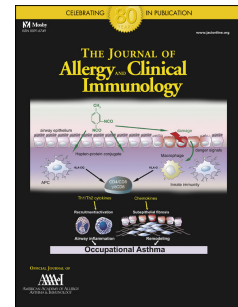


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Human amniotic fluid antibodies protect the neonate against respiratory syncytial virus infection

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Conflict of interest

R. J. Joost van Neerven is also employed by FrieslandCampina (Amersfoort, The Netherlands), although FrieslandCampina was not financially involved in this study.

Capsule summary

Virus-specific antibodies in human amniotic fluid protect mice against respiratory syncytial virus (RSV) infection. Presence of these antibodies in the lungs of neonates could provide protection against RSV infection during the first weeks after birth.

Key words:

RSV; amniotic fluid; neutralizing antibodies; IgG.

Abbreviations:

RSV, respiratory syncytial virus; ALRI, acute lower respiratory tract infection; AF, amniotic fluid; IgG, immunoglobulin G; nAbs, neutralizing antibodies; BALF, bronchoalveolar lavage fluid.

To the Editor:

Respiratory syncytial virus (RSV) is a leading cause of acute lower respiratory tract infections (ALRI), hospitalizations and mortality in young children. Nearly all children are infected at least once with RSV by two years of age.¹ Early RSV infection plays an important role in the pathogenesis of recurrent wheeze in the first year of life and possibly asthma development later in life.^{2,3} In a single year, an estimated 34 million episodes of RSV-associated ALRI may occur in children younger than five years.⁴

Despite the high incidence of RSV infections during early childhood, RSV infection in the first weeks of life is uncommon.⁵ Newborns are likely to be protected against RSV infection by maternal RSV neutralizing antibodies (nAbs).⁶ Previous studies have demonstrated that serum RSV nAbs correlate with protection against RSV infection.⁷ These RSV nAbs are frequently present at protective levels in serum of infants in the first six months after birth.⁸ Nevertheless, the highest burden of RSV infections is in infants from approximately four weeks to six months of age.^{1,4,5} Thus, serum RSV nAbs do not fully explain why healthy term babies are resistant to RSV infection during neonatal age. Since serum RSV nAbs only partly reflect the RSV protective capacity at the site of infection, which is the respiratory mucosa, better local correlates of humoral immune protection are needed. Indeed nasal RSV-specific antibodies were recently shown to better correlate with RSV protection in adults than serum RSV-specific antibodies.⁹ Amniotic fluid (AF) contains maternal antibodies and is in direct contact with the respiratory tract of the fetus. However, nothing is known on the presence of RSV nAbs in AF.

Therefore, we investigated the presence of RSV nAbs in a panel of 28 AF samples from healthy term infants and hypothesized that 1) RSV nAbs are present in human AF and that 2) presence of these antibodies in the lungs contributes to protection against RSV infection. First, we measured by ELISA the total levels of IgG (mean 96 $\mu\text{g/mL}$ [21-589 $\mu\text{g/mL}$]) and IgA (mean 41 $\mu\text{g/mL}$ [4-361 $\mu\text{g/mL}$]) in AF (Fig 1, A, and see Fig E1, A, in this article's Online Repository at www.jacionline.org). Further details of the methods used in this study can be found in the Methods section in this article's Online Repository at www.jacionline.org. All the AF samples contained RSV-specific IgG (mean: 2.8 $\mu\text{g/mL}$ [0.5-10.5 $\mu\text{g/mL}$]) and lower levels of RSV-specific IgA (mean: 0.1 $\mu\text{g/mL}$ [0-1 $\mu\text{g/mL}$]) (Fig 1, B, and see Fig E1, B, in this article's Online Repository at www.jacionline.org). AF RSV-specific IgG also bound to RSV-infected HEp-2 cells in a FACS-based antibody-binding assay, but not to uninfected cells (see Fig E2 in this article's Online Repository at www.jacionline.org). In an RSV neutralization assay with palivizumab (human IgG1 against the RSV F protein) used as control antibody (Fig 1, C) we detected RSV neutralizing activity in all AF samples (Fig 1, D). However, all RSV neutralizing activity was removed when AF was preincubated with prefusion, but not postfusion, RSV F protein (Fig 1, E, and see Fig E1, C-F, in this article's Online Repository at www.jacionline.org), suggesting specificity of RSV nAbs in AF for prefusion RSV F protein surfaces. AF RSV neutralizing activity was mainly IgG-dependent, as demonstrated by an IgG depletion assay (Fig 1, F, and see Fig E3, A and B, in this article's Online Repository at www.jacionline.org), and to a lesser extent presumably on other immunoglobulin isotypes (i.e. IgA) and non-immunoglobulin AF constituents (i.e. cytokines and oligosaccharides). We analyzed matched cord blood (CB) samples of 20 donors for total IgG and RSV-specific IgG concentrations as well as for RSV neutralizing activity (see Fig E4 in this article's Online

Repository at www.jacionline.org). No correlation was found between CB and AF total IgG concentration, RSV-specific IgG concentration, or RSV neutralizing activity (unpublished observations).

