Letter to the Editor

Human newborn B cells mount an interferon-α/β receptor-dependent humoral response to respiratory syncytial virus

To the Editor:

Newborns express distinct immunity from adults, resulting in age-specific susceptibility to infection.[1] Defining the ontogeny of host defense to pathogens provides insight into age-specific susceptibility and may inform translation of new biomarkers and therapeutic modalities. During viral respiratory tract infections, B cells play an important role in the antiviral immune response and contribute to the development of adaptive immunity.[2] Respiratory syncytial virus (RSV) infection induces local pulmonary B-cell activation and virus-specific antibodies, including IgM that may mitigate disease severity.[3] Maternal IgG antibodies may provide protection against RSV infection in newborns and infants.[4] In addition, innate IgM-based B-cell mechanisms may function in early life, though it is unclear whether they contribute to anti-RSV immunity.

To assess whether human newborns might mount an innate humoral response against RSV, we used newborn cord blood mononuclear cells (CBMCs) and adult PBMCs. Furthermore, we investigated the presence of RSV-IgM in newborn plasma and its ability to inhibit RSV infection of epithelial cells. Methodologic details are found in the Methods section in this article's Online Repository at www.jacionline.org.

CD69 or CD86 were used as B-cell activation markers (see Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org). CBMCs and PBMCs demonstrated similar RSV-induced CD69 expression (Fig 1, *A*). Because RSV activates Toll-like receptor 4 (TLR4) signaling and production of type I IFNs that could contribute to B-cell activation, we compared RSV-induced activation of mononuclear cells (MCs) to that induced by LPS (TLR4 agonist), cytosine-phosphate-guanine dideoxynucleotide (type I IFN-inducing TLR9 agonist), or live influenza

