Neutrophils in respiratory syncytial virus disease
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Citation for published version (APA):
Cortjens, B. (2017). Neutrophils in respiratory syncytial virus disease: Untangling the NET

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Chapter 8

Broadly Reactive anti-RSV G Antibodies from Exposed Individuals Effectively inhibit Infection of Primary Airway Epithelial Cells


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Journal of Virology, 2017 April; 91(10): e02357-16
RSV G protein specific antibodies

Abstract

Respiratory syncytial virus (RSV) causes severe respiratory disease in young children. Antibodies specific for the RSV prefusion F protein have guided RSV vaccine research and in human serum these antibodies attribute to >90% of the neutralization response, however detailed insight in the composition of the human B cell repertoire against RSV is still largely unknown. In order to study the B cell repertoire of 3 healthy donors for specificity against RSV, CD27+ memory B cells were isolated and immortalized using BCL6 and Bcl-xL. Of the circulating memory B cells 0.35% recognized RSV-A2 infected cells, of which 59% were IgA and 41% were IgG expressing cells. When we generated monoclonal B cells selected for high binding to RSV infected cells, 44.5% of IgGs and 56% of IgAs reacted to the F protein, while unexpectedly 41.5% of IgG and 44% of IgA expressing B cells reacted to the G protein. Analysis of the G-specific antibodies revealed that 4 different domains on the G protein were recognized. These epitopes predict cross-reactivity between RSV-A and RSV-B and matched with their potency to neutralize RSV in HEp-2 cells and in primary epithelial cell cultures. G-specific antibodies were also able to induce antibody dependent cellular cytotoxicity and antibody dependent cellular phagocytosis of RSV-A2 infected cells. However, these processes did not seem to dependent on a specific epitope. In conclusion, healthy adults harbor a diverse repertoire of RSV glycoprotein specific antibodies with a broad range of effector functions that likely play an important role in anti-viral immunity.

Importance

Human RSV remains the most common cause of severe lower respiratory tract disease in premature babies, young infants, the elderly and immunocompromised patients, and plays an important role in asthma exacerbations. In developing countries RSV lower respiratory tract disease is a disease with a high mortality. Without an effective vaccine, only passive immunization with palivizumab is approved for prophylactic treatment. However, highly potent RSV specific monoclonal antibodies could potentially serve as a therapeutic treatment and contribute to disease control and mortality reduction. In addition, these antibodies could guide further vaccine development. In this study, we isolated and characterized several novel antibodies directed at the RSV G protein, which can add to our understanding and treatment of RSV disease.
Introduction

Human respiratory syncytial virus (RSV) remains the most common cause of lower respiratory tract disease (LRTD) hospital admissions in the US and RSV-LRTD is accompanied by high mortality in low and middle income countries. Without an approved vaccine, prevention of severe RSV-LRTD has focused on RSV fusion (F) protein specific antibodies, especially since the prefusion F structure has been solved using stabilizing prefusion F specific antibodies. The F protein specific antibody, palivizumab, has been shown to be able to prevent severe disease in high risk infants. However, it is not cost-effective to administer it to all young children and is currently only used in a high-dose, prophylactic manner in high risk infants. Therefore, the search for more potent antibodies continues as effective therapeutic options are still lacking. In this context the role of other RSV surface proteins, such as the G protein, in the prevention of severe disease is less defined and could prove useful in the development of new therapies.

The G protein is produced in two forms: a membrane bound and a soluble form (sG). The latter is composed of the extracellular domain of the G protein and is shed within hours after infection in vitro. Although modified RSV strains lacking G protein are still infectious in vitro, infection in vivo is highly attenuated, underscoring the importance of the G protein. Successful infection in vivo thus seems to depend on the presence of a functional G protein. Compared to the highly conserved F protein, the G protein is highly variable, with low identity (53%) between RSV strain A and B. The extracellular domains (amino acid: 66 – 298) of the sG are even less conserved (44%). Despite this variability, the extracellular domains of sG have one central conserved region between amino acid (aa) 164-176 followed by a region with four conserved cysteine residues (aa 173-186) which form a ‘cysteine noose’ containing a CX3C-motif. This motif is similar to the only known CX3C-chemokine called fractalkine. Tripp and colleagues have shown that the G-protein can influence immune signaling by interaction with the fractalkine-receptor (CX3CR1), a receptor present on leukocytes, and that blocking this interaction abrogated inflammation and viral replication in mice. Recent reports support the hypothesis that CX3CR1 is a cellular receptor for RSV in primary human epithelial cell cultures.

In this study, we evaluated the diversity of the RSV specific B cell repertoire in healthy child day care providers (adults) using a FACS based screening assay. Our aim was to map RSV specific antibody diversity and to search for highly potent neutralizing G protein specific antibodies with immune-modulating properties.
Materials & Methods

Viruses and cells

RSV reference strain, A2 (VR-1540, ATCC, subgroup A), RSV-X (GenBank FJ948820.1, subgroup A, kindly provided by M. Widjojoatmodjo, RIVM, the Netherlands), GFP expressing RSV-A2 (rgRSV224, originally described by Hallak et al.\textsuperscript{17}, was a kind gift from L. Bont, WKZ, Utrecht, the Netherlands) and RSV-B strain (VR-1580, kindly provided by F. Coenjaerts, University of Utrecht, the Netherlands) were grown on HEp-2 cells in Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco) with 8% fetal calf serum and 100 IU/mL penicillin, 100 µg/mL streptomycin.

HAE cell culture

Primary human airway epithelial cells were obtained from patients undergoing an elective lobectomy in the Academic Medical Center, Amsterdam, The Netherlands. Healthy tracheobronchial tissue was obtained from the margin of the surgical resection by a pathologist. The informed consent was obtained from the patient before sampling. The Institutional Review Board of Academic Medical Center approved the study protocol (2015_122#A2301550). Epithelial cells were isolated following a modified Fulcher’s protocol.\textsuperscript{18} Briefly, the tissue was cleaned by removing the excess connective tissue and sliced into 1-2 cm segments. These segments were treated for 48 hours with 0.001% DNase and 0.1% protease in 40 mL MEM (Life Technology) with 0.25 ug/mL AmphotericinB (Sigma), 50 ug/mL Gentamicin (Sigma), 100 U/mL Penicillin (Sigma) and 100 ug/mL Streptomycin (Sigma) at 4°C. Then 4 mL FBS (Thermo Scientific) was added and epithelial cells were gently scraped off with a #10 scalpel blade, followed by washing with DPBS and resuspension in BEGM. The primary epithelial cells are either cultured in VitroCol (Advanced BioMatrix) coated T75 flasks for expansion or in human type IV placental collagen (Sigma) coated porous support Transwell inserts in 24-well plates (Corning). When the cells were confluent, the lower chamber medium was changed into PneumaCult ALI medium (StemCell Technologies) and the upper chamber medium was removed (air-liquid interface). After 31-32 days of culture the cells were fully differentiated and ready for neutralization experiments.

B cell isolation, culture and immortalization

The use of peripheral blood samples was approved by the medical ethical committee of the Academic Medical Center, Amsterdam, The Netherlands (NL.19303.018.07) and were obtained after written informed consent. Memory B cells were isolated from peripheral blood of 3 healthy child day care providers. The B cells were immortalized using BCL6 and BCL-XL, and were sorted in a CD27*IgA+ and CD27*IgG+ population using flow cytometry (FACS ARIA,
The transduced IgA⁺ and IgG⁺ B cell populations were maintained in IMDM with 8% FCS, in the presence of mouse recombinant IL-21 and irradiated CD40L expressing L cells for prolonged periods of time and supernatants of the mini-bulk cultures (MBC, 20 B cells per well) were tested for specificity against RSV-A₂, by binding to intact RSV-A₂ infected HEp-2 cells analyzed by flow cytometry (FACS Canto II, BD Biosciences). The RSV reactive MBCs were single cell cultured and retested for RSV-A₂ antigen binding.

**Sequencing and Cloning of selected antibodies**

A selection of the MBCs, producing antibodies with the highest binding to RSV-A₂ infected cells based on flow cytometry, were selected for further cloning. From total RNA of the selected B cell clones, cDNA was generated and a Variable Heavy (VH) and Light (VL) chain PCR was performed. VH and VL variable regions were sequenced and antibodies of interest were cloned into the pCR2.1 TA cloning vector (Invitrogen). Sequences were checked for reverse transcriptase or DNA polymerase induced mutations before the VH and VL regions were cloned in frame with human IgG₁ and Kappa or Lambda constant domains into pcDNA3.1 (Invitrogen). For protein production, the vectors were transiently transfected into 293 cells. Antibodies were subsequently purified using HiTrap Protein A or G columns on an ÄKTA instrument (GE healthcare). Accession numbers are deposited in GenBank (IgGs no. KY249684 up to and including KY249697 and IgAs no. KY249698 up to and including KY249704).

**Antibody specificity**

The RSV reactive clones were screened for binding to RSV surface proteins: F, G or SH protein by flow cytometry on intact transfected 293 cells. The 293 cells were transfected with a construct expressing the RSV-A₂ F protein, G protein (RSV-X derived) or SH protein (PCAGGS SH-RSV-A containing construct; kind gift of Dr. X. Saelens, University of Gent, Belgium). In a separate experiment all antibodies were tested for cross reactivity to RSV-B strain. The RSV specific antibody panel was added to the transfected cells and binding was detected with a PE-labeled goat anti-human F(ab’)₂ antibody (Southern Biotech). The data were analyzed using FlowJo™ software (FlowJo LLC).

**Surface plasmon resonance analysis**

Surface plasmon resonance (SPR) analysis was performed on an IBIS Mx96 instrument (IBIS technologies). Purified antibodies were chemically immobilized on amine-functionalized chips (Ssens technologies) as described before.²⁰ Epitope mapping was done by immobilizing biotinylated peptides on a streptavidin-coated SPR chip in a CFM spotter (Wasatch Microfluidics), using a 2.0 µM peptide solution diluted in capture buffer (PBS, 0.05 % Tween20 and 0.1 % sodium azide). Before inject-
ing samples, chips were extensively washed with capture buffer. All injected samples were prepared in capture buffer containing 0.01% HSA. During the course of the experiments, the temperature in the IBIS flow-cell was kept at 25 °C. Data was processed with SprintX software (IBIS Technologies). Kinetic constants were fitted to the binding curves, by making a global fit to all curves using a 1:1 binding model, with Scrubber2 software (BioLogic).

Murine antibody 131-2G was purchased from EMD Millipore. For binding to purified RSV G protein we used the extracellular domain of strain A2: aa 66-297 (11070-V08H), and B: aa 67-299 (13029-V08H) from Sino Biological and a library of N-terminally biotinylated 12-mer peptides, comprising amino acids 150-200 of the RSV-A2 G protein (NKI, Dutch Cancer Institute, Amsterdam).

Complement dependent and independent RSV neutralization

Complement dependent neutralization (CDN) was performed in vitro by using a similar method described by Zielinska et al.21 In short, 25 PFU/well of RSV-A2 was mixed and pre-incubated with G-specific antibodies (from B cell culture supernatants) for 60 min at 37°C, with 10% rabbit complement-serum (Sigma-Aldrich) and added to HEp-2 cells. After 48 hrs of culture at 37°C the monolayers were fixed with 1:1 acetone/methanol. Infected cells were visualized by incubating the samples overnight with polyclonal goat RSV-specific AlexaFluor-647-labeled antibody (Biodesign). Fluorescent images were acquired and analyzed using the Operetta (PerkinElmer). Complement independent neutralization on primary human epithelial cell (HAE) cultures was done in a similar fashion by incubating 50 PFU/well of RSV-GFP with the G-specific antibody panel (final concentration of 20 and 0.2 µg/mL) for 60 min at 37°C, after which the inoculum was added to HAE cultures for 30 min at 37°C. Next, the inoculum was removed from the surface of the HAE cultures and GFP expression was evaluated after 30 hours of infection, using the Operetta (PerkinElmer). Membranes were fixed in 4% PFA for 30 min. Confocal microscopy (LEICA SP-8 X) was performed on whole membranes stained with: anti-β-tubulin (Sigma), MUC5B (Santa Cruz) and Hoechst 33342 (Sigma) overnight at 4°C. Other membranes were embedded in paraffin and 5 µm thick sections were cut. Immunohistochemic staining of CX3CR1 on HAE cultures was performed as described before22, with an anti-CX3CR1 antibody (eBioscience, kind gift of C. Verseijden, Tytgat institute, Amsterdam, the Netherlands).