In vivo, we show that monoclonal RSV neutralizing antibodies (0.005-0.5 mg/kg palivizumab) administered into the lungs of naïve wild-type BALB/c mice protected against RSV infection in a dose-dependent manner (Fig 2, A) and that this protection lasted at least one week (Fig 2, B). We found no role for activating Fc gamma receptors (FcγRs) in providing protection during the first three days against RSV infection, since Fc receptor γ-chain knockout (*Fcγr1g^{-/-}*) BALB/c mice, which lack activating FcγRs, were protected against RSV infection (Fig 2, C). This indicates that mechanisms other than activating FcγRs contributed to persistence of the passively acquired antibodies in the respiratory mucosa early in the infection.

In humans, it is impossible to distinguish between the protective effects of maternal antibodies acquired into the respiratory mucosa via serum and AF. Therefore, to investigate the specific contribution of antibodies in AF to protection against RSV infection we performed a proof-of-concept study in mice by administering human AF into the lungs one day before RSV challenge. Exposure of the airways of naïve wild-type BALB/c mice to human AF protected against RSV infection (Fig 2, D, and see Fig E3, D, in this article's Online Repository at www.jacionline.org). This effect was mostly IgG dependent, because IgG depleted AF was significantly reduced in protective capacity and purified AF IgG was sufficient for protection (Fig 2, D, and see Fig E3, C and D, in this article's Online Repository at www.jacionline.org).

Based on the results obtained in the present study, we propose a new mechanism to explain how healthy term infants are protected against respiratory viral infection during the first month after birth, but become susceptible to infection in the following months. This protection is dependent on RSV nAbs in the lungs of neonates originating from AF. As the AF RSV nAb levels in the airways drop below a protective threshold (probably after the first month of life), post-neonatal infants become susceptible to RSV infection despite having high serum RSV nAb levels. In this regard, we also propose that AF RSV nAbs would be a better correlate of protection for newborns than serum RSV nAbs.

In summary, we have evaluated a panel of human AF for RSV nAbs and found protective levels of RSV nAbs targeting the prefusion RSV F protein. Our studies in mice suggest that *in utero* acquired AF antibodies are sufficient to prevent virus infection of the lung for at least one week and, thus, aid in understanding why neonates are generally protected against life-threatening RSV infection in the first weeks of life.

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Disclosure of potential conflict of interest: R. J. J. van Neerven is also employed by

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Figure legends

Fig 1.

Human amniotic fluid (AF) contains RSV neutralizing antibodies. **A**, Total IgG and **B**, RSV-specific IgG concentrations in AF. **C**, RSV neutralizing activity of palivizumab and **D**, AF. **E**, Fusion protein competition neutralization assay. **F**, AF RSV-GFP neutralization. AF 6 and AF 7 refer to different AF donors. Results in **C and F** represent mean $\pm$ SD of triplicate measurements.

Fig 2.

Intranasal administration of amniotic fluid (AF) protects against RSV infection. RSV titers in bronchoalveolar lavage fluid (BALF). Palivizumab prophylaxis **A**, 1d; **B**, 1-7d (0.5 mg/kg); **C**, 3d (0.5 mg/kg) before infection. **D**, Palivizumab and AF prophylaxis 1d before infection. WT mice

206 were used, except for *Fcer1g*^{-/-} mice in **C**. N=4-5 mice/group. (median $\pm$ IQR; Mann-Whitney test,
207 * P<0.05 ** P<0.01).

