NK cell activation by RSV-specific antibodies is decreased in infants with severe respiratory infections and correlates with Fc-glycosylation

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Abstract

Background

Respiratory syncytial virus (RSV) is a major cause of severe acute lower respiratory tract infections in infants and there is no vaccine available. In early life, the most important contributors to protection against infectious diseases are the innate immune system and maternal antibodies. However, the mechanisms by which antibodies can protect against severe RSV disease are incompletely understood, as both antibody levels and neutralization capacity correlate poorly with protection.

Methods

We performed an observational case-control study including infants hospitalized for RSV infection (n=43), hernia surgery (n=16), or RSV-negative viral respiratory tract infections (n=18). First, we determined RSV antigen-specific antibody levels in infant plasma using a multiplex immunoassay. Subsequently, we measured the capacity of these antibodies to activate natural killer (NK) cells. Finally, we assessed Fc-glycosylation of the RSV-specific antibodies by mass spectrometry.

Results

We found that the concentrations of RSV antigen-specific antibodies did not differ between cases and controls. Importantly, RSV-specific maternal antibodies potently activated NK cells *in vitro*. Furthermore, antibodies from infants hospitalized for severe lower respiratory tract infections (RSV and/or other) induced significantly less NK cell interferon gamma production than those from uninfected controls. Finally, NK cell activation correlated with Fc-fucosylation of RSV-specific antibodies, but their glycosylation status did not significantly differ between cases and controls.

Conclusions

Our results suggest that Fc-dependent antibody function and quality, exemplified by NK cell activation and glycosylation, contribute to protection against severe RSV disease and warrant further studies to evaluate the potential of harnessing these activities to develop an effective vaccine.

Introduction

Respiratory syncytial virus (RSV) is a major cause of severe lower respiratory tract disease in young children, with an estimated 118 200 deaths worldwide in children below 5 years of age annually (1, 2). Despite decades of research, there are currently no market-approved vaccines available against this virus and their development is hampered by the lack of a well-defined correlate of protection. Severe RSV disease is most prevalent in the first six months of life (3, 4), when infants mainly rely on their innate immune system and maternal antibodies for protection against infectious diseases. Considering that maternal vaccination is a potential strategy to prevent RSV disease in infants, it is of pivotal importance to obtain a thorough understanding of the mechanisms by which maternal antibodies might protect against disease, both directly and through their interaction with innate immune cells.

The role of maternal antibodies in RSV infection and disease is unclear. Some studies show that high RSV-specific maternal antibody titers are associated with protection against RSV infection or (severe) disease (5-8). In contrast, other studies do not show a protective effect (9-12) or even indicate an association between high maternal antibody titers and an increased risk of recurrent wheezing (13). Strikingly, the vast majority of studies investigating the role of (maternal) antibodies in RSV infection only look at *in vitro* binding or neutralization capacity, while additional antibody effector functions are not taken into account.

Natural killer (NK) cells are important innate immune cells in the early response to viral infection and their activity is tightly regulated via their interaction with antigen-specific antibodies (14). Engagement of the main NK cell Fc gamma receptor (FcγRIIIa) by antibodies bound to virus-infected cells leads to the release of cytotoxic granules containing perforins and granzymes, a process known as antibody-dependent cell-mediated cytotoxicity (ADCC) (15). In addition, antibody-dependent activation of NK cells is known to result in the secretion of pro-inflammatory cytokines, including interferon gamma (IFN-γ) (16).

Several groups have shown clearance of RSV-infected cells by peripheral blood mononuclear cells (PBMCs) in the presence of monoclonal antibodies, or antibodies from different natural sources, including breast milk, cord blood, nasopharyngeal secretions, and serum (17-21). However, none of these studies shows whether killing was specifically NK cell-mediated. In addition, antibodies from RSV patients and controls have to our knowledge never been compared regarding functional properties other than neutralization, except for antibody-dependent enhancement of infection (22).

In recent years, it has become increasingly clear that the glycan present at the conserved *N*-linked glycosylation site in the Fc-tail of immunoglobulin G (IgG) is variable, and significantly affects antibody functionality (23). Moreover, in some immune responses, such as with HIV (24), dengue (25), and alloimmune diseases (26, 27), reduced fucosylation of the elicited antigen-specific antibodies can affect disease outcome by increasing the affinity for FcγRIIIa. In autoimmune diseases, IgG antibodies can display atypical galactosylation which affects disease severity through mechanisms that are not yet understood (28). The impact of IgG glycosylation on (protection from) RSV disease, if any, has not been studied to date.

Here, we used samples from an observational case-control study of 84 infants (<7 months of age) that were hospitalized for RSV (cases), an inguinal hernia surgery (uninfected controls), or viral respiratory infections other than RSV (RSV-negative infected controls). We first measured plasma levels of antigen-specific IgG against the RSV attachment (G) and fusion (F) proteins. We then assessed the capacity of RSV-specific antibodies to activate NK cells *in vitro* as measured by CD107a surface expression, which is a marker for degranulation, and IFN-γ production. Finally, we assessed the Fc-glycosylation status of total and RSV-specific antibodies by mass spectrometry. Our findings suggest that antibody-mediated NK cell activation contributes to protection against severe RSV disease and should be further investigated as a potential means to improve vaccine efficacy.

102 ## Methods

103 **Study design**
104 Plasma samples from hospitalized infants were collected in an observational case-control study in
105 2010-2014 and have been described before (22). For the current study, hospitalized infants below 7
106 months of age with PCR-confirmed RSV infections were included as RSV cases. Age-matched infants
107 admitted for inguinal hernia repair surgery were included as uninfected controls, whereas infants
108 admitted to the hospital for viral respiratory tract infections other than RSV were included as RSV-
109 negative infected controls. The ePlex System (Genmark Dx, Carlsbad, CA, USA) was used for
110 identification of respiratory viral pathogens and RSV subtype in nasopharyngeal aspirates. No RSV
111 was detected in uninfected controls and RSV-negative infected controls. Blood samples were taken
112 within 24 h after admission. Patients with congenital heart or lung disease, immunodeficiency, or
113 glucocorticoid use were excluded. The study protocols were approved by the Regional Committee on
114 Research Involving Human Subjects Arnhem-Nijmegen (serving as the IRB) and were conducted in
115 accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained
116 from the parents of all infants.
117
118 **Cells and viruses**
119 Peripheral blood mononuclear cells (PBMCs) were obtained from healthy adult volunteers at the
120 National Institute for Public Health and the Environment (RIVM, the Netherlands). Blood was
121 collected in heparin tubes and the mononuclear fraction was isolated by density gradient
122 centrifugation (Lymphoprep, Nycomed). Isolated cells were cultured in Roswell Park Memorial
123 Institute medium (RPMI, Gibco) supplemented with 10% heat-inactivated fetal calf serum (hiFCS,
124 Hyclone, GE Healthcare), 1% penicillin/streptomycin/glutamine (PSG, Gibco), and 5 ng/mL
125 recombinant human IL-15 (Biolegend). Before use, PBMCs were rested overnight at a density of
126 $1x10^6$ PBMCs/mL at 37°C and 5% CO_2. Vero cells (ATCC CCL-81) were cultured in Dulbecco's Modified
127 Eagle Medium (DMEM, Gibco), supplemented with 5% hiFCS and 1% PSG.
128 Recombinant RSV-X (GenBank FJ948820), used as a coat in the NK cell activation assay, was
129 propagated in Vero cells and purified between layers of 10% and 50% sucrose by ultracentrifugation.
130 The 50% tissue culture infectious dose (TCID50) per mL was determined on Vero cells using the
131 Spearman and Karber method (29). RSV-A2 (ATCC VR1302), used as a coat for the enrichment of RSV-
132 specific antibodies for mass spectrometry, was propagated in Vero cells, purified and concentrated
133 by filtration, and inactivated by the addition of 2% Triton X-100.
134
135 **Control sera/plasma**
136 The International Standard for Antiserum to Respiratory Syncytial Virus (16/284, National Institute
137 for Biological Standards and Control) was used as control in the NK cell activation assay and
138 glycosylation study. An in-house adult reference serum pool (RIVM) was used as standard in the
139 multiplex immunoassay. Additionally, cord blood (CB) plasma pooled from >10 individual donors was
140 (mock-) depleted for RSV-specific antibodies by incubation with mock- or RSV-infected Vero cells,
141 respectively. Depletion of RSV-specific antibodies was confirmed by multiplex immunoassay as
142 described below. Cord blood samples were collected from umbilical cords of healthy neonates born
143 by cesarean delivery at Radboudumc Nijmegen (the Netherlands). All mothers provided written
144 informed consent.
145
146 **RSV-specific multiplex immunoassay**
147 To quantify the concentration of RSV-specific IgG and IgA, a multiplex immunoassay was performed
148 as described before (30). Briefly, plasma samples were diluted 200 and 4000 times and incubated
149 with RSV antigen-coupled beads, including G_A, G_B, post-F, and pre-F (DS-CAV1 mutant) (31). All
150 antigens were produced in eukaryotic cells. Captured antibodies were detected with secondary R-
151 phycoerythrin-labeled goat-anti-human IgA $F(ab')_2$ (Southern Biotech) or IgG $F(ab')_2$ (Jackson