Antibody-dependent cellular cytotoxicity assay

We determined antibody-dependent cellular cytotoxicity (ADCC) as described before.23 Briefly, PBMCs were thawed and rested overnight in IMDM with 8% FCS at 37°C. Serial dilutions of the G-specific antibody panel were added to 1.25 × 10⁴ Calcein AM (Corning) labeled RSV-A2 infected cells in 96-well plates, then PBMCs (5 × 10⁵ cells) were added in a ratio of 40 PBMCs
per 1 RSV infected HEp-2 cell, and incubated for 4 hours at 37°C. The cells were washed once with PBS 1 mM EDTA, detached with PBS 0.5% Trypsin and transferred to a CellCarrier Spheroid ULA 96-well plate (PerkinElmer). DAPI and Accudrop beads (BD Biosciences) were added and the cells were analyzed by flow cytometry (LRS Fortessa, BD Biosciences). The stopping gate was set to 5000 beads per sample. The number of remaining RSV-A2 infected HEp-2 cells in each condition was related to the number of RSV-A2 infected HEp-2 cells in the control wells without antibody.

**Antibody-dependent cellular phagocytosis assay**

RSV-A2 infected HEp-2 cells were green fluorescent labeled with the PKH kit (Sigma-Aldrich). Next, 2 x 10⁴ RSV-A2 infected HEp-2 cells were added in a 96-well plate containing serial dilutions of the G-specific antibody panel, with AT12-009 (hepatitis C virus E2 specific antibody, IgG1) as a negative control for 1 hour at 37°C. Followed by adding 2 x 10⁴ Ficoll isolated human blood neutrophils. The plates were incubated overnight at 37°C. The next day the neutrophils were stained with DAPI and CD45-PE-Cy7 (BD Biosciences) for 30 min on ice and the percentage FITC positive neutrophils (Viable/CD45 positive) was calculated.
Results

Isolation and characterization of RSV specific antibodies

The frequency of RSV specific memory B cells was determined in the CD27^+IgG^+ and CD27^+IgA^+ memory B cell fraction of the child day care providers. After immortalization of the B cells with BCL6 and Bcl-xL, the potency of antibodies present in culture supernatant to bind RSV-A2 infected HEp-2 cells was tested by flow cytometry. From the total number of IgG^+ (±57,000 cells) and IgA^+ (±54,000 cells) memory B cells screened, 208 cultures were producing IgG and 185 cultures produced IgA specific for RSV infected cells (table 1). In these child day care providers who most likely encounter RSV regularly, the frequency of RSV-A2 specific B cells was thus approximately 1 in 282. In two donors we could compare the immunoglobulin isotype distribution of RSV specific antibodies. As shown in table 1, circulating IgA^+ memory B cells were dominating the RSV response compared to IgG^+ memory B cells (59% vs. 41%, respectively).

We selected the most potent RSV-A2 binders of the IgA (9 clones) and IgG MBCs (65 clones) and determined their reactivity against RSV surface proteins (F, G and SH), which was divided between reactivity against F protein and G protein (Fig. 1) while no antibodies specific for RSV SH protein were found. Of nine IgG antibodies (14%, 9/65, fig. 1B) we were unable to determine the antigen specificity. Since they specifically bind RSV infected cells, the antigen will probably be one of the other RSV proteins.

![Figure 1](image_url)

Figure 1: Specificity of selected RSV reactive antibodies.

(A) Distribution of selected IgA^+ B cells (N = 9) specific for G (dark grey) or F protein (light grey)  (B) Distribution of selected IgG^+ B cells (N = 65) specific for G- (dark grey), F-protein (light grey), SH-protein (black) or directed against an unknown antigen (white).
Table 1: RSV-A2-specific antibody repertoire and repertoire of strong RSV-A2-binding clones

<table>
<thead>
<tr>
<th>Donor</th>
<th>B cells screened</th>
<th>RSV-A2 reactive MBCs(^a) (%)</th>
<th>IgA+ / IgG+ (%)</th>
<th>No. of clones selected</th>
<th>IgA+ / IgG+ (G/F/unknown)</th>
<th>IgA+ Reactive to G/F/unknown</th>
<th>IgG+ Reactive to G/F/unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46,000</td>
<td>153 (0.33%)</td>
<td>94 (61%) / 59 (39%)</td>
<td>21</td>
<td>6 / 15</td>
<td>3 / 3 / 0</td>
<td>6 / 5 / 4</td>
</tr>
<tr>
<td>2</td>
<td>46,000</td>
<td>161 (0.35%)</td>
<td>91 (57%) / 70 (43%)</td>
<td>19</td>
<td>3 / 16</td>
<td>1 / 2 / 0</td>
<td>4 / 9 / 3</td>
</tr>
<tr>
<td>3</td>
<td>19,000</td>
<td>79 (0.42%)</td>
<td>ND / 79 (100%)</td>
<td>34</td>
<td>ND / 34</td>
<td>ND</td>
<td>17 / 15 / 2</td>
</tr>
<tr>
<td>Total</td>
<td>111,000</td>
<td>393 (0.35%)</td>
<td>185 / 208</td>
<td>74</td>
<td>9 / 65</td>
<td>4 / 5 / 0</td>
<td>27 / 29 / 9</td>
</tr>
</tbody>
</table>

\(^a\) Clones were selected by their binding to RSV-A2-infected intact cells.  
\(^b\) MBC, mini-bulk culture (20 cells per well).  
\(^c\) ND, not determined
Since RSV G-specific antibodies derived from humans have been less well studied compared to F-specific antibodies, we chose to focus on G-specific antibodies in our study. We selected 7 G-specific IgG⁺ B cell clones, based on binding response to RSV-A2 infected cells by flow cytometry, of which six were IgG₁, and one was of the IgG₂ subclass. All antibodies showed high levels of somatic hypermutation in the VH and VL region (average 15.1 and 7.6 mutations, respectively), indicative of antigen selection (table 2). Regarding VH usage, it has been described that for infectious disease targets such as the influenza hemagglutinin stem region²⁴, RSV²⁵, and HCV²⁶, the VH1-69 family is predominantly used. In this study however, we did not observe this, since only 1 of the 7 selected clones used VH1-69 (table 2).

