Glycan variants of a respiratory syncytial virus antibody with enhanced effector function and in vivo efficacy

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Edited* by Charles J. Arntzen, Arizona State University, Tempe, AZ, and approved March 17, 2014 (received for review February 10, 2014)

Respiratory syncytial virus (RSV) can cause devastating lower respiratory tract infections in preterm infants or when other serious health problems are present. Immunoprophylaxis with palivizumab (Synagis), a humanized IgG_1 mAb, is the current standard of care for preventing RSV infection in at-risk neonates. We have explored the contribution of effector function to palivizumab efficacy using a plant-based expression system to produce palivizumab N-glycan structure variants with high homogeneity on different antibody isotypes. We compared these isotype and N-glycoform variants with commercially available palivizumab with respect to both in vitro receptor and C1q binding and in vivo efficacy. Whereas the affinity for antigen and neutralization activity of each variant were indistinguishable from those of palivizumab, their Fcγ receptor binding profiles were very different, which was reflected in either a reduced or enhanced ability to influence the RSV lung titer in challenged cotton rats. Enhanced Fcγ receptor binding was associated with reduced viral lung titers compared with palivizumab, whereas abrogation of receptor binding led to a drastic reduction in efficacy. The results support the hypotheses that classic antibody neutralization is a minor component of efficacy by palivizumab in the cotton rat and that antibody-dependent cell-mediated cytotoxicity activity can significantly enhance the efficacy of this antiviral mAb.

immunotherapy | glycoengineering | afucosylated | ADCC

Respiratory syncytial virus (RSV) is one of the most important pathogenic infections for both children and the elderly and is associated with significant morbidity and mortality (1, 2). The histopathologic characteristics of RSV infection are acute bronchiolitis, mucosal and submucosal edema, and luminal occlusion caused by epithelial cells, macrophages, fibrin, and mucin (3). There is a single RSV serotype with two major antigenic subgroups, A and B. Strains of both subtypes often cocirculate, but one subtype usually predominates. RSV infections exhibit a distinct seasonality, especially in temperate climates, with onset in late fall or early winter (4).

Most children experience at least one RSV infection by 2 y of age, but infected older children generally suffer little consequence. However, healthy young infants and children who have other serious health problems are known to be at higher risk of complications from RSV infection (5). Data from a variety of studies have suggested that the preventive use of the humanized mAb palivizumab has a favorable effect in children who are at higher risk of acquiring severe RSV infection, compared with placebo. Children treated with palivizumab were found to be hospitalized less often, to spend fewer days in the hospital, to be admitted to an intensive care unit less often, and to have fewer days of oxygen therapy than children who received a placebo (6). Although palivizumab was shown to be effective in reducing hospitalizations, its cost-effectiveness is not easy to determine because large differences in pharmacoeconomics have been observed in different studies (6–18). Recent evidence of a reduction in subsequent wheezing in palivizumab-treated children may improve the pharmacoeconomics of palivizumab use (19).

We previously reported the prophylactic and therapeutic testing of a plant-derived (*Nicotiana benthamiana*) palivizumab (palivizumab-N) as well as other mAbs that have been identified as potent RSV neutralizers (20). The inability of some potent neutralizing Abs to mediate protection in vivo is an indication that factors other than in vitro neutralization are important contributors to in vivo efficacy. Some factors that could contribute to in vivo efficacy might involve biodistribution, serum half-life, or interactions with effector cells and receptors. Palivizumab is reported to act by binding to the RSV envelope fusion protein (RSV F) on viruses and infected cells, blocking virus–cell and cell–cell fusion (21, 22). Another common mechanism of action that has been reported for virus-neutralizing antibodies involves the Fc-related functions of the antibody (23). Therefore, we designed experiments to evaluate the FcγR interactions, in vitro affinity and neutralization, and in vivo efficacy and biodistribution of isotype and glycoform variants of palivizumab. The variants were purified from *Nicotiana* plants that had been modified to be devoid of xylosyl- and fucosyl-transferase activity (24). The N-glycans on these variants consisted predominantly of the G0 glycoform (76–81%), a form that is underrepresented in NS0 cell culture-derived palivizumab (5%), regardless of the Fc backbone isotype. Our results revealed a significant alteration in in vivo efficacy, depending on receptor binding activity, and strongly