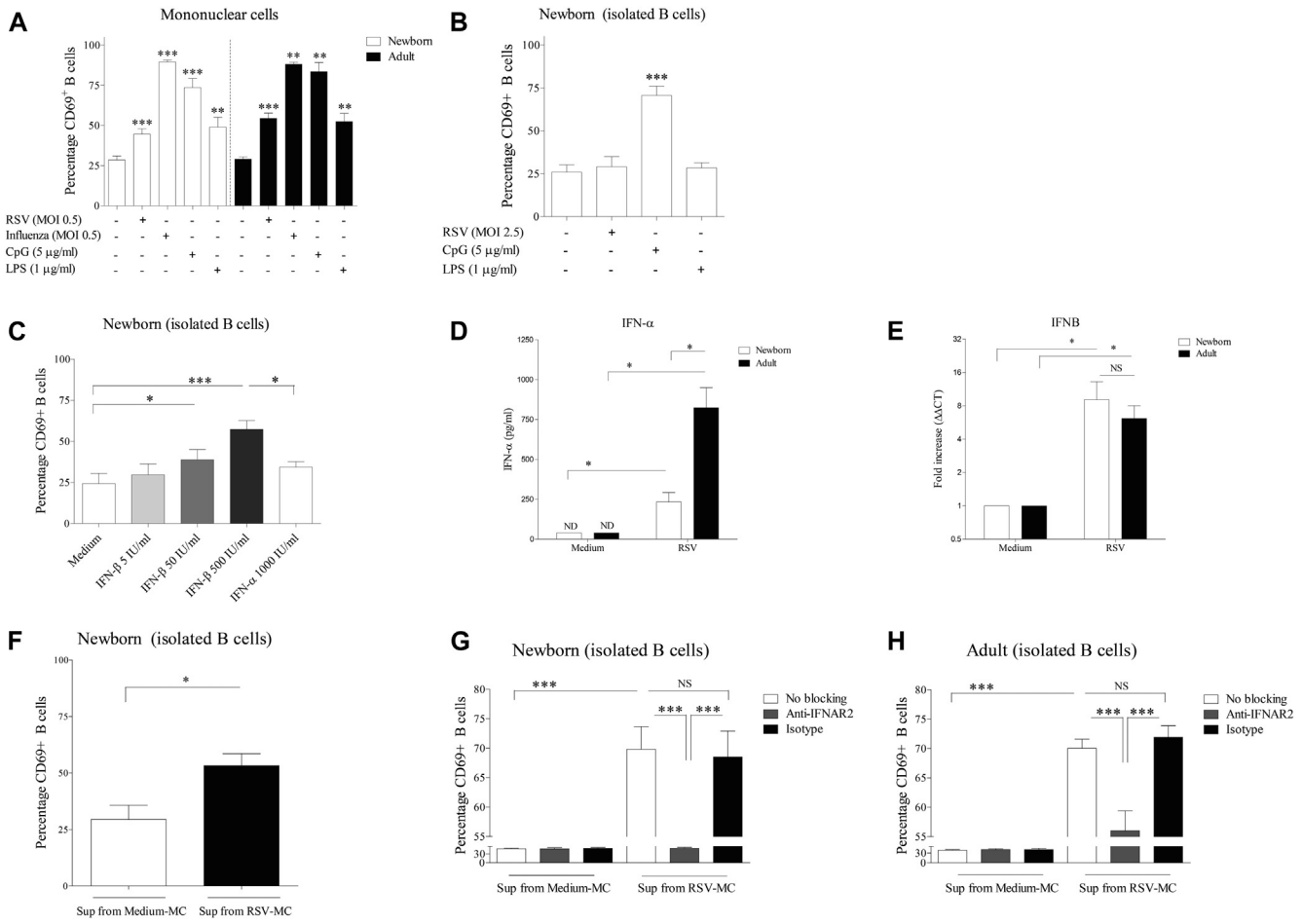

FIG 1. A-H, RSV induces IFNAR-dependent activation of newborn B cells. $CD69^+$ B cells after 18-hour stimulation of (Fig 1, *A*) MCs or (Fig 1, *B-C*) newborn B cells (N = 5-11). Induction of (Fig 1, *D*) IFN-α and (Fig 1, *E*) IFN-β in MCs. $CD69^+$ B cells after 18-hour stimulation of (Fig 1, *F* and *G*) newborn B cells or (Fig 1, *H*) adult B cells in the absence or presence of anti–IFNAR-blocking antibodies or isotype controls (N = 3-5) (mean ± SEM). *INFAR*, Interferon-α/β receptor; *NS*, not significant. $*P < .05$. $**P < .01$. $***P < .001$.

virus (GFP-PR8/34; H1N1 strain, known to activate TLR7/8). Each of these stimuli induced newborn and adult B-cell activation (Fig 1, *A*). To investigate direct B-cell activation without potential interactions with other types of MCs, experiments were conducted on isolated newborn B cells. Neither RSV nor LPS induced B-cell activation as measured by the expression of CD69 and CD86. Cytosine-phosphate-guanine dideoxynucleotide and IFN-β directly activated newborn B cells, whereas IFN-α was less potent (Fig 1, *B* and *C*, and Fig E1, *C*). Similar results regarding IFN-α and IFN-β were obtained with adult B cells (see Fig E1, *D*). These data suggest that RSV-induced newborn B-cell activation may result from IFN-β production and is not dependent on direct interactions between RSV and TLR4 on B cells. The activation of B cells upon exposure to RSV and influenza virus indicates that a general antiviral immune response is present in newborns. Simultaneous exposure of B cells to LPS and IFN-β did not result in increased B-cell activation compared with IFN-β alone (data not shown). However, the observed IFN-β–mediated B-cell activation in newborns suggests that IFN-β may potentiate B-cell activation during infectious diseases.

To further characterize RSV-induced B-cell activation, we measured induction of IFN-α and IFN-β. Supernatants from RSV-stimulated CBMCs and PBMCs contained IFN-α and IFN-β (Fig 1, *D* and *E*) and activated newborn and adult B cells that express interferon-α/β receptor 1 and interferon-α/β receptor 2 (Fig 1, *F*, and see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org) Moreover, blocking interferon-α/β receptor on isolated newborn and adult B cells prevented the induction of $CD69^+$ B cells by IFN-β (see Fig E2, *C* and *D*) and by supernatants from RSV-stimulated MCs (Fig 1, *G* and *H*).

We next assessed whether RSV functionally activates B cells by measuring total-IgM, RSV-IgM, and total-IgG antibody-secreting cells (ASCs) (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). RSV-activated newborn and adult MCs demonstrated increased total-IgM and RSV-IgM ASCs (Fig 2, *A* and *B*). Beta-propiolactone–inactivated RSV also increased the amount of total-IgM ASCs, suggesting that induction of IgM ASCs occurs independently of viral replication (Fig 2, *A*). RSV induced an increase in total-IgG ASCs in adult MCs. In contrast, RSV-stimulated newborn MCs demonstrated low numbers of total-IgG ASCs with no increase in total-IgG ASCs (see Fig E3, *B*). Consistent with RSV-IgM ASCs in the newborn MC fraction, detectable levels of RSV-IgM were present in newborn plasma with even higher concentrations in infant and adult plasma (Fig 2, *C*). Finally, we measured the neutralizing capacity of purified newborn IgM from pooled plasma (IgM purity confirmed by ELISA; data not shown) on RSV infection of A549 cells, a bronchial epithelial cell line used to investigate RSV neutralization.[5] IgG concentrations in purified IgM were undetectable (<4,000,000-fold diluted standard). Newborn IgM reduced RSV infection of A549 cells (Fig 2, *D* and *E*). Newborn plasma, also containing maternal IgG, completely neutralized RSV infection (Fig 2, *D* and *E*). Purified IgM from 4 newborn samples each reduced RSV infection of A549 cells (see Fig E3, *C*).

Overall, we demonstrate for the first time that (1) RSV and IFN-β induce surface upregulation of CD69 on newborn and adult B cells to a similar extent in a model of MCs, (2) RSV-induced IFN-β derived from mixed MCs directly activates newborn and adult B cells via interferon-α/β receptor signaling, and (3) RSV increases the amount of newborn and adult total-IgM and RSV-IgM ASCs. Finally, we demonstrate the neutralizing capacity of human newborn plasma IgM on RSV infection of human epithelial cells (Fig 2, *F*).

Because IgM does not cross the placental barrier, RSV-IgM ASCs and plasma RSV-IgM titers may reflect newborn innate B cells and natural antibodies.[6] Natural antibodies include polyreactive pathogen-binding IgM antibodies that are present without previous exposure and are part of the innate immune system, opsonize pathogens, and promote phagocytosis.[7] RSV envelope proteins include fusion protein (F protein) and attachment protein (G protein), both glycosylated and potentially targeted by natural IgM antibodies.

Our study is novel but also has limitations, including the uncertain applicability of our *in vitro* model to the clinical setting. Future studies should investigate additional factors including immune cells and immunoglobulin transport, potentially by using a murine model of RSV infection. Clinically, RSV infections have a broad range of disease and only approximately 1% of those with RSV infections develop symptoms resulting in hospitalization.[8] These epidemiologic observations indicate that the early life immune system does not prevent RSV infection but, in most cases, does protect against severe disease. Our data suggest that innate IgM-based B-cell mechanisms may contribute to early life protection against RSV. Of note, IgM-enriched fractions have demonstrated potential efficacy to prevent or treat septic shock,[9] suggesting potential IgM-based translational opportunities.

In conclusion, human newborns mount robust RSV-induced and IFN-β–mediated B-cell activation and IgM production, indicating that this humoral mechanism is present at birth. Although detection of neutralizing RSV-IgM in human newborns suggests the presence of natural anti-RSV IgM antibody at birth, the higher levels of RSV-IgM in infants indicate that the RSV/IFN-induced pathways we have described may be relevant *in vivo*, providing a first line of defense during RSV infections in early life. Future studies should examine whether the relative presence of RSV-IgM could predict the frequency and severity of RSV infection and assess the potential utility of anti-RSV IgM as a prophylactic or therapeutic agent.

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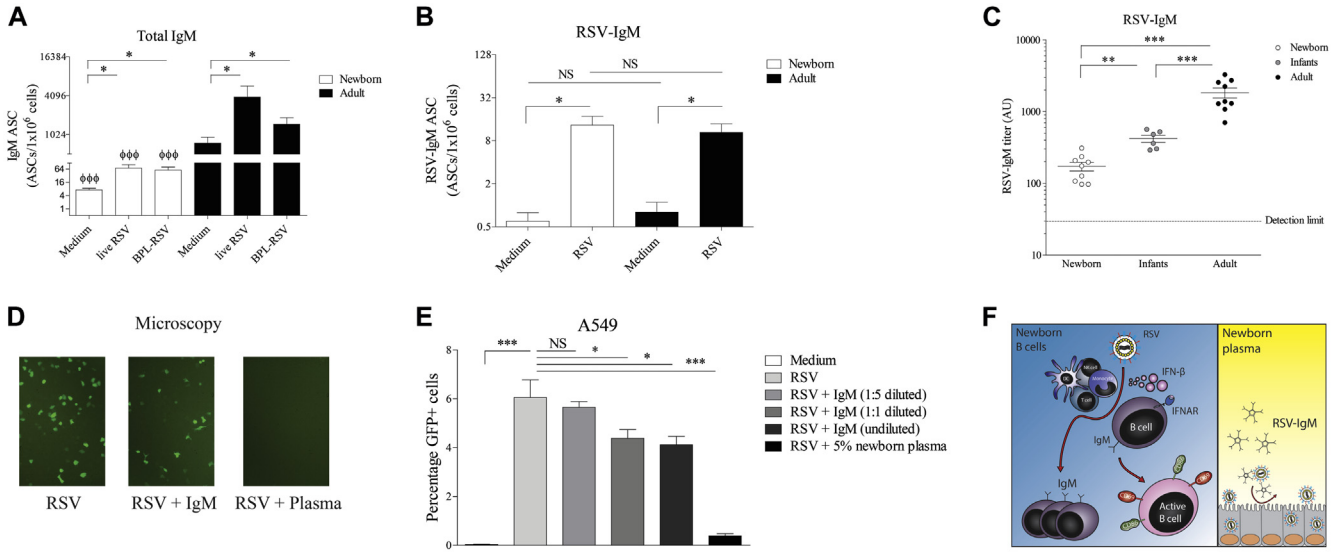

FIG 2. A-F, RSV-IgM ASCs in newborns and functional RSV-IgM in neonatal plasma that reduces RSV infection. (Fig 2, *A*) Total-IgM ASC or (Fig 2, *B*) RSV-IgM ASCs determined by Enzyme-linked Immunospot. N = 5-8. Fig 2, *C*, RSV-IgM plasma concentrations. N = 6-9. (Fig 2, *D*) Microscope images and (Fig 2, *E*) quantification of fluorescent A549 cells after exposure to RSV in medium, purified newborn IgM (RSV + IgM), or newborn plasma (RSV + plasma). N = 3. (mean ± SEM). Fig 2, *F*, Graphical summary of the IFNAR-dependent humoral response to RSV in newborns. *INFAR*, Interferon-α/β receptor; *NS*, not significant. **P* < .05. ***P* < .01. ****P* < .001. ϕϕϕNewborn vs adult: *P* < .001.

cases involving pediatric infection; has recently submitted an NIH (NIAID) grant relating to the Human Immunology Project Consortium; has a patent through Boston Children's Hospital for the use of the antimicrobial protein Bactericidal Permeability Increasing protein as a radiation mitigation agent; and has received travel support from the International Neonatal and Maternal Immunization Symposium. G. Ferwerda has received research support from the Dutch Government (project FES0908) and the Netherland Genomics Initiative Project (050-060-452). The rest of the authors declare that they have no relevant conflicts of interest.

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METHODS

Cell isolation and stimulation

Human newborn cord blood was collected from the umbilical cord after Cesarean section. Peripheral blood was collected from healthy adult volunteers. After centrifugation at 800*g*, plasma was collected from cord blood, healthy infants (between age 2 and 4 months), or healthy adults. MCs were collected using Ficoll-Hypaque gradient and B cells with CD19 microbeads (Miltenyi Biotec, Leiden, The Netherlands; purity >93%). When indicated, cells were incubated with anti-interferon-α/β receptor-blocking antibodies (MMHAR-2, PBL Interferon Source, 5 μg/mL) or isotype controls. MCs (5×10^5/well) or isolated B cells (1×10^5/well) were stimulated for 18 hours for flow cytometry or 5 days for the Enzyme-Linked Immunospot (ELISpot) assay. Recombinant green fluorescent protein–expressing RSV A2 (Rhinella-green RSV30), kindly provided by Dr M. E. Peeples, was cultured as previously described[E1] and used at an multiplicity of infection of 0.5 unless otherwise indicated. When indicated, Rhinella-green RSV was inactivated using β-propriolactone.

Cytokine induction

IFN-α concentrations were measured by ELISA (R&D Systems, Minneapolis, Minn) with a lower limit of detection of 39 pg/mL. Induction of IFN-β was determined by RT-qPCR using TaqMan Assays for beta-2-microglobulin (Hs00187842_m1) and IFNB (Hs0621180_s1) (ThermoFisher Scientific, Waltham, Mass). IFNB gene expression was normalized to the housekeeping gene beta-2-microglobulin. ΔΔCT was calculated by subtracting normalized gene expression in unstimulated cells from RSV-stimulated cells.

Total IgM and RSV-IgM B-cell ELISpot

ASCs were determined using ELISpot assay (R&D Systems). Membrane plates (Millipore, Amsterdam, The Netherlands) were coated with goat anti-human IgM or β-propriolactone-RSV (4×10^5/well) in PBS overnight at 4°C and blocked with PBS/1% BSA/5% sucrose. MCs were added in triplicate at 2×10^4 to 1×10^6 cells/well for 18 hours and ASCs were determined according to the manufacturer's protocol (R&D Systems). Spots were calculated as the number of ASCs/1×10^6 plated cells.

RSV-IgM ELISA

RSV-specific IgM concentrations in plasma samples were determined by coating ELISA plates with Rhinella-green RSV (2×10^4/well) in PBS. Plasma samples were added and subsequently incubated with AP-conjugated anti-human IgM in PBS/1% BSA for 2 hours (1:500; Southern Biotech, Birmingham, Ala). Detection of AP activity used 10 mM diethanolamine with 0.5 mM $MgCl_2$. The ODs of PBS-coated wells were subtracted from those of RSV-coated parallel samples. Serial dilutions of healthy adult pooled plasma ranging from 1:10 to 1:10,240 (vol/vol) were used as standard to calculate arbitrary units. The detection limit for RSV-IgM was 32 arbitrary units.

Neutralization assay

Human bronchial epithelial A549 cells (ATCC CCL-185) were seeded in 96-well plates at 2×10^4 cells/well and incubated in Dulbecco modified Eagle medium + 10% FCS for 24 hours at 37°C and 5% CO_2 to adhere. Anti-human IgM (μ-chain specific)-Agarose beads (Sigma, St Louis, Mo) were used to purify IgM from newborn plasma. Cells were incubated with RSV (multiplicity of infection 5). When indicated, purified IgM or 5% newborn plasma was added. After 20 hours, cells were detached with trypsin and infection quantified by flow cytometry as green fluorescent protein$^+$ cells.

Statistics

Statistical analyses used the Wilcoxon matched-pairs signed rank test for paired analysis between 2 conditions and repeated-measures ANOVA or 1-way analysis of variance with Bonferroni multiple comparison test for, respectively, paired and unpaired analysis between more than 2 conditions. Comparative testing between newborns and adults was performed with Mann-Whitney *U* test. Statistical analysis was performed with GraphPad Prism version 6 software (La Jolla, Calif).

Study approval

Guidelines of the US Department of Health and Human Services, the Brigham & Women Hospital, Beth Israel Hospital, Boston Children's Hospital, and the Regional Committee on Research involving Human Subjects Arnhem-Nijmegen were observed, following protocols approved by the local institutional review boards.

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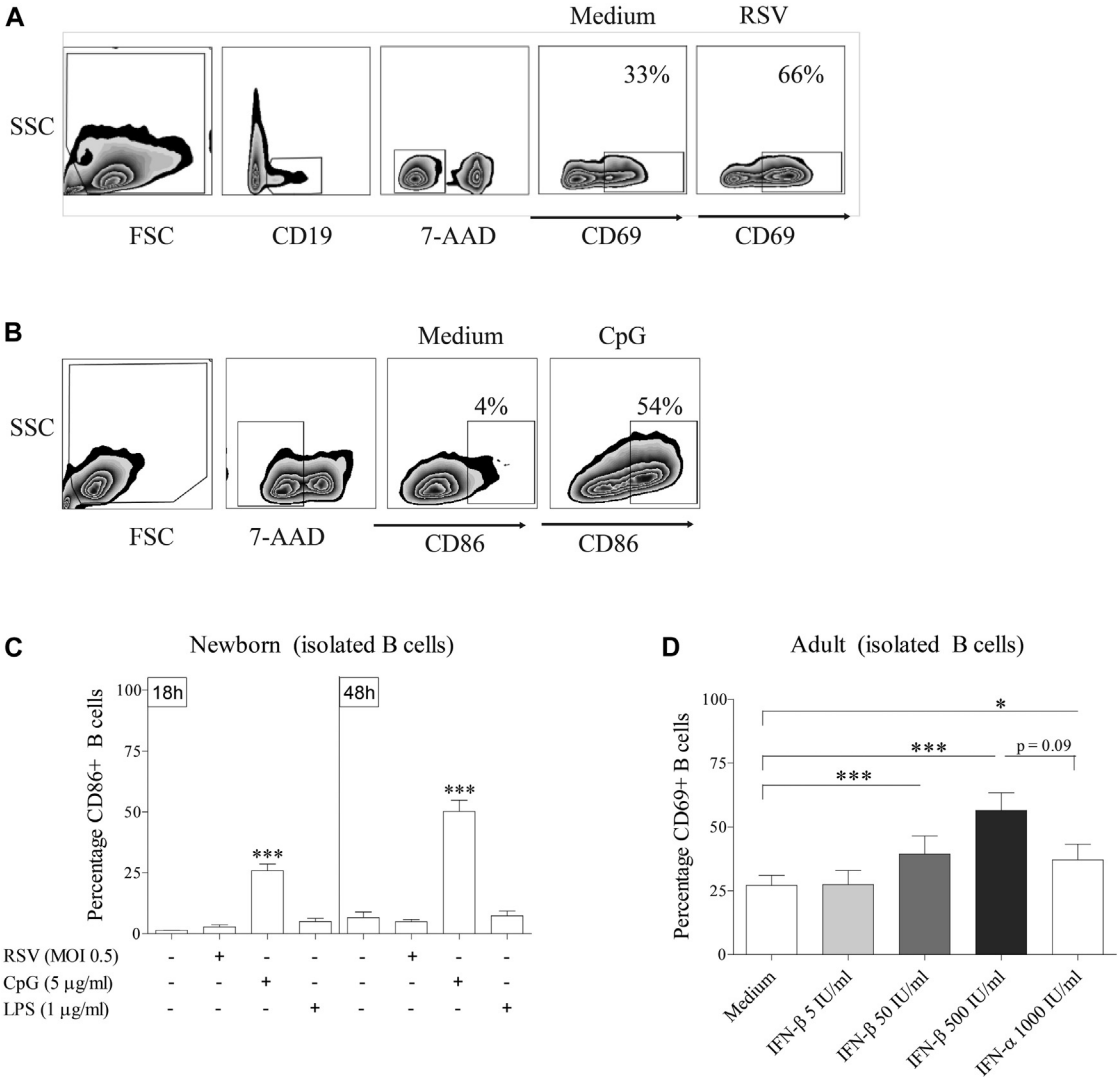

FIG E1. A-D, Soluble mediators activate adult isolated B cells. Representative gating strategy to determine (Fig E1, *A*) $CD69^+$ B cells or (Fig E1, *B*) $CD86^+$ cells. Fig E1, *C*, Percentage of $CD86^+$ B cells after stimulation of newborn isolated B cells (N = 5). Fig E1, *D*, Percentage of $CD69^+$ B cells after stimulation of adult isolated B cells with IFN-α or IFN-β (N = 5) (mean ± SEM). *7-AAD*, 7-Aminoactinomycin D; *FSC*, forward-scattered light; *SSC*, side-scattered light. $*P < .05$. $***P < .001$.