From the RSV-specific IgA repertoire we selected the B cell clones that were the strongest binders to intact RSV-A2 infected cells, measured by flow cytometry. We selected 9 IgA⁺ B cell clones. From these 9 antibodies, 2 were of the IgA₂ subclass, 7 were IgA₁ (table 2) and only AT62 used a kappa light chain. Of the IgA antibodies, 5 were specific for the RSV F protein and 4 were specific for RSV G protein. Compared to the IgG clones, the majority of the IgA clones have an amino acid composition matching more closely to germline sequences. Although IgAs can activate complement via the mannose-binding lectin pathway²⁷, none of the IgA antibodies neutralized, irrespective whether complement was added or not (data not shown). Because IgA antibodies lack potent neutralizing capacity we choose to focus on IgG G-specific antibodies in the subsequent functional experiments. However, to determine whether absence of neutralization of IgA G-specific antibodies was due to the IgA isotype, we re-cloned two IgA antibodies (AT61G and AT64G) into an IgG background.

**G-specific antibody affinity**

We determined the affinity of our G-specific IgG antibody panel by surface plasmon resonance (SPR) analysis. A chip was coated with the G-specific antibodies, and full-length secreted G protein was injected over the chip. Corresponding kinetic parameters of antibody-antigen binding ($k_a$, $k_d$, and $K_D$) are shown in table 3. AT33, AT64G and AT34 showed the highest affinity to the RSV-A2 G protein indicated with low KD’s of 11.1, 14.7 and 12.8 pM. From the G-specific antibodies, four (AT34, AT40, AT42, and AT64G) recognize both the RSV-A2 and RSV-B G-protein. Except for AT42, affinity of the RSV A/B cross-reactive antibodies was higher for the RSV-A2 G protein then for the RSV-B G protein (table 3). 131-2G, a mouse G-specific antibody displayed low affinity for both RSV-A and RSV-B G protein (3640 and 1420 pM respectively, table 3).
### Table 2: mAb gene segment analysis of IgG and IgA RSV G-specific antibodies

<table>
<thead>
<tr>
<th>Clone</th>
<th>Isotype</th>
<th>V</th>
<th>D</th>
<th>J</th>
<th>CDR3</th>
<th>λ/κ</th>
<th>V</th>
<th>J</th>
<th>CDR3</th>
<th>H-chain</th>
<th>L-chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT32</td>
<td>IgG1</td>
<td>1-24*01</td>
<td>6-13*01 (1)</td>
<td>4*02</td>
<td>CAAEAR YCDNSR CSPNFDHW</td>
<td>κ</td>
<td>KV4-1*01</td>
<td>KJ5*01</td>
<td>CQQYYDPLTF</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>AT33</td>
<td>IgG1</td>
<td>1-69*01</td>
<td>5-24*01 (3)</td>
<td>4*02</td>
<td>CARDAEWAAG SDYFFDYW</td>
<td>λ</td>
<td>LV3-25*03</td>
<td>LJ2*01</td>
<td>CQSTDTSPLF</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>AT34</td>
<td>IgG1</td>
<td>3-30*03</td>
<td>3-22*01 (2)</td>
<td>6*02</td>
<td>CASQGAKGQH ELSFYCALDVW</td>
<td>κ</td>
<td>KV1-5*03</td>
<td>KJ1*01</td>
<td>CQQYNSHTWF</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>AT40</td>
<td>IgG1</td>
<td>3-30-3*01</td>
<td>4-17*01 (2)</td>
<td>4*02</td>
<td>CARGRALDFA FYG GYYFDYW</td>
<td>κ</td>
<td>KV1-12*01</td>
<td>KJ3*01</td>
<td>CQQANTFPFTF</td>
<td>24</td>
<td>8</td>
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<tr>
<td>AT42</td>
<td>IgG2</td>
<td>4-39*09</td>
<td>2-21*01 (2)</td>
<td>4*02</td>
<td>CARHWAGLY FDSW</td>
<td>κ</td>
<td>KV3-20*01</td>
<td>KJ4*01</td>
<td>CQYGDSPGSF</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>AT50</td>
<td>IgG1</td>
<td>1-18*01</td>
<td>5-24*01 (1)</td>
<td>6*02</td>
<td>CARGGAQEMVRH JYGGMDVW</td>
<td>λ</td>
<td>KV1-9*01</td>
<td>KJ3*01</td>
<td>CQLNTPFLTF</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>AT51</td>
<td>IgG1</td>
<td>1-18*01</td>
<td>3-3*01 (2)</td>
<td>4*02</td>
<td>CARPASYDDLRS GYLNCDYW</td>
<td>κ</td>
<td>KV1-9*01</td>
<td>KJ4*01</td>
<td>CQFHTYPLTF</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>AT61</td>
<td>IgA1</td>
<td>3-33*01</td>
<td>3-22*01 (2)</td>
<td>4*02</td>
<td>CAR DGYDSSG YPPYW</td>
<td>λ</td>
<td>LV3-21*01</td>
<td>LJ3*02</td>
<td>CQVWDSSDHVF</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>AT62</td>
<td>IgA1</td>
<td>3-30*03</td>
<td>2-8*01(2)</td>
<td>4*02</td>
<td>CATDPPKFY DIDLDPVPW</td>
<td>κ</td>
<td>KV2-24*01</td>
<td>KJ4*01</td>
<td>CMQATHLLTF</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>AT63</td>
<td>IgA1</td>
<td>4-31*03</td>
<td>3-10*01 (1)</td>
<td>2*01</td>
<td>CAREVLFWVG E QNPWYFDLW</td>
<td>λ</td>
<td>LV7-43*01</td>
<td>LJ2*01</td>
<td>CIFYFGNRVF</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td>AT64</td>
<td>IgA1</td>
<td>4-4*02</td>
<td>3-10*01 (3)</td>
<td>3*01</td>
<td>CARSKRTVPVA KGGGADW</td>
<td>λ</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>15</td>
<td>9</td>
</tr>
</tbody>
</table>