Figure 1:

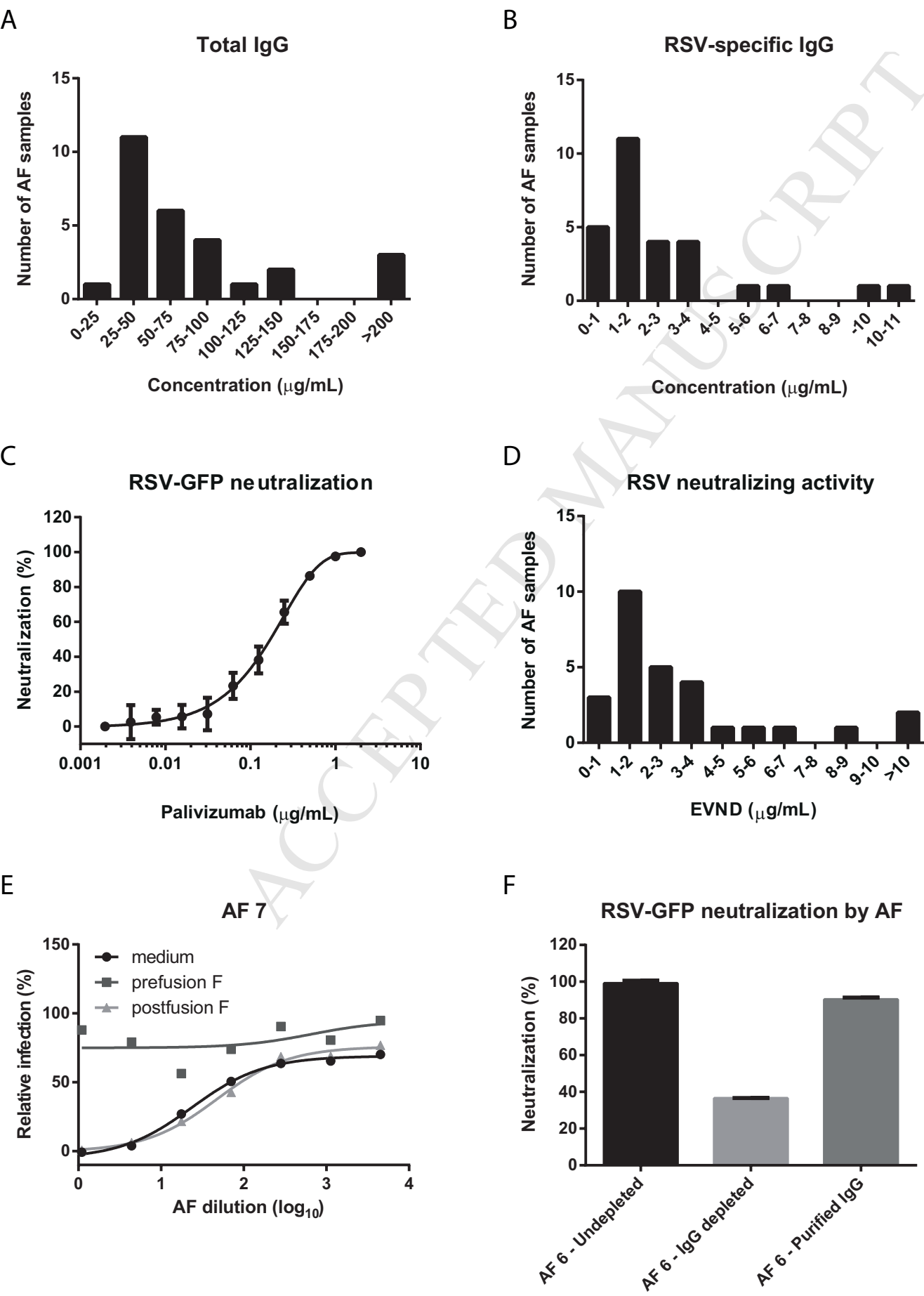
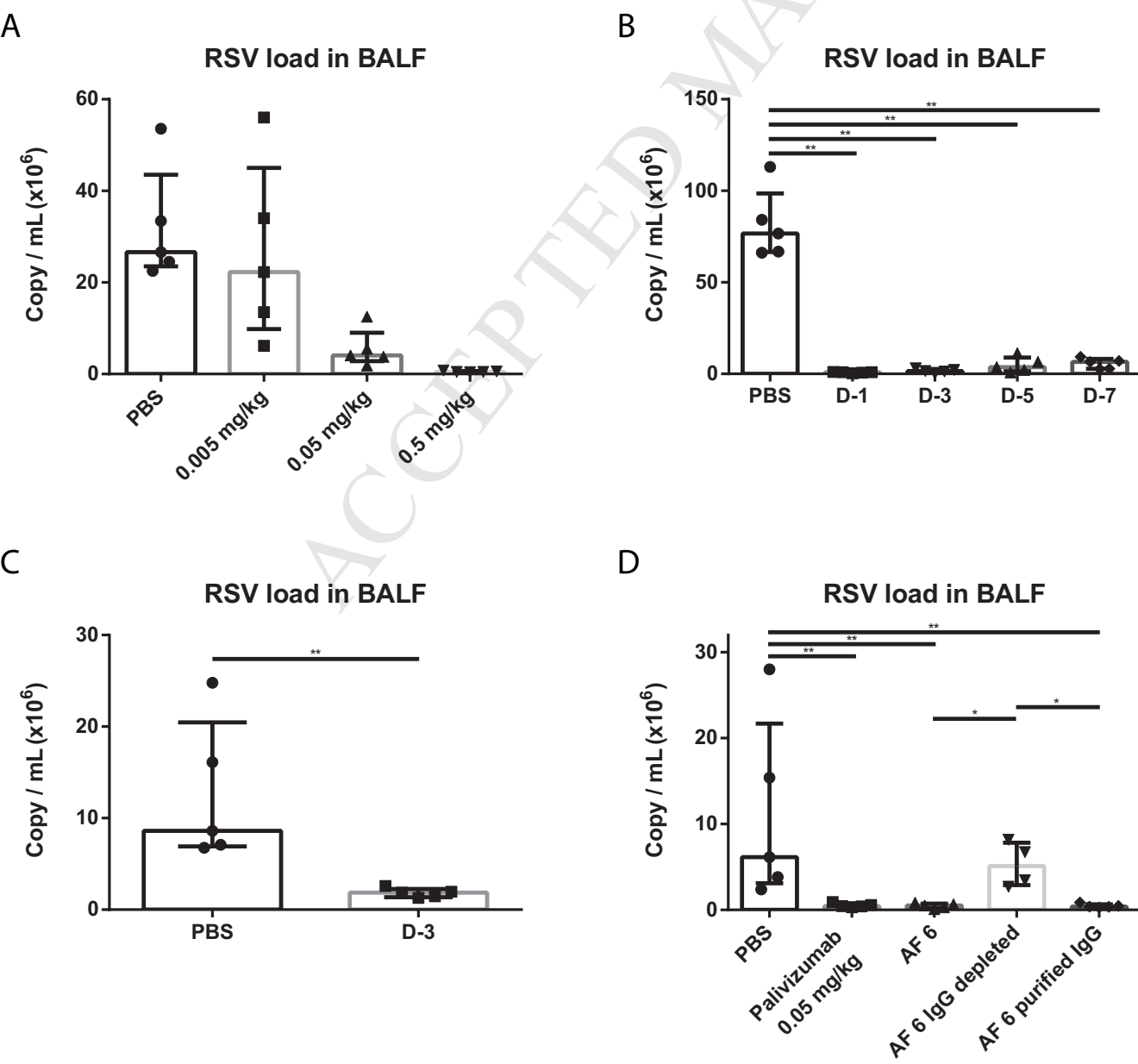