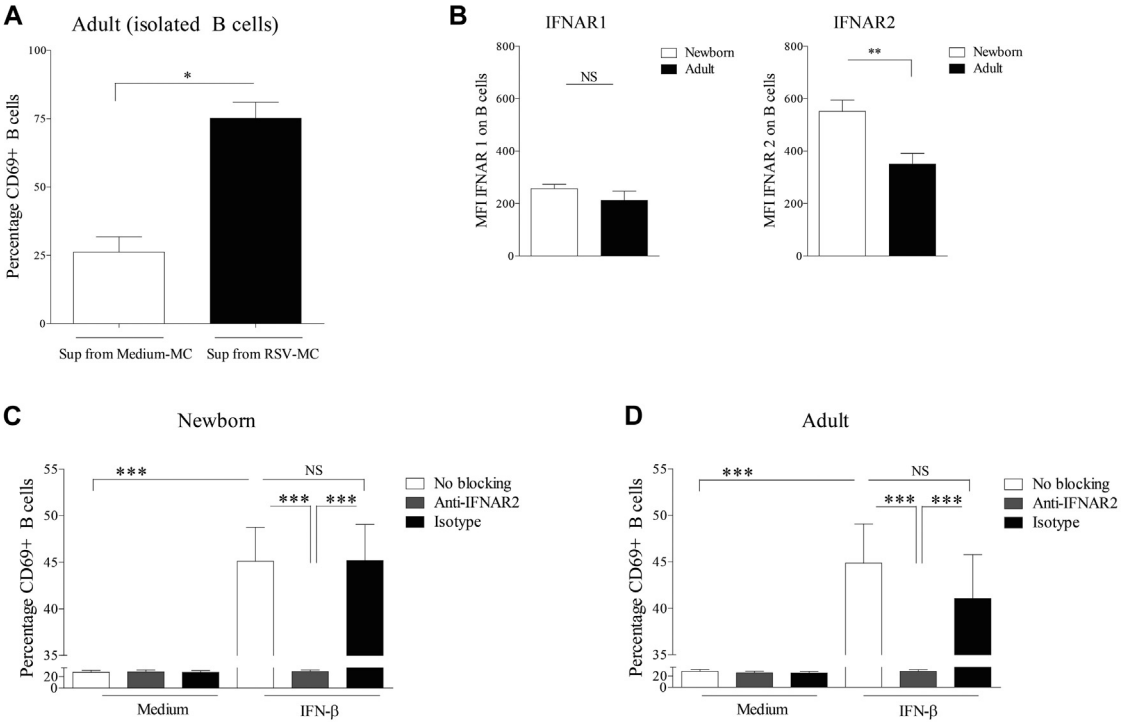

FIG E2. A-D, IFNAR-dependent activation of newborn and adult B cells after exposure to IFN-β. Fig E2, *A*, Percentage of $CD69^+$ B cells (N = 6). Fig E2, *B*, Expression of IFNAR1 and IFNAR2 on newborn and adult B cells (N = 5-6). Percentage of $CD69^+$ B cells after stimulation of (Fig E2, *C*) newborn or (Fig E2, *D*) adult MCs with IFN-β (500 IU/mL) in the absence or presence of anti–IFNAR-blocking antibodies or isotype controls (N = 5) (mean ± SEM). *INFAR*, Interferon-α/β receptor; *NS*, not significant. **P* < .05. ***P* < .01. ****P* < .001.

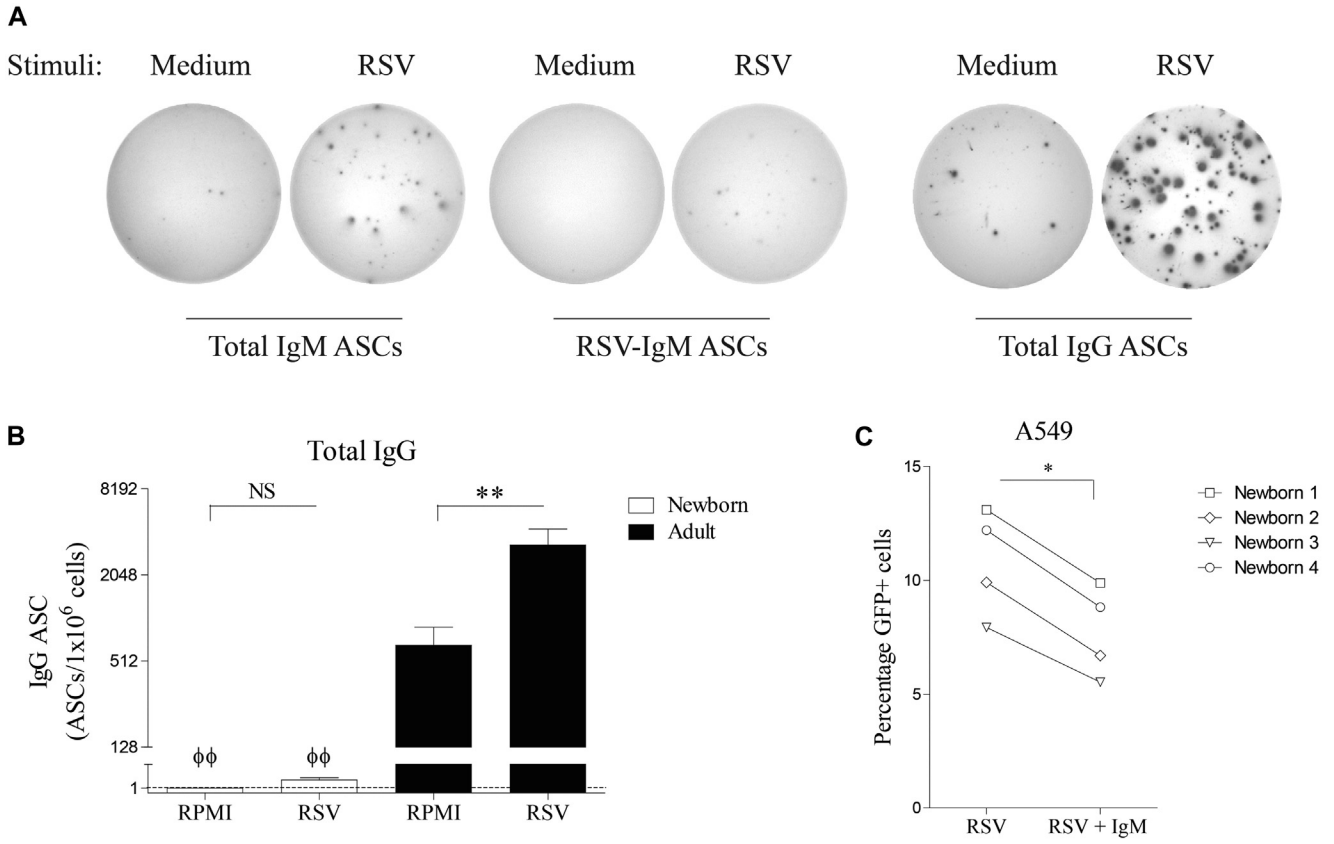

FIG E3. A-C, Purified IgM reduces RSV infection of epithelial cells. Fig E3, *A*, Representative ELISpot of total-IgM, RSV-IgM, and total-IgG ASCs. Fig E3, *B*, Total-IgG ASC (ASC/1 $\times$ 10^6 plated cells) after stimulation of MCs with RSV (MOI, 0.5) (N = 5-9). Fig E3, *C*, Percentage of RSV-infected GFP^+ A549 cells after exposure to RSV (MOI, 2.5) in medium or to RSV in medium with purified newborn IgM from 4 different donors (mean $\pm$ SEM). *GFP*, Green fluorescent protein; *MOI*, multiplicity of infection; *NS*, not significant. $^*P < .05$. $^{**}P < .01$. ϕϕNewborn vs adult: $P < .01$.