* Displayed is the immunoglobulin buildup separated in V-D-J sections of the heavy and light chains, including somatic hypermutation. ND, not determined.
RSV G-specific antibody epitope mapping

The fact that not all antibodies in our panel have a similar specificity and affinity indicates they recognize different epitopes. To determine the epitope of the G-specific antibodies we used a library of short peptide fragments (12aa) of the RSV-A2 strain (aa 164-199, peptides shifted by 1 residue), which were immobilized on a SPR chip. Antibody mapping revealed four different binding domains on the G protein (Fig. 2). One antibody, AT42, did not recognize any of the peptides and did not react with denatured G protein in western blot (data not shown). However, this antibody did bind to G protein transfected cells and to G protein in ELISA and SPR. These results suggest a conformational epitope that cannot be reproduced in a 12 amino acid peptide. We have named this conformational epitope, epitope A. The 3 other regions consisted of linear epitopes surrounding the CX3C binding domain (aa 169-191, fig. 2). Epitope B consists of the amino acid sequence NDFHFEVFNF and is recognized by AT34, AT40, and AT64G but also by well-known antibodies like 131-2G and 3D3. The epitope B sequence is highly conserved between RSV strains, which explains why antibodies specific for epitope B recognize RSV strain A and B (Fig. 2 and table 3). A third epitope (epitope C), which is recognized by AT50, AT51, and AT61G overlaps with the last 2 cysteine residues that flank the cysteine noose region (Fig. 2). Yet another epitope, epitope D, is located just outside the cysteine noose region. This epitope is specifically recognized by AT32 and AT33. Antibodies specific for epitope C and D only recognize RSV-A (table 3).

Figure 2: Epitope mapping of RSV G-specific antibodies.

(A) An SPR array of 12 amino acids long peptides, partially overlapping, was used to determine the binding epitope of G-specific antibodies. Lines depict binding response (in mdeg) to the corresponding peptide numbers. Three linear epitopes can be distinguished: epitope B (131-2G, AT34, AT40, AT64G), epitope C (AT50, AT51, AT61G) and epitope D (AT32, AT33). (B) Amino acid sequence of the RSV-A2 G protein, indicating the binding epitope of each antibody.
### Table 3: Kinetic constants for binding RSV-A2 G protein

<table>
<thead>
<tr>
<th>Strain and epitope</th>
<th>Antibody</th>
<th>$k_a$ (s$^{-1} \cdot M^{-1}$)</th>
<th>$k_d$ (s$^{-1}$ [10$^5$])</th>
<th>$K_D$ (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV-A2</td>
<td>AT42</td>
<td>79.8 ± 3.3</td>
<td>65.0 ± 13.2</td>
<td>809 ± 132</td>
</tr>
<tr>
<td></td>
<td>AT34</td>
<td>31.3 ± 4.6</td>
<td>0.4 ± 0.2</td>
<td>12.8 ± 6.9</td>
</tr>
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<td></td>
<td>AT40</td>
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<td>5.8 ± 0.4</td>
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<tr>
<td></td>
<td>AT64G</td>
<td>35.7 ± 6.2</td>
<td>0.5 ± 0.2</td>
<td>14.7 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>131-2G</td>
<td>28.5 ± 2.0</td>
<td>103 ± 2</td>
<td>3640 ± 320</td>
</tr>
<tr>
<td>C</td>
<td>AT50</td>
<td>21.8 ± 2.5</td>
<td>35.1 ± 7.7</td>
<td>1610 ± 340</td>
</tr>
<tr>
<td></td>
<td>AT51</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AT61G</td>
<td>18.9 ± 4.0</td>
<td>200 ± 40</td>
<td>10800 ± 3000</td>
</tr>
<tr>
<td>D</td>
<td>AT32</td>
<td>42.5 ± 8.3</td>
<td>18.2 ± 2.3</td>
<td>459 ± 122</td>
</tr>
<tr>
<td></td>
<td>AT33</td>
<td>73.5 ± 5.2</td>
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</tr>
<tr>
<td>RSV-B</td>
<td>AT42</td>
<td>22.2 ± 1.2</td>
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<td>208 ± 33</td>
</tr>
<tr>
<td></td>
<td>AT34</td>
<td>3.76 ± 0.42</td>
<td>2.7 ± 0.7</td>
<td>719 ± 174</td>
</tr>
<tr>
<td></td>
<td>AT40</td>
<td>5.99 ± 0.50</td>
<td>2.3 ± 0.4</td>
<td>379 ± 45</td>
</tr>
<tr>
<td></td>
<td>AT64G</td>
<td>6.33 ± 0.99</td>
<td>4.8 ± 1.1</td>
<td>776 ± 169</td>
</tr>
<tr>
<td></td>
<td>131-2G</td>
<td>111 ± 5</td>
<td>159 ± 20</td>
<td>1420 ± 120</td>
</tr>
<tr>
<td>C</td>
<td>AT50</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td></td>
<td>AT51</td>
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<tr>
<td></td>
<td>AT33</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Data represent mean ± standard deviations from at least two independent measurements. -, no binding detected.

AT51 did not bind in this setup (the immobilized antibody is inactive).