152 Immunoresearch Laboratories). IgG was measured on a Bio-Plex 200 (Luminex Corporation) in
153 combination with Bio-Plex Manager software version 6.1 (Bio-Rad). IgA was measured on a Flexmap
154 3D (Luminex Corporation) in combination with Xponent version 4.2 (Luminex Corporation). For each
155 analyte, median fluorescence intensity (MFI) was converted to arbitrary units/ml (AU/ml) by
156 interpolation from a 5-parameter logistic standard curve from an in-house reference serum pool.
157 Whereas all samples were tested in the NK cell activation assay, for some samples not enough
158 volume remained to be tested in the IgG (n=8) and/or IgA (n=11) multiplex immunoassay. These
159 samples were spread equally across the 3 different groups.
160
161 **NK cell activation assay**
162 Sterile Immulon ELISA plates (ThermoScientific) were coated with intact RSV-X particles at a
163 concentration of $1.7x10^5$ TCID50/well. After overnight coating at 4°C, wells were washed with PBS
164 and blocked with 10% hiFCS in PBS for 30 min at RT. After washing with PBS, coated plates were
165 incubated for 2 hours at 37°C with 10-fold diluted plasma, to allow for antigen-specific immune
166 complex formation. Subsequently, unbound antibodies and other plasma constituents were washed
167 away and $1x10^6$ PBMCs were added per well, together with Brefeldin A (BD Bioscience) and anti-
168 human CD107a-PerCP/Cy5.5 (clone H4A3; BioLegend). PBMCs were incubated with the opsonized
169 virions for 4 hours at 37°C, after which the cells were stained for flow cytometric analysis as
170 described below. Incubation of PBMCs in RSV-coated plates in the absence of antibodies was used to
171 determine the background signal. The PBMC supernatants were stored at -80°C and used for
172 detection of perforin secretion by ELISA (Mabtech). The ELISA was performed following the
173 manufacturer's protocol and optical density was measured at 450 nm using an ELISA reader (BioTek).
174 Due to the limited amount of fresh PBMCs per donor, we were unable to test all samples using
175 cells of all 6 donors. For this reason, we could test 63 of the 84 plasma samples on cells from all 6
176 PBMC donors. For 18 samples, we were able to test with PBMCs from 2-5 different donors. Three
177 samples were only tested on a single donor; these were all RSV-negative infected controls. Excluding
178 these 3 samples had no effect on the subsequent statistical analysis. The mock-depleted CB pool was
179 used for normalization between the different PBMC donors.
180
181 **Flow cytometric analysis**
182 PBMCs that were incubated with opsonized virions as described above were stained for flow
183 cytometric analysis. Extracellular staining was performed with anti-human CD3-FITC (clone UCHT1,
184 Biolegend), anti-human CD56-PE (clone HCD56, Biolegend), and Fixable Viability Staining-eFluor780
185 (eBioscience). After fixation and permeabilization, cells were intracellularly stained with anti-human
186 IFN-γ-PECy7 (clone B27, Biolegend). Flow cytometric analysis was performed using the FACS LSR
187 Fortessa X20 (BD Bioscience). In all experiments, NK cells were gated as the $CD3^-$, $CD56^+$ population.
188 The gating strategy is depicted in **Fig S1**. FlowJo software V10 (FlowJo, LLC) was used for data
189 analysis.
190
191 **Purification of plasma antibodies for glycosylation analysis**
192 Total IgG antibodies were captured from 2 µL of plasma using Protein G Sepharose 4 Fast Flow beads
193 (GE Healthcare, Uppsala, Sweden) in a 96-well filter plate (Millipore Multiscreen, Amsterdam, The
194 Netherlands) as described previously (11). To isolate RSV-specific antibodies from plasma, maxisorp
195 ELISA plates were coated with RSV-A2 antigen and washed with PBS containing 0.05% Tween20
196 (PBST). Secondly, plasmas were diluted five times in PBST and incubated for 1 hour at room
197 temperature while shaking. WHO RSV standard serum and adalimumab in 1% bovine serum albumin
198 in PBST was used as a positive and negative control, respectively. Plates were then washed once with
199 PBST, twice with PBS and twice with 200 µl ammonium bicarbonate. Elution of RSV-specific
200 antibodies was performed by incubating with 100 mM formic acid for 5 minutes.

201 **Mass spectrometric IgG Fc-glycosylation analysis**
202 Eluates containing either total or RSV-specific IgGs were collected in V-bottom plates, dried by
203 vacuum centrifugation for 2.5 hours at 50°C and then dissolved in 20 µL RA buffer (0.4% sodium
204 deoxycholate, 10 mM TCEP, 40 mM chloroacetamide, 100 mM TRIS pH8.5) followed by shaking for
205 10 minutes and heating for 5 minutes at 95°C. Antibody samples were then subjected to proteolytic
206 cleavage by adding 20 µL trypsin (10 ng/µL) and incubating for 20 hours at 37°C.
207 Analysis of IgG Fc-glycosylation was performed with nanoLC reverse phase (RP)-electrospray
208 (ESI)-MS on an Ultimate 3000 RSLCnano system (Dionex/Thermo Scientific, Breda, The Netherlands)
209 coupled to a Maxis HD quadrupole-time-of-flight MS equipped with a nanoBooster (Bruker Daltonics)
210 using acetonitrile-doped nebulizing gas. (Glyco-)peptides were trapped with 100% buffer A (0.1%
211 trifluoroacetic acid in water) and separated on a 4.5 min 3.0-21.7% buffer B (95% acetonitrile, 5%
212 water) linear gradient (32). In the current study we focused on IgG1, without analyzing IgG3 due to
213 its possible interference with IgG2 and IgG4 at the glycopeptide level (21). Mass spectrometry results
214 were extracted and evaluated using Skyline software.
215
216 **Statistical analysis**
217 Comparison of two groups or data points was performed by using a nonparametric Mann-Whitney
218 test. Multiple comparisons were analyzed by using a nonparametric Kruskal-Wallis test, followed by
219 Dunn's multiple comparisons test. Multiple comparisons of categorical values were tested by Chi-
220 square test. Correlations were assessed using a nonparametric Spearman's rank-order correlation.
221 Paired samples were analyzed using a nonparametric Wilcoxon matched-pairs test. *P* values <0.05
222 were considered statistically significant. Statistical analyses were performed and graphs were
223 produced with Prism 8 software (GraphPad).

224 ## Results

225 **Clinical characteristics of study subjects**
226 Plasma samples were obtained from a total number of 84 infants below 7 months of age that were
227 hospitalized for RSV infection (cases), hernia surgery (uninfected controls), or viral respiratory
228 infections other than RSV (RSV-negative infected controls). Since we were mostly interested in the
229 antibodies already present at the time of infection and the presence of pathogen-specific IgA is
230 indicative of a newly elicited antibody response, we excluded those children (n=5) that tested
231 positive for IgA (AU/mL > 0.2) against a minimum of 2 RSV antigens (**Fig 1A**). Excluded samples are
232 indicated with white squares. In addition, we excluded 2 children that had presumably received
233 Palivizumab, based on the fact that they displayed unusually high IgG levels for both pre-F and post-
234 F, while G-specific levels were low or even below detection level.
235 The clinical and demographic characteristics of the children included in further analyses are
236 depicted in **Table 1**. There was no significant difference in age, gestational age or breastfeeding
237 between the three groups. However, there was a significant difference in sex between the three
238 groups (P = 0.0459). A higher percentage of infants was infected with RSV-A (70%) compared to RSV-
239 B (30%). The group of RSV patients contained both RSV mono-infections (n=28) and co-infections
240 (n=15) with other respiratory viral pathogens, with rhinovirus being the most prevalent.

241 **Table 1. Demographic and clinical characteristics of study subjects**

	RSV cases (n=43)	Uninfected controls (n=16)	RSV-negative controls (n=18)	P Value
Age in days (range)	70 (11-185)	79 (28-175)[a]	89 (16-202)	0.3260[b]
Gestational age in weeks (range)[c]	38 (34-41)	NA	38 (35-41)	0.9749 [d]
Sex (M/F)	22/21	12/4	5/11[e]	0.0459[f]
Breastfeeding (%)[g]	21 (50%)	NA	7 (39%)	0.8246[f]
RSV types				
A	30 (70%)	NA	NA	
B	13 (30%)	NA	NA	
Co-infections (%)	15 (35%)	NA	NA	

242 [a] Age of one infant was not recorded
243 [b] Comparisons between RSV patients, uninfected controls, and RSV-negative controls; Kruskal-Wallis test followed by
244 Dunn's test to adjust for multiple comparisons.
245 [c] Gestational age of 1 RSV patient, all uninfected controls, and 3 RSV-negative controls was not recorded.
246 [d] Comparisons between RSV patients, uninfected controls, and RSV-negative controls; Mann-Whitney test
247 [e] Sex of two infants was not recorded
248 [f] Comparisons between RSV patients, uninfected controls (only for sex), and RSV-negative controls; Chi-square test
249 [g] Breastfeeding was not recorded for 1 RSV patient, all the uninfected controls, and 3 RSV-negative controls
250

251 **The concentration of RSV-specific IgG does not differ between RSV cases and controls**
252 We used a multiplex immunoassay to quantify the concentration of IgG specific for the RSV
253 attachment (G) and fusion (F) proteins. G protein from both an A- and B-strain was included, as these
254 sequences are highly variable between the two RSV subtypes (33). Both the pre- and post-fusion
255 conformations of the F protein were included, as these harbor partly overlapping but also distinct
256 epitopes (34). We could detect antibodies to all four antigens in all samples and observed substantial
257 spread between individuals (**Fig 1B**). Antibody levels were lower than those in the adult reference
258 serum pool (triangles) in all samples. Despite the cross-sectional design of the study, we could
259 observe a gradual waning of antibody concentrations with increasing age, as exemplified by
260 antibodies targeting pre-F and G_A (**Fig 1C**). As expected, there was a positive correlation between the
261 concentrations of antibodies targeting different RSV antigens, as exemplified for pre-F and G_A (**Fig**
262 **1D**). To assess a possible relation with RSV disease, we compared the concentrations of RSV antigen-
263 specific antibodies between RSV-infected cases, uninfected controls, and RSV-negative infected
264 controls (**Fig 2A-D**). The concentrations of antigen-specific antibodies did not significantly differ
265 between the three groups, confirming previous findings in a subset of this cohort (12).
266