Significance

Recently developed plant hosts that have been genetically engineered to produce mammalian glycosylation enzymes are capable of producing mAbs with specific glycans that satisfy effector function or homogeneity requirements. When produced in this type of plant system, an antirespiratory syncytial virus mAb containing nearly homogeneous glycans with N-acetylglucosamine termini was found to significantly enhance the effectiveness of the approved antibody drug palivizumab. The improvement was because of augmented effector function via binding to antibody Fc receptors, suggesting that classic viral neutralization plays a secondary role in the in vivo function of this antibody. We conclude that enhanced antibody-dependent cell-mediated cytotoxicity activity can be a potent contributor to the in vivo efficacy of palivizumab.

Author contributions: N.B., O.B., C.G., D.K., M.H.P., J.V., K.J.W., P.A.P., B.E.G., and L.Z. designed research; N.B., O.B., C.G., D.K., M.H.P., J.V., P.A.P., B.E.G., and L.Z. performed research; N.B. contributed new reagents/analytic tools; A.H., D.K., M.H.P., K.J.W., P.A.P., B.E.G., and L.Z. analyzed data; and A.H., K.J.W., P.A.P., B.E.G., and L.Z. wrote the paper.

Conflict of interest statement: K.J.W. and L.Z. are co-owners of Mapp Biopharmaceutical.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

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www.pnas.org/cgi/doi/10.1073/pnas.1402458111

Table 1. In vitro properties of the anti-RSV mAbs

Palivizumab variant	Microneutralization IC_{50} (μg/mL) Without complement* (mean ± SD)	With complement†	Affinity ($\times 10^{-10}$ M) (mean ± SD)
Palivizumab	1.5 ± 0.8	1.2	1.6 ± 0.4
Palivizumab-N	1.8 ± 0.8	2.1	1.6 ± 0.1
Palivizumab-N-IgG_2	3.6	2.6	3.1 ± 1.1

*Summary of five to seven tests for palivizumab and palivizumab-N; palivizumab-N-IgG_2 was tested only once.
†Guinea pig complement was added at the time of virus addition to the assay. Each monoclonal antibody was tested only once.

suggest that viral protection in vivo is dependent upon effector functions.

Results

Production of mAbs. Palivizumab, a humanized murine mAb, was expressed as either IgG_1 (palivizumab-N) or IgG_2 (palivizumab-N-IgG_2) using a viral-based transient expression system (magnICON) (25, 26). Transgenic *N. benthamiana* in which plant-specific N-glycans (with core α-1,3-fucose and β-1,2-xylose) are greatly reduced by RNAi-mediated inhibition of plant-specific glycosyl-transferases (24) was used as the host plant for transient expression after infection with recombinant *Agrobacterium tumefaciens*. The purification yield was ~200 mg/kg, as previously reported (20). The purified mAbs were greater than 95% pure, as assessed by SDS/PAGE and HPLC–size-exclusion chromatography, and all mAbs had low levels of aggregation (≤3%).

Neutralization Testing of mAbs. Microneutralization assays were conducted as previously described (20) and revealed that palivizumab, palivizumab-N, and palivizumab-N-IgG_2 had comparable microneutralization IC_{50} values (Table 1). In addition, the affinities of each mAb for glycoprotein F, measured using a Biacore 100X system, were not significantly different, ranging from 1.6 to 3.1 $\times 10^{-10}$ M (Table 1).

Glycanalysis. Anthranilic acid (2-AA) glycan analysis (27) was performed on each mAb. In keeping with previously published results, palivizumab had a heterogeneous population of N-glycans, the majority of which were fucosylated. Only 5% of the glycans were of the G0 variety (Fig. 1). Approximately 6–7% of the total glycan species were minor structures and are not represented in Fig. 1. Of the fucosylated structures on palivizumab, G0F and G1F accounted for ~76% of the structures. In contrast, the mAbs produced in *Nicotiana* contained a far less heterogeneous glycan population that was dominated by the G0 glycan (76–81%) on both palivizumab-N and palivizumab-N-IgG_2. The remainder of the major glycans on the *Nicotiana*-derived mAbs consisted of branched mannose residues.

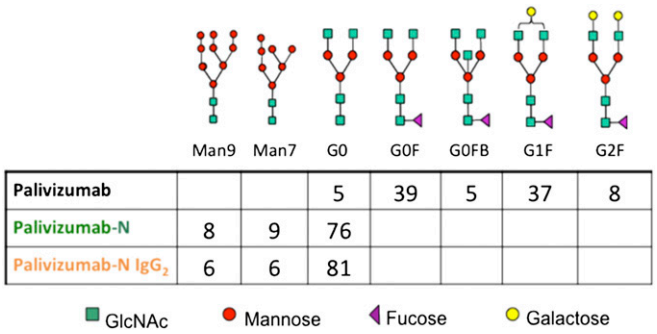

Fig. 1. N-linked glycans on the anti-RSV mAbs. N-glycosylation profile of palivizumab, palivizumab-N, and palivizumab-N-IgG_2, as determined by 2-AA glycanalysis. Numbers represent the presence of the different glyco-species in percent of total glycan (mol/mol). Minor glycoforms (below 5%) are not indicated. The N-glycan nomenclature used was from www.proglycan.com.

Binding to Fcγ Receptors. Because rodent FcγRs efficiently bind human IgG subclasses (28), we measured the binding of the palivizumab variants to murine Fc receptors by surface plasmon resonance. Recombinant HIS-tagged Fcγ receptors were captured on an NTA chip, and a fixed concentration of mAb was then flowed over the chip surface and analyzed using a Biacore 100X system. Binding was expressed as a percentage of that of palivizumab, which was defined as 100%. The results demonstrated significant differences in FcγR binding depending on both the Fc isotype (i.e., palivizumab-N vs. palivizumab-N-IgG_2) and glycan composition (i.e., palivizumab vs. palivizumab-N). Palivizumab-N-IgG_2 displayed negligible binding to all tested FcγRs. In contrast, palivizumab-N showed significantly enhanced binding to FcγRIIb and FcγRIII compared with palivizumab (100% and 350% greater, respectively) (Fig. 2).

C1q Binding. C1q binding to the Fc region of antibodies, the first step in the classic complement cascade, is isotype- and glycosylation-dependent (29). The ability of the three different versions of palivizumab to bind human C1q was compared (Fig. 3) using a standard ELISA (30). As expected, palivizumab-N-IgG_2 displayed minimal binding to human C1q. In contrast, binding of both palivizumab and palivizumab-N was observed, although palivizumab was a more potent binder.

In Vivo mAb Distribution. The mAb levels in the serum, nasal wash, and lung lavage of cotton rats were measured by neutralization assay (serum) and ELISA (serum, nasal wash, lung lavage) to assess the mAb distribution of the palivizumab variants (Table 2). The two mAb variants had similar serum, nasal wash, and lung lavage titers compared with palivizumab.

In Vivo Evaluation of mAb Protective Efficacy. The protective efficiency of the mAbs was evaluated using the cotton rat model of RSV infection (31). The animals received mAb (5 mg/kg) 1 d before challenge with the RSV/A strain Tracy. The pulmonary viral titer (PFU/g) was determined 4 d postchallenge. The results revealed significant differences in pulmonary viral load depending on the isotype and glycan profile of the mAb. In the absence of mAb prophylaxis, pulmonary virus was detected at levels of 1.7 ± 0.3 $\times 10^5$ PFU/g. This level was dramatically reduced over 100-fold to 1.2 ± 0.2 $\times 10^3$ PFU/g by palivizumab (Fig. 4) and was even further reduced to 4.0 ± 0.7 $\times 10^2$ PFU/g in animals treated with palivizumab-N ($P < 0.01$ compared with palivizumab by unpaired t test, two-sided). In contrast, the IgG_2 isotype displayed only a modest viral reduction of fivefold to 3.7 ± 0.8 $\times 10^4$ PFU/g.