Figure 2:



Methods

Amniotic fluid (AF), cord blood (CB), cells, and viruses. AF and CB samples were collected during normal delivery at term and were filtered and stored at -80°C until further use, as described previously.^{E1} HEp-2 cells (ATCC) were maintained in IMDM (Gibco) supplemented with Penicillin (100 Units/mL), Streptomycin (100 µg/mL) and 10% fetal calf serum (FCS). RSV-A2 and RSV-GFP were propagated in HEp-2 cells (70-80% confluent), purified by polyethylene glycol 6000 precipitation, resuspended in PBS supplemented with 10% sucrose and stored in liquid nitrogen. RSV-A2 carrying an mKate reporter was used in the RSV F protein competition neutralization assay.^{E2}

Quantification of antibody concentrations. ELISA was conducted to determine the total and RSV-specific IgG and IgA concentrations in AF. 96 wells plates (NUNC Maxisorp) were coated O/N at 4°C with either 50 ng goat-anti-hKappa antibody (Southern Biotech) in PBS for total IgG/IgA ELISA, or 1×10^6 plaque-forming units (PFU) RSV-A2 in PBS for RSV-specific IgG/IgA ELISA. In between steps, plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T). Plates were blocked for 1 h at 37°C with 1% BSA (Roche) in PBS-T. AF was added (100 µL/well) and incubated for 1.5 h at room temperature (RT). Palivizumab (Synagis®; MedImmune) was used as calibration standard in the IgG ELISA and recombinant palivizumab-IgA was used as calibration standard in the IgA ELISA. HRP-labeled goat-anti-hIgG (0.5 µg/mL, Jackson) and HRP-labeled goat-anti-hIgA (0.5 µg/mL, Southern Biotech) were used as detection antibody for 1 h at RT. Plates were developed with ABTS substrate (Roche) and the absorbance was measured at 405 nm with a Multiscan RC (Thermolab systems).

