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Serum Antibodies Critically Affect Virus-Specific CD4+/CD8+ T Cell Balance during Respiratory Syncytial Virus Infections

Debby Kruijsten,* Mark J. Bakkers,* Nathalie O. van Uden,* Marco C. Viveen,† Tetje C. van der Sluis,* Jan L. Kimpen,* Jeannette H. Leusen,‡,§ Frank E. Coenjaerts,¶,‖ and Grada M. van Bleek*

Following infection with respiratory syncytial virus (RSV), reinfection in healthy individuals is common and presumably due to ineffective memory T cell responses. In peripheral blood of healthy adults, a higher CD4+/CD8+ memory T cell ratio was observed compared with the ratio of virus-specific effector CD4+/CD8+ T cells that we had found in earlier work during primary RSV infections. In mice, we show that an enhanced ratio of RSV-specific neutralizing to nonneutralizing Abs profoundly enhanced the CD4+ T cell response during RSV infection. Moreover, FcγRs and complement factor C1q contributed to this Ab-mediated enhancement. Therefore, the increase in CD4+ memory T cell response likely occurs through enhanced endosomal Ag processing dependent on FcγRs. The resulting shift in memory T cell response was likely amplified by suppressed T cell proliferation caused by RSV infection of APCs, a route important for Ag presentation via MHC class I molecules leading to CD8+ T cell activation. Decreasing memory CD8+ T cell numbers could explain the inadequate immunity during repeated RSV infections. Understanding this interplay of Ab-mediated CD4+ memory T cell response enhancement and infection mediated CD8+ memory T cell suppression is likely critical for development of effective RSV vaccines.

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Address correspondence and reprint requests to Dr. Grada M. van Bleek, Department of Pediatrics, Wilhelmina Children’s Hospital, University Medical Center, Utrecht, The Netherlands. E-mail address: g.vanbleek@umcutrecht.nl

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Abbreviations used in this paper: AS, autologous serum; AS-IgG, IgG-depleted autologous serum; BM-DC, bone marrow–derived dendritic cell; DC, dendritic cell; γc−/−, γ-chain deficient; h, human; HI, heat inactivation; moi, multiplicity of infection; N, nucleocapsid; NS, nonstructural protein; RSV, respiratory syncytial virus; RT, room temperature; UV-RSV, UV-inactivated respiratory syncytial virus; WT, wild-type.

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RSV infections are a major health burden in infants and elderly people (1–3). Although severe RSV bronchiolitis and pneumonia usually occur in children during primary infections, symptomatic reinfections are frequent in healthy individuals (4). These reinfections can occur with the same RSV strain, indicating that acquired immunity is not effectively established or does not completely protect against reinfection. Precise correlates of protection for RSV-induced respiratory tract infections are not defined, and the reason for incomplete immune protection is not clear. Abs provide partial protection against severe RSV bronchiolitis and pneumonia (5–7). Prophylactic treatment with palivizumab (Synagis, MedImmune, Gaithersburg, MD), a neutralizing Ab specific for the F protein of RSV, is used to protect high-risk infants against severe lower respiratory tract disease (8). Furthermore, children with T cell deficiencies are unable to efficiently clear the virus, which shows that T cells play a role in virus eradication (9, 10). The susceptibility of elderly people to severe RSV pneumonia might be the consequence of T cell senescence and/or decreased RSV-specific memory T cell numbers (11–13). However, no detailed information is available on the immune status of individuals in different age groups with respect to RSV.

The role of RSV-specific Abs is likely diverse. Neutralizing Abs can contribute to the reduction of viral load and, as a result, decrease innate immune responses. Nonneutralizing Abs might enhance infection and might cause immune complex deposition, leading to enhanced respiratory disease (14). Abs play a role in the uptake of immune complexes by APCs via FcRs or complement receptors and influence CD4+ and CD8+ T cell activation (15–19). In addition, Abs might alter innate immune activation by targeting viral particles to endosomal compartments where TLRs get activated or by interacting with viral proteins or carbohydrates that bind to immune receptors [e.g., chemokine and pattern recognition receptors (20, 21)] that play regulatory roles during innate and adaptive immune responses.

In the present work, we studied the impact of RSV-specific Abs on initiation of adaptive immunity during Ag presentation. We observed that a higher ratio of neutralizing versus nonneutralizing Abs enhanced the balance of responding virus specific CD4+/CD8+ T cells in vitro as well as in vivo. A possible role for RSV-specific Abs in shaping the human virus-specific memory T cell pool is discussed.

Materials and Methods

Mice

Pathogen-free 6–8-wk-old C57BL/6j wild-type (WT) mice were purchased from Charles River Laboratories (Maastricht, The Netherlands). FCγR common γ-chain–deficient (γC−/−) mice and FcγRIIb−/− mice on a C57BL/6 background (22–24) were bred and maintained at the central animal facility at Utrecht University. The mouse study protocols were approved by the Animal Ethics Committee of the University Medical Center Utrecht (Utrecht, The Netherlands).
RSV A2 strain was grown in HEp-2 cells, purified by polyethylene glycol precipitation, and stored in liquid nitrogen in 10% sacrose in PBS. The 50% tissue culture-infective dose was determined posttitration in HEp-2 cells. Influenza virus strain A Nanchang/9335/95 (H3N2) was grown in fertilized chicken embryos. For heat inactivation of IgGs, stocks that were determined to be heat-inactivated by titration on Madin-Darby canine kidney cells (25).