### Strain specific neutralization of G-specific antibodies correlates with epitope specificity

Antibody efficacy is determined by multiple variables ranging from antibody and target glycosylation, binding on and off rate, epitope and the angle the target is approached by the antibody. Here, we hypothesize that for the G-specific antibodies the neutralization potency may be determined by the binding site on the G protein. Therefore, we performed in vitro complement dependent neutralization (CDN). The seven selected IgGs and the two IgA—IgG G-specific antibodies were tested (Fig. 3 and table 4). Antibodies binding epitope A, B and D all neutralized in the presence of complement on HEp-2 cells (Fig. 3). There were differences in the 50% inhibitory concentration ($IC_{50}$, table 4) between the selected antibodies, with AT32 being the most potent in the presence of complement ($IC_{50}$: 14.7 ng/mL, table 4) and AT33 being the least potent ($IC_{50}$: 249 ng/mL, table 4). Both the IgG1 antibodies and the IgG2 antibody neutralized RSV in the presence of complement when purified directly from B cell supernatant. On the contrary, antibodies specific for epitope C did not neutralize in the pres-
ence of complement, this included the IgA antibody (AT61G) that was re-cloned onto an IgG backbone and was specific for epitope C. However, the AT64G antibody, which originated from an IgA epitope B specific antibody, did neutralize although neutralization never reached > 70% (Fig. 3, IC$_{50}$ mean + 95% CI: 109 ng/mL; 36.3 - 329 ng/mL). The RSV-B reactive antibodies (AT34, AT40, AT42, AT64G) were also tested for neutralization of RSV-B infected HEp-2 cells. Neutralization with complement resulted in even lower IC$_{50}$ values compared to RSV-A neutralization (Table 4, fig. 3B). Together these results may suggest that the binding domain of the antibodies on the G protein contributes to the complement-induced neutralization potency. None of the G-specific antibodies neutralized RSV-A or RSV-B for >50% in the absence of complement in the HEp-2 cell assay, whereas the prefusion F specific AM22 did potently neutralize (Fig. 3C-D).

![Figure 3](image3.png)

**Figure 3:** Complement dependent and independent RSV neutralization by G-specific antibodies.

In vitro neutralization of RSV-A2 or RSV-B on HEp-2 cells, by increasing concentration of antibodies specific for RSV G protein with 10% rabbit complement (A and B) and without complement (C and D). AT12-009, a HCV E2 specific antibody was used as negative control, AM22 is a prefusion F protein specific antibody. All experiments were performed twice in triplicate.
RSV G protein specific antibodies

Because HEp-2 cells probably lack the RSV-binding receptor CX3CR1 and infection depends on the surrogate receptors (e.g. heparan sulfates), we tested complement independent neutralization on primary human airway epithelial (HAE) cells that do express CX3CR1. After, we confirmed the expression of CX3CR1 in our HAE culture system on ciliated cells but not goblet cells (Fig. 4A-B), we then evaluated RSV-A2 infection and found infection of only apical ciliated cells (Fig. 4C-E). We found complete neutralization of all the G-specific antibodies at 20 µg/mL, including 131-2G (Fig. 5), and partial neutralization at 0.2 µg/mL for antibodies binding epitope A (AT42: 69%) and C (AT50: 92% and AT51: 68%, fig. 5). Antibodies binding epitope B and D did not neutralize at low concentrations (Fig. 5). In contrast to the mechanism of complement-induced neutralization, which probably relies on destroying virus particles; neutralization on HAE cells is likely dependent on blocking the G protein-CX3CR1 interaction. Consistent with this idea, epitope C specific antibodies show potent neutralization since they bind an epitope very close to the CX3CR1 binding domain on the G protein (Fig. 1). Altogether these findings suggest that antibodies against different epitopes on the G protein exhibit different neutralization potencies in the absence of complement.

Table 4: CDN, ADCC, and ADCP

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Clone</th>
<th>Strain(s) specificity</th>
<th>K_D (pM) for G protein from: RSV-A</th>
<th>RSV-B</th>
<th>IC_{50} (ng/mL) for RSV A/B</th>
<th>% neutralization of HAE cells in culture at 0.2 µg/mL</th>
<th>EC_{50} (ng/mL)</th>
<th>ADCC</th>
<th>ADCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>AT42</td>
<td>A/B</td>
<td>809 ± 132</td>
<td>208 ± 33</td>
<td>90.3 / 11.1</td>
<td>69 ± 7</td>
<td>-</td>
<td>609</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>AT34</td>
<td>A/B</td>
<td>12.8 ± 6.9</td>
<td>719 ± 174</td>
<td>158 / 37.2</td>
<td>1 ± 0</td>
<td>1352</td>
<td>304</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>AT40</td>
<td>A/B</td>
<td>106 ± 29</td>
<td>379 ± 45</td>
<td>30.6 / 15.1</td>
<td>7 ± 2</td>
<td>456</td>
<td>336</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>AT64G</td>
<td>A/B</td>
<td>14.7 ± 2.8</td>
<td>776 ± 169</td>
<td>109 / 39.9</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>131-2G</td>
<td>A/B</td>
<td>3640 ± 320</td>
<td>1420 ± 120</td>
<td>-</td>
<td>92 ± 11</td>
<td>ND</td>
<td>ND</td>
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<td>C</td>
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<td>1610 ± 340</td>
<td>-</td>
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<td>92 ± 4</td>
<td>791</td>
<td>798</td>
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<tr>
<td>C</td>
<td>AT51</td>
<td>A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>68 ± 13</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>C</td>
<td>AT61G</td>
<td>A</td>
<td>10800 ± 3000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>D</td>
<td>AT32</td>
<td>A</td>
<td>459 ± 122</td>
<td>-</td>
<td>14.7 / -</td>
<td>23 ± 33</td>
<td>3866</td>
<td>265</td>
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<tr>
<td>D</td>
<td>AT33</td>
<td>A</td>
<td>11.1 ± 4.8</td>
<td>-</td>
<td>249 / -</td>
<td>41 ± 21</td>
<td>-</td>
<td>279</td>
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</tr>
<tr>
<td>-</td>
<td>AM22</td>
<td>A/B</td>
<td>-</td>
<td>2.6 / 41.1</td>
<td>99 ± 2</td>
<td>222 / 119</td>
<td>-</td>
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</table>

* Neutralization and opsonization experiments were performed with antibodies purified from B cell supernatants. Data represent mean ± standard deviations from at least three independent measurements. IC_{50}, 50% Inhibitory concentration, the concentration of antibody which inhibits 50% of RSV-plaque formation in vitro. EC_{50}, 50% effective concentration, the concentration of antibody which results in 50% of neutrophils with phagocytized FITC labeled RSV-A2 infected HEp-2 cells or 50% ADCC. - , no binding to RSV G protein detected in SPR experiment or < 50% neutralization, ADCC or phagocytosis was measured. ND, not determined. AM22, antibody specific for the RSV pre-fusion F protein.
Figure 4: RSV-A2 infection of primary human airway epithelial cell cultures.