FACS-based antibody binding assay. HEp-2 cells (70-80% confluent in T175 flasks) were infected with 1×10^5 PFU RSV-A2 and incubated O/N at 37°C. Cells were then harvested by trypsinization and 1×10^5 cells/well were seeded in V-bottom 96 wells plates (Greiner bio-one). The cells were subsequently incubated with several dilutions of palivizumab, AF diluted 10x in PBS or PBS for 45 min at RT. After three washes with PBS, RPE-labeled goat-anti-hIgG (0.5 µg/mL, Southern Biotech) was added for 45 min at RT. Finally, cells were washed with PBS, fixed with 1% formaldehyde and resuspended in PBS to analyze antibody binding by FACS (BD Bioscience, Canto II and FACS Diva software).

FACS-based RSV neutralization assay. HEp-2 cells (5×10^4 cells/well) were seeded in flat bottom 96 wells plates (Nunc Microwell) and infected the next day with RSV-GFP (1×10^5 PFU) that was pre-incubated for 1 h at 37°C with several dilutions of palivizumab, AF or IMDM culture medium. After 20-24 h, cells were trypsinized, fixed with 1% formaldehyde and resuspended in PBS to analyze the GFP expression by FACS (BD Bioscience, Canto II and FACS Diva software). To calculate the neutralization percentages the mean fluorescence intensity (MFI) values of uninfected control cells and infected control cells were set to 100% and 0% neutralization, respectively. The RSV neutralizing activity of AF samples in figure 1 D and CB samples in figure E4 C are expressed as equivalent virus neutralization dose (EVND). 1 EVND was set as 1 µg/mL palivizumab.

IgG depletion assay. For the RSV neutralization assay IgG depletion was conducted with a ProteoPrep Immunoaffinity Albumin & IgG depletion kit (Sigma-Aldrich) as described by the manufacturer. AF IgG that bound to the ProteoPrep columns was eluted using 0.1 M Glycine pH

2.5 and the pH was immediately neutralized by adding 1 M Tris pH 8.8. The eluate was 5x diluted compared to the initial volume of AF loaded on the ProteoPrep columns. Purified AF IgG, the depleted and undepleted AF samples were all tested in a 10x final dilution for RSV neutralization activity by the FACS based RSV neutralization assay. For the *in vivo* RSV challenge study IgG of AF 6 was depleted with a Protein G HP SpinTrap column (GE Healthcare) as described by the manufacturer. AF IgG that bound to the Protein G column was eluted as described above. The eluate was 2x diluted compared to the initial volume of AF loaded on the Protein G column and was dialysed to PBS using a Slide-A-Lyzer Dialysis Cassette (Thermo Scientific). Purified AF 6 IgG, the depleted and undepleted AF 6 samples were used in a 2x final dilution in the RSV challenge study.

Prefusion and postfusion F protein competition neutralization assay. Four-fold dilutions of AF (from undiluted to 1:4096 diluted) were preincubated for 1 h at 37°C with either MEM medium (GE Life Sciences) supplemented with 10% FBS or stabilized prefusion/postfusion RSV F (10 µg/mL final concentration) and then incubated 1:1 for an additional hour at 37°C with RSV-A2 carrying an mKate reporter, as described previously.^{E2} 50 µl of AF-F protein/medium-virus mixture was added to 1.8×10^4 HEp-2 cells (in 30 µl MEM supplemented with 10% FBS) seeded in 384-well black clear-bottom plates (Thermo Scientific), and plates were incubated for 22-26 hours at 37°C. Spectrophotometric measurement was done at 588 nm excitation and 635 nm emission (Molecular Devices LLC).

RSV challenge studies in mice. For the human AF prophylaxis experiment wild-type female BALB/c mice aged 3 weeks were purchased from Janvier Labs. For the palivizumab prophylaxis

experiments wild-type female BALB/c mice aged 8-10 weeks and *Fcer1g*^{-/-} female BALB/c mice aged 6 weeks were purchased from Taconic. Anesthetized mice (4% isoflurane) were treated i.n. with 50 µL 2x diluted human AF (AF 6 [246 µg/mL IgG], AF 6 IgG depleted [4 µg/mL IgG], AF 6 purified IgG [241 µg/mL IgG]) or palivizumab at doses varying from 0.005 to 0.5 mg/kg in a volume of 50 µL PBS and were challenged 1-7 days later with 3x10⁶ PFU RSV-A2 in a volume of 50 µL PBS. At day five post infection the mice were euthanized by i.p. injection of sodium pentobarbital (200 µL 60 mg/mL). Lungs were lavaged with 1 mL PBS and the bronchioalveolar lavage fluid (BALF) or lung homogenate were used to determine the viral load, as described previously.^{E3}

Statistics. Statistical analysis (Mann-Whitney test, two-tailed) and non-linear regression analysis were performed with GraphPad Prism 6 software.