267 **RSV-specific maternal antibodies activate NK cells *in vitro***
268 As NK cells play an important role in the control of viral lung infections, we next assessed whether
269 RSV-specific maternal antibodies are capable of activating NK cells in an *in vitro* assay (schematically
270 depicted in **Fig 3A**). To control for RSV-specificity, the assay was set up with a cord blood plasma pool
271 that had been (mock-) depleted for RSV-specific antibodies by incubation with (mock-) infected cells.
272 Successful depletion was confirmed by multiplex immunoassay (**Fig 3B**). Incubation of NK cells with
273 RSV-antibody complexes formed with a dilution series of mock-depleted cord blood plasma, naturally
274 containing RSV-specific maternal antibodies, resulted in a concentration-dependent activation as
275 measured by IFN-γ production (**Fig 3C**) and CD107a surface expression (**Fig 3D**), the latter being an
276 established marker for NK cell degranulation (35). Importantly, cord blood plasma largely lacking
277 RSV-specific antibodies (RSV-depleted) showed a strongly reduced NK cell activation.

RSV-specific antibodies from infants with severe viral respiratory tract infections induce less IFN-γ production by NK cells than those from uninfected controls

Next, we assessed whether the capacity of RSV-specific antibodies to activate NK cells differed between RSV cases and controls. Since we observed considerable donor variation in primary NK cell activation in our initial experiments, we included the mock-depleted cord blood plasma pool for normalization (**Fig S2**). Interestingly, we found that RSV-specific antibodies from both RSV patients and RSV-negative infected controls showed significantly decreased induction of NK cell IFN-γ production compared to uninfected controls (**Fig 4A**). Slightly less induction of CD107a expression was also observed for plasma from both RSV patients and RSV-negative infected controls compared to those from uninfected controls, but this difference was not statistically significant (**Fig 4B**). Since NK cell CD107a surface expression is used as a proxy for degranulation, we assessed its correlation with perforin secretion in the supernatant of the NK cell activation assay for one donor and found a strong positive correlation (**Fig 4C**). Finally, since the capacity of antibodies to activate NK cells is expected to be related to their concentration, we assessed the correlation between antibody levels and IFN-γ production. As exemplified for pre-F-specific antibody concentrations, there was only a moderate correlation with IFN-γ production (**Fig 4D**), suggesting that additional factors besides concentration contribute to the functional capacity of these antibodies.

RSV-specific antibodies are differently glycosylated than total infant antibody pool

We next assessed the Fc-glycosylation status of total and RSV-specific antibodies in our cohort. To this end, we enriched samples for RSV-specific antibodies by adsorption to inactivated RSV-A2-coated plates and subsequent elution (schematically depicted in **Fig 5A**). Overall, we found a significantly higher abundance of galactosylation and sialylation in RSV-specific antibodies compared to total antibodies (**Fig 5B**). In addition, we noticed that for both RSV-specific and total antibodies the abundance of fucosylation increased with age (**Fig 5C-D**), while the abundance of galactosylation, sialylation, and bisecting *N*-acetylglycosamine (bisection) decreased with age (**Fig 5E-J**).

Fucosylation of RSV-specific antibodies correlates with NK cell activation

Afucosylation of the core glycan present on the antibody Fc-tail has been shown to increase binding affinity for the main Fc gamma receptor (FcγRIIIa) present on NK cells, thereby increasing the capacity of the antibody for activation (23, 36). We therefore asked whether fucosylation of RSV-specific antibodies correlated with the antibody-induced NK cell activation observed in our assay. Indeed, although not very strong, we did find a significant negative correlation between fucosylation and the induction of NK cell IFN-γ production (**Fig 6A**) and CD107a surface expression (**Fig 6B**). Galactosylation of RSV-specific antibodies did not significantly correlate with IFN-γ production (**Fig 6C**), but it did show a small but significant positive correlation with CD107a surface expression (**Fig 6D**). Sialylation showed a small but significant positive correlation with both IFN-γ production (**Fig 6E**) and CD107a surface expression (**Fig 6F**). Bisection did not show a significant correlation with NK cell activation. As RSV-specific antibodies from infants with severe respiratory tract infections induced significantly less NK cell IFN-γ production compared to antibodies from uninfected controls, we asked whether this difference was reflected in the glycosylation status of these antibodies. However, none of the glycoforms showed a significant difference in abundance between cases and controls (**Fig 7A-D**).

321 ## Discussion

322 Studies investigating the role of (maternal) antibodies in RSV infection mostly focus on neutralization
323 and binding titers. Whereas antibody-mediated protection against RSV disease likely depends (at
324 least in part) on virus neutralization, there is increasing evidence that Fc-mediated effector functions
325 also play an important role during RSV infection (reviewed in (37, 38)). In two independent *in vivo*
326 studies, modification of the Fc-domain of RSV-specific antibodies has shown major effects on lung
327 viral titer (39, 40). These results strongly support the idea that Fc-dependent mechanisms contribute
328 to the protective efficacy of RSV-specific antibodies. In the present study, we found that RSV-specific
329 antibodies from infants hospitalized for RSV and/or other respiratory viral infections less potently
330 induced NK cell IFN-γ production than those from uninfected controls. In addition, we found that NK
331 cell activation significantly correlated with RSV antigen-specific antibody concentration and
332 fucosylation, although neither of these factors alone significantly differed between cases and
333 controls.
334 Antibody-mediated NK cell activation has gained increasing attention for its role in protection
335 against a variety of infectious diseases. The capacity of antibodies to induce NK cell ADCC correlated
336 with the control of mycobacterium tuberculosis (41) and the killing of chlamydia trachomatis (42).
337 For HIV, ADCC-inducing antibodies have been identified as a key correlate of protection in the RV144
338 HIV vaccine trial (43-45). For influenza, the role of ADCC was shown to be protective in some studies
339 (46, 47), but others point to the involvement of ADCC in exaggeration of the immune response (48-
340 50). Taken together, these examples show that the capacity of pathogen-specific antibodies to
341 engage NK cells can significantly contribute to the outcome of infection. Regarding RSV infection, in
342 mice NK cells have been shown to be sufficient to eliminate infection (51) and NK cell depletion was
343 found to increase viral titers (52). However, NK cells have also been found to contribute to
344 inflammatory lung injury in mice (52, 53). In humans, NK cells have been reported to be both
345 decreased (54, 55) and increased (56, 57) compared to controls upon RSV infection. Thus, the exact
346 role of NK cells during RSV infection remains to be established but our results suggest their activation
347 positively contributes to the outcome of infection.
348 We found that RSV-specific antibodies from both RSV-infected and RSV-negative infected controls
349 showed a decreased propensity to activate NK cells compared to those from uninfected controls.
350 Since we only have samples that were obtained during hospitalization, and not before infection, we
351 cannot exclude that the difference we observe is due to the severe inflammation that these infants
352 are experiencing. Although it has been shown that chronic infection and inflammation can affect
353 functionality of the newly produced antibody repertoire (58), acute inflammation is unlikely to affect
354 the antibodies that are already in circulation. Alternatively, the observed effect might be unrelated to
355 antibody functionality per se. Before addition of PBMCs to the antibody-opsonized virus particles, we
356 washed away unbound plasma constituents such as cytokines and non-RSV-specific antibodies.
357 However, it is possible that a particular plasma component, e.g. complement, that differs between
358 infected and uninfected infants was deposited on the RSV-specific antibodies during opsonization,
359 which could potentially affect the outcome of the assay. Future experiments should include a heat
360 inactivation or IgG purification step to circumvent this problem. In addition, a prospective study in
361 which children are sampled before and during infection would provide more insight in the effects of
362 inflammation.
363 Glycosylation analysis of the total antibody pool revealed changes related to infant age, where
364 especially galactosylation showed a strong negative correlation with age. Fucosylation, on the other
365 hand, showed a slight but significant increase with age. These observations are in line with the
366 gradual replacement of maternal with infant antibodies. Changes occurring during pregnancy result
367 in an increased IgG Fc-galactosylation of maternal antibodies (59, 60). In addition, like all adult
368 antibodies, maternal antibodies show a slight skewing towards lower fucosylation (94%) compared to
369 highly fucosylated infant antibodies, reaching adult levels around the age of 20 years (59). We
370 observed similar correlations between glycosylation and age for RSV-specific antibodies. This

371 suggests that, despite the young age of the infants, our analysis is not entirely restricted to maternal
372 antibodies. However, the gradual waning of RSV-specific antibody concentrations seen with age does
373 suggest that we are looking mainly at maternal antibodies.
374 Antibody functionality, and ultimately protective efficacy, is determined by a wide range of
375 factors, including concentration, antigen and epitope specificity, glycosylation, isotype, and subclass.
376 Each of these factors contributes to a greater or lesser extent to protection, which might explain why
377 we did find a small but significant difference between cases and controls in antibody-mediated NK
378 cell IFN-γ production, but not in CD107a surface expression, antibody concentration, and
379 glycosylation. The multitude of factors contributing to antibody-mediated protection from severe
380 RSV disease complicates the definition of a correlate of protection for this pathogen, which in turn
381 hampers vaccine development, as efficacy has to be demonstrated in large-scale clinical trials. The
382 use of a systems serology approach for RSV may offer an unbiased and comprehensive way to
383 systematically investigate antibody characteristics and effector functions on a multidimensional level.
384 It has already proven effective in identifying antibody features that contribute to protection against
385 various other (viral) pathogens (41, 61, 62).
386 In conclusion, our results support the idea that antibody-mediated NK cell activation contributes
387 to protection against severe RSV disease and warrant further investigations into the potential of
388 harnessing Fc-mediated effector functions for improving the effectiveness of future RSV vaccines. A
389 thorough understanding of the protective capacity, in its broadest sense, of (maternal) RSV-specific
390 antibodies will be invaluable for the development of a safe and effective vaccine against this elusive
391 pathogen.

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402 ## Figure legends

403 **Figure 1. Characterization of the infant RSV-specific (maternal) antibody repertoire.** An RSV-specific
404 multiplex immunoassay was performed to quantify the concentration of IgA and IgG specific for RSV
405 G (A and B), pre-F, and post-F in plasma. (A) Concentration of plasma IgA for each of the RSV
406 antigens. Children that tested positive for IgA against a minimum of 2 RSV antigens (n=5) are
407 excluded for subsequent analyses (white squares). (B) Concentration of plasma IgG for each of the
408 RSV antigens. Black triangles indicate the IgA/IgG levels of the adult reference serum pool. Graphs
409 depict geometric mean and SD. (C) Concentration of RSV pre-F- and G_A-specific plasma IgG plotted
410 against age. (D) Correlation analysis of pre-F- and G_A-specific maternal antibody levels.
411 Nonparametric Spearman's correlation analysis was used to assess correlations. Abbreviations: AU,
412 arbitrary units; IgA, immunoglobulin A; IgG, immunoglobulin G; RSV, respiratory syncytial virus; SD,
413 standard deviation.
414
415 **Figure 2. RSV-specific antibody concentrations do not differ between RSV cases and controls.** An
416 RSV-specific multiplex immunoassay was performed to quantify the amount of RSV antigen-specific
417 IgG specific in plasma. Comparison of the antibody concentration against RSV-A attachment protein
418 G (A), RSV-B attachment protein G (B), pre-fusion F protein (C), and post-fusion F protein (D)
419 between uninfected controls, RSV patients, and RSV-negative infected controls. All graphs depict
420 geometric mean and SD. Kruskal-Wallis test with Dunn's multiple comparisons test was used for
421 comparison between multiple groups, no significant differences were found. Abbreviations: AU,
422 arbitrary units; IgG, immunoglobulin G; RSV, respiratory syncytial virus; SD, standard deviation.
423
424 **Figure 3. RSV-specific maternal antibodies activate NK cells *in vitro*.** (A) Schematic representation of
425 the NK cell activation assay. (B) RSV-specific multiplex immunoassay of mock-depleted (white bar)
426 compared to RSV-depleted (hatched bar) cord blood plasma. NK cell-specific IFN-γ production (C) and
427 CD107a surface expression (D) of three individual PBMC donors after 4 hour incubation with viral
428 particles opsonized with mock-depleted or RSV-depleted cord blood plasma. Graphs depict
429 geometric mean and SD. Kruskal-Wallis test with Dunn's multiple comparisons test was used for
430 comparison between mock- and RSV-depleted cord blood plasma with the same dilution (*P<0.05,
431 **P<0.01). Abbreviations: AU, arbitrary units; CB, cord blood; IFN-γ, interferon gamma; NK cells,
432 natural killer cells; RSV, respiratory syncytial virus.
433
434 **Figure 4. NK cell activation in RSV cases compared to controls**. Normalized NK cell IFN-γ production
435 (A) and CD107a surface expression (B) after 4 hour incubation of PBMCs (≤6 individual donors per
436 sample) with viral particles opsonized with 10-fold diluted plasma samples from uninfected controls,
437 RSV patients, or RSV-negative infected controls. Graphs depict geometric mean and SD. Kruskal-
438 Wallis test with Dunn's multiple comparisons test was used for comparison between multiple groups
439 (*P<0.05, **P<0.01). (C) Correlation analysis between perforin secretion and CD107a surface
440 expression of one donor. (D) Correlation analysis between RSV pre-F-specific antibody levels and IFN-
441 γ production. Uninfected controls are indicated in black, RSV patients in grey, and RSV-negative
442 infected controls in white. Nonparametric Spearman correlation analysis was used to assess
443 correlations. Abbreviations: IFN-γ, interferon gamma; NK cells, natural killer cells; RSV, respiratory
444 syncytial virus; SD, standard deviation.

445 **Figure 5. Fc-glycosylation pattern of total and RSV-specific antibodies.** (A) Schematic representation
446 of RSV-specific antibody enrichment. (B) Glycoform abundance of total compared to RSV-specific
447 antibodies. Graph depicts geometric mean and SD. Nonparametric Wilcoxon matched-pairs test was
448 used for comparison between total and RSV-specific antibodies (***P<0.001, ****P<0.0001).
449 Correlation analysis between glycoform abundance (fucosylation, galactosylation, sialylation,
450 bisection) and age for total antibodies (C, E, G, I) and RSV-specific antibodies (D, F, H, J).
451 Nonparametric Spearman correlation analysis was used to assess correlations. AU, arbitrary units;
452 IFN-γ, interferon gamma; NK cells, natural killer cells; RSV, respiratory syncytial virus; SD, standard
453 deviation.

454 **Figure 6. Fc-glycosylation correlates with NK cell activation.** Correlation analysis between glycoform
455 abundance of RSV-specific antibodies (fucosylation, galactosylation, and sialylation) and the capacity
456 to induce IFN-γ production (A, C, E) and CD107a surface expression (B, D, F). Nonparametric
457 Spearman correlation analysis was used to assess correlations. AU, arbitrary units; IFN-γ, interferon
458 gamma; IgG, immunoglobulin G; RSV, respiratory syncytial virus.

459 **Figure 7. Fc-glycosylation does not differ between RSV cases and controls.** Comparison of the
460 abundance of fucosylation (C), galactosylation (D), sialylation (E), and bisection (F) between
461 uninfected controls, RSV patients, and RSV-negative infected controls. Graphs depict geometric mean
462 and SD. Kruskal-Wallis test with Dunn's multiple comparisons test was used for comparison between
463 multiple groups, no significant differences were found. IgG, immunoglobulin G; RSV, respiratory
464 syncytial virus; SD, standard deviation.
465
466 **Supplementary Figure 1. Gating strategy for NK cell activation assays.** Gating strategy for the
467 measurement of CD107a surface expression and IFN-γ production in CD3(-)CD56(+) NK cells. Dot
468 plots and histograms are depicted for one representative PBMC donor. Abbreviations: IFN-γ,
469 interferon gamma; PBMCs, peripheral blood mononuclear cells; NK cells, natural killer cells.
470
471 **Supplementary Figure 2. Normalization of donor variation in NK cell activation assay.** NK cell-
472 specific IFN-γ production (A) and CD107a surface expression (B) of six individual PBMC donors after 4
473 hour incubation with viral particles opsonized with the indicated concentrations WHO RSV reference
474 serum. NK cell-specific IFN-γ production (C) and CD107a surface expression (D) after normalization to
475 a mock-depleted cord blood plasma sample. All graphs depict geometric mean and SD.
476 Abbreviations: AU, arbitrary units; IFN-γ, interferon gamma; NK cells, natural killer cells; RSV,
477 respiratory syncytial virus; SD, standard deviation.

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658

Figure 1

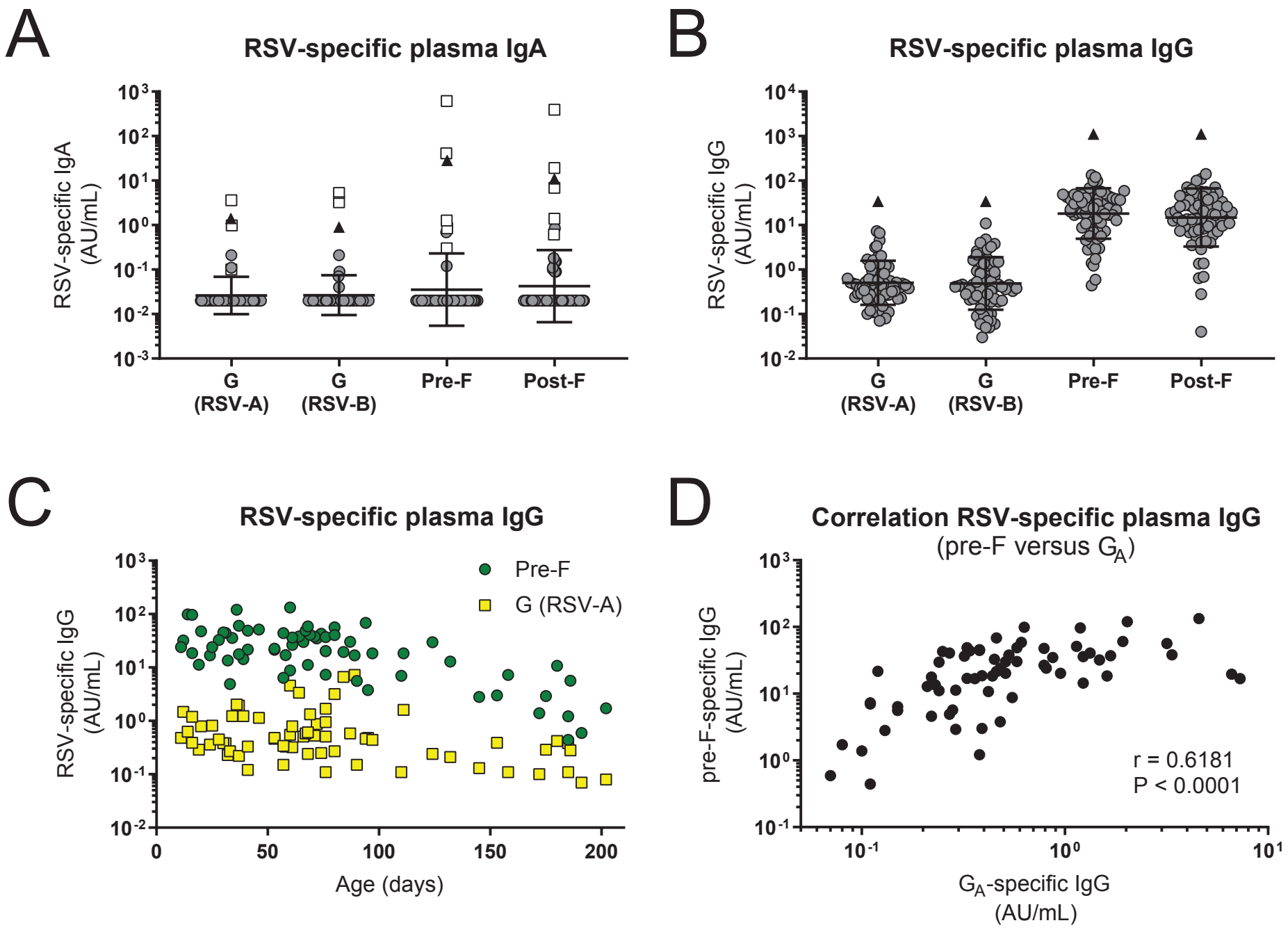

A
RSV-specific plasma IgA
RSV-specific IgA (AU/mL)
G (RSV-A)
G (RSV-B)
Pre-F
Post-F
B
RSV-specific plasma IgG
RSV-specific IgG (AU/mL)
G (RSV-A)
G (RSV-B)
Pre-F
Post-F
C
RSV-specific plasma IgG
Pre-F
G (RSV-A)
RSV-specific IgG (AU/mL)
Age (days)
D
Correlation RSV-specific plasma IgG
(pre-F versus G_A)
pre-F-specific IgG (AU/mL)
G_A-specific IgG (AU/mL)
r = 0.6181
P < 0.0001

Figure 2

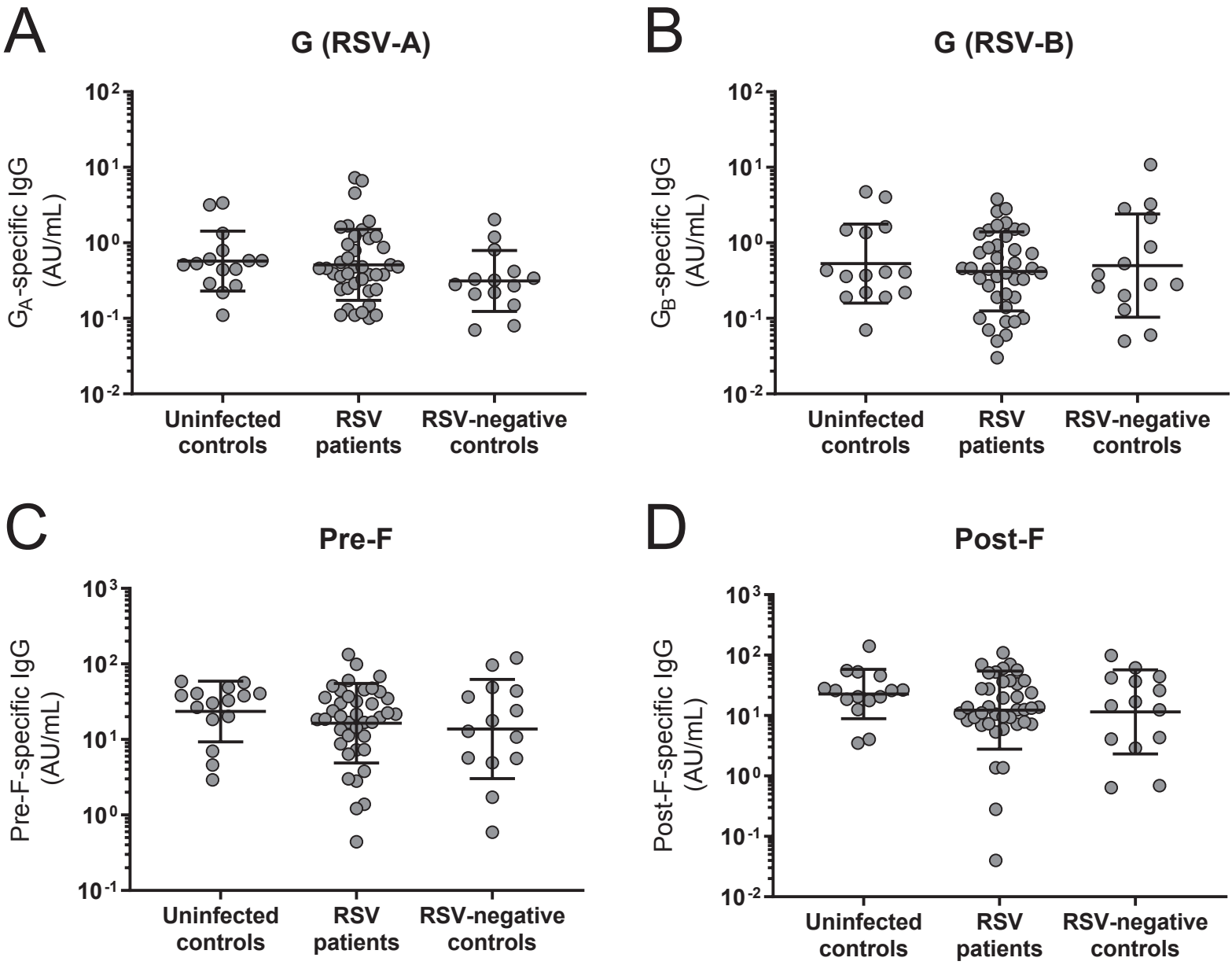

A
G (RSV-A)
G_A-specific IgG (AU/mL)
10^2
10^1
10^0
10^{-1}
10^{-2}
Uninfected controls
RSV patients
RSV-negative controls
B
G (RSV-B)
G_B-specific IgG (AU/mL)
10^2
10^1
10^0
10^{-1}
10^{-2}
Uninfected controls
RSV patients
RSV-negative controls
C
Pre-F
Pre-F-specific IgG (AU/mL)
10^3
10^2
10^1
10^0
10^{-1}
Uninfected controls
RSV patients
RSV-negative controls
D
Post-F
Post-F-specific IgG (AU/mL)
10^3
10^2
10^1
10^0
10^{-1}
10^{-2}
Uninfected controls
RSV patients
RSV-negative controls

Figure 3

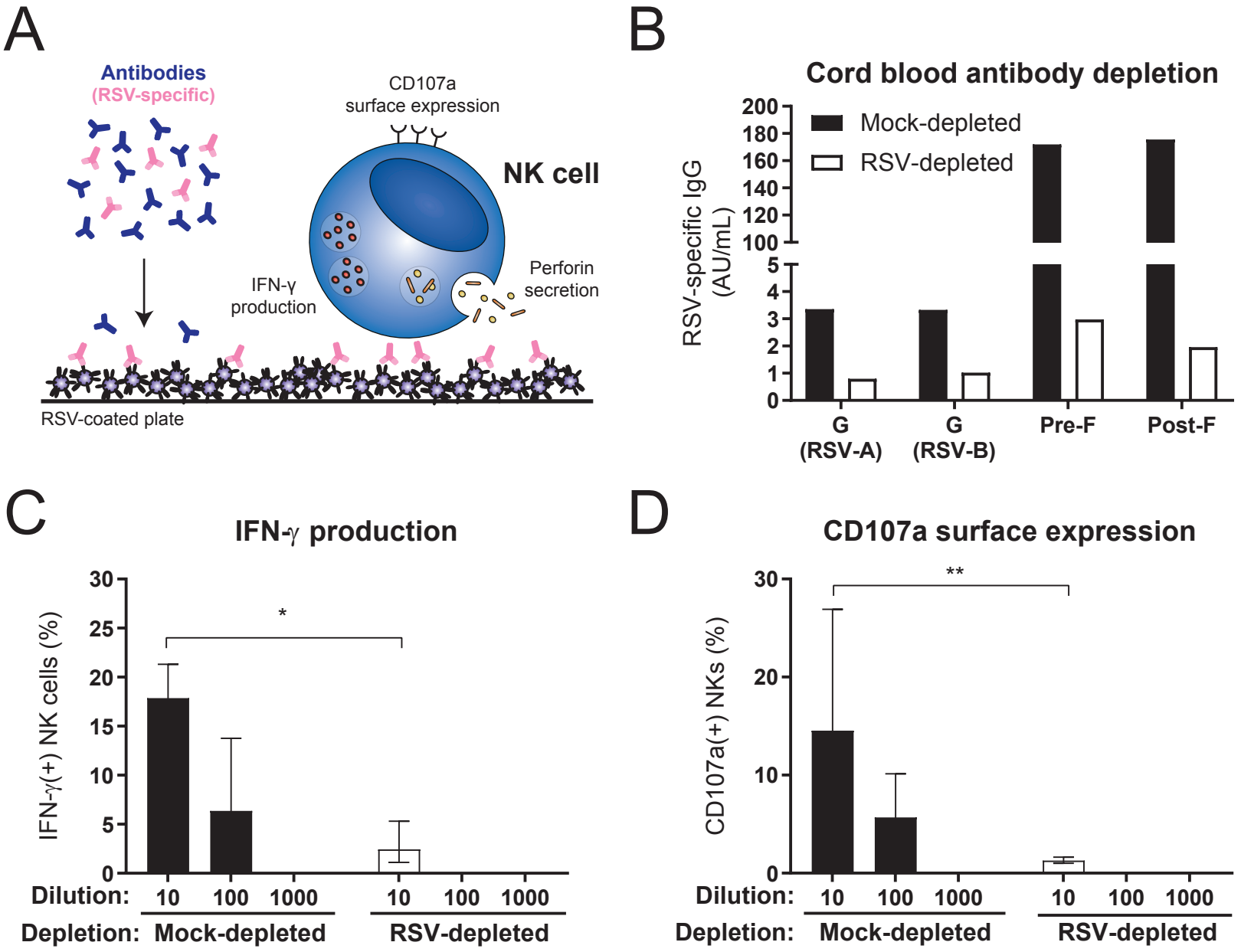

A
Antibodies
(RSV-specific)
CD107a
surface expression
NK cell
IFN-γ
production
Perforin
secretion
RSV-coated plate
B
Cord blood antibody depletion
Mock-depleted
RSV-depleted
RSV-specific IgG
(AU/mL)
200
180
160
140
120
100
5
4
3
2
1
0
G
(RSV-A)
G
(RSV-B)
Pre-F
Post-F
C
IFN-γ production
IFN-γ(+) NK cells (%)
30
25
20
15
10
5
0
*
Dilution:
10
100
1000
10
100
1000
Depletion:
Mock-depleted
RSV-depleted
D
CD107a surface expression
CD107a(+) NKs (%)
30
20
10
0
**
Dilution:
10
100
1000
10
100
1000
Depletion:
Mock-depleted
RSV-depleted

Figure 4

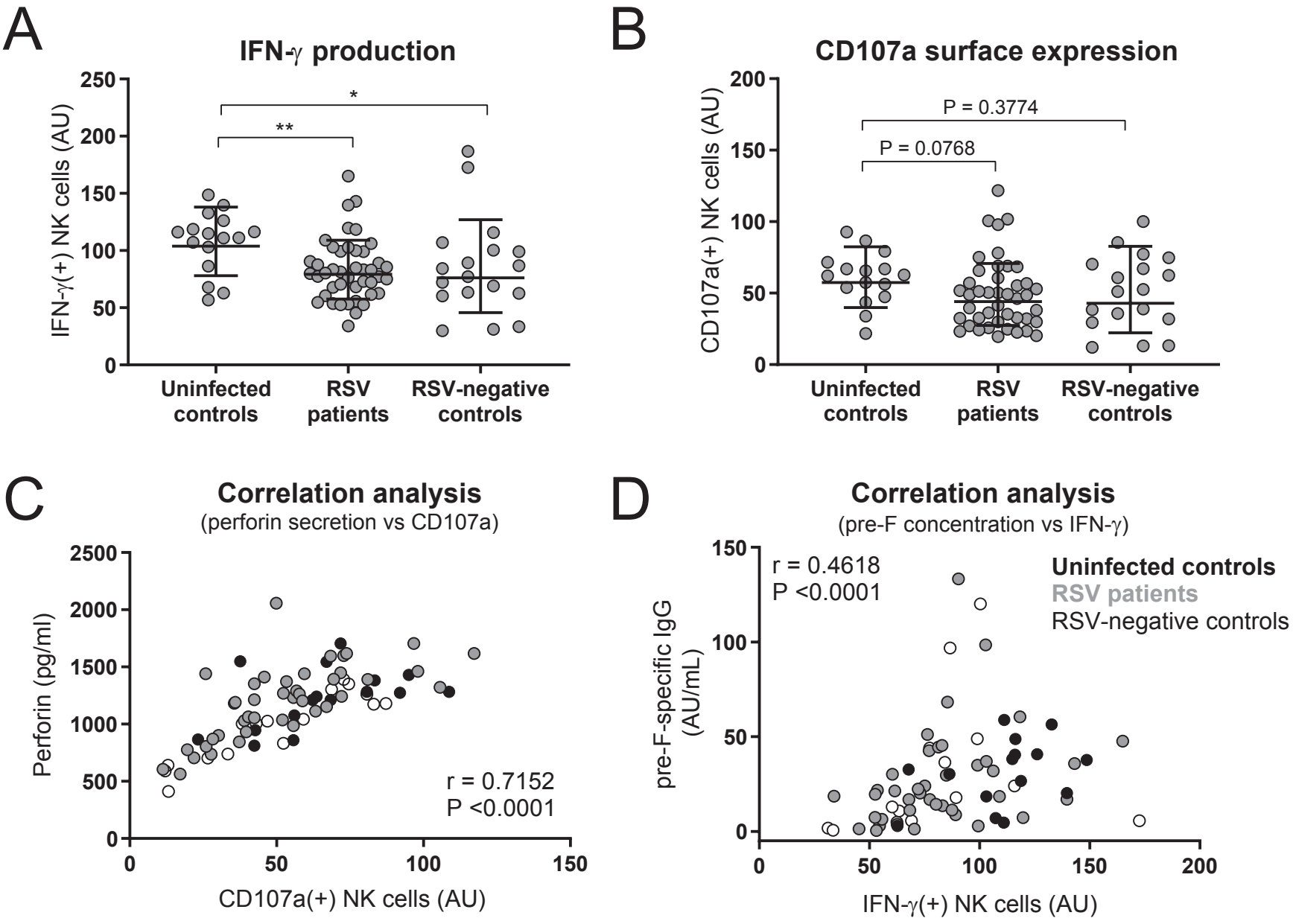

A
IFN-γ production
IFN-γ(+) NK cells (AU)
250
200
150
100
50
0
*
**
Uninfected controls
RSV patients
RSV-negative controls
B
CD107a surface expression
CD107a(+) NK cells (AU)
200
150
100
50
0
P = 0.3774
P = 0.0768
Uninfected controls
RSV patients
RSV-negative controls
C
Correlation analysis
(perforin secretion vs CD107a)
Perforin (pg/ml)
2500
2000
1500
1000
500
0
r = 0.7152
P <0.0001
0
50
100
150
CD107a(+) NK cells (AU)
D
Correlation analysis
(pre-F concentration vs IFN-γ)
pre-F-specific IgG (AU/mL)
150
100
50
0
r = 0.4618
P <0.0001
Uninfected controls
RSV patients
RSV-negative controls
0
50
100
150
200
IFN-γ(+) NK cells (AU)