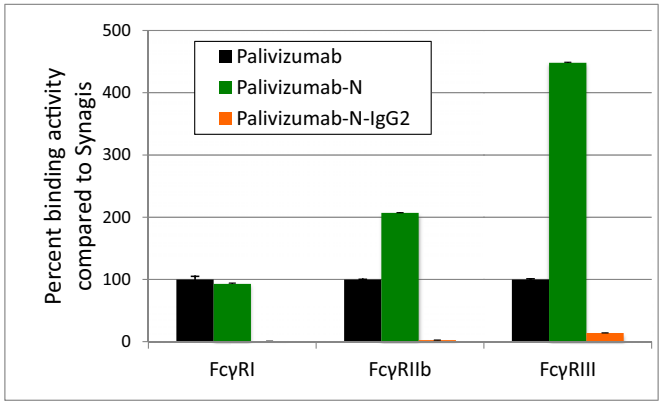

Fig. 2. Relative binding activity of the anti-RSV mAbs to murine Fcγ receptors. Binding of the anti-RSV mAbs to murine Fcγ receptors was assessed via surface plasmon resonance using a Biacore 100X system. Recombinant HIS-tagged Fcγ receptors were captured on an NTA chip, and a fixed concentration of mAb was then flowed over the chip surface. Binding was compared with that of palivizumab, which was defined as 100%. $n = 3$ replicates per data point. Error bars indicate the SD.

Discussion

We previously showed that a variety of *Nicotiana*-derived anti-RSV mAbs have significant neutralizing activity in vitro and high affinity for recombinant RSV glycoprotein F (20). Of the mAbs tested in vivo, they all were found to reduce the viral titer in the cotton rat lung, but none except palivizumab-N were as effective as palivizumab. In the present report, the functional superiority of palivizumab-N compared with palivizumab, albeit not dramatic, was nonetheless significant. Combined with previous reports that the binding activity of *Nicotiana*-derived mAbs is indistinguishable from its parental mAbs (32–35), the present report suggests that the additional gains achieved by manipulating the glycans in the transient plant system can potentially be applied to other mAbs. For the mAbs produced here, a transgenic *N. benthamiana* line was used that yields mAbs with mammalian N-glycans that are predominantly of the G0 glycoform (24). Although this glycoform can enhance antibody-dependent cell-mediated cytotoxicity (ADCC) (36), it does not influence other effector functions or the long serum half-life of IgG conferred by binding to the FcRn receptor (20).

The potential benefits of enhancing the efficacy of mAb prophylaxis for RSV are numerous (37, 38). First and foremost, the marginal cost-effectiveness of the regimen for infants may be improved if efficacy is augmented. Second, inclusion of additional at-risk populations (e.g., healthy infants, patients with cystic fibrosis or immunodeficiency, lung transplant recipients, the elderly) may become possible. Finally, at-risk populations in developing countries may finally benefit from RSV prophylaxis if the regimen can be made less expensive (6). To begin evaluating the immunological factors that may contribute to improved RSV prophylaxis, we produced glycoform and isotype variants of palivizumab and evaluated their efficacy in both in vitro and in vivo experiments.

Although it had neutralization and biodistribution characteristics that were indistinguishable from palivizumab and palivizumab-N, the IgG_2 isotype variant (palivizumab-N-IgG_2) was severely compromised in its ability to reduce the titer of RSV in the lungs of cotton rats. The absence of a correlation of C1q binding (palivizumab > palivizumab-N > palivizumab-N-IgG_2) with efficacy in vivo (palivizumab-N > palivizumab > palivizumab-N-IgG_2) suggests that complement activation via the classic pathway is not a major mechanism of protection by these mAbs in the cotton rat model. Because human IgG_2 is an isotype that has minimal interaction with either human or rodent FcγR (Fig. 2) (28), it is an ideal isotype to assess the potential contribution of the FcγR interaction to the potency of a mAb prophylactic. FcγR binding can have profound effects on a number of mAb properties that contribute to therapeutic or prophylactic efficacy (39). These effects include the half-life in both serum and target tissues, the overall biodistribution, and the initiation of a variety of secondary effector functions. One can conclude from the results presented above that an interaction with FcγR is an integral component of the efficacy of RSV prophylaxis with palivizumab. To our knowledge, this has never previously been reported.

The role of glycans in the clinical performance of approved mAbs has been the subject of intense investigation for a number of years. In particular, the role of fucose residues on N-linked glycans, or their absence, has been shown to disproportionately contribute to the biological activity of a number of mAbs that are either in preclinical testing or have been approved by the Food and Drug Administration (40). In 2000, Clynes et al. (41) studied the antitumor effects of several therapeutic antibodies, including trastuzumab and rituximab, in the presence of immune effector cells. The interaction of Fc with activating Fcγ receptors leads to increased cellular cytotoxicity, but the interaction of Fc with inhibitory Fcγ receptors leads to the opposite effect. The authors noted that activated ADCC contributes substantially to the antitumor effects of the antibodies. Soon after, Shields et al. (42) found that fucose-depleted trastuzumab results in (*i*) improved binding to the activating Fc receptors on NK cells and (*ii*) a 43-fold increase in the potency of ADCC against breast cancer cells in vitro. Nearly simultaneously, in experiments involving fucose-depleted rituximab, Shinkawa et al. (43) found that the removal of fucose produces a significant enhancement of cytotoxic potency against the Raji CD20-overexpressing human B lymphoma cell line. Fucose-depleted rituximab has a markedly greater affinity for the FcγRIII receptor compared with standard rituximab (44). More recently, afucosylated mAbs have been found to possess enhanced potency against infectious pathogens, including HIV and Ebola virus (36, 45).

In addition to enhancing the potency of palivizumab by structural manipulation, it is clear that cost reduction can be achieved by reducing the cost of one or more components of the production process (46). The production of mAbs using transient expression systems in *Nicotiana* is becoming a common component of mAb research, in part because of the ease with which an iterative process of mAb evaluation and improvement can be

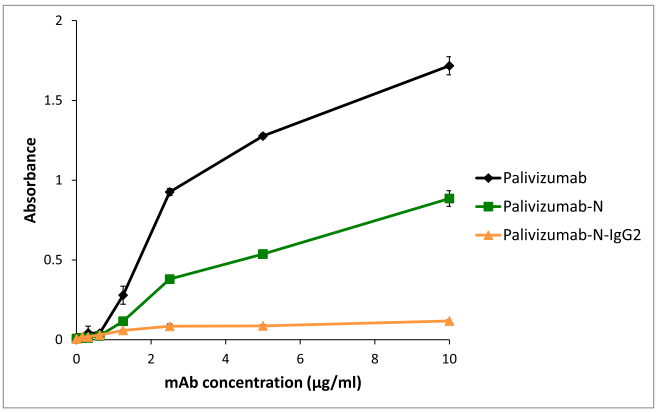

Fig. 3. Binding to human C1q by ELISA. Various concentrations of mAb were coated onto ELISA plates. After blocking, 2 μg/mL of human C1q was added. The binding of C1q to the mAb was detected using a goat anti-human C1q polyclonal antibody followed by a rabbit anti-goat (human adsorbed) HRP-conjugated antibody. Error bars indicate the SD ($n = 3$).

Table 2. Concentrations of the mAbs in the cotton rat serum, nasal wash, and lung lavage

Treatment group	Neutralization assay	ELISA			
		Serum (mean ± SD, μg/mL)			
	Serum day 4 (mean ± SD, μg/mL)	Day 0	Day +4	Nasal wash, day +4 (mean ± SD, ng per nose)	Lung lavage, day +4 (mean ± SD, ng per lungs)
Untreated	0	0	0	0	0
Palivizumab, 5 mg/kg	43 ± 2	23.2 ± 4.4	16.7 ± 2.2	29.0 ± 17.8	381 ± 48
Palivizumab-N, 5 mg/kg	31 ± 2	21.5 ± 2.0	19.0 ± 2.9	14.3 ± 9.0	412 ± 148
Palivizumab-N-IgG_2, 5 mg/kg	39 ± 4	45.8 ± 9.6	29.8 ± 7.6	39.0 ± 19.3	578 ± 92

achieved (25, 26). In addition, the ability to glyco-engineer antibody drug products may allow for greater clinical opportunities. In the case of palivizumab, dramatically heightened ADCC activity may allow for a therapeutic application in addition to the prophylactic protocol that is currently followed.

Methods

***N. benthamiana* Expression Vectors.** The palivizumab variable region sequences were used as reported and codon optimized (VL: KC283077; VH: KC283078) (31). Genes containing these sequences were synthesized (Life Technologies) and subsequently cloned into plant expression vectors (TMV and PVX; Icon Genetics) containing codon-optimized human κ and the constant regions of either IgG_1 or IgG_2, followed by transformation into *A. tumefaciens* strain ICF320 (47). Palivizumab (MedImmune) was purchased from a commercial-supplier.

Production of Anti-RSV mAbs in *N. benthamiana*. We used the "magnifection" procedure (48) with minor modifications. Plants grown for 4 wk in an enclosed growth room (20–23 °C) were used for vacuum infiltration. Equal volumes of *Agrobacterium* cultures grown overnight were mixed in infiltration buffer (1 mM Mes/10 mM $MgSO_4$, pH 5.5), resulting in a 1:1,000 dilution for each individual culture. Infiltration solution was transferred into a 20-L custom built (Kentucky Bioprocessing) vacuum chamber. The aerial parts of the plants were inverted into the bacterial/buffer solution. A vacuum of 0.5 bars was applied for 2 min, and the plants were returned to the growth room. Seven days postinfiltration, leaf tissue was extracted in a juicer (Model GS-1000, Green Star) using 250 mL of chilled extraction buffer (100 mM Tris/40 mM ascorbic acid/1 mM EDTA) per kilogram of green leaf tissue. The extract was clarified by lowering the pH to 4.8 with 1 M phosphoric acid then readjusting to pH 7.5 with 2 M Tris base to insolubilize the plant polymers. The supernatant was transferred and centrifuged at 16,000 × *g* for 30 min. The clarified extract was filtered (0.2 μm) before concentration using the Minim Tangential Flow Filtration System (Pall) and then 0.2 μm was filtered immediately before loading onto a 5-mL HiTrap MabSelect SuRe (GE Healthcare) Protein A column at 2 mL/min. The column was washed with running buffer (50 mM Hepes/100 mM NaCl, pH 7.5) and eluted with 0.1 M acetic acid, pH 3.0. The resulting eluate was neutralized to pH 7 using 2 M Tris, pH 8.0, and supplemented with 0.01% Tween 80. The mAb solution was polished via Q filtration (Mustang Acrodisc Q membrane; Pall), aliquoted, and stored at −80 °C until use. All mAbs were fully assembled and of greater than 98% purity, as determined by SDS/PAGE. Endotoxin levels were measured with Endosafe PTS (Charles River) and were < 100 EU/mg.

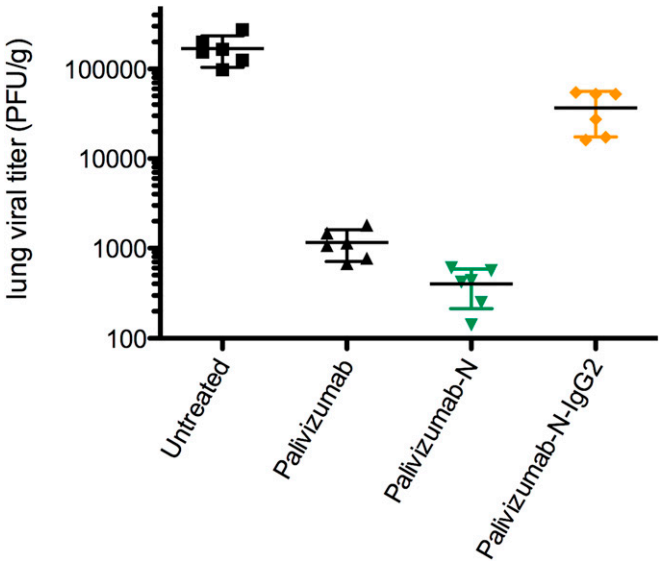

Fig. 4. Relative reduction in the RSV lung titer in cotton rats treated prophylactically with an anti-RSV mAb (5 mg/kg). The animals received mAb one day before challenge with RSV (strain Tracy), and the viral titer was determined four days postchallenge. Error bars denote the SD.

Glycanalysis. N-linked glycans were released by digestion with N-glycosidase F (PNGase F), and subsequent derivatization of the free glycan was achieved with 2-AA. The 2-AA-derivatized oligosaccharide was separated from any excess reagent via normal-phase HPLC. The column was calibrated with 2-AA–labeled glucose homopolymers and glycan standards. The test samples and 2-AA–labeled glycan standards were detected fluorometrically. Glycoforms were assigned either by comparing their glucose unit (GU) values with those of the 2-AA–labeled glycan standards or by comparing with the theoretical GU values (49). Confirmation of glycan structure was accomplished with LC/MS.

Kinetic Testing of mAb Binding to Recombinant RSV F. The kinetics of mAb binding to recombinant RSV F were evaluated using a Biacore ×100 system. An NTA chip was used to capture recombinant gF (strain A2). Each mAb (diluted in NTA running buffer) was then flowed over the chip at five different concentrations (with the highest concentration having an Rmax between 30 and 80 RUs), and kinetic analyses were performed using BIAEvaluation software (1:1 fit). Fast flow rates and controls, including a flow cell with no gF and immobilized gF with the flow of buffer only, were used to ensure against acquiring mass transfer-limited data.

Relative Binding to Fcγ Receptors. Recombinant human Fcγ receptors (Sino Biological) were captured on the surface of NTA chips (GE Healthcare), with a target capture level of 1000 RUs. Each mAb (5 μg/mL) was then flowed over the chip. Fast flow rates and controls (including a flow cell with no receptor and immobilized receptor with the flow of buffer only) were used to ensure against acquiring mass transfer-limited data.

C1q Binding ELISA. The binding of human C1q (Calbiochem) to IgG mAb was assessed using a previously described method (30). High-binding 96-well plates (Costar) were coated overnight at 4 °C with various concentrations of mAb diluted in coating buffer (PBS). After blocking [PBS/2% (wt/vol) BSA] for 1 h, 2 μg/mL of human C1q was added. The binding of C1q to the mAb was detected using a 1:1,000 dilution of goat anti-human C1q polyclonal antibody (Mybiosource.com), followed by a 1:5,000 dilution of rabbit anti-goat (human adsorbed) HRP conjugated antibody (Southern Biotech) with 0.1% goat serum. The plates were developed with TMB (KPL). The reaction was halted with 2.5 N H_2SO_4, and the absorbance at 450 nm was measured.