Study approval. Ethical approval for human AF and CB sample collection was obtained by the Medical Ethics Committee of the University Medical Centre Utrecht (UMCU). AF and CB samples were collected at UMCU from women who provided written informed consent prior to inclusion in this study.^{E1} Animal studies were approved by the local Committee for Animal Experimentation.

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Figure legends

Fig E1.

Human amniotic fluid (AF) contains RSV-specific IgA antibodies. **A**, Total IgA and **B**, RSV-specific IgA concentrations in AF. **C-F**, Fusion protein competition neutralization assay. The prefusion RSV F protein concentration used in this assay was not sufficient to fully inhibit the RSV neutralizing activity of AF 3 and AF 10. AF3, 10, 11 and 15 refer to different AF donors.

104

105 **Fig E2.**

106 Amniotic fluid (AF) antibodies bind specifically to RSV-infected HEp-2 cells. **A**, AF IgG binding to
107 uninfected HEp-2 cells and **B**, RSV-infected HEp-2 cells. The red line indicates background
108 binding of AF IgG to uninfected cells. Results represent mean $\pm$ SD of triplicate measurements.

109

110 **Fig E3.**

111 Amniotic fluid (AF) IgG neutralizes RSV. **A and C**, Total IgG concentrations. **B**, RSV neutralizing
112 activity. **D**, RSV lung titers of WT mice treated with palivizumab or AF 1d before infection. N=5
113 mice/group (median $\pm$ IQR; Mann-Whitney test, ** P<0.01). AF2, 4 and 6 refer to different AF
114 donors. Results in **A-C** represent mean $\pm$ SD of triplicate measurements.

115

116 **Fig E4.**

117 RSV neutralizing activity of human cord blood (CB). **A**, Total IgG and **B**, RSV-specific IgG
118 concentrations in CB. **C**, RSV neutralizing activity of CB. N=20 donors.

Figure E1:

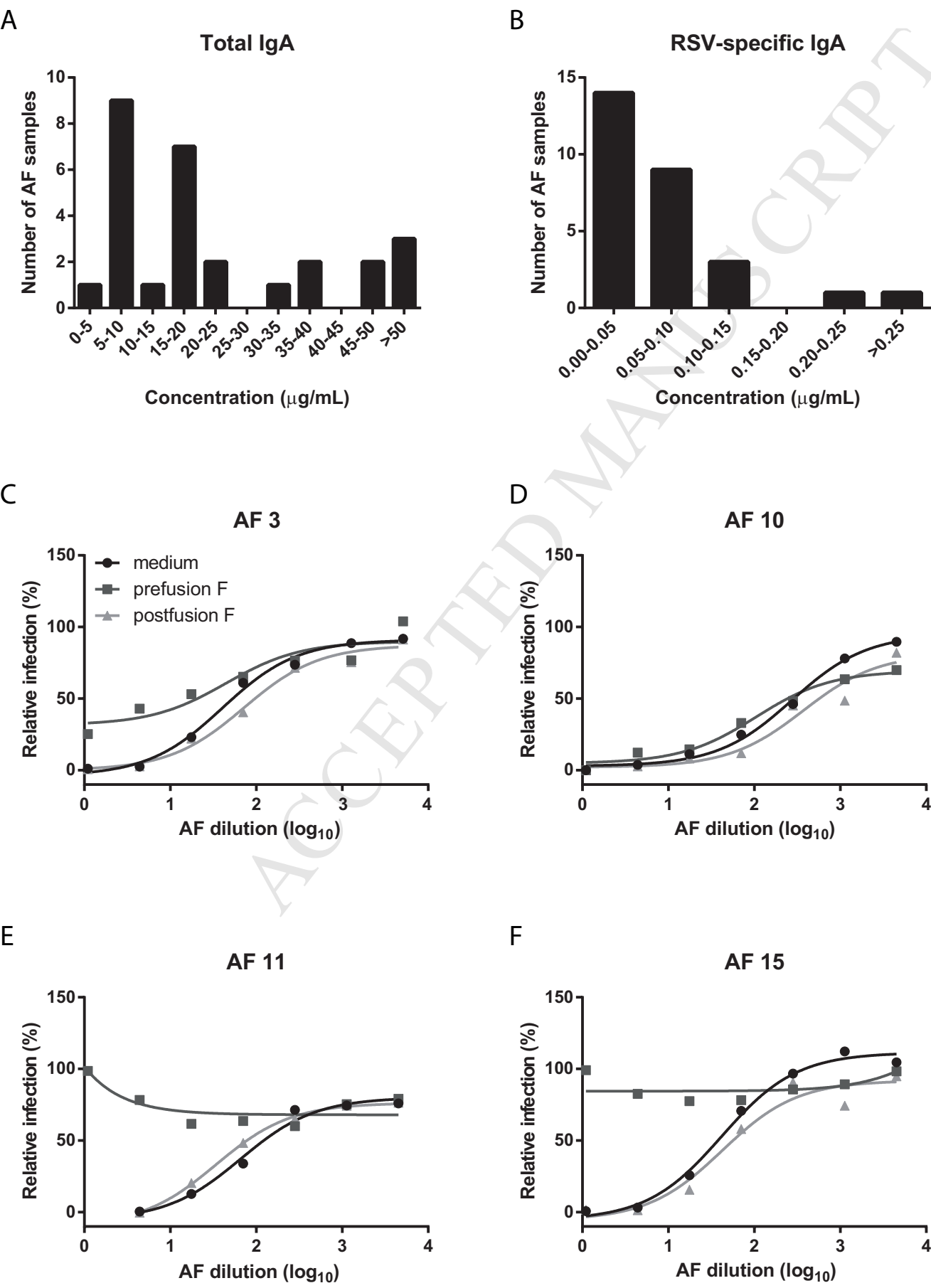
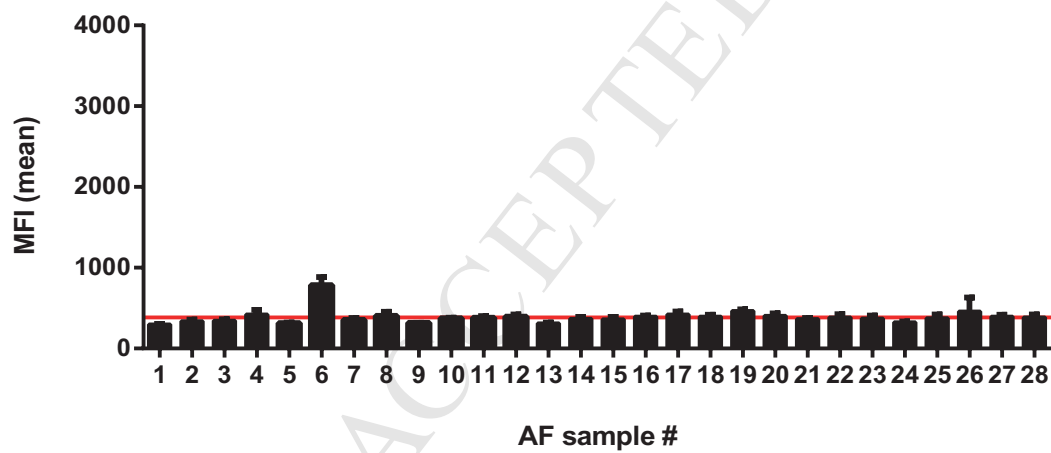


Figure E2:

A

AF-IgG binding to uninfected HEp-2



B

AF-IgG binding to RSV-infected HEp-2

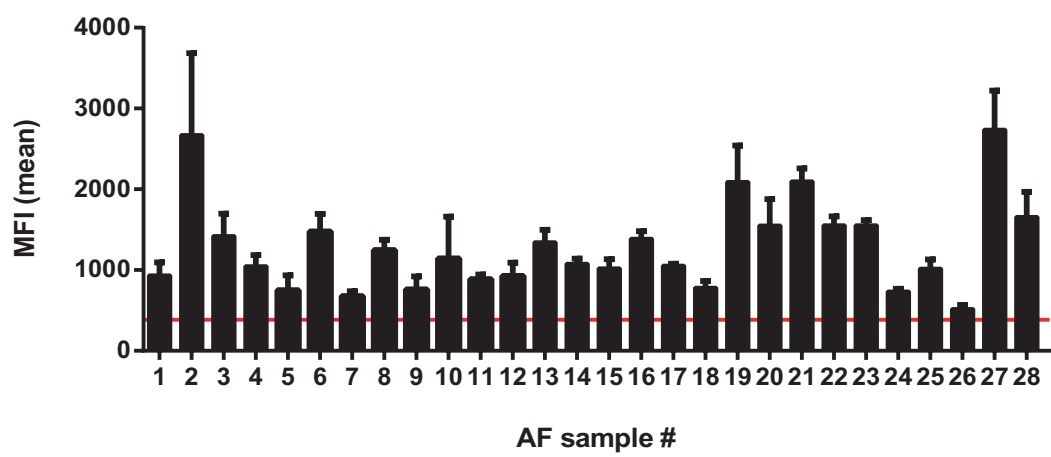


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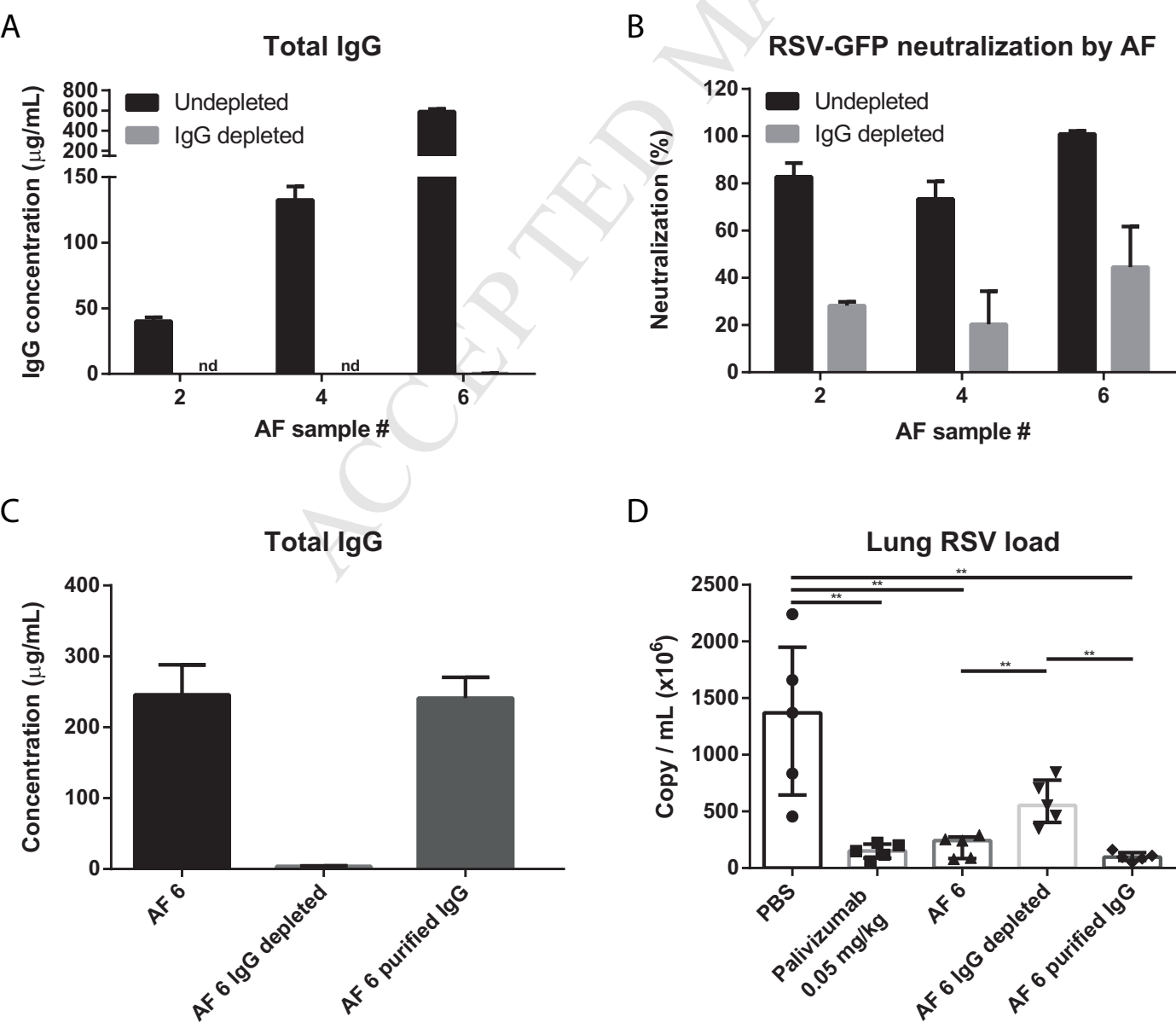


Figure E4:

