl 0·14 M; KH_2PO_4 1·47 mM; Na_2HPO_4 7·81 mM; KCl 2·68 mM), and challenged intranasally with 1×10^7 PFU of hRSV in 75 μl. Similarly, for hRSV re-infection assays (herein, second challenge), 10 days post-infection mice were challenged with hRSV without receiving a second dose of palivizumab. Mouse weight was monitored daily after starting palivizumab administration until euthanasia.

Analysis of infiltrating cells in bronchoalveolar lavages

On day 6 after infection, mice were terminally anaesthetized and lungs were washed through the trachea three times with 500 μl of sterile PBS. Recovered cells were separated in two aliquots, one was centrifuged at 300g for 6 min, re-suspended in 300 μl and stained with a 1 : 1000 dilution of anti-CD11b-FITC and anti-Gr-1 (Ly-6G)-APC antibodies (BD Pharmingen) for 40 min on ice. Alternatively, bronchoalveolar lavages (BALs) were stained with anti-CD11b-FITC, anti-Siglec-F-PE, anti-Ly-

6C-PerCP, anti-Ly-6G-APC and anti-CD11c-PE-Cy7. Data acquisition was performed with FACSCalibur and FACSCanto-II flow cytometers (BD Biosciences). The second aliquot of cells was spun onto glass slides, air dried and stained with May–Grünwald and Giemsa stains (Merck, Kenilworth, NJ, USA).

Kinetics of airway inflammation after hRSV challenge

Wild-type and FcγRIII$^{-/-}$ mice were challenged at day 0 with 1×10^7 PFU of hRSV in 75-μl inocula and control mice were challenged with supernatants of non-infected Hep-2 cells. After 1, 3 and 5 days of infection, mice were terminally anaesthetized and BALs were obtained on the different days (see above). The obtained cells were centrifuged at 300g for 6 min, resuspended in 300 μl and stained with 1 : 1000 dilution of anti-CD11b-FITC and anti-Gr-1 (Ly-6G)-APC (BD Pharmingen, San Jose, CA, USA) for 40 min on ice. Data acquisition was performed on a FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA).

Lung histopathology

Lungs of control and infected mice were surgically removed. The upper lobules were conserved in Tissue-Tek OCT Compound (Sakura, AJ Alphen aan den Rijn, Netherlands) at −80°. Slices of 5 μm thickness were prepared on a cryostat. For immunofluorescence assays, slides were fixed and permeabilized in 70% ethanol for 2 hr at −20°. Then, tissue sections were changed to 100% ethanol for 30 min and dried for another 30 min at room temperature. Before staining, lung sections were hydrated, starting with 95% ethanol for 30 min, transferred to 75% ethanol for 5 min, then to 0·4% Triton X-100 in PBS for 5 min and finally rinsed twice in PBS. Tissue sections were incubated in blocking solution (4% BSA in 1 × PBS) for at least 30 min at room temperature. Then, slides were incubated over night at 4° with a biotin-conjugated rabbit polyclonal anti-hRSV antibody, diluted 1 : 100 in blocking solution (United States Biologicals, Swampscott, MA). Tissue sections were then incubated with FITC-conjugated streptavidin (1 : 200) for 1 hr at room temperature and washed with PBS. Nuclei were stained with Hoescht 33342 (5 μg/ml) for 30 min. Coverslips were mounted and examined in a Fluoview FV1000 Laser Scanning Confocal Microscope.

Results

Human RSV neutralization with anti-F IgG

To evaluate the capacity of neutralizing antibodies against hRSV to block virus entry into DCs, first we assessed the antibody concentrations that were sufficient to block the infection of epithelial cells (HEp-2 and A549). Unlike DCs, HEp-2 and A549 cells do not express FcγRs on their surface, as determined by flow cytometry (data not shown). Increasing concentrations of neutralizing monoclonal antibodies against the fusion protein of hRSV, antibody RS-348 or 50 μg/ml of palivizumab, were used to form IgG–hRSV immune complexes (hRSV-IC or hRSV-IC Pali) and tested on HEp-2 and A549 cells. As determined by bright-field microscopy (cytopathic effect, Fig. 1a) and flow cytometry (surface staining of the fusion protein or intracellular staining of the hRSV N protein, Fig. 1b–d or hRSV-derived GFP expression in the Supplementary material, Fig. S1C, D), increasing amounts of anti-F RS-348 (Fig. 1c and Supplementary material, Fig. S1A–C) and palivizumab (see Supplementary material, Fig. S1C, D) prevented syncytia formation, as well as the expression of both virus-derived proteins within A549 and HEp-2 cells. To ensure complete virus neutralization, we used a final concentration equal to 0·86 mg/ml of anti-F antibody throughout the following experiments.

DCs undergo abortive infection with hRSV-IC

To assess the infective capacity of hRSV-IC on immature murine bone marrow-derived DCs, we evaluated DC infection after challenge with hRSV and hRSV-IC. Expression of the hRSV fusion protein on DCs was measured at 1, 24 and 48 hr after virus inoculation using an MOI equal to 1 (Fig. 2 and Supplementary material, Fig. S1E). As shown in Fig. 2(b), 48 hr after inoculation, over 25% of hRSV-inoculated DCs expressed the hRSV F protein on the surface, which was consistent with our previous findings.[7] Unexpectedly, an equivalent proportion of DCs inoculated with hRSV-IC expressed the hRSV fusion protein on the surface, which suggested equivalent levels of virus entry and viral protein expression within these cells, as compared to free virus (Fig. 2b and Supplementary material, Fig. S1E). Dendritic cells inoculated with UV-inactivated hRSV did not display significant surface expression of the viral protein (data not shown). Additionally, we assessed the effect of the anti-F antibodies on hRSV entry into DCs using the GFP-expressing recombinant hRSV mentioned above. Because of the low infectivity of this virus in murine DCs regardless of the MOI used (< 5% GFP$^+$ CD11c$^+$ MHC-II$^+$ cells), no significant differences were observed in the presence of anti-F IgG (data not shown). It is noteworthy that DCs pre-incubated with an FcγR-blocking antibody (clone 2.4G2, Fc Block™) and then challenged with hRSV-IC did not display significant surface expression of the F protein, and fluorescence was equivalent to background levels displayed by uninfected DCs (Fig. 2b). The DCs pre-treated with 2.4G2 and then challenged with free hRSV displayed a discrete reduction for the surface expression of the F protein compared with DCs infected with hRSV alone,

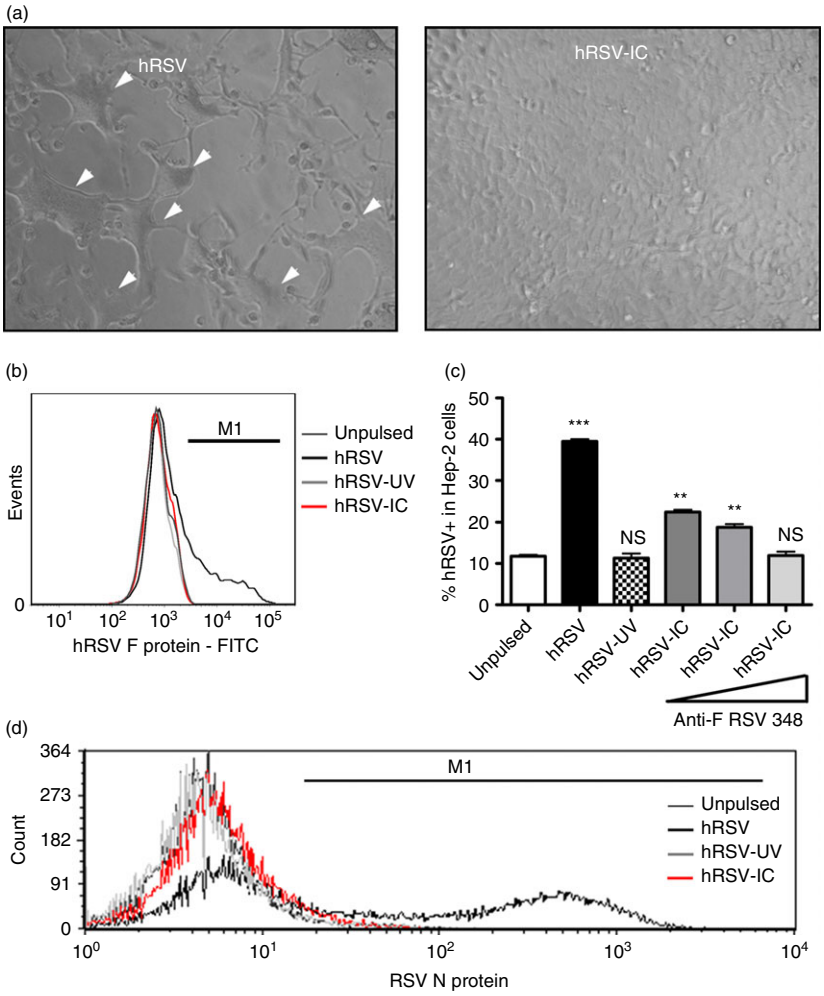


Figure 1. Human respiratory syncytial virus (hRSV) coated with an IgG1 neutralizing antibody displays impaired capacity to infect HEp-2 cells. HEp-2 cells were inoculated with hRSV and IgG-coated hRSV (hRSV-IC) at a multiplicity of infection (MOI) of 1 for 48 hr and then analysed for RSV infection. (a) Cytopathic effect (syncytia formation, arrowheads) on HEp-2 cells inoculated with hRSV and hRSV-IC. Images were taken at 40 × magnification. (b) Expression of hRSV F protein on the surface of HEp-2 cells determined by flow cytometry. Representative histogram showing the expression of F protein on the surface of HEp-2 cells pulsed either with hRSV (black), hRSV-UV (grey) or hRSV-IC (red). Uninfected cells were included as a control (thin black line). (c) Quantification of HEp-2 cells positive for F protein expression after infection with hRSV, hRSV-UV and hRSV-IC. The hRSV-IC was prepared with increasing amounts of a monoclonal neutralizing antibody. (d) FACS analysis of intracellular N protein expression in HEp-2 cells pulsed with hRSV (black), hRSV-UV (grey) and hRSV-IC (red) using an antibody dilution equal to 0·86 mg/ml with hRSV. Data are means ± SEM of two independent experiments $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$; data were analysed by one-way analysis of variance and Bonferroni post-test.

although differences between both treatments were not significant (Fig. 2b and see Supplementary material, Fig. S1E).

Real-time quantitative PCR quantification of hRSV nucleoprotein RNA in DCs was consistent with the data obtained by flow cytometry. As shown in Fig. 2(c) and the Supplementary material, Fig. S1F, DCs inoculated with hRSV-IC or free hRSV displayed significant amounts of hRSV N protein RNA, which only decreased significantly at 48 hr when DCs were pre-treated with 2.4G2 and then inoculated with IgG-coated hRSV (Fig. 2c). Dendritic cells inoculated with UV-inactivated hRSV displayed an insignificant expression of hRSV N protein RNA (Fig. 2c), which is consistent with previous data from our group.[7,44]

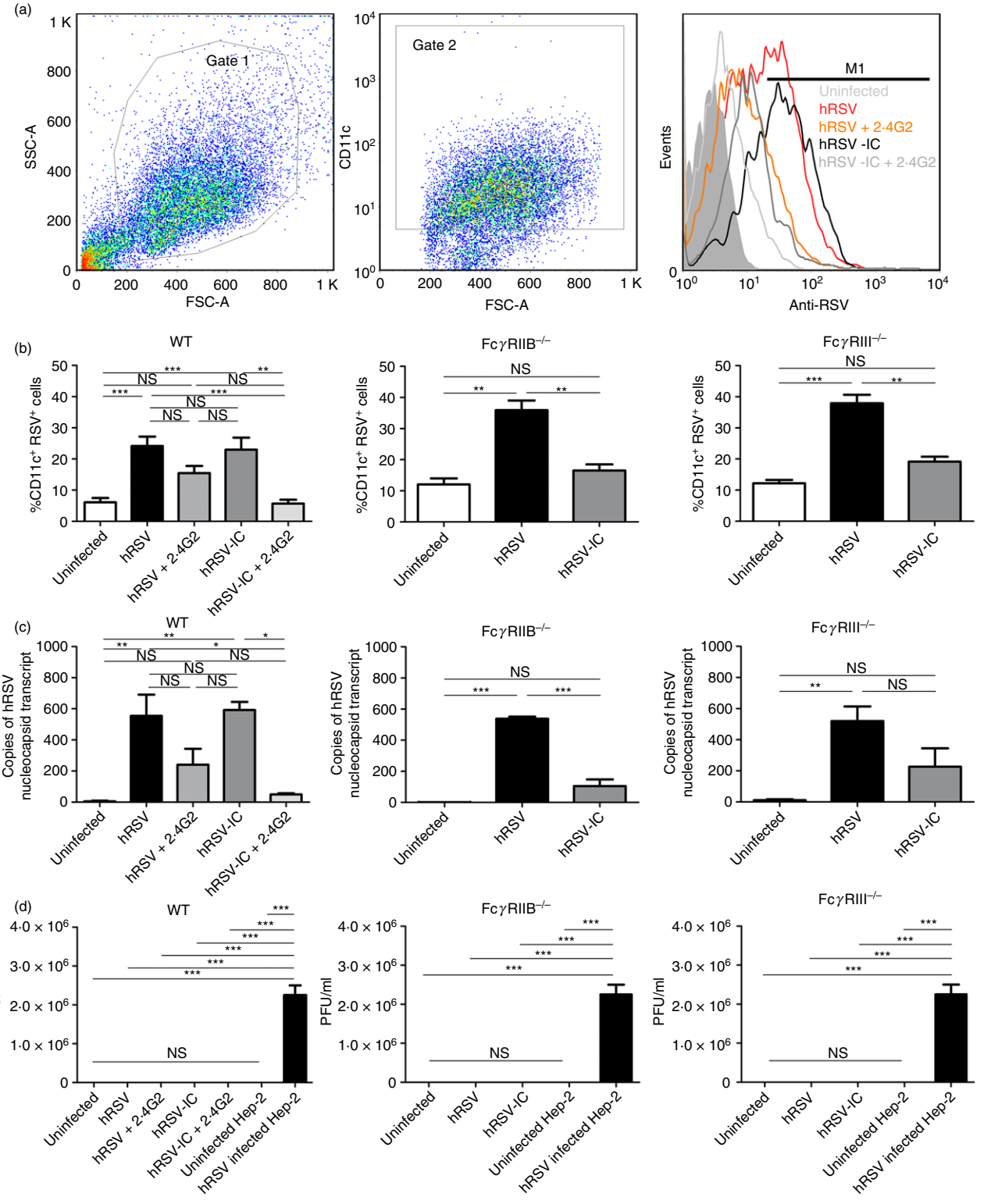
To further characterize the type of interaction between DCs and hRSV, we determined the amount of infectious viral particles released into the supernatants of DCs treated with hRSV and hRSV-IC. As shown in Fig. 2(d), DCs inoculated with hRSV and hRSV-IC did not release

Figure 2. IgG-coated human respiratory syncytial virus (hRSV-IC) infects murine dendritic cells (DCs). DCs derived from wild-type (WT), FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice were incubated overnight with hRSV at a multiplicity of infection (MOI) equal to 1 and analysed 48 hr later for hRSV infection. Pre-treatment with the Fc-blocking antibody 2.4G2 was applied when indicated. (a) Gate strategy used for analysing hRSV-infected DCs. CD11c^{+} cells were subsequently analysed on histogram overlays for F protein expression. The M1 marker was used to measure F-derived fluorescence beyond the baseline of unstained cells. The grey-filled histogram represents control CD11c^{+} cells stained with the secondary antibody. (b) Expression of RSV F protein on the surface of CD11c^{+} DCs pulsed either with hRSV or hRSV-IC measured by FACS. Uninfected DCs were included as control. (c) Real-time quantitative PCR for the detection of RSV nucleoprotein RNA in DCs. Graphs show number of copies of N RNA molecules per 5000 copies of β-actin. (d) Generation of infective hRSV particles by plaque assay on HEp-2 cells. As a control we included supernatants of uninfected or hRSV-infected HEp-2 cells. Plaque-forming units per ml (PFU/ml) from supernatants of hRSV and hRSV-IC inoculated. Data are means ± SEM of three to nine independent experiments $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, ns: non-significant; data were analysed by one-way analysis of variance and Bonferroni post-test.

significant levels of infectious viral particles into the supernatants, regardless of the treatment. Therefore, consistent with previous studies, these data suggest that hRSV-IC undergoes abortive infection in DCs, similar to free hRSV[7,8,45] or to hRSV-IC in macrophages.[46]

DC maturation and cytokine secretion induced by free and IgG coated-hRSV

Engagement of activating FcγRs by antibodies contained in immune complexes can trigger DC maturation and



promote antimicrobial adaptive immune responses.[27,47,48] Importantly, the masking of particular viral antigens by the antibodies within immune complexes can also skew or alter DC activation.[49] Hence, to assess the effect of hRSV-IC over DCs, we evaluated DC maturation in response to free and IgG-coated virus. With this aim, we assessed surface expression of maturation markers such as CD40, CD80, CD86, MHC-I (H-2K^b) and MHC-II (I-A^b) in uninfected, hRSV-, hRSV-IC- and hRSV-UV-inoculated DCs in the presence or absence of the FcγR-blocking antibody 2.4G2 (see Supplementary material, Fig. S2). At 24 hr post-inoculation, only CD40, CD80 and H-2K^b were significantly upregulated in hRSV-inoculated DCs, whereas CD86 remained similar in all groups (see Supplementary material, Fig. S2A). MHC-II only varied significantly for hRSV-UV-treated DCs. Interestingly, hRSV-IC-treated DCs did not display significant differences compared with uninfected DCs for any of the maturation markers assessed (see Supplementary material, Fig. S2A). The DCs pre-treated with 2.4G2 and then inoculated with hRSV-IC significantly up-regulated the expression of CD40 and CD80 (see Supplementary material, Fig. S2A). As controls, we evaluated uninfected DCs pre-treated with 2.4G2 and DCs inoculated with hRSV-UV. Controls displayed expression levels similar to those of uninfected DCs for the maturation markers assessed, except for MHC-II which was reduced both in 2.4G2-treated and hRSV-UV-inoculated DCs (see Supplementary material, Fig. S2A). These results suggest that anti-F IgG–hRSV immune complexes elicit poor DC maturation, comparable to free virus.

Next, we measured the secretion of IL-6, IL-10 and IL-12p70 by DCs challenged with hRSV and hRSV-IC (see Supplementary material, Fig. S2B). As previously reported, DCs infected with free hRSV produced significant amounts of IL-6,[7] regardless of whether FcγRs were blocked or not with 2.4G2. On the contrary, DCs did not secrete measurable amounts of IL-6 in response to hRSV-IC (see Supplementary material, Fig. S2B). Compared with uninfected control cells, DCs inoculated either with hRSV or hRSV-IC secreted only minor amounts of IL-10 and IL-12p70. Furthermore, inoculation with hRSV-UV failed to promote significant secretion of IL-6, IL-10 and IL-12p70 by DCs (see Supplementary material, Fig. S2B). These data support the notion that hRSV-IC does not promote significant DC maturation.

Abortive infection with hRSV-IC requires surface expression of FcγRs on DCs

To identify the FcγR responsible for mediating abortive infection by hRSV-IC, DCs were generated from either FcγRIIb$^{-/-}$ or FcγRIII$^{-/-}$ knockout mice and challenged with IgG-coated hRSV (Fig. 2b–d). Dendritic cells derived from FcγRIII$^{-/-}$ and FcγRIIb$^{-/-}$ mice showed a significant reduction in viral protein expression in hRSV-IC-treated DCs (Fig. 2b). Similarly, FcγRIII$^{-/-}$ and FcγRIIb$^{-/-}$ DCs displayed reduced expression of viral nucleoprotein RNA after challenge with hRSV-IC compared with cells challenged with free hRSV (Fig. 2c). UV-inactivated hRSV was included as a control, showing reduced expression of viral nucleoprotein RNA (Fig. 2c). These results suggest that both receptors, FcγRIII (activating) and FcγRIIb (inhibitory), contribute to facilitating abortive infection of DCs with hRSV-IC *in vitro*.

Abortive infection by hRSV-IC impairs the activation of CD8$^+$ and CD4$^+$ T cells by DCs

To determine whether abortive infection with hRSV-IC can modulate the capacity of DCs to activate naive T cells, as previously reported for free hRSV,[7] *in vitro* DC–T-cell co-culture assays were performed. Transgenic OT-I or OT-II T cells were cultured with ovalbumin peptide-loaded DCs that were either challenged with hRSV or hRSV-IC. T-cell activation in response to control, hRSV- or hRSV-IC-inoculated DCs was determined by measuring the secretion of IL-2 for CD4$^+$ T cells and IFN-γ for CD8$^+$ T cells (Fig. 3a–f). As previously described,[7,8] hRSV-pulsed DCs showed a significantly reduced capacity to induce IL-2 secretion by CD4$^+$ T cells (Fig. 3a). Importantly, CD4$^+$ T cells co-cultured with hRSV-IC-challenged DCs also displayed a reduced capacity to produce IL-2 (Fig. 3a). T-cell activation was restored when DCs were inoculated with hRSV-IC in the presence of 2.4G2 (Fig. 3a). As expected, T cells stimulated either with uninfected, 2.4G2- or hRSV-UV-pulsed DCs secreted significant amounts of IL-2 (Fig. 3a and data not shown).

To assess the effect of hRSV-IC on the capacity of DCs to activate CD8$^+$ T cells, IFN-γ secretion by these cells was measured. Interferon-γ secretion was also significantly diminished when CD8$^+$ T cells were stimulated with DCs pulsed either with hRSV or hRSV-IC, compared with peptide-pulsed uninfected DCs (Fig. 3b). However, FcγR blockade with 2.4G2 did not restore IFN-γ secretion by CD8$^+$T cells when DCs were challenged with hRSV-IC (Fig. 3b). Interleukin-2 secretion by CD8$^+$ T cells showed a similar pattern to that observed for IFN-γ when cells were stimulated with hRSV-or hRSV-IC-pulsed DCs (data not shown). Trypan blue exclusion assays performed during these experiments did not show any significant decrease in T-cell viability, suggesting that the reduced T-cell activation described above was not due to cell death (data not shown).

Next, FcγRIII- and FcγRIIb-deficient DCs were used to determine the contribution of individual FcγRs to hRSV-IC-induced inhibition of T-cell activation. As shown in Fig. 3, FcγRIII- and FcγRIIb-deficient DCs challenged with free hRSV were unable to activate T cells (Fig. 3c–f). On the contrary, FcγRIII- and FcγRIIb-deficient DCs

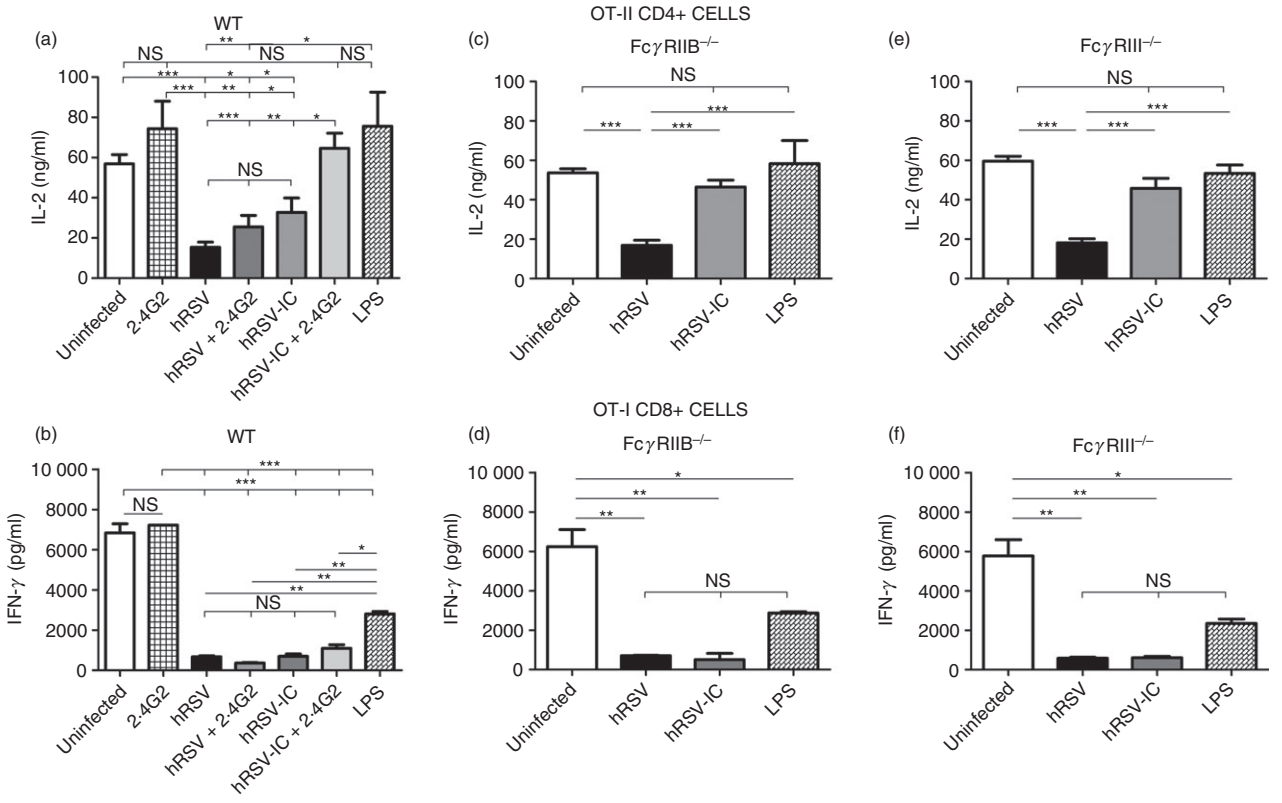


Figure 3. IgG-coated human respiratory syncytial virus (hRSV-IC) impairs the capacity of dendritic cells (DCs) to activate naive T cells. (a, c and e) Secretion of interleukin-2 (IL-2) by OT-II $CD4^+$ and (b, d and f) interferon-γ (IFN-γ) by OT-I $CD8^+$ T cells stimulated with pOVA-pulsed DCs (a and b wild-type (WT) DCs: c and d $Fc\gamma RIIb^{-/-}$ DCs; and e and f $Fc\gamma RIII^{-/-}$ DCs), either uninfected, hRSV- or hRSV-IC-inoculated. When indicated, DCs were pre-treated with 2.4G2 to block FcγRIIB and FcγRIII. Data are means ± SEM of at least three independent experiments $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$, ns: non-significant; data were analysed by one-way analysis of variance and Bonferroni post-test.

challenged with hRSV-IC induced IL-2 secretion by OT-II T cells in an antigen-dependent manner (Fig. 3c and e). However, IFN-γ secretion by $CD8^+$ OT-I T cells was not induced by FcγRIII- and FcγRIIb-deficient DCs pulsed with hRSV-IC (Fig. 3d and f). These data suggest that hRSV-IC differentially affects the capacity of DCs to activate $CD4^+$ and $CD8^+$ T cells and that this process is FcγR-dependent. Moreover, inhibition of DC function by hRSV and hRSV-IC indicates that limited viral replication, such as that seen in the abortive infection of DCs, generates sufficient levels of viral proteins and RNA to interfere with the fundamental function of these antigen-presenting cells.

Human RSV infects lung DCs and modulates T-cell responses after challenge

To assess DC infection *in vivo*, control or palivizumab-treated WT, $Fc\gamma RIII^{-/-}$ and $Fc\gamma RIIb^{-/-}$ mice were challenged with hRSV and their lungs were analysed by flow cytometry (Fig. 4 and see Supplementary material, Fig. S3). We analysed the recruitment of $CD11c^+$ $MHC\text{-}II^+$ cells to the lungs 6 days after primary infection with hRSV (Fig. 4a) and then 6 days after secondary infection with the same virus (Fig. 4b). The population assessed and the time-point analysed (day 6) were consistent with previous reports showing that $CD11c^+$ $MHC\text{-}II^+$ cells are recruited to the lungs of infected mice at this time-point.[50,51] As shown in Fig. 4(a), DCs obtained from the lungs of mice pre-treated with palivizumab and challenged with hRSV expressed significant amounts of viral nucleoprotein (hRSV-N) on the surface, compared with DCs obtained from mice that did not receive this antibody (Fig. 4a). However, the opposite was observed for FcγR knockout mice, in which hRSV-challenge in the presence of palivizumab resulted in reduced hRSV-N expression on the surface of DCs compared with animals receiving hRSV alone (Fig. 4a). These data suggest that FcγRs contribute to the capture of hRSV-IC by DCs *in vivo*. As expected, lung DCs from hRSV-infected WT and $Fc\gamma RIII^{-/-}$ mice showed increased expression of N protein compared with uninfected animals, although differences were not statistically significant (Fig. 4). These differences might be due to the migration of infected DCs

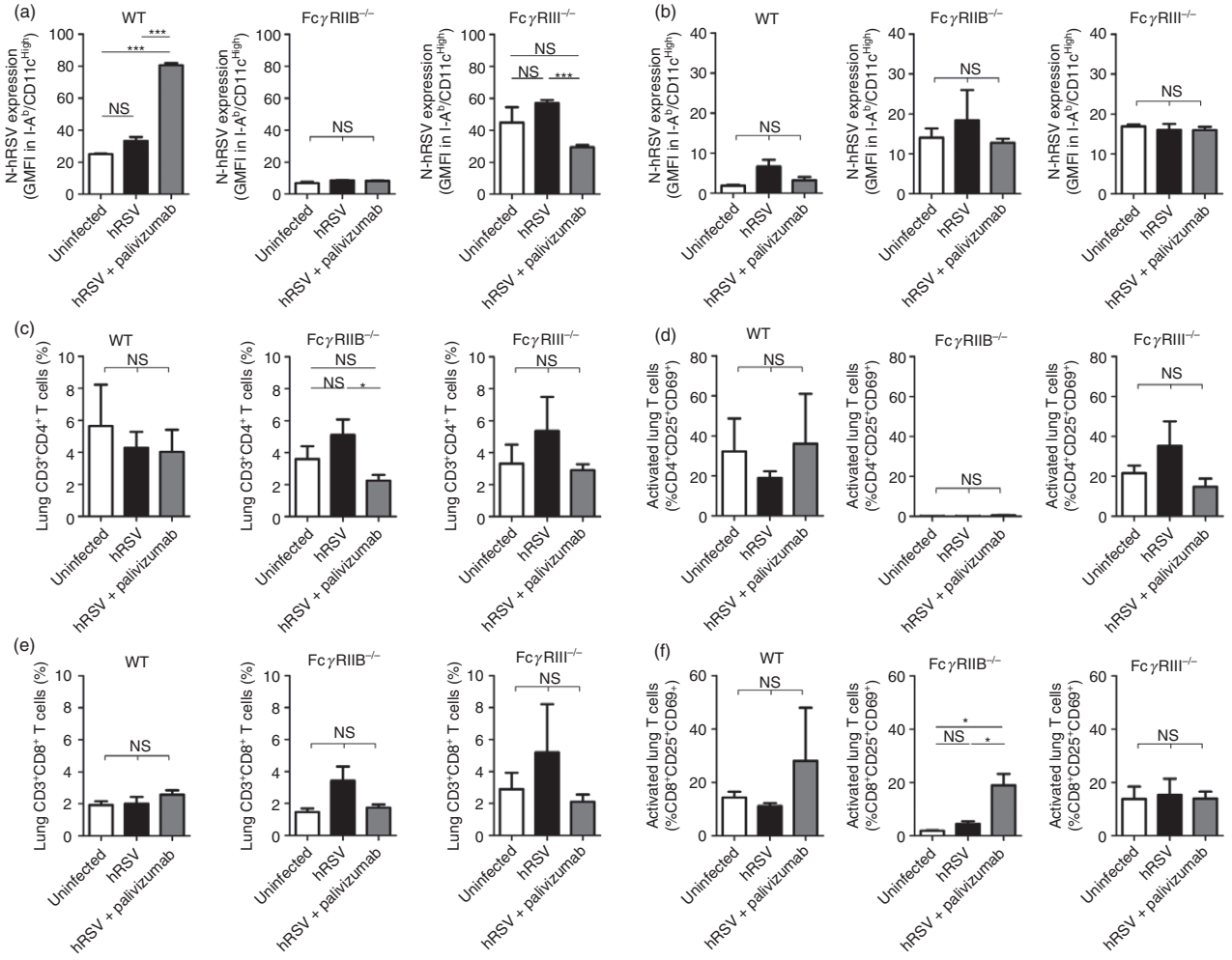


Figure 4. Pulmonary dendritic cell (DC) infection and *in vivo* T-cell responses after human respiratory syncytial virus (hRSV) challenge. Wild-type (WT), FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice received an intraperitional dose of palivizumab (50 mg/kg, ~ 1·25 mg/mouse) and 1 day later were infected with 1×10^7 plaque-forming units (PFUs) of hRSV. Uninfected mice were included as control in all groups. (a) Expression of hRSV nucleoprotein (N) in CD11c$^+$ MHCII$^+$ cells was analysed by FACS at day 6 post infection in the lungs of WT, FcγRIII$^{-/-}$ and FcγRIIb$^{-/-}$ mice. (b) Ten days after primary infection, animals were re-challenged with hRSV and 6 days later CD11c$^+$ MHCII$^+$ lung cells from WT, FcγRIII$^{-/-}$ and FcγRIIb$^{-/-}$ mice were analysed for nucleoprotein expression. (c and e) Percentages of total CD4$^+$ CD3$^+$ and CD8$^+$ CD3$^+$ cells were measured in the lungs after first challenge and (d and f) percentage of activation of CD4$^+$ CD25$^+$ CD69$^+$ (gated on CD4$^+$) and CD8$^+$ CD25$^+$ CD69$^+$ (gated on CD8$^+$) on the lungs from WT, FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice after first challenge were analysed by flow cytometry. Data are means ± SEM of two independent experiments. *$P < 0{\cdot}05$, ***$P < 0{\cdot}001$, ns: non-significant. Data were analysed by one-way analysis of variance and Bonferroni post-test.

to the lymph nodes at earlier time-points than those assessed herein, as previously described by others.[51] Lung DCs from WT and FcγRs$^{-/-}$ animals exposed to a secondary infection with hRSV overall did not display any differences in viral protein expression regardless of palivizumab treatment (Fig. 4b).

Next we evaluated the effect of palivizumab treatment over T-cell phenotype and migration into the airways. First, we assessed the migration of CD4$^+$ (Fig. 4c) and CD8$^+$ (Fig. 4e) T cells into the lungs after primary infection. Interestingly, in most cases treatment with palivizumab did not significantly alter the percentage of CD4$^+$ and CD8$^+$ T cells in the lungs after hRSV challenge in WT and FcγRIII$^{-/-}$ mice, (Fig. 4c and e). Only, FcγRIIb$^{-/-}$ mice displayed a reduced percentage of CD4$^+$ T cells in the lungs after palivizumab treatment (Fig. 4c). Additionally, we analysed the phenotype of the T cells located at the lungs by measuring surface expression of CD25 and CD69, two T-cell activation markers (Fig 4d and f).[7] Although WT mice challenged with hRSV alone exhibited a slightly lower percentage of activated CD4$^+$ T cells in the lungs than uninfected animals (Fig. 4d), treatment with palivizumab did not significantly increase the activation of CD4$^+$ and CD8$^+$ T cells in the lungs of WT

and FcγRIII$^{-/-}$ mice (Fig 4d and f). Interestingly, lungs of FcγRIIb$^{-/-}$ mice pre-treated with palivizumab and then infected with hRSV displayed an increased percentage of activated CD8$^+$ T cells when compared with untreated or uninfected animals (Fig. 4f).

As mentioned above, similar experiments were performed in mice that were re-infected with hRSV 10 days after the primary infection to recall naturally occurring hRSV-specific memory T cells in the lungs. Similar to the results obtained after primary infection, treatment with palivizumab did not elicit increased percentages of activated CD4$^+$ or CD8$^+$ T cells after secondary encounter with the virus (see Supplementary material, Fig. S3). Furthermore, we observed that the lungs of FcγRIIb$^{-/-}$ mice displayed significantly fewer CD4$^+$ and CD8$^+$ T cells than did WT and FcγRIII$^{-/-}$ mice after a second challenge with hRSV (see Supplementary material, Fig. S3).

These findings suggest that FcγRs are probably involved in the entry of IgG-coated hRSV into lung DCs and that CD4$^+$ and CD8$^+$ T-cell responses are somewhat equivalent, either in the presence or absence of hRSV-neutralizing antibodies. Therefore, it would seem that there was no significant improvement in T-cell immunity against hRSV as a result of passive anti-F IgG therapy with a simultaneous hRSV infection.

FcγRIIb- and FcγRIII-deficient mice show differential lung hRSV replication

To determine whether FcγRs modulate hRSV replication in the lungs in the presence or absence of palivizumab, we evaluated lung infection 6 days after viral challenge. This time-point was chosen based on viral replication kinetics that showed sustained virus replication in the lungs at this time (data not shown). Lungs from WT, FcγRIII$^{-/-}$ and FcγRIIb$^{-/-}$ mice were collected 6 days after hRSV challenge to determine the expression of both viral RNA and viral proteins. As expected, WT mice challenged with hRSV expressed large amounts of viral RNA for the virus nucleoprotein, as measured by quantitative real-time PCR (Fig. 5a). Importantly, WT mice treated with palivizumab before hRSV infection expressed significantly less ($P < 0{\cdot}01$) viral RNA than did mice challenged only with hRSV (Fig. 5a). Similar results were obtained for FcγRIII$^{-/-}$ mice, although lower levels of viral RNA were detected overall when compared with WT mice (Fig. 5a). On the other hand, FcγRIIb$^{-/-}$ mice challenged with hRSV, displayed significantly higher levels of viral RNA in the lungs after hRSV challenge, compared with WT mice. Finally, FcγRIII$^{-/-}$ mice displayed lower levels of viral RNA compared with WT and FcγRIIb$^{-/-}$ mice, regardless of palivizumab treatment (Fig. 5a).

In addition to measuring hRSV RNA, we performed immunofluorescence experiments to detect viral proteins in the lungs of infected mice (Fig. 5b, c). Consistent with the quantitative PCR assays described above, lungs from hRSV-challenged WT and FcγRIIb$^{-/-}$ mice expressed viral proteins in the airway epithelium (Fig. 5b, c). On the contrary, viral proteins were hardly detectable in the lungs of palivizumab-treated WT and FcγRIIb$^{-/-}$ mice. Remarkably, viral proteins were also virtually undetectable in the lungs of FcγRIII$^{-/-}$ mice, independent of palivizumab treatment (Fig. 5b, c).

These results are consistent with previous reports describing the capacity of neutralizing antibodies, such as palivizumab to prevent lung epithelial cell infection with hRSV.[18,19] However, our data strongly suggest that FcγRIII plays an important role in promoting the infection of lung epithelial cells by free hRSV (Fig. 5). Interestingly, FcγRIIb seems to have a somewhat protective effect on infection, in contrast to FcγRIII (Fig. 5).

FcγRIII$^{-/-}$ mice display reduced airway inflammation

To evaluate whether FcγRs modulate hRSV pathology in the presence of anti-hRSV neutralizing antibodies, WT, FcγRIII$^{-/-}$ and FcγRIIb$^{-/-}$ mice were passively immunized with palivizumab before hRSV challenge. As expected, WT mice infected with hRSV displayed massive immune cell infiltration into the lungs, as determined by flow cytometry analyses of BAL fluid (Fig. 6). Bright-field microscopy analyses of BAL cytospins, as well as flow cytometry analyses of these samples revealed that most infiltrating cells were neutrophils and monocytes expressing CD11b and Gr-1 on their surfaces (Fig. 6a, b). Further analyses revealed that a significant fraction of infiltrating cells expressed the eosinophil marker Siglec-F (data not shown). Strikingly, the majority of the CD11b-positive cells were also Siglec-F-positive in FcγRIIb$^{-/-}$ mice (data not shown). Nevertheless, visual analyses of BALs (cytospins) did not reveal significant numbers of eosinophils in these samples (Fig. 6a).

Treatment with palivizumab in WT and FcγRIIb$^{-/-}$ mice reduced inflammatory cell infiltration caused by hRSV challenge to levels equivalent to those in uninfected animals (Fig. 6b, upper panels). These data are consistent with previous studies showing the capacity of palivizumab to protect against hRSV.[18,19] However, untreated and palivizumab-treated FcγRIII$^{-/-}$ mice showed completely different responses after hRSV challenge, compared with WT and FcγRIIb$^{-/-}$ mice. More specifically, hRSV-infected FcγRIII$^{-/-}$ mice that did not receive palivizumab displayed significantly fewer infiltrating cells in BALs (Fig. 6a, b) than WT and FcγRIIb$^{-/-}$ mice. In contrast, treatment with palivizumab significantly increased cellular infiltrates in BALs of FcγRIII$^{-/-}$ mice (Fig. 6b). These data suggest that FcγRIII plays an important role at regulating cellular infiltration into the airways during hRSV infection and mediates the protective effect of palivizumab. The inflammation observed in FcγRIII-deficient

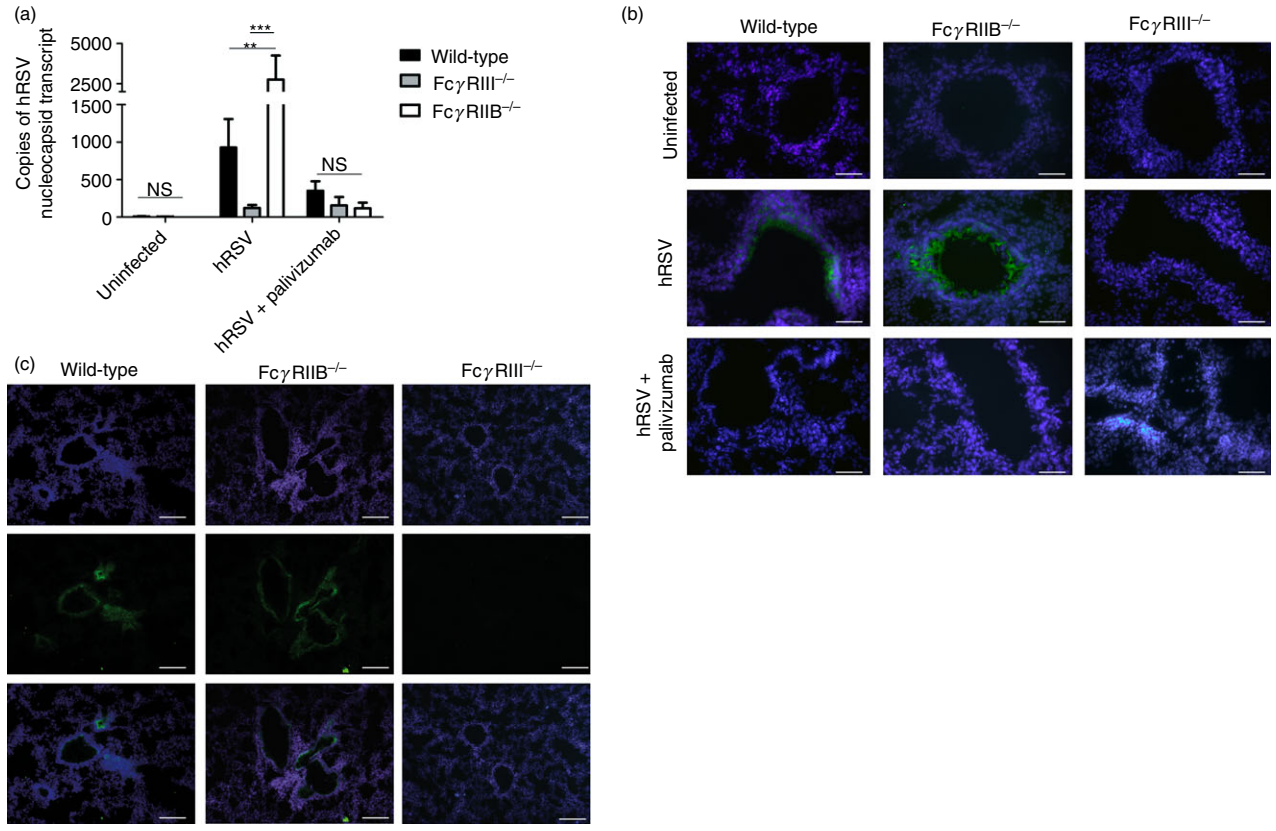


Figure 5. FcγRIII$^{-/-}$ mice display reduced human respiratory syncytial virus (hRSV) replication in the lungs. Wild-type (WT), FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice were passively immunized with palivizumab (50 mg/kg, ~ 1·25 mg per mouse) and infected 1 day after with hRSV. (a) At day 6 post infection, total RNA from lungs of control and hRSV-infected animals (three mice per group) were obtained and reverse-transcribed to quantify the number of hRSV-nucleoprotein RNA copies by real-time PCR. Data are expressed as the number of hRSV-nucleoprotein gene copies per 5000 copies of the β-actin gene. Data are means ± SEM of four independent experiments *P < 0·05, **P < 0·01, ns: non-significant. Data were analysed by two-way analysis of variance and Bonferroni post-analysis. (b and c) Six days after infection, lungs were removed, fixed onto slides, permeabilized and stained with a biotin-conjugated anti-hRSV antibody followed by streptavidin-FITC. Nuclei were stained with Hoescht 33342 as a counterstain. (b) shows lung microphotographs for all treatments at 40 × magnification, (bar represent 50 μm) (c) shows only microphotographs for infected animals taken at 10 × magnification (upper panels: counterstain, middle panels: hRSV (green fluorescence) and lower panels: (counterstain and hRSV stain merged). (Bars represent 200 μm).

mice treated with palivizumab could be the result of impaired immune-complex clearance in these animals, as previously described.[52]

Cellular infiltration in the lungs of FcγRIII$^{-/-}$ mice during hRSV infection

To better define the contribution of FcγRIII to the lung pathology caused by hRSV infection, which is characterized by the infiltration of immune cells into the lungs, Gr-1$^+$ CD11b$^+$ cells infiltrating this tissue were measured at different time-points after virus challenge. The WT and FcγRIII$^{-/-}$ mice were mock-treated (HEp-2 non-infectious supernatants) or hRSV-infected and killed 1, 3 and 5 days later to collect and characterize lung infiltrates (Fig. 7a).

Upon infection, WT and FcγRIII$^{-/-}$ mice displayed contrasting cellular infiltration patterns in the lungs, compared with mock-treated animals. Although at day 1 FcγRIII$^{-/-}$ mice showed slightly increased infiltration of Gr-1$^+$ CD11b$^+$ cells in BALs, compared with WT mice, by day 5 this effect was reversed in FcγRIII$^{-/-}$ mice, which showed a sharp decrease in the number of lung infiltrating cells (Fig. 7a). In contrast, WT mice displayed a gradual increase in cellular infiltrates at days 3 and 5. These data suggest that expression of FcγRIII is associated with an early increase in inflammatory cell infiltration into the lungs after inoculation with free hRSV.

Next, to evaluate whether reduced lung pathology in FcγRIII$^{-/-}$ mice was the result of reduced viral replication in the lungs, quantitative PCR analyses were performed at the same time-points as indicated above. Significant viral RNA was detected in the lungs of FcγRIII$^{-/-}$ mice, which peaked at 5 days post-infection (Fig. 7b) but then decreased by day 6 (Fig. 5). Therefore, decreased lung pathology in FcγRIII$^{-/-}$ mice, despite the

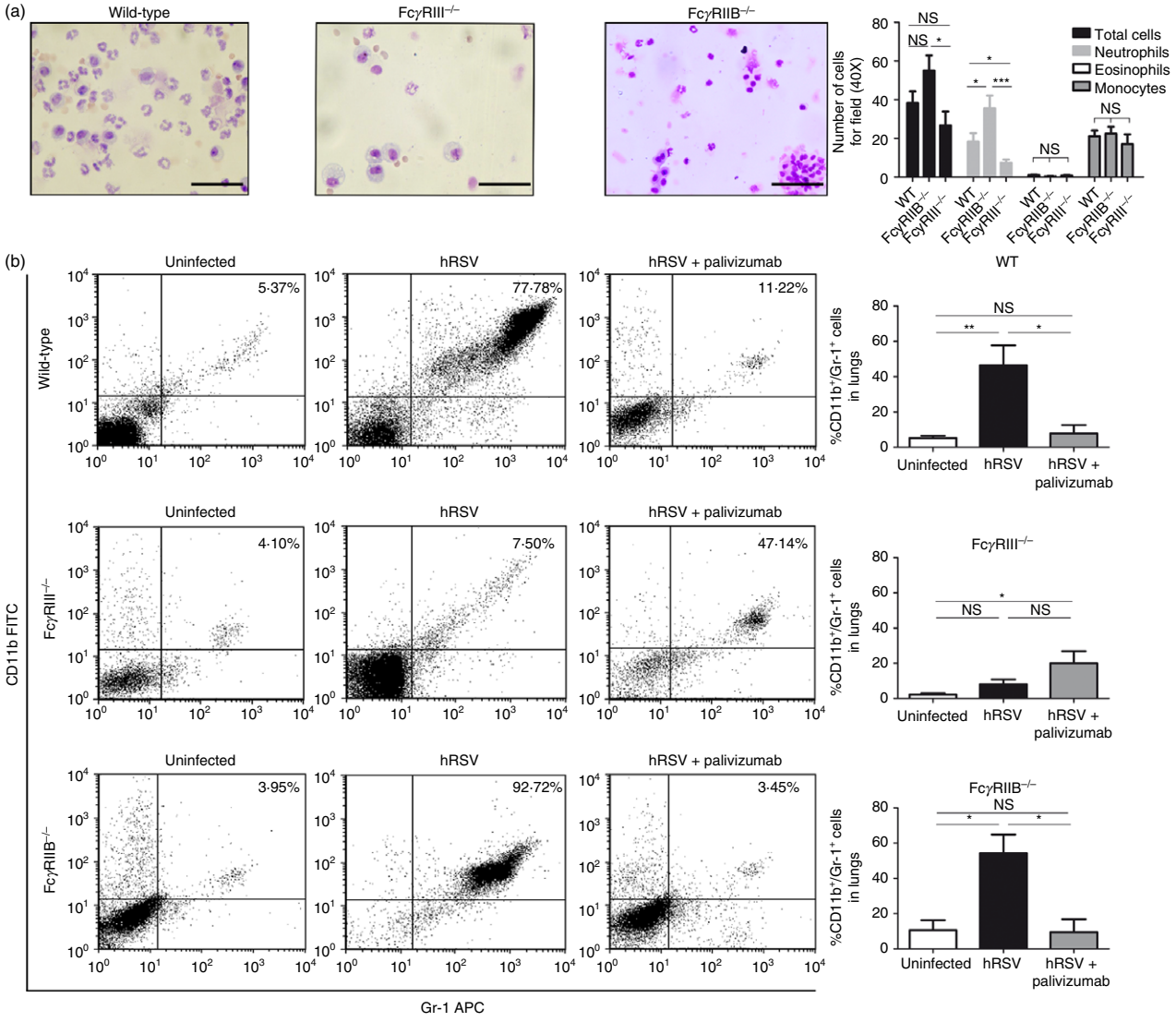


Figure 6. FcγRIII$^{-/-}$ mice display reduced recruitment of inflammatory cells into the airways. Wild-type (WT), FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice received an intraperitional dose of palivizumab (50 mg/kg, ~ 1·25 mg per mouse) and were later infected with 1×10^7 plaque-forming units (PFU) human respiratory syncytial virus (hRSV). Uninfected mice were included as control in all groups. (a) Six days after RSV challenge, bronchoalveolar lavages (BALs) were obtained from WT, FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice inoculated with hRSV and spun on glass slides, stained with May–Grunwald and Giemsa stains, and observed under the microscope at 40 × magnification. Graph on the right shows the quantification of neutrophils, eosinophils, monocytes and total cells observed in cytospin slides obtained from BALs of hRSV-infected mice. Data are total number ± SEM and were analysed by Student's *t*-test. (*$P < 0{\cdot}05$, ***$P < 0{\cdot}001$, ns: non-significant) Bars represent 50 μm. (b) Percentage of CD11b and Gr-1 positive cells in BALs 6 days after inoculation. Data are means ± SEM of four independent experiments analysed by one-way analysis of variance and Bonferroni's Multiple Comparison Test (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ns: non-significant).

presence of viral RNA, suggests that lesser pulmonary disease is probably a result of reduced recruitment of inflammatory cells into the airways, rather than reduced viral replication. To evaluate whether decreased hRSV replication on day 6 post-infection in FcγRIII$^{-/-}$ mice could be the result of pre-existing or newly synthesized anti-hRSV antibodies, ELISA were performed with sera derived from these animals. As shown in Fig. 7c, no-significant hRSV-specific antibodies were detected in the sera of these animals.

Discussion

Neutralizing antibodies are essential for the clearance of most viruses.[28,53] The coating of viruses with antibodies to form immune complexes can enable phagocytic cells, such as neutrophils, DCs and macrophages, to capture and eliminate these pathogens, avoiding further replication and dissemination in the host.[28] Furthermore, capture of ICs containing microbes by DCs can significantly contribute to establishing robust and effective

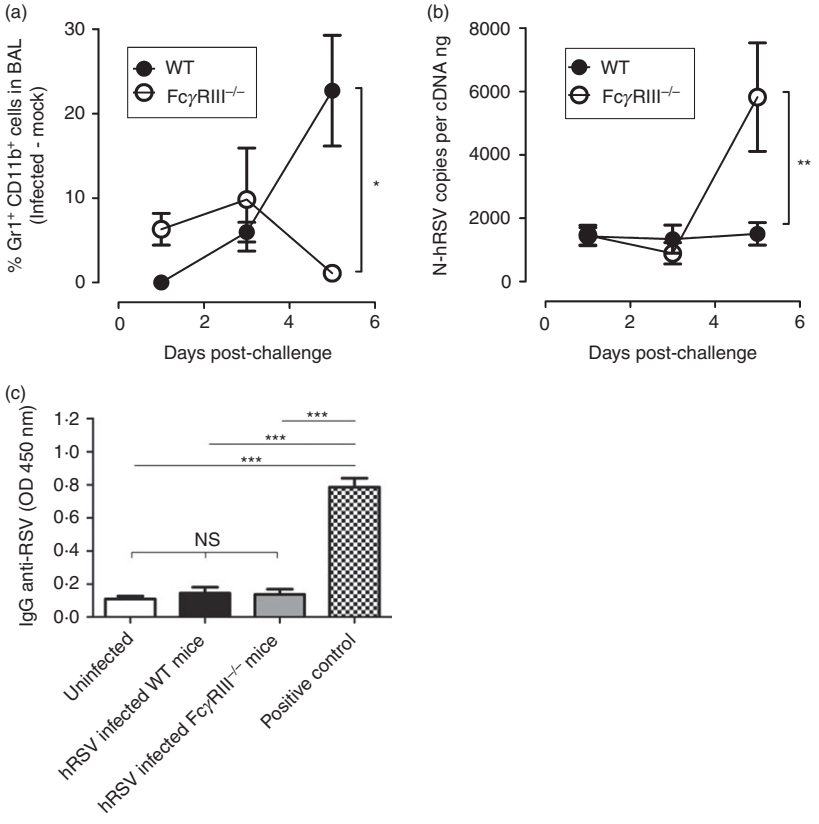


Figure 7. FcγRIII$^{-/-}$ mice display early inflammatory cell recruitment into the lungs, as compared with wild-type (WT) mice. WT and FcγRIII$^{-/-}$ C57BL/6 mice were mock-inoculated (HEp-2 non-infectious supernatants) or human respiratory syncytial virus (hRSV) infected (1×10^7 PFU). (a) Flow cytometry for CD11b$^+$GR-1$^+$ cells in bronchoalveolar lavage (BAL) was performed at different time-points. Data are represented as the differences in the percentage of inflammatory cells, between hRSV-infected and mock-inoculated mice for each day analysed. WT mice: closed circles; FcγRIII$^{-/-}$ mice: open circles. Data are means ± SEM of two pooled independent experiments and were analysed by one-way analysis of variance using Bonferroni's Multiple Comparison Test (*$P < 0{\cdot}05$) (b) Quantitative real-time PCR analyses of lung from hRSV-infected mice. The graph shows number of RSV-nucleoprotein RNA copies detected per ng of cDNA in lungs from WT (close circles) and FcγRIII$^{-/-}$ (open circle) mice. Data are means ± SEM of two independent experiments analysed by Student's *t*-test (**$P < 0{\cdot}01$). (c) Sera from WT and FcγRIII$^{-/-}$ mice were collected 6 days after infection with hRSV to assess specific antibodies against hRSV by ELISA. Data represent 1/20 dilution of the serum. A serum from uninfected WT mouse (Uninfected) was used as a negative control and anti-F RS-348 mouse antibody as a positive control. Data are means ± SEM of two independent experiments. Data were analysed by one-way analysis of variance and Bonferroni's Multiple Comparison Test (***$P < 0{\cdot}0001$, ns: non-significant).

antigen-specific T-cell responses that elicit memory and protective immunity.[25,27,54]

Antibodies targeting the antigenic site II of the hRSV F protein[39,55,56] have been developed as prophylactic strategies to reduce viral infection with equivalent results in humans and animal models.[18,19,39,57–59] Indeed, palivizumab (a humanized IgG antibody) and the anti-F RS-348 antibody (mouse IgG1 antibody) have been shown to decrease hRSV viral loads and hRSV-associated immunopathology in mice.[18,19,40] Nevertheless, whether virus encounter with such neutralizing antibodies can elicit enhanced antiviral T-cell responses or protective immune memory and immunity against hRSV has not been fully evaluated.[20–23] Hence, we assessed whether hRSV coated with anti-F neutralizing antibodies could mediate virus entry into DCs or modulate their phenotype and ability to activate T cells.

Here, we showed that DCs treated with hRSV-IC underwent, abortive infection *in vitro,* similar to previous findings with free hRSV.[8,45] Importantly, this process was mediated by surface FcγRs and was enough to negatively modulate the function of DCs. Modulation of DCs by hRSV and hRSV-IC could be mediated by viral proteins expressed within these cells. Indeed, DCs displayed sustained increases in the expression of viral proteins (F and N) and viral RNA over time, implying productive virus entry, viral protein expression and nucleic acid synthesis. We have recently reported that the hRSV nucleoprotein alone can prevent immunological synapse assembly between T cells[44] and the DCs treated with UV-

inactivated virus corroborated that we were in the presence of abortive infection, rather than DC capture of inactive viral particles. Concomitantly, few infectious virus particles were obtained from these cells, suggesting that DCs may be deficient for determinants required for virus egress or actively restrain this process. It is worth mentioning that other viruses have also been described as undergoing similar types of infection processes in DCs while retaining their virulence.[60–62]

Other viruses, such as dengue virus, have been shown to be infective in the form of ICs by exploiting the human FcγRIIA activating receptor, a homologue of FcγRIII in the mouse.[63] Similarly, immune-complexed porcine reproductive and respiratory syndrome virus can increase its infectivity by binding to swine FcγRII, a homologue of human FcγRIIb.[30] Hence, the infectivity of IgG-coated viruses depends on, among other things, the microbe itself and FcγRs expressed on the surface of target cells.

