

## INFECTIOUS DISEASE

## A highly potent extended half-life antibody as a potential RSV vaccine surrogate for all infants

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Prevention of respiratory syncytial virus (RSV) illness in all infants is a major public health priority. However, no vaccine is currently available to protect this vulnerable population. Palivizumab, the only approved agent for RSV prophylaxis, is limited to high-risk infants, and the cost associated with the requirement for dosing throughout the RSV season makes its use impractical for all infants. We describe the development of a monoclonal antibody as potential RSV prophylaxis for all infants with a single intramuscular dose. MEDI8897\*, a highly potent human antibody, was optimized from antibody D25, which targets the prefusion conformation of the RSV fusion (F) protein. Crystallographic analysis of Fab in complex with RSV F from subtypes A and B reveals that MEDI8897\* binds a highly conserved epitope. MEDI8897\* neutralizes a diverse panel of RSV A and B strains with >50-fold higher activity than palivizumab. At similar serum concentrations, prophylactic administration of MEDI8897\* was ninefold more potent than palivizumab at reducing pulmonary viral loads by >3 logs in cotton rats infected with either RSV A or B subtypes. MEDI8897 was generated by the introduction of triple amino acid substitutions (YTE) into the Fc domain of MEDI8897\*, which led to more than threefold increased half-life in cynomolgus monkeys compared to non-YTE antibody. Considering the pharmacokinetics of palivizumab in infants, which necessitates five monthly doses for protection during an RSV season, the high potency and extended half-life of MEDI8897 support its development as a cost-effective option to protect all infants from RSV disease with once-per-RSV-season dosing in the clinic.

## INTRODUCTION

Respiratory syncytial virus (RSV) is the most prevalent cause of viral lower respiratory tract disease among infants and young children worldwide (1–4). RSV circulates seasonally in temperate regions, usually between late fall and early spring. In developing countries, it is among the leading causes of infant death (2). Although deaths due to RSV are rare in the developed world, it is a significant cause of infant hospitalization and is also responsible for a large outpatient health care burden (1, 4, 5). In addition to acute disease, RSV lower respiratory infections in infancy have been associated with long-term wheezing and asthma (6–10).

There is no licensed vaccine available for RSV despite 50 years of research and development. One reason for this lack of progress is that the highest rates of RSV hospitalization are in infants ≤2 months of age (11, 12) when the ability to elicit strong anti-RSV responses may be compromised by the presence of maternal antibodies and the immaturity of the infant immune system. In addition, there are concerns that nonlive vaccine compositions could result in vaccine-enhanced RSV disease similar to what was observed with a formalin-inactivated vaccine candidate administered to seronegative infants in the 1960s (13). Live-attenuated RSV vaccines and chimeric viruses expressing RSV antigens (14, 15) are not considered a risk for inducing enhanced disease; however, achieving a proper balance between attenuation (safety) and immunogenicity, as

well as genetic stability, has been difficult. To date, these types of vaccines have been either over- or underattenuated when tested in infants (16, 17).

An alternative approach to RSV prevention is passive immunoprophylaxis with neutralizing antibody. RSV encodes 11 proteins, including the fusion (F) and attachment (G) surface glycoproteins that are targets for virus-neutralizing antibodies. The mature F protein is a trimer of heterodimers consisting of disulfide-linked F1 and F2 subunits. This highly conserved protein exists on the surface of virions in a prefusion conformation that drives an irreversible conformational change that brings the viral and host cell membranes together as it adopts a stable postfusion conformation (18). Preventing this conformational change from occurring blocks viral entry, and most of the neutralizing activity detected in a human immunoglobulin (IgG) preparation capable of protecting at-risk infants from RSV disease was found to be directed against the prefusion conformation of RSV F (19).

Palivizumab, a humanized monoclonal antibody (mAb) directed against a neutralizing epitope found on both the pre- and postfusion forms of the RSV F protein, is effective in preventing RSV hospitalization in infants and children at highest risk for serious disease (that is, premature infants, children with chronic lung disease of prematurity, or children with congenital heart disease) (20). This antibody is administered at a dose of 15 mg/kg body weight by intramuscular injection once a month throughout the RSV season. The current cost of this mAb prophylaxis, along with the inconvenience of once-a-month injections, makes immunoprophylaxis with palivizumab unfeasible for healthy infants who nonetheless would benefit from RSV prevention. Since the licensure of palivizumab almost 20 years ago, new methods of antibody generation have led to identification of broadly neutralizing mAbs targeting the prefusion form of the RSV F protein that are much more potent than palivizumab (21, 22). Technologies have also been invented to extend the serum half-lives of antibodies (23–25), and

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advances in manufacturing sciences have resulted in lower costs of production. Together, these advances in mAb technology may allow for the generation of a highly potent RSV-neutralizing mAb that could be administered to all infants by intramuscular injection once per RSV season at vaccine-comparable pricing.

Here, we describe MEDI8897\*, an in vitro-optimized human mAb that binds a highly conserved epitope present on the prefusion conformation of the RSV F protein and exhibits >2 logs greater potency in vitro and about ninefold better activity in vivo against RSV laboratory strains when compared with palivizumab. The Fc region of MEDI8897\* was engineered to extend the serum half-life, and pharmacokinetic (PK) analysis of the resulting molecule MEDI8897 in cynomolgus monkeys (*Macaca fascicularis*) confirmed the expected prolonged half-life and demonstrated acceptable preclinical safety to support clinical development. With the high potency and extended serum half-life of MEDI8897, the simulated PK profiles of MEDI8897 based on published palivizumab PK in infants and young children (26) predict that efficacious exposure is achievable and protection of all infants from RSV disease throughout their first RSV season may be possible with a single intramuscular injection of this antibody.

## RESULTS

### Selection and optimization of MEDI8897\*

To develop an immunoprophylactic agent for all infants, it was necessary to identify a highly potent and broadly RSV-neutralizing mAb. Previously, a panel of anti-RSV IgG1 mAbs was selected directly from the memory B cells of human donors through functional screening (22). The four mAbs from this panel with the greatest potency against laboratory strains of RSV (mAbs AM14, AM22, AM23, and D25) were further evaluated for potency and breadth of activity in a microneutralization assay against 25 clinical isolates (12 RSV A and 13 RSV B). Motavizumab, an affinity-optimized derivative of the humanized IgG1 mAb palivizumab with approximately 10-fold improved in vitro-neutralizing activity, was used as a benchmark mAb in this study. All mAbs (AM14, AM22, AM23, D25, and motavizumab) showed potent neutralizing activity against RSV A isolates, with calculated median half-maximal inhibitory concentration (IC<sub>50</sub>) values of 13.4, 32.4, 11.5, 7.2, and 44.7 ng/ml, respectively (Fig. 1A). When tested against RSV B isolates, AM23 failed to neutralize 10 of 11 viruses even at high concentrations. In contrast, AM14 and D25 exhibited similar and potent neutralizing activity with calculated median IC<sub>50</sub> values of 13.9 ng/ml (range, 1.52 to 89.2 ng/ml) and 10.3 ng/ml (range, 1.2 to 84.4 ng/ml), respectively, whereas AM22 and motavizumab had median IC<sub>50</sub> values of 172.7 ng/ml (range, 11.6 to 1254 ng/ml) and 47.0 ng/ml (range, 9.2 to 202.4 ng/ml), respectively. Overall, D25 showed the highest potency against both RSV A and B isolates with more than fivefold greater antiviral activity than motavizumab.