(A) Immuno-histochemistry staining of CX3CR1 (5 µg/mL, indicated by red staining) on HAE membrane. Goblet cells are marked using an asterisks. (B) IgG isotype control staining (5 µg/mL). (C) Top and side views of RSV-A2 infected HAE cells, showing RSV infected cells in green (GFP), goblet cells in yellow (MUC5B) and nuclei in blue (Hoechst 33342). (D) Top and side views of RSV-A2 infected HAE cells, showing RSV infected cells in green (GFP), cilia in red (β-tubulin) and nuclei in blue (Hoechst 33342).
Figure 5: Complement independent RSV neutralization by G-specific antibodies on primary epithelial cell cultures.

(A) Representative images of RSV-A2-GFP infected HAE cultures 30 hours after infection. RSV infection indicated by green GFP positive cells in the left panel, in the middle panel after pre-incubation with AT12-009 (negative control) or after pre-incubation with 131-2G (positive control), which resulted in 100% neutralization (i.e. no RSV infected cells). (B) Quantification of percentage RSV neutralization using 20 µg/mL (grey bars) and 0.2 µg/mL (black bars) of the G-specific antibodies. All G-specific antibodies neutralize at 20 µg/mL. Antibodies specific for epitope A and C still neutralize at 0.2 µg/mL. Data are expressed as mean ± SEM of two experiments in duplo. Data was generated using an Operetta (PerkinElmer) and analyzed using Columbus software (PerkinElmer).
RSV G-specific antibodies facilitate ADCC and ADCP

Antibodies can, besides direct neutralization, also facilitate the destruction of infected cells. For example, by antibody-dependent cellular cytotoxicity (ADCC), where antibodies bind to infected cells and flag natural killer (NK) cells to lyse the infected cells. The efficacy of ADCC induction depends on antibody subclass (IgG1 > IgG2, table 2). We determined the capacity of the G-specific antibodies to induce ADCC using RSV-A2 infected HEp-2 cells and human PBMCs. All G-specific antibodies induced ADCC albeit with different potency (Fig. 6A). Antibodies directed against epitope B (AT34: EC\textsubscript{50} 1352 ng/mL and AT40: EC\textsubscript{50} 456 ng/mL) and AT50 were the most potent inducers of ADCC (AT50: EC\textsubscript{50} 791 ng/mL), while AT40 was equally potent compared to AM22 at low antibody concentrations (Fig. 6A). AT42 (epitope A), AT33 (epitope D) and AT51 (epitope C) had the lowest ADCC induction and did not reach > 50% target cell killing (Fig. 6A).

Infected cells or infectious debris can also be phagocytized by neutrophils. Thus, besides ADCC we also tested antibody-dependent cellular phagocytosis (ADCP) of RSV infected HEp-2 cells by neutrophils, finding that G-specific antibodies induced phagocytosis with EC\textsubscript{50} levels between 200 to 900 ng/mL, with the high-affinity antibodies AT32 and AT33 demonstrating higher phagocytosis activity compared to the antibodies with lower affinities (Table 4, fig. 6B). The IgG2 antibody induced similar phagocytosis compared to the IgG1 G-specific antibodies. Antibodies from epitope A and C (AT42, AT50/51) do demonstrate opsonization activity, although with a lower capacity compared to the other epitopes (Fig. 6B). The results from the ADCC and ADCP assay suggest that, in contrast to the neutralization, the ability of G-specific antibodies to promote effector cell functions is probably not epitope dependent.

Figure 6: ADCC and phagocytosis of RSV-A2 infected HEp2 cells

(A) Antibody-dependent cellular cytotoxicity (ADCC) assay. Lines depict the mean percentage of RSV-A2 infected HEp-2 cells killed by human PBMCs. AT12-009 is used as negative control. Experiments were performed twice in duplicate. (B) Neutrophil phagocytosis assay (ADCP), lines depict the mean percentage of neutrophils that have phagocytized FITC labeled RSV-A2 infected HEp-2 cells after 24 h incubation in the presence of increasing concentration of antibodies. All experiments were performed in triplicate in three different neutrophil donors.
Discussion

In this study we investigated the RSV specific B cell repertoire of 3 healthy donors in order to find potential therapeutic antibodies. All donors had high numbers (0.35%) of memory B cells producing antibodies against intact RSV-A2 infected cells. These antibodies mainly targeted RSV-A2 surface proteins G and F. The majority of RSV specific antibodies were of the IgA isotype (59%), while 41% were of the IgG isotype.

Other groups that studied naturally occurring RSV specific antibodies in healthy adults have found either much lower frequencies (<0.01%) of RSV G reactive B cells or comparable frequencies (0.04-0.18%) of B cells producing F-specific antibodies, compared to the 1 in 282 (0.35%) memory B cells we found. The variation might be explained by the efficiency of our B cell culture method combined with the sensitivity of the flow cytometry based selection method, but could also be due to the selection of our donors. By using B cells from child day care providers who are almost certainly exposed to multiple RSV carriers each year we aimed to increase the chance of finding highly potent antibodies. In addition, we may even have underestimated the frequency of RSV specific B cells, since we screened on RSV-A2 but not on RSV-B infected cells. Others have found that serum neutralizing capacity mainly depends on pre-fusion F protein specific antibodies and not on G protein specific antibodies. However, this study determined neutralization on HEp-2 cells without addition of complement, thereby missing the G-specific antibodies which neutralize in the presence of complement and in HAE cultures.

In our study, we focused on the G-specific IgG antibody panel. We found four dominant epitopes with distinct functional properties. Antibodies specific for epitope A and B are cross-reactive between RSV strain A and B, but antibodies specific for epitope C and D only recognize RSV-A (table 4). This could be explained by the high amino acid homology between strain A and B around epitope A and B, also termed ‘conserved region’, whereas the amino acid sequence after this region is more variable.