Figure 5

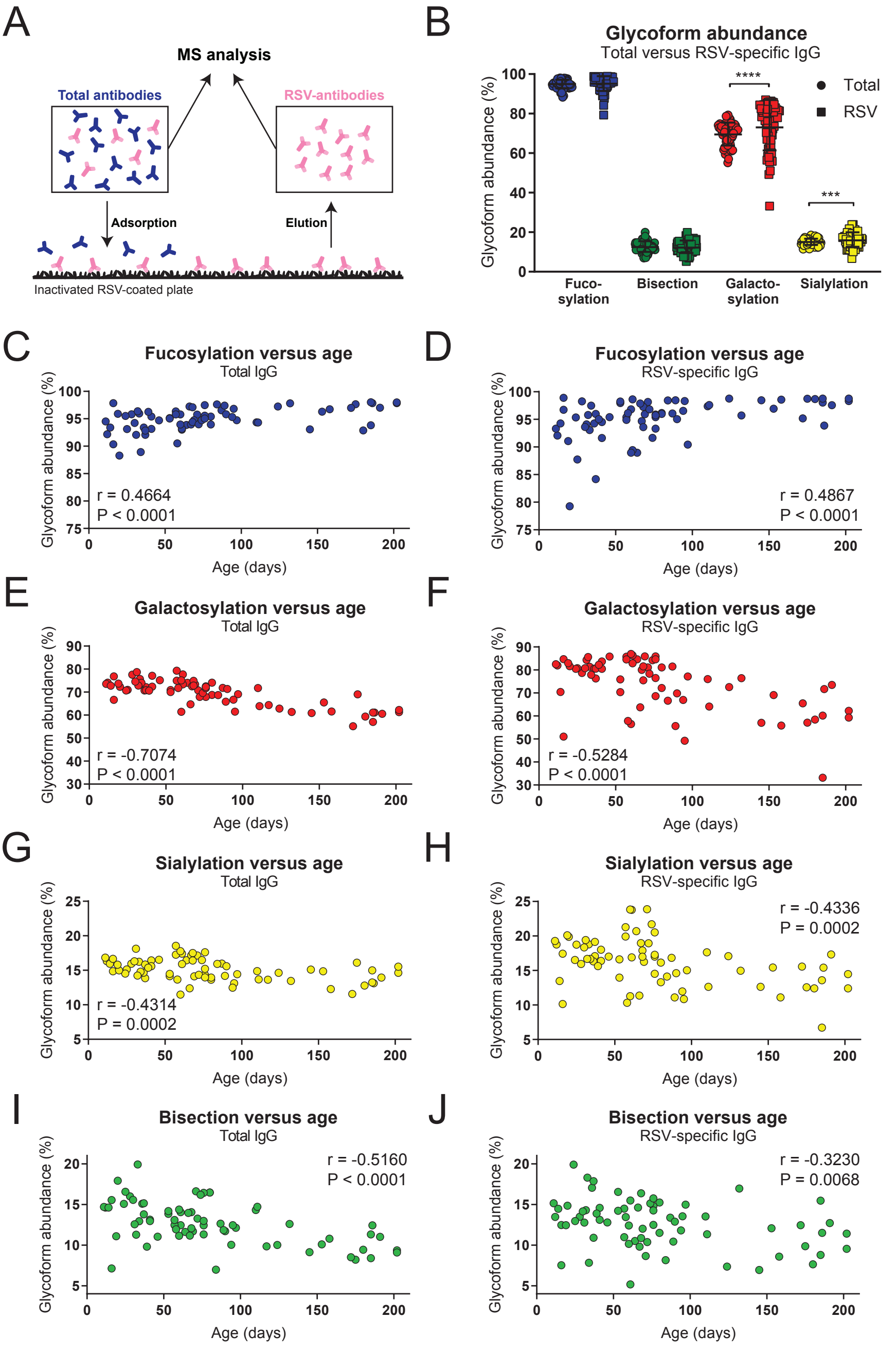

A
MS analysis
Total antibodies
RSV-antibodies
Adsorption
Elution
Inactivated RSV-coated plate
B
Glycoform abundance
Total versus RSV-specific IgG
Glycoform abundance (%)
Total
RSV
****
***
Fuco-sylation
Bisection
Galacto-sylation
Sialylation
C
Fucosylation versus age
Total IgG
r = 0.4664
P < 0.0001
D
Fucosylation versus age
RSV-specific IgG
r = 0.4867
P < 0.0001
E
Galactosylation versus age
Total IgG
r = -0.7074
P < 0.0001
F
Galactosylation versus age
RSV-specific IgG
r = -0.5284
P < 0.0001
G
Sialylation versus age
Total IgG
r = -0.4314
P = 0.0002
H
Sialylation versus age
RSV-specific IgG
r = -0.4336
P = 0.0002
I
Bisection versus age
Total IgG
r = -0.5160
P < 0.0001
J
Bisection versus age
RSV-specific IgG
r = -0.3230
P = 0.0068
Glycoform abundance (%)
Age (days)