Our *in vitro* results showed that DCs inoculated with hRSV-coated with anti-F neutralizing antibodies were impaired at activating both $CD4^+$ and $CD8^+$ T cells, in a similar way to free virus.[7] Plaque formation assays confirmed that the antibody concentrations used in our experiments fully blocked F-mediated infection of HEp-2 cells, suggesting that hRSV-IC infection of DCs is FcγR-mediated. Although the participation of these receptors was evidence for $CD4^+$ T cells when using 2.4G2, we were unable to restore $CD8^+$ T-cell activation using this blocking agent. Interestingly, a recent study that also reported impaired T-cell activation for $CD8^+$, but not $CD4^+$, T cells using peripheral blood mononuclear cells and palivizumab-coated hRSV, found that opsonization with antibodies directed against another viral antigen, namely glycoprotein G, restored $CD8^+$ T-cell activation.[23] This last observation suggests that hRSV G protein might interfere with $CD8^+$ T-cell activation, and so could explain why DCs inoculated with hRSV-IC and 2.4G2 did not restore $CD8^+$ T-cell activation in our assays.

Although recent studies have evaluated the effect of hRSV on DCs *in vivo,* they have mainly approached the question as to how the virus can modulate DC migration from the lungs to the lymph nodes, rather than directly assessing infection of these cells.[51,64,65] Indeed, whether hRSV infects or modulates DC function *in vivo* has not been conclusively defined. Furthermore, the question as to hRSV-IC could impair the capacity of DCs to activate T cells had not been approached. Here, we were able to detect hRSV-infected DCs in the lungs of WT and $Fc\gamma R^{-/-}$ mice pre-treated with palivizumab and challenged with hRSV. These results are in agreement with our *in vitro* results, which also showed DC infection with hRSV-IC in the presence of FcγRs. These results suggest that hRSV can gain access within lung DCs through FcγRs, despite neutralization of the fusion protein. It is important to note that the infectious capacity of IgG-coated hRSV in the lungs was somewhat limited only to $CD11c^+$ $MHCII^+$ cells analysed in our study, as palivizumab-treatment significantly reduced epithelial cell infection in the airways, as previously reported for this passive immunization strategy.[18] Although we were able to detect signs of hRSV infection in $CD11c^+$-$MHCII^+$ pulmonary cells at day 6 post-infection, it remains to be determined what specific DC subsets are more susceptible to infection and functionally impaired by hRSV.[50,66,67] Moreover, it would be important to determine in future studies the migratory capacity of these cells in and out of the lungs after infection in FcγR-deficient mice. This aspect has been previously assessed for WT mice after hRSV infection.[50,51]

Further, we observed that FcγRs can significantly contribute to the pathogenesis of hRSV infection either in the absence or presence of neutralizing antibodies. Indeed, in the absence of palivizumab lack of FcγRIII significantly reduced lung pathology caused by hRSV infection, despite the fact that mice showed significant viral RNA early after infection in the lungs. These data are in agreement with studies suggesting that FcγRIII can promote T helper type 2 inflammatory immune responses in the lungs of mice, a type of adaptive immunity known to increase susceptibility to hRSV-induced pathology.[68] Consistent with this notion, hRSV challenge of FcγRIII-deficient mice showed a significant reduction of inflammatory cells in the lungs at day 6 post-infection, as well as reduced viral RNA and nearly undetectable levels of hRSV proteins in the lungs at this time-point. These results suggest that the activating FcγRIII receptor is likely to promote immune cell migration into the airways during infection with hRSV. In contrast, the inhibitory FcγRIIb receptor may play a protective role against non-IgG-coated hRSV by reducing lung inflammation. Indeed, we observed that FcγRIIb-deficient mice challenged with hRSV displayed significant levels of inflammatory cell infiltration in the lungs, particularly increased eosinophil infiltration, as well as increased levels of both hRSV RNA and proteins in the lungs. These data are in agreement with previous studies describing that the deficiency of this receptor increases eosinophil migration in a rhinitis model.[69] These data also suggest that in the absence of neutralizing antibodies, FcγRIII could be deleterious to the host upon hRSV infection. FcγRIII-mediated pathology could promote viral infection of lung cells, whereas FcγRIIb could play a protective role by reducing viral replication in these cells.

In the presence of hRSV-neutralizing antibodies, variant results were obtained for FcγRIII-deficient mice. Although palivizumab treatment reduced the amount of hRSV RNA in the lungs of FcγRIII-deficient mice, equivalent to WT mice, palivizumab induced a slight increase in infiltrating cells in FcγRIII-deficient mice, when compared with WT mice. This observation is consistent with a

recent study describing that the antiviral effect of passively transferred anti-hRSV antibodies requires inflammatory cells that express FcγRs on their surface.[70] It is important to point out that other groups have reported that FcγRIII-deficient mice display difficulties at eliminating immune complexes, such as IgG1-coated particles by macrophages.[52] Yet, these animals have been described overall as responding in a similar way to WT mice in IgG-independent inflammatory processes.[71] However, we cannot rule out that this knockout mouse may harbour uncharacterized immune abnormalities that could influence the results observed.

The findings described here, which show that hRSV remains infective in DCs and alters their capacity to activate naive T cells despite being bound to neutralizing antibodies is consistent with the notion that naturally occurring anti-hRSV antibodies can fail at establishing an effective immunity to the virus, although there are conflicting results.[23,24,72–75] Along these lines, the contribution of maternal antibodies to protection against hRSV remains controversial. Although some studies conclude that maternal neutralizing antibodies are beneficial against hRSV infection in the newborns,[76,77] others have suggested that these antibodies fail to protect against hRSV infection and can lead to detrimental effects, such as recurrent wheezing, airway hypersensitivity and re-infection.[78–80] These latter observations are more consistent with our data, which suggest that coating hRSV with a neutralizing antibody fails to prevent some of the virulent features of hRSV, such as the infection of DCs that interferes with the establishment of protective T-cell responses. The lack of agreement on the contribution of antibodies to hRSV pathogenesis highlights the importance of understanding the role of FcγRs that bind to IgG-coated hRSV, such as maternally derived or newly elicited antibodies. However, because neutralizing antibodies effectively reduce epithelial cell infection, palivizumab remains the best available alternative to prevent hRSV infections in high-risk infants.[18,20,21] Nevertheless, more studies assessing the role of palivizumab in modulating the immune response are needed to properly dissect the biological functions of this neutralizing antibody. Further, our data suggest that removing the Fc portion of antibodies could prevent hRSV-ICs from binding to FcγRs and subsequently infecting DC, eventually enhancing the effectiveness of neutralizing antibodies. Whether such an approach would help to restore T-cell activation and trigger long-term protective memory responses in individuals remains to be determined.

In summary, our results support the notion that FcγRs play a relevant role in hRSV-induced pathogenesis, both in the presence and in the absence of neutralizing antibodies. We found that, although anti-F antibodies blocked viral infection of epithelial cells, hRSV immune complexes remained infective in FcγR-expressing cells, such as DCs. Importantly, DC infection with hRSV-IC led to poor maturation and impaired T-cell activation capacity *in vitro*, as well as to poor T-cell responses *in vivo*. Furthermore, we report that in the absence of neutralizing antibodies there seems to be a balance on the roles of FcγRIIb and FcγRIII, which down-regulate or up-regulate hRSV pathogenesis, respectively. Future studies should contribute at determining the specific roles of these FcγRs in hRSV infection. Finally, the biological properties of IgG-coated hRSV should be further evaluated on FcγR-expressing cells to improve the effectiveness of current and future antibody therapies.

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Author contributions

RG, BR, PC, KC, and CP performed experimental work, data analyses and wrote the manuscript. SR and PG contributed to the experimental design, data analyses and writing the manuscript. AMK participated in planning the experiments and writing the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. HEp-2, A549 and dendritic cell infection with human respiratory syncytial virus (hRSV), hRSV–immune complex (hRSV-IC), hRSV-green fluorescent protein (hRSV-GFP) and hRSV-GFP-IC.

Figure S2. Dendritic cell maturation and cytokine secretion in response to IgG-coated human respiratory syncytial virus (hRSV).

Figure S3. Pulmonary $CD4^+$ and $CD8^+$ T-cell responses after secondary challenge with human respiratory syncytial virus (hRSV).