We determined the epitopes of the selected G-specific antibodies by binding to short 12 aa long peptides. It is likely that most peptides have a linear or minimal conformational structure, therefore it is well possible that the binding domain of the antibodies is outside the G protein part that we studied (aa 150 to 200) or covers an area larger than the 12 aa sequences. For example, 131-2G has an epitope between aa 163-168, which is similar to the epitope found by Kauvar and colleagues (aa 164-168). However, Johnson et al. found defective binding of 131-2G to RSV with a mutated 186 aa, indicating that the epitope might span a larger portion of the G protein. This could explain how 131-2G is capable to neutralize in our HAE system, while other antibodies binding epitope B do not at lower concentrations.
All human IgG RSV G-specific antibodies specific for epitope A, B, and D are strong neutralizers in the presence of complement (table 4). Complement components play a critical role in anti-viral antibody efficacy. For instance, component C1q enhances neutralization activity of monoclonal antibodies against influenza virus. These antibodies may induce potent complement activation because they form hexameric clusters. Antibodies binding epitope C and the mouse 131-2G antibody do not neutralize on HEp-2 cells in the presence of complement. This could be the result of their lower affinity for the G protein compared to the other epitopes, which could result in less antigen-driven antibody hexameric clustering. The epitope C specific antibodies may thus not be able to fully activate complement and induce neutralization. CDN relies on lysis of the virion membrane after signaling and activation of the complement system by virus-bound antibodies. High antibody affinity will result in high levels of bound antibody potentiating CDN. This relation is most clear in RSV-B CDN, where higher affinity translates to more potent neutralization (table 4). This phenomenon is less clear for RSV-A CDN, where some lower-affinity antibodies still potently neutralize (table 4). This suggests that, although affinity certainly potentiates the neutralization capacity, there are other factors (e.g. antibody cross-linking, Fc tail presentation and epitope) influencing the availability and presentation of the Fc tail to complement.

Fc tail presentation could also play a role during other immune effector functions activated through bound antibodies, such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) of infected cells, as has been shown in influenza disease. Some of our antibodies were able to induce substantial ADCC (mainly epitope B and C: AT34, AT40 & AT50), while others were more potent inducers of ADCP (mainly epitope D: AT32, AT33). Especially ADCP seems to depend on affinity rather than epitope, as antibodies to different epitopes induce similar EC50 values (table 4). Similar to CDN, both ADCC and ADCP require an accessible epitope on the target protein and antibodies bound to the epitope to activate effector cells to initiate cellular cytotoxicity or phagocytosis. Taken together, G-specific antibodies may play an important role in anti-viral immunity in vivo via ADCC and ADCP.

The role of the RSV G-protein in cell tropism, infectivity, and attachment of RSV to its target cells still has to be fully elucidated. Recent studies reported an important role for G protein and its receptor CX3CR1 in the pathogenesis of RSV. In addition, it was shown that genetic ablation of the G protein reduces RSV infectivity in mice and cotton rats, indicating that viral entry of airway epithelial cells might be hampered without a functional G protein. The G protein expresses a CX3C-motif which can bind to the CX3CR1 receptor and hence can facilitate viral entry in vivo in airway epithelial cells and in vitro in HAE cultures. Inhibition of this interaction by G-specific antibodies may thus lead to reduced viral infection in HAE cultures. Indeed, we found complete neutralization at 20 µg/mL, while at lower concentrations
RSV G protein specific antibodies

(0.2 µg/mL) neutralization potency seemed grouped by antibody epitope specificity. Interestingly the antibodies specific for epitope C, who did not show strong efficacy and had a relative low binding affinity, showed potent neutralization in the HAE culture system. The epitope C specific antibodies seem to recognize an epitope located on the apex of the cysteine noose and therefor they may be best suited to inhibit the G – CX3CR1 interaction. Together, this suggests that antibody induced inhibition of the RSV G protein interaction with the CX3CR1 receptor does prevent viral entry into airway epithelial cells. Notwithstanding that besides the G-specific antibodies also AM22, a pre-fusion F protein specific antibody3-19 completely neutralized virus infection in HAE cultures, suggesting that a functional F protein is also required for HAE infection.

Furthermore, the fractalkine binding motif in the G protein is also thought to play a role in modulating the immune response as it is able to block fractalkine signaling through binding to the CX3CR1 receptor on human PBMC’s.11 In vivo and in vitro studies have shown that inhibition of this interaction can inhibit immune-cell trafficking and dampens viral replication and pathology in mice infected with RSV.11,13 This also suggests that the G-specific antibodies could play an immune-modulating role by inhibition of soluble G-CX3CR1 signaling in vivo.

In conclusion, healthy adults harbor a diverse repertoire of high affinity RSV specific antibodies, with a predominant IgA+ B cell response. The IgG G-specific antibodies are divided in four epitopes, of which antibodies to two epitopes, A and B, cross-react with both RSV strain A and B, but antibodies to epitopes C and D are specific to strain A. The antibodies have a broad range of distinct functions, including: CDN, ADCC, ADCP and complement independent neutralization in primary HAE cultures. While neutralization in HAE cultures is highly dependent on epitope, ADCC and ADCP are more related to affinity. Our results indicate that G-specific antibodies are part of the natural antibody response to RSV and that these antibodies could play an important part in anti-viral immunity. Future studies should analyze the potency of these G-specific antibodies in vivo to boost therapeutic antibody regime development and structure based vaccine design.

Acknowledgements

AIMM Therapeutics employed: E. Yasuda, K. Wagner, Y.B. Claassen, A.Q. Bakker and T. Beaumont. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript, except AIMM Therapeutics who discovered, patented, and furthered the development of the described RSV G-specific antibodies. They supported the research collaboration of E. Yasuda, K. Wagner, Y.B. Claassen, A.Q. Bakker and T. Beaumont with the other authors and were consulted prior to the decision to publish. AIMM Therapeutics agreed to publish these results. We would also like to thank K. de Haan
for her excellent support setting up the HAE cultures and H. Hilkmann for the 12-mer peptide generation.

Statement of contribution

BC, JW, TB and EY designed the study. BC, EY, Kw, YC and AB conceived and carried out the experiments. BC, EY and KW analysed the data. BC, XY and EY carried out the in vitro experiments and analysed the data. BC, EY, YC, AB, KW, XY and TB interpreted the results. All authors were involved in writing the paper and had final approval of the submitted and published versions.

References