Figure 6

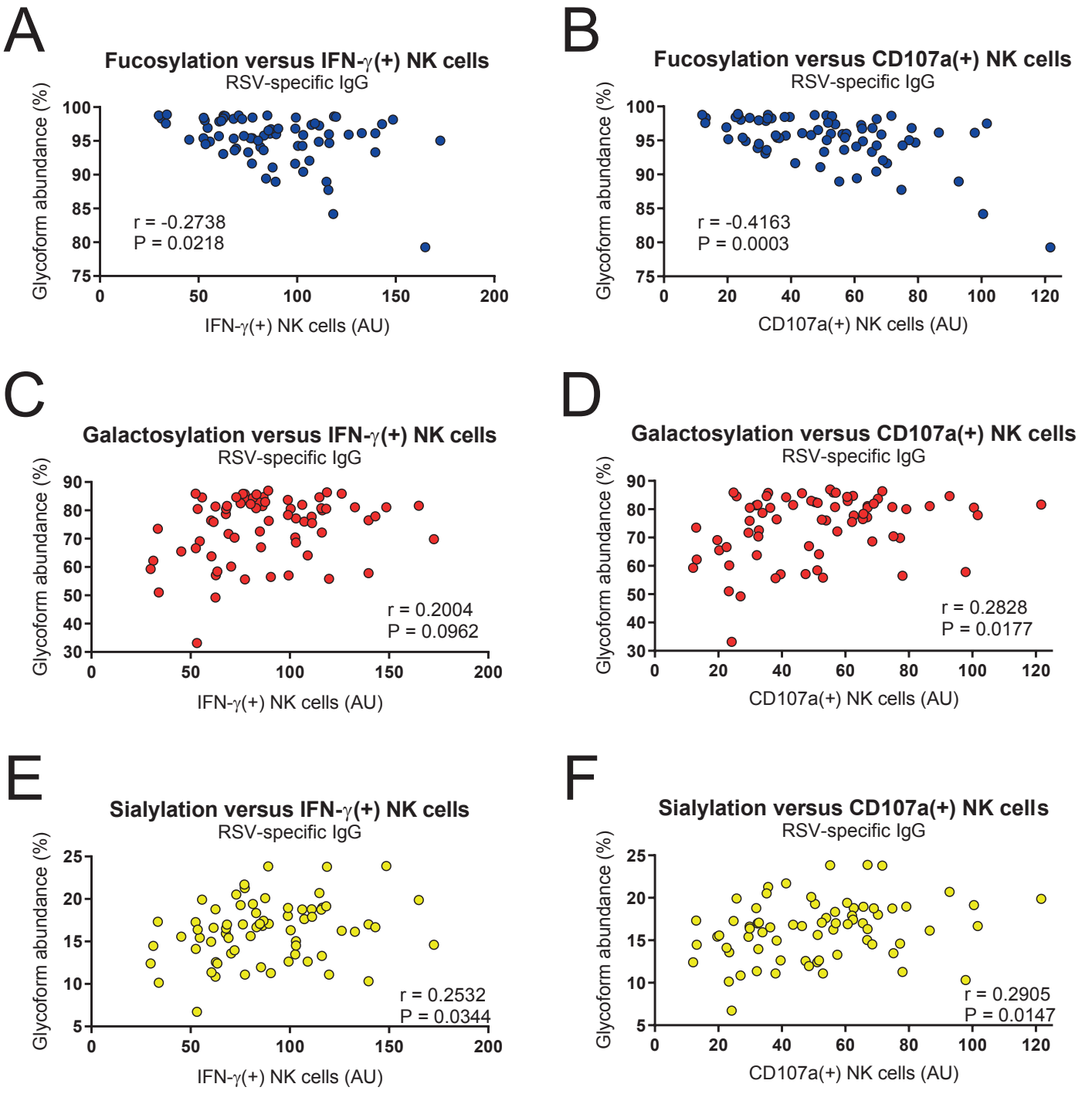
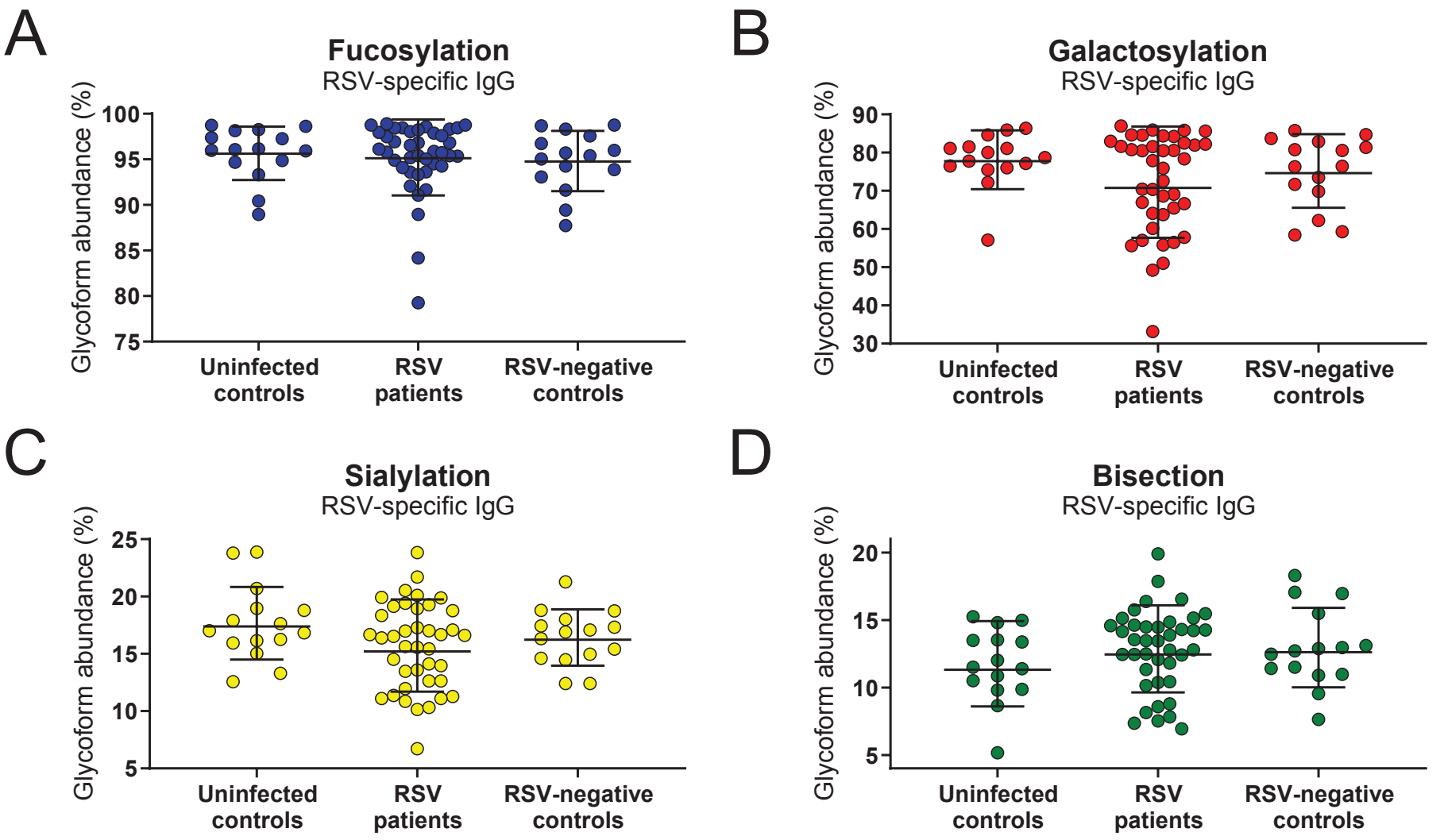
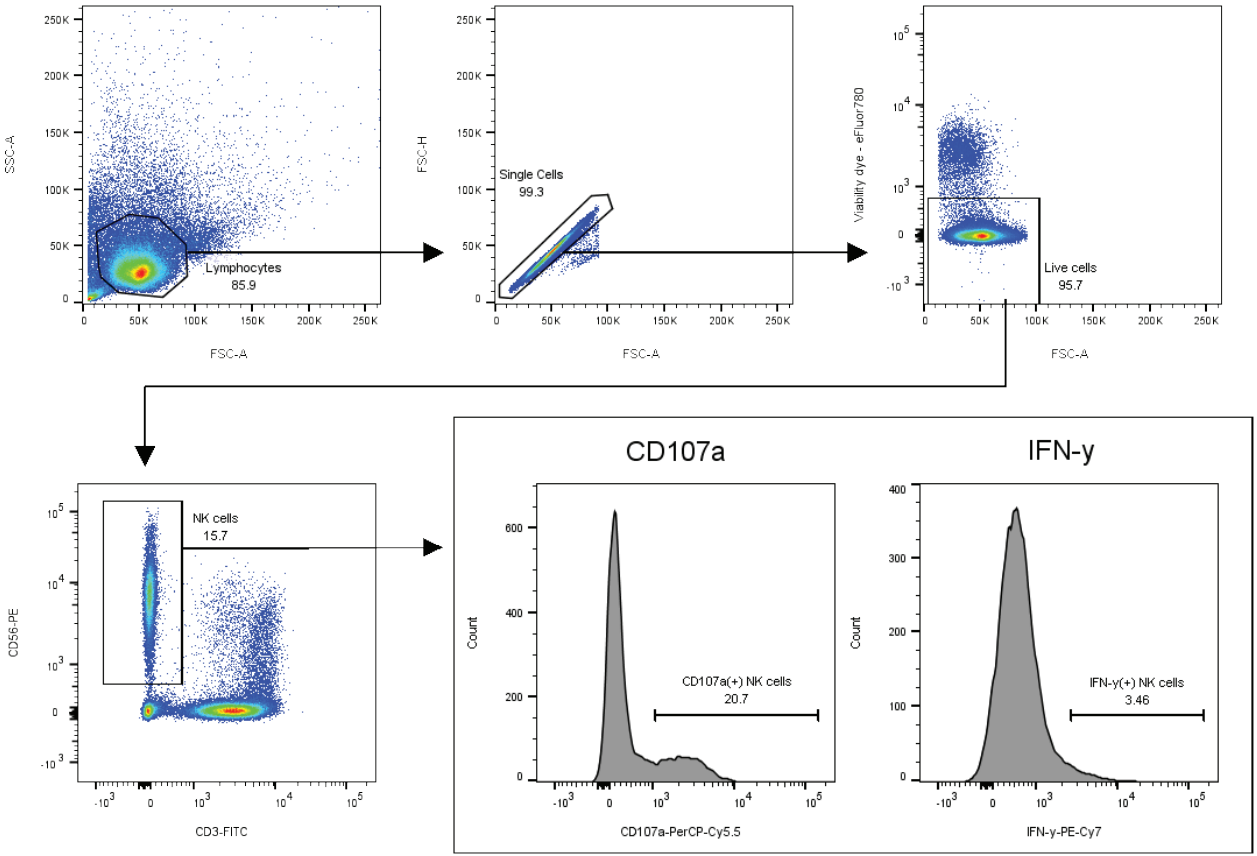
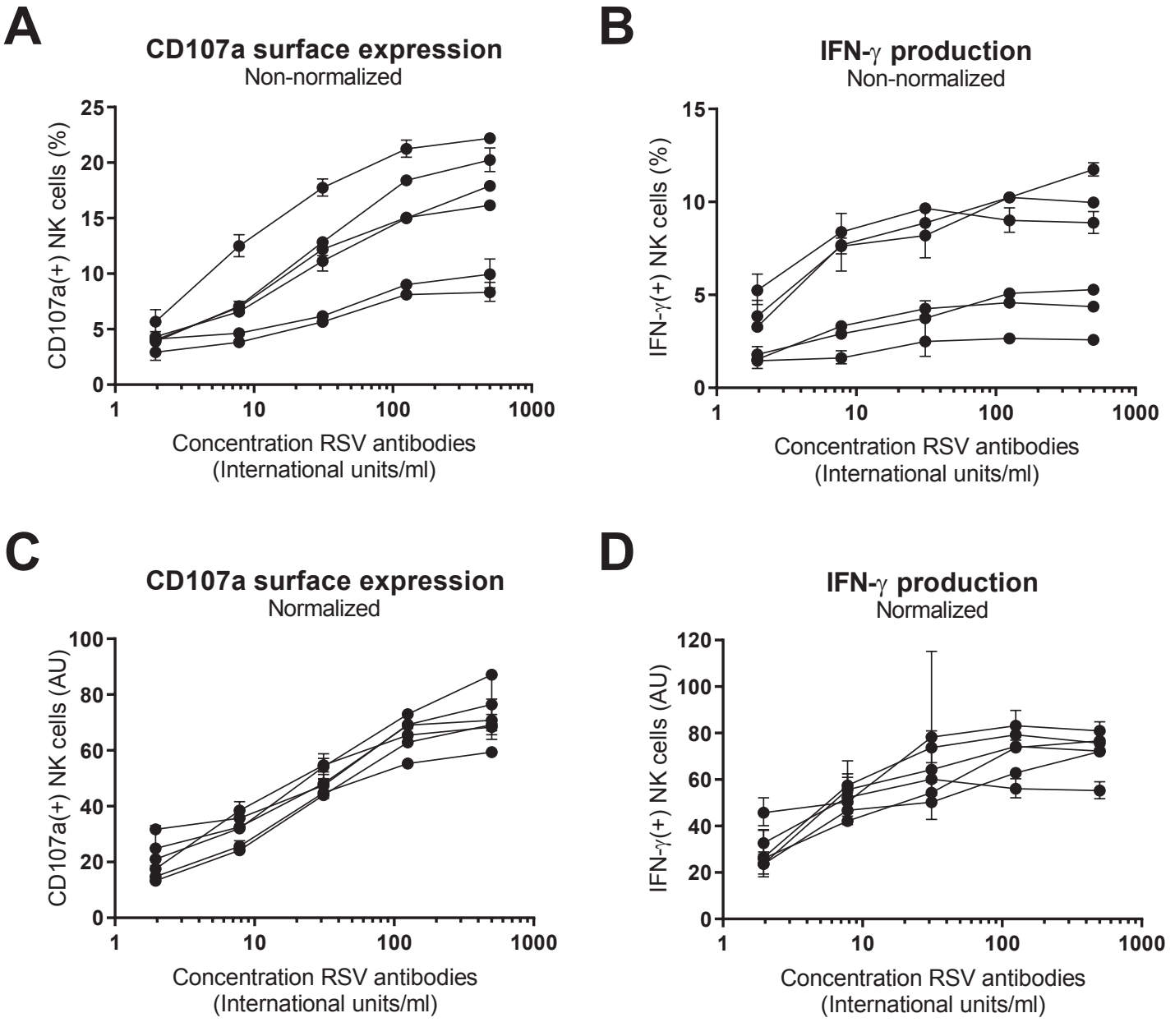

A
Fucosylation versus IFN-γ(+) NK cells
RSV-specific IgG
Glycoform abundance (%)
IFN-γ(+) NK cells (AU)
r = -0.2738
P = 0.0218
B
Fucosylation versus CD107a(+) NK cells
RSV-specific IgG
Glycoform abundance (%)
CD107a(+) NK cells (AU)
r = -0.4163
P = 0.0003
C
Galactosylation versus IFN-γ(+) NK cells
RSV-specific IgG
Glycoform abundance (%)
IFN-γ(+) NK cells (AU)
r = 0.2004
P = 0.0962
D
Galactosylation versus CD107a(+) NK cells
RSV-specific IgG
Glycoform abundance (%)
CD107a(+) NK cells (AU)
r = 0.2828
P = 0.0177
E
Sialylation versus IFN-γ(+) NK cells
RSV-specific IgG
Glycoform abundance (%)
IFN-γ(+) NK cells (AU)
r = 0.2532
P = 0.0344
F
Sialylation versus CD107a(+) NK cells
RSV-specific IgG
Glycoform abundance (%)
CD107a(+) NK cells (AU)
r = 0.2905
P = 0.0147

Figure 7


A
Fucosylation
RSV-specific IgG
Glycoform abundance (%)
100
95
90
85
80
75
Uninfected controls
RSV patients
RSV-negative controls
B
Galactosylation
RSV-specific IgG
Glycoform abundance (%)
90
80
70
60
50
40
30
Uninfected controls
RSV patients
RSV-negative controls
C
Sialylation
RSV-specific IgG
Glycoform abundance (%)
25
20
15
10
5
Uninfected controls
RSV patients
RSV-negative controls
D
Bisection
RSV-specific IgG
Glycoform abundance (%)
20
15
10
5
Uninfected controls
RSV patients
RSV-negative controls

Supplemental Figure 1



Supplemental Figure 2