HEp-2 and A549 cells were cultured in IMDM (21908-065, Life Technologies, Rockville, MD) supplemented with 2 mM l-glutamine, 25 mM HEPES buffer, 5% FCS, and 1% penicillin/streptomycin. RSV viral cultures and titration assays on HEp-2 cells were performed in IMDM containing 1% FCS and 1% penicillin/streptomycin. D1, a mouse dendritic cell (DC) line derived from C57BL/6 mice (26) used in Ag-viral cultures and titration assays on HEp-2 cells were performed in IMDM, 5% HyClone FCS (SH30080.03, Perbio, Lausanne, Switzerland), 1% penicillin/streptomycin, and 50 μM 2-ME supplemented with 30% conditioned medium from GM-CSF–producing R1 cells [mouse fibroblast NIH3T3, transfected with GM-CSF gene (26)].

**IFN-γ ELISPOT**

Human PBMCs were isolated by Ficoll-Paque (17-1440-02, Pharmacia Biotech, Piscataway, NJ) gradient centrifugation. A total of 2 × 10^6 total PBMCs or 1 × 10^6 purified APCs plus 1 × 10^6 enriched CD4+ or CD8+ T cell populations were infected with RSV (multiplicity of infection [ moi] 2), infected with influenza virus ( moi 0.5) or stimulated with the same virus with 10% FCS (HyClone, Breda, The Netherlands), 500 U/ml GM-CSF (a gift of Schering-Plough, Kenilworth, NJ), and 250 U/ml IL-4 (Strathmann, Carpinteria, CA) to analyze IFN-γ production by human PBMCs or purified CD4+ or CD8+ T cells. Multiscreen-IP filter plates (MS1PN4510, Millipore, Bedford, MA) were used in IFN-γ ELISPOT assay. An ELISPOT assay was performed as described before (27) to detect IFN-γ production by human PBMCs or purified CD4+ or CD8+ T cells. Multiscreen-IP filter plates (MS1PN4510, Millipore, Bedford, MA) were coated overnight with anti-IFN-γ–coating Ab 1-DIK (100 μl, 15 μg/ml 3002831, Mabtech, Nacka Strand, Sweden) in 0.1 M carbonate-bicarbonate buffer (pH 9.6) at 4°C. Before adding the plates, the cell were blocked for 1 h at 37°C with RPMI 1640 (52400-041, Life Technologies) containing 10% FCS. After blocking, PBMCs (2 × 10^6/well) were stimulated with virus preparations in 200 μl RPMI 1640 containing 5% FCS and penicillin/streptomycin for 24 h at 37°C in 5% CO2. Cells were removed, and 100 μl/well containing free RPA-7.6 (1-biotin (3420-6-1000, Mabtech) in PBS-0.5% FCS was added for 2 h. After 2 h, 100 μl 1:1000 dilution of ExtrAvidine alkaline phosphatase conjugate for an additional 5 min at 37°C with C1q (C1740, Sigma-Aldrich, St. Louis, MO; amounts given in the figure legends). The amount of C1q necessary to reconstitute complement in heat-inactivated serum varied. We used 75 μl/ml in experiments in which this is a variable physiological amount present in human serum. When indicated, IgGs were depleted using Protein G (P3296, Sigma-Aldrich).

**ELISA**

ELISA plates (Nunc, Roskilde, Denmark) were coated with denatured RSV lysate from RSV-infected HEp-2 cells in PBS for 18 h at 4°C. Postremoval of unbound RSV lysate, plates were blocked with 200 μl 1% BSA in 0.05% Tween-20/PBS for 1 h at 37°C. A 10% serum of healthy donors, 0.1% BSA, 0.05% Tween-20/PBS, was added to the wells for 15 min. The enzymatic activity was stopped by 9.8% H2SO4 and measured at OD 450.

Mouse ELISAs were performed with serum of either naive or RSV-infected mice diluted in 0.1% BSA/0.05% Tween-20/PBS. Serum batches from RSV-infected mice were prepared from blood derived 6 d after secondary RSV infection. Dilutions used in the assays were four-step serial dilutions starting at 1/2% or three-step serial dilutions starting at 3% as indicated in the figure legends. The secondary Abs were HRP-labeled Abs, anti-mouse IgG1 (64-6120, Invitrogen, Carlsbad, CA), or anti-mouse IgG2c (GG2c-9OP, Immunology Consultant Laboratory, Newburg, OR) diluted in 0.1% BSA/0.05% Tween-20/PBS and incubated for 2 h at RT.