Microneutralization Assay. Microneutralization assays were performed in 96-well microtiter plates with HEp-2 cells grown in 2% (vol/vol) FBS-MEM. Plaque-purified RSV strains were used in these assays. Duplicate serial twofold dilutions (50 μL; starting at a 1:8 dilution) were used to determine the neutralizing titer for each sample. The neutralizing antibody titer was defined as the serum dilution at which a > 50% reduction in the viral cytopathic effect (CPE) was observed. The

CPE was determined visually after the wells were fixed with 10% (vol/vol) neutral buffered formalin and stained with Crystal violet. The neutralizing antibody titers were categorical log numbers and not continuous values. The lowest detectable titer was 2.5 $\log_2$. Samples with nondetectable titers were assigned a value of 2 $\log_2$.

Cotton Rat Experiments. Cotton rats (male and female *Sigmodon hispidus*, 60–125 g body weight) were bred and housed in cages in covered with barrier filters in the vivarium of the Baylor College of Medicine and given food and water ad libitum. All animal experiments followed the National Institutes of Health and US Department of Agriculture guidelines, and the experimental protocols were approved by the Baylor College of Medicine Investigational Animal Care and Use Committee. Groups of five to six animals were used in all studies. MAbs were injected intramuscularly. Challenge virus (RSV Tracy, 2×10^5 PFU) was administered intranasally (100 μL) to cotton rats that were lightly anesthetized with isoflurane. The animals were killed 4 d post-challenge. Following killing with CO_2, each cotton rat was weighed, and its sex and age was recorded. The left and one of the large right lobes of the lungs were removed, rinsed in sterile water to remove external blood contamination, and weighed. The left lobe was transpleurally lavaged using 3 mL of Iscove's medium with 15% glycerin mixed with 2% (vol/vol) FBS-MEM (1:1) in a 3 mL syringe with a 26-G × 3/8-inch needle by injecting at multiple sites to totally inflate the lobe. The lavage fluid was recovered by gently flattening the inflated lobe and used to transpleurally lavage the right lobe following the same technique. The lavage fluid was collected and stored on ice until titered. For nasal washes of the upper respiratory tract, the jaw was disarticulated. The head was removed, and 1 mL of Iscove's medium with 15% glycerin mixed with 2% FBS-MEM (1:1, vol:vol) was pushed through each nare (total of 2 mL). The effluent was collected from the posterior opening of the pallet and stored on ice until titered at the end of all sample collections.

RSV Tracy lung lavage titers (PFU/g lung) and nasal wash titers (total PFU) were determined by plaque assay. Plaque assays were performed using 24-well tissue culture plates containing nearly confluent monolayers ($20–40 \times 10^4$ cells per well) of HEp-2 cells grown in 10% FBS for 24 h before the start of the assay. At the start of each assay, dilutions (serial $\log_{10}$) were made of the test samples. A volume of 0.2 mL of each sample was added to the wells in duplicate and allowed to adsorb for 90 min in an incubator at 36 °C and 5% CO_2 with occasional gentle agitation. After the inoculum was removed, monolayers were overlaid with 0.75% methylcellulose in 2% (vol/vol) FBS-MEM containing antibiotics, vitamins, and other nutrients. Tissue culture and positive virus controls were included in each assay. The plates were placed in an incubator at 36 °C and 5% CO_2. On day 7, the plates were stained with a 0.01% Crystal violet/10% formalin solution (~1.5 mL per well) and allowed to sit for 24–48 h at room temperature. The wells were rinsed with water. All of the plaques in wells containing ~20–80 plaques were enumerated and averaged, and the virus titers were calculated as total $\log_{10}$ PFU for the nasal wash fluid or $\log_{10}$ PFU/g for the lung lavage. The lower limit of detection by this method was ~0.7 total $\log_{10}$ PFU for the nasal wash fluid or 1.5 $\log_{10}$ PFU/g for the lung lavage.

ACKNOWLEDGMENTS. The authors thank Sonnie Kim Grossman, for her assistance with this research, Dr. Yuri Gleba for providing access to the magnICON system, and Dr. Herta Steinkellner for the glycosylation-modified plants. This work was supported by the National Institutes of Health Grants R41AI063681 (to L.Z.) and HHSN272201000004I (to B.E.G.).

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