**Human T cell expansion assay**

RSV-specific CD8+ T cells present in peripheral blood were expanded with or without prior CFSE labeling of PBMCs as described before (29). In short, PBMCs were washed twice with RPMI without FCS. A total of 2.5 μM CFSE was added to 1 × 10^6 cells/ml for 10 min at 37°C. Cells were washed twice with cold (4°C) PBS. Cells were stimulated with 1 × 10^5 depleted RSV-infected immature DCs incubated with RSV, RSV opsonized by 5 μg/ml corresponding peptide (NS1 41–49 or M2 64–72) as a control for 2% immune serum from RSV-infected mice or preimmune serum (room temperature [RT]) and washing (0.05% Tween-20/PBS). Serum batches from RSV-infected mice were prepared from blood derived 6 d after secondary RSV infection. Dilutions used in the assays were four-step serial dilutions starting at 1/2% or three-step serial dilutions starting at 3% as indicated in the figure legends. The secondary Abs were HRP-labeled Abs, anti-mouse IgG1 (64-6120, Invitrogen, Carlsbad, CA), or anti-mouse IgG2c (GG2c-9OP, Immunology Consultant Laboratory, Newburg, OR) diluted in 0.1% BSA/0.05% Tween-20/PBS and incubated for 2 h at RT.

**MACS**

To study CD4+ and CD8+ T cell activation in a human ELISPOT assay: 1) CD4+, 2) CD8+ T cells; and 3) APCs were negatively selected from total PBMCs using MACS. PBMCs were incubated with a mixture of FITC-labeled Abs at 4°C for 30 min in PBS with 0.5% BSA and 2 mM EDTA (MACS buffer): 1) anti-CD8 (clone SK1, BD Biosciences, San Jose, CA), anti-CD4 (clone 25723.11), 2) anti-CD2 (clone 12-2C12, BD Biosciences), anti-CD44 (clone M499, BD Biosciences), and anti-CD19 (clone 4G7, BD Biosciences); 2) anti-CD4 (clone SK3, BD Biosciences), 3) anti-CD56 (clone NCAM16.2, BD Biosciences), anti-CD54 (clone M20, BD Biosciences), and anti-CD14 (clone M2-ME, BD Biosciences), 4) anti-CD14 (clone M2-ME, BD Biosciences), and 5) anti-CD4 (clone SK3, BD Biosciences), and 6) anti-CD8 (clone RPA-T8, BD Biosciences) Abs. Before intracellular staining, cells were fixed and permeabilized with CytoFix/CytoPerm (B7651, Sigma-Aldrich). Intracellular cytokines were detected with anti–IFN-γ (554722, BD Biosciences), anti–IL-2 (554824, BD Biosciences), anti–IL-4 (554725, BD Biosciences), and anti–IL-12 (554722, BD Biosciences) Abs. Intracellular cytokines were detected with anti–IFN-γ (554722, BD Biosciences), anti–IL-2 (554824, BD Biosciences), and anti–IL-12 (554722, BD Biosciences) Abs. Before intracellular staining, cells were fixed and permeabilized with CytoFix/CytoPerm (B7651, Sigma-Aldrich). Intracellular cytokines were detected with anti–IFN-γ (554722, BD Biosciences), anti–IL-2 (554824, BD Biosciences), and anti–IL-12 (554722, BD Biosciences) Abs. Before intracellular staining, cells were fixed and permeabilized with CytoFix/CytoPerm (B7651, Sigma-Aldrich). Intracellular cytokines were detected with anti–IFN-γ (554722, BD Biosciences), anti–IL-2 (554824, BD Biosciences), and anti–IL-12 (554722, BD Biosciences) Abs.
serum from naive mice. In parallel, RSV was preincubated with 1 µg/ml anti-F [humanized IgG1 mAb, Syngis, MedImmune (8)] or 1 µg/ml anti-G (mouse IgG1, MAB858-2-5, Chemicon International, Temecula, CA). After 24 h, total RNA was extracted from these cells using MagnaPure LC equipment, cDNA was synthesized, and viral loads were determined by real-time PCR as recently described (30). In short, RNA extraction was performed using a MagnaPure LC total nucleic acid kit (Roche Diagnostic Systems, Somerville, NJ). Extracted RNA was reverse transcribed using a MultiScribe reverse transcriptase kit and random hexamers (Applied Biosystems, Foster City, CA) according to the manufacturer’s guidelines. RT inactivation was performed at 95°C for 5 min. Real-time PCR was performed with primers specific for the N gene of RSV: RSA-1: 5'-AG-ATCAACCTTCTGTCATCAGCA-3'; RSA-2: 5'-TTGTGCAACATATA-ATTAGGATGAT-3'; RSB-1: 5'-AAATGACAAATCAATGAACTTCA-CAGGA-3'; RSB-2: 5'-TGATATCCAGCATCTTTAAGTATCTTTATAG-TG-3'; RSA probe: 5'-ACCATCCTAACCAGGACAGCAGGATAT-3'; and RSB probe: 5'-TCTTCTCTTCAACTCCTGAGACTATATACCATACCT-3'. RT-PCR was performed with 20 µl cDNA, TaqMan universal PCR mastermix (Applied Biosystems), primers (RSA 900 nM each, RSB 300 nM each), and fluorogenic probes (58.3 and 66.7 nM for RSA and -B reporter dye FAM and the 3' terminator dye TAMRA. Amplification and detection were performed with a ABI Prism 7000 (Applied Biosystems). 

**Tissue sampling mice**

Mice were sacrificed by i.p. injection of 300 µl pentobarbital. Prior to removal, the lungs were perfused with PBS containing 100 µM heparin. Lungs were cut to 1 × 1 mm pieces and incubated with collagenase (2.4 mg/ml; 10103586001, Roche Applied Science, Burgess Hill, U.K.) and DNease (1 mg/ml; 10104159001, Roche Applied Science) for 20 min at 37°C. Single-cell suspensions were prepared by processing the tissue through 70-µm cell strainers (BD Falcon, BD Biosciences).

**Mouse bone marrow-derived DC cultures**

Bone marrow-derived DCs (BM-DCs) were prepared as described before (31). Bone marrow was depleted for erythrocytes using erythrocyte lysis mix (155 mM NH₄Cl, 10 mM KHCO₃, and 1 mM EDTA [pH 7.4]). BM-DCs were enriched for 7 d in RPMI 1640 (61870-044, Life Technologies) supplemented with 2 mM l-alanyl-glutamine, 5% HyClone FCS and 10% IL-4 (31). BM-DCs were enriched for 7 d in RPMI 1640 (61870-044, Life Technologies) supplemented with 2 mM l-alanyl-glutamine, 5% HyClone FCS and 10% IL-4. BM-DCs were enriched for 7 d in RPMI 1640 (61870-044, Life Technologies) supplemented with 2 mM l-alanyl-glutamine, 5% HyClone FCS and 10% IL-4. BM-DCs were enriched for 7 d in RPMI 1640 (61870-044, Life Technologies) supplemented with 2 mM l-alanyl-glutamine, 5% HyClone FCS and 10% IL-4.

**Mouse T cell activation assay**

In vitro mouse Ag-presentation assays were performed with D1 cells and in experiments to study the role of FcyRs and FcγRII with BM-DCs cultured from bone marrow derived from knockout mice and compared with C57BL/6 WT BM-DCs. To study Ag presentation of RSV immune complexes by BM-DC or D1 cells, RSV or UV-RSV at moi 10, 3, 1 or 0.1 were preincubated with either plasma derived from secondary RSV-infected mice or naive mice (preimmune serum) for 15 min at 37°C. When indicated, the IgG fraction was depleted from serum using protein G beads. In parallel, RSV or UV-RSV were preincubated with anti-F [humanized IgG1 mAb, Syngis, MedImmune (8)] or anti-G (mouse IgG1, MAB858-2-5, Chemicon International). RSV immune complexes were incubated with 5 × 10⁶ BM-DC/D1 cells per condition for 24 h. After 24 h, the APCs were incubated with 5 × 10⁶ total lung cells in the presence of 25 U/ml rhIL-2 (11147528001, Roche) and 10 µg/ml brefeldin A (B7651, Sigma-Aldrich) for 5 h at 37°C in 5% CO₂. Lung cells were obtained from C57BL/6 mice 8 d after primary RSV infection, at the peak of the T cell response (32). Ag presentation of RSV was analyzed by measuring IFN-γ production by lung CD4⁺ and CD8⁺ T cells by intracellular FACS staining. Cells were washed with FACS buffer and stained for surface markers with anti-CD8 (clone 53-6.7, BD Biosciences) and anti-CD4 (clone RM4-5, BD Biosciences). Before intracellular staining, cells were fixed and permeabilized with CytoFix/Cytoperm (554722, BD Biosciences) solution and Perm/Wash buffer (554723, BD Biosciences). Intracellular cytokines were detected with anti-IFN-γ (clone XMG1.2, BD Biosciences). Stained samples were acquired on an FACSCan flow cytometer (BD Biosciences), and the data were analyzed using FACS Diva software (BD Biosciences).

**In vivo RSV infection experiments**

Mice were lightly anesthetized with isoflurane and intranasally infected with 2 to 3 × 10⁶ PFU RSV in a volume of 50 µl at day 0. At day 28, mice were challenged with RSV. Six days postchallenge, T cell responses in the lung were analyzed. Single-cell suspensions of lung cells (10⁶) from RSV-infected WT, FcyRII-/- mouse were stimulated for 5 h at 37°C, 5% CO₂, with 2 × 10⁵ RSV-infected D1 cells or uninfected D1 cells in 200 µl IMDM (21908-065, Life Technologies) supplemented with 2 mM t-glutamine, 25 mM HEPEs buffer, 5% FCS, penicillin/streptomycin, 50 µg/ml 2-ME, and 25 U/ml rhIL-2 (11147528001, Roche). Brefeldin A 10 µg/ml (B7651, Sigma-Aldrich) was added for the duration of the stimulation to facilitate intracellular accumulation of cytokines. D1 cells were infected for 48 h with RSV moi 2 before addition to the lung cell suspension. IFN-γ production by CD4⁺ and CD8⁺ T cells was analyzed by intracellular cytokine staining.

**Statistical analysis**

Data were analyzed for statistical significance using a Student t test or ANOVA, as indicated in the figure legends. Data are expressed as the mean ± SEM. A p value <0.05 was taken as the level of significance.

**Results**

AS enhances in vitro-induced RSV-specific IFN-γ responses and suppresses influenza virus-specific IFN-γ production by PBMCs

To determine the effect of virus-specific Abs on T cell activation, we performed in vitro T cell stimulation assays with PBMCs derived from healthy adult donors. Antigenic stimulus was achieved by treatment of PBMCs with RSV alone or RSV treated with AS. We performed IFN-γ ELISPOT assays and compared the response against RSV with the response against influenza virus. RSV-specific IFN-γ responses increased in the presence of AS, whereas influenza virus-specific responses strongly decreased in the presence of AS (Fig. 1A). To confirm that Abs present in AS caused the altered IFN-γ responses, RSV and influenza virus were preincubated with IgG-depleted AS. Depletion of IgG significantly reduced the number of IFN-γ-producing cells specific for RSV. In contrast, the IFN-γ response against influenza virus, which was strongly suppressed in the presence of AS, increased after IgG depletion (Fig. 1A). To determine a possible role for complement during the Ag-presentation process, we first used heat-inactivated human serum to repeat IFN-γ ELISPOT assays. Fig. 1B (a representative experiment with one donor) and Fig. 1C (the mean of three individual donors) show that HI of human serum diminished the IFN-γ production induced by opsonized live and UV-inactivated RSV. Replenishing heat-inactivated serum with C1q, the initiator of the classical complement pathway, reconstituted the heat labile factor in serum. These experiments further showed that the increased T cell response in the presence of C1q depended on the presence of virus-specific serum Abs, because enhanced IFN-γ production was not observed when C1q was added to PBMCs stimulated with RSV in FCS (Fig. 1B) or in AS devoid of IgG (Fig. 1C). Fig. 1D shows that serum of all the adult donors contained RSV-specific (IgG) Abs, as measured by an RSV-specific ELISA using lysate of RSV-infected HEp-2 cells as a source of Ag. The polyclonal virus-specific Abs found in adult donors potentially recognize neutralizing and nonneutralizing viral epitopes. We established the neutralizing capacity of the RSV-specific
serum IgG Abs used in the ELISPOT experiments by testing the ability of the donor sera to block RSV infection of A549 lung epithelial cells and PBMCs. Using the same serum-to-virus ratio applied in the ELISPOT experiments, we observed a complete suppression of viral replication in A549 cells, as determined by real-time PCR (Fig. 1E). This neutralizing capacity was most likely a primary result of serum IgG. HI of AS had no effect on the RSV Ag recognition or neutralizing capacity of serum Abs (Fig. 1D, E). We further determined that replication was completely abrogated by UV treatment of the virus. In comparison with A549 cells, PBMCs were less permissive to viral infection (Fig. 1E), which could be explained in part by the fact that a smaller fraction of cells was infected. Specifically, confocal microscopy experiments (data not shown) revealed that only HLA class II-positive, mostly CD14+ cells such as monocytes and possibly DCs (10–15% of PBMCs), were infected by RSV. In contrast to A549 cells, we detected low levels of RSV in PBMC samples exposed to serum-treated RSV, which might represent viral material internalized by APCs rather than viral infection. We found that IFN-γ responses were similar poststimulation with live and UV-inactivated RSV in ELISPOT assays performed in FCS and in AS (Supplemental Fig. 1 and data not shown).

**AS enhances the IFN-γ response of RSV-specific CD4+ T cells and decreases influenza virus and RSV-specific CD8+ T cell activation**

Presentation of virus-derived antigenic peptides to CD8+ T cells occurs after productive infection, whereby peptides derived from viral proteins synthesized in the cytoplasm are processed by proteasomes and translocated into the endoplasmic reticulum. Within the endoplasmic reticulum, viral peptides are loaded onto nascent HLA class I molecules. In contrast, the default route for HLA class II peptide presentation to CD4+ T cells is internalization of antigenic material, followed by degradation in late endosomal compartments or lysosomes. A possible explanation for our contrasting observations with the RSV and influenza virus-specific IFN-γ response in the presence of serum IgG could be the relative fraction of CD4+ T cells and CD8+ T cells in the antiviral memory pool. Thus, we hypothesized that neutralizing Abs in serum decreased influenza-specific CD8+ T cell responses.
through inhibition of viral infection and HLA class I-mediated Ag presentation, whereas the RSV-specific CD4+ T cell response was enhanced by Ab-mediated endosomal uptake and HLA class II Ag presentation.

To test this hypothesis, we performed ELISPOT experiments using CD4+ or CD8+ T cells purified from PBMCs. The T cell subsets were stimulated with the monocyte/DC fraction of PBMCs from the same donor. The monocyte/DC fraction was exposed to live RSV, live influenza virus, or UV-inactivated RSV in the presence of AS or FCS. This experiment showed that the influenza virus-specific IFN-γ production by CD8+ T cells was indeed strongly suppressed postexposure to serum treated and supposedly Ig-opsonized virus (Fig. 2A). CD8+ T cell responses specific for RSV were absent or barely detectable by ELISPOT. For RSV, the IFN-γ response was dominated by CD4+ T cells, and AS caused an increase in the RSV-specific IFN-γ production by these cells (Fig. 2A).

In earlier work, we and others have shown that RSV-specific memory CD8+ T cells are present in adult PBMCs, although responses against single epitopes were low and, in most donors,
not detectable without in vitro expansion (36–42). However, the virus-specific T cell response might be directed against a broad panel of HLA-presented peptides derived from the viral proteome (36, 39). In experiments presented in this study (Fig. 2A and data not shown), it also appeared that total RSV-specific CD8+ T cells were low or undetectable. To exclude the possibility that technical reasons resulted in underestimated CD8+ memory T cell numbers (i.e., that HLA class I presentation was somehow inefficient after RSV infection of the APCs), we first expanded CD8+ T cells specific for single viral epitopes. The ability of these T cell lines to respond to APCs loaded with the same synthetic peptide used for expansion or RSV-infected APCs was then evaluated. Fig. 2B shows an example of in vitro T cell expansion poststimulation of PBMCs with peptide NS1 41–49 derived from the nonstructural protein NS1, a peptide that is presented in the context of HLA-B51 (36). More than eight cell divisions were required to detect substantial numbers of IFN-γ–producing CD8+ T cells in PBMCs. Similar observations were made for epitopes derived from structural proteins (M, Np, M2, data not shown). Expansion of NS1 41–49–specific CD8+ T cells performed with live RSV was less efficient than expansion with the synthetic peptide. This finding illustrates the well-documented inhibitory effect of RSV infection on T cell proliferation (28, 36, 43–46). Using expanded virus-specific CD8+ T cell populations, we found similar IFN-γ responses induced by HLA class I molecules on both virus-infected APCs and peptide-loaded APCs. Both the APC fraction in PBMCs (Fig. 2C, 2D) and monocyte-derived DCs (Fig. 2E) presented viral peptide to expanded CD8+ T cells. This indicated that virus infection did not suppress HLA class I-mediated Ag presentation. Moreover, in contrast to the suppression of T cell proliferation, we found no indication for a suppressive effect of infectious virus on the production of IFN-γ by T cells.

Therefore, we concluded that results from IFN-γ ELISPOT assays showing low IFN-γ production from the RSV-specific CD8+ T cell fraction could not be attributed to a lack of Ag presentation, but instead reflected a low frequency of RSV-specific CD8+ memory T cells. Furthermore, RSV infection was required for effective HLA class I–mediated Ag presentation in the in vitro ELISPOT assay because the IFN-γ response of CD8+ T cells was absent when APCs were stimulated with replication-defective UV-treated RSV or RSV in the presence of neutralizing serum Abs (Fig. 2C–E).

Activating FcγRs are involved during Ag presentation to RSV-specific mouse CD4+ T cells in vitro and in vivo

To determine whether IgG plays a significant role during T cell activation in vivo, we initiated a series of experiments in mice. We first used an in vitro T cell stimulation assay to test preimmune serum and serum from mice obtained after secondary RSV infection. In these experiments, we used a mouse DC line, D1, as the APCs. As a source of polyclonal RSV-specific CD4+ and CD8+ T cells, lung cells were harvested at day 8 after a primary RSV infection administered by intranasal inoculation. T cell activation was measured by intracellular staining for IFN-γ. Similar to the results observed with human serum, we found that mouse immune serum, in comparison with preimmune serum, enhanced RSV-specific CD4+ T cell responses (Fig. 3A). IgG depletion experiments confirmed the role of IgG.

To determine a possible role for FcγR in Ag uptake during virus-specific Ag presentation, we proceeded with in vitro Ag presentation studies using BM-DCs from WT and γ−/− mice lacking expression of activating FcγRI, -III, and -IV (Supplemental Fig. 2) (23). As shown in Fig. 3B, immune serum caused enhanced presentation of RSV Ag to virus-specific CD4+ T cells when activating FcγRs were expressed. In contrast, using DCs cultured from γ−/− bone marrow, CD4+ T cell responses were unaltered when virus Ag was presented in the presence of preimmune serum. UV-inactivated RSV was used to completely rule out infection-related effects in these experiments. Similar experiments performed with DCs derived from mice that lack expression of FcγRIIb, the only inhibitory FcγR in mice, did not show differences in Ag presentation in the presence of immune serum compared with WT BM-DCs (Fig. 3C). From these experiments, we concluded that activating FcγR played a role during Ag presentation when RSV-specific Abs were present.

To test the relevance of these observations during in vivo T cell responses, we performed RSV infection studies in C57BL/6 WT, γ−/−, and FcγRIIb−/− mice. T cell responses in the lungs of these mice were measured after ex vivo restimulation with RSV-infected and -uninfected D1 cells. After secondary RSV infections, similar RSV-specific Ab responses (IgG1 and IgG2c) were elicited in WT
and γ−/− mice (Fig. 4A). RSV-specific CD4+ T cell responses were significantly higher in WT compared with γ−/− mice (Fig. 4B), which was consistent with the enhanced Ag presentation observed during in vitro assays in the presence of immune serum. No differences were found in the level of the RSV-specific CD4+ T cell response in FcγRIIb−/− mice compared with WT mice (Fig. 4B), whereas IgG2c Ab levels were slightly higher in the mice lacking the inhibiting FcγRIIb (Fig. 4A). During primary infection, no differences in immune responses were observed in WT, γ−/−, or FcγRIIb−/− mice (data not shown).

**The ratio of neutralizing and nonneutralizing Abs in serum determines the activation of the CD8+ T cell response**

In different in vivo experiments, we observed a variable effect of virus-specific Abs on CD8+ T cell responses. These responses were sometimes equal and sometimes slightly lower in γ−/− mice compared with WT mice (two examples shown in Supplemental Fig. 3). It is possible that RSV-specific Abs might affect RSV-specific CD8+ T cell responses in vivo via different mechanisms such as virus neutralization (resulting in lower antigenic load), altered innate immune responses, or Ab-mediated cross presentation of viral Ag. The resulting in vivo T cell response might therefore be determined by the sum of different effects of Abs that work synergistically or antagonistically. We tested different serum batches from immune mice in the in vitro Ag presentation assay and found that some sera inhibited in vitro CD8+ T cell responses whereas others did not. An example is shown in Fig. 5A, depicting immune sera A and B obtained from mice after secondary response, whereas serum B did not change the CD8+ T cell response. The lower CD8+ T cell response found when RSV was opsonized with serum A was caused by the IgG fraction of serum (Fig. 5A). We reasoned that serum A might be a better neutralizing serum. Indeed, a virus neutralization assay confirmed that the neutralizing capacity of serum A was at least 4-fold stronger than the neutralizing capacity of serum B (Fig. 5B). Based on an RSV-specific ELISA, the virus-specific Ab levels were similar in both serum samples (Fig. 5C).

To confirm the different effects of neutralizing and nonneutralizing Abs on the activation of CD4+ and CD8+ T cells and to exclude a potential contribution of other serum components, we repeated in vitro T cell stimulation assays using two mAbs. We used nonneutralizing murine IgG1, specific for the G protein of RSV, and palivizumab, a strongly neutralizing humanized IgG1 Ab specific for the F protein (Fig. 6A). Despite their distinct origin, both Abs show similar binding affinities to FcRs. Again, both mAbs increased CD4+ T cell responses to a similar extent, whereas only opsonization with neutralizing palivizumab lowered the CD8+ T cell response (Fig. 6B). This result supports the conclusion that different ratios of neutralizing and nonneutralizing RSV-specific serum Abs might influence the relative ratio of RSV-specific CD4+ and CD8+ T cell responses.

**Discussion**

In the present work, we have shown that virus-specific Abs play a crucial role in the regulation of RSV-specific T cell responses. We base this conclusion on in vitro stimulation assays with human PBMCs and murine lung-derived effector T cells as well as experiments with an in vivo mouse model. Polyclonal virus-specific Abs generated in vivo are a complex mixture of neutralizing and nonneutralizing Abs. We showed that the ratio of neutralizing and nonneutralizing RSV-specific Abs in serum may affect the efficacy of Ag presentation to virus-specific CD4+ and CD8+ T cells. Both neutralizing and nonneutralizing Abs enhanced Ag presentation to RSV-specific CD4+ T cells, whereas neutralizing Abs lowered RSV- and influenza virus-specific CD8+ T cell responses. Therefore, the presence of virus-specific maternal Abs in neonates before natural RSV infection, or induced by a RSV vaccination, was calculated using a Student t test. These experiments were performed three times with similar results. *p < 0.05; **p < 0.001.

**FIGURE 4.** Activating FcγRs are involved during in vivo Ag presentation of RSV-derived Ag. WT, γ−/−, or FcγRIIb−/− mice were infected with RSV at day 0 and challenged with RSV at day 28. Six days postchallenge, blood was collected to analyze serum Abs, and lungs were analyzed for T cell responses. A. RSV-specific IgG1 and IgG2c levels in serum (three-step serial dilution of serum starting at 3%). B. Percentage of IFN-γ–producing CD4+ T cells in lung tissue responding to RSV-infected or uninfected D1 cells. Error bars represent the SEM of five individual mice per group. Results are shown for five mice per group of one representative experiment. In A, significance was calculated using two-way ANOVA. In B, significance was calculated using a Student t test. These experiments were performed three times with similar results. *p < 0.05; **p < 0.001.
might affect the activation of virus-specific T cells in vivo. In a vaccine trial with a formalin-inactivated RSV vaccine, poorly neutralizing RSV-specific Abs were induced (47–49), illustrating that during intervention with vaccines, Ab-mediated effects on T cell activation might differ from B and T cell responses during natural infection. Moreover, maternal Abs present during primary infections, or Abs delivered as a prophylactic, might affect the induction of the T cell response during natural infection.

From the present work, it appears that RSV-specific memory CD8+ T cell numbers are low when compared with RSV-specific CD4+ T cell numbers and influenza-specific memory CD8+ T cell numbers. Extensive studies on the RSV specific T cell immune status in healthy individuals are sparse. However, one elegant study by Lee et al. (12) compared CD4+ and CD8+ T cell responses specific for RSV and influenza virus in a group of healthy young adults and elderly people. Similar to our observations, this group found significantly higher ratios for IFN-γ-producing CD4+/CD8+ T cells specific for RSV than for influenza virus in both young and elderly groups. In contrast, during severe primary RSV infections, CD8+ T cell responses dominate during the acute response (50). Because the immune status during the first year after primary infection and before a secondary RSV exposure has not been monitored, it is currently not clear whether a shift in the CD4+/CD8+ memory T cell ratio results from an ineffective differentiation or poor survival of RSV-specific CD8+ memory T cells. An alternative explanation could be that the switch in the CD4+/CD8+ memory T cell ratio occurs during multiple exposures to RSV in older children and adults. Our studies suggest that neutralizing RSV-specific Abs could potentially play a role in such a process. Because CD8+ T cells are likely important for RSV clearance, diminished CD8+ T memory cell numbers could be a reason why RSV-specific immune memory wanes and individuals become prone to reinfection.

In our studies, in vitro MHC class I-mediated Ag presentation presumably occurred via the classical proteasome-dependent pathway of Ag presentation that required viral infection of APCs
(Fig. 2C, 2D). It is unclear how these in vitro observations might translate to the in vivo situation in humans. The route of viral entry and access of virus-derived antigenic material to different subsets of DCs in vivo might differ from in vitro experiments performed with PBMC. Cross presentation, the process by which acquired exogenous Ag gains access to the class I Ag-processing pathway of DCs (34), did not play a significant role in the in vitro experiments (Figs. 2, 5). However, this process might contribute in vivo via uptake of infected epithelial cell debris or immune complexes by DC subsets specialized in cross presentation. In fact, our previous work in a murine intranasal RSV infection model showed that both RSV-infected lung-derived DCs and uninfected lymph node resident DCs presented Ag in the context of both MHC class I and class II molecules (51). Thus, uninfected lymph node resident DCs cross-presented virus Ag via MHC class I molecules and could therefore contribute in vivo to Ag presentation to CD8+ T cells.

DCs specialized in class I cross presentation mainly reside in tissue draining lymph nodes and spleen. Recently, human CD141+ DCs in peripheral blood, and closely related cells in lymph nodes, were found to be related to murine CD8+ splenic DCs, a DC type specialized in cross presentation (52–55). However, the CD141+ DC type in peripheral blood is present in extremely low numbers (0.03–0.05% of PBMCs) and might not play a significant role during the in vitro Ag presentation assays performed with human PBMCs.

The contribution of cross presentation might be important for effective activation and restimulation of RSV-specific CD8+ T cell responses in vivo. We and others have shown that RSV infection of APCs results in impaired induction of T cell proliferation (28, 49, 50), (Fig. 2B). Therefore, it can be envisaged that for an effective in vivo expansion of RSV-specific CD4+ and CD8+ T cells, indirect routes of Ag presentation are most effective for efficient induction of T cell responses whereby DC acquire noninfectious material like opsonized virus particles or necrotic infected cells. Cross presentation is facilitated by stimulation of innate immune receptors expressed by DCs (56–60). The efficacy of the cross presentation route via MHC class I molecules and the relative contribution of MHC class II presentation for in vivo RSV-specific T cell activation might depend on several factors. These include the local inflammatory milieu induced by RSV infection, the access route of viral material, and the innate immune process triggered by, for instance, Ab-opsonized virus or infected necrotic cells. Insight into these mechanisms is important to develop effective and safe intervention procedures for RSV disease.

Our experiments showed that FcγR and/or complement contributed to more effective RSV-specific CD4+ T cell responses when virus was opsonized with Abs. These results indicated that the enhanced CD4+ T cell response was caused by enhanced uptake and processing of viral antigenic material. Alternatively, C1q can induce cytokine production when added to APCs in the absence of immune complexes (61, 62). However, our experiments (Fig. 1B, 1C) showed that C1q did not contribute significantly to enhanced responses when IgGs were not present.

Most published work on CD8+ T cell memory status in adults or effector CD8+ T cell responses during primary disease in infants has been performed with expanded T cell populations, either using RSV-infected APCs or a specific method for T cell expansion (37–39, 41, 42). These methods confirm the presence of RSV CD8+ T cells in individuals previously exposed to RSV, but are inadequate to measure the exact in vivo frequencies of these cells. IFN-γ ELISPOT assays performed on total PBMCs do not distinguish between the CD4+ and CD8+ T cells contributing to the response. Due to the low frequency of RSV-specific T cells in peripheral blood, intracellular cytokine staining methods are difficult to perform. Moreover, for effective expression of MHC class I-presented viral epitopes representing the total virus proteome, RSV infection of autologous APCs needs to be initiated at least 24 h before exposure to T cells. This makes such a procedure cumbersome for large studies.

Importantly, the current study underscores the crucial need to carefully develop in vitro T cell assays useful for monitoring RSV-specific T cell responses. Such assays could be employed for the evaluation of the efficacy of future RSV vaccine candidates. Furthermore, understanding the reason for frequent reinfections with RSV and the susceptibility of elderly people to severe morbidity caused by RSV infections requires careful analysis of the RSV-specific immune status of different age groups. The dynamics of the antiviral adaptive immune responses during, and in between, RSV seasons should also be carefully considered. The observation that RSV-specific CD8+ T cells might be present in low numbers in the peripheral memory pool and the role that virus-specific Abs could play during in vivo T cell activation are important issues that need to be understood in detail when considering the development of safe and effective vaccines and evaluating correlates of protection against severe RSV-induced respiratory infections.

Disclosures
The authors have no financial conflicts of interest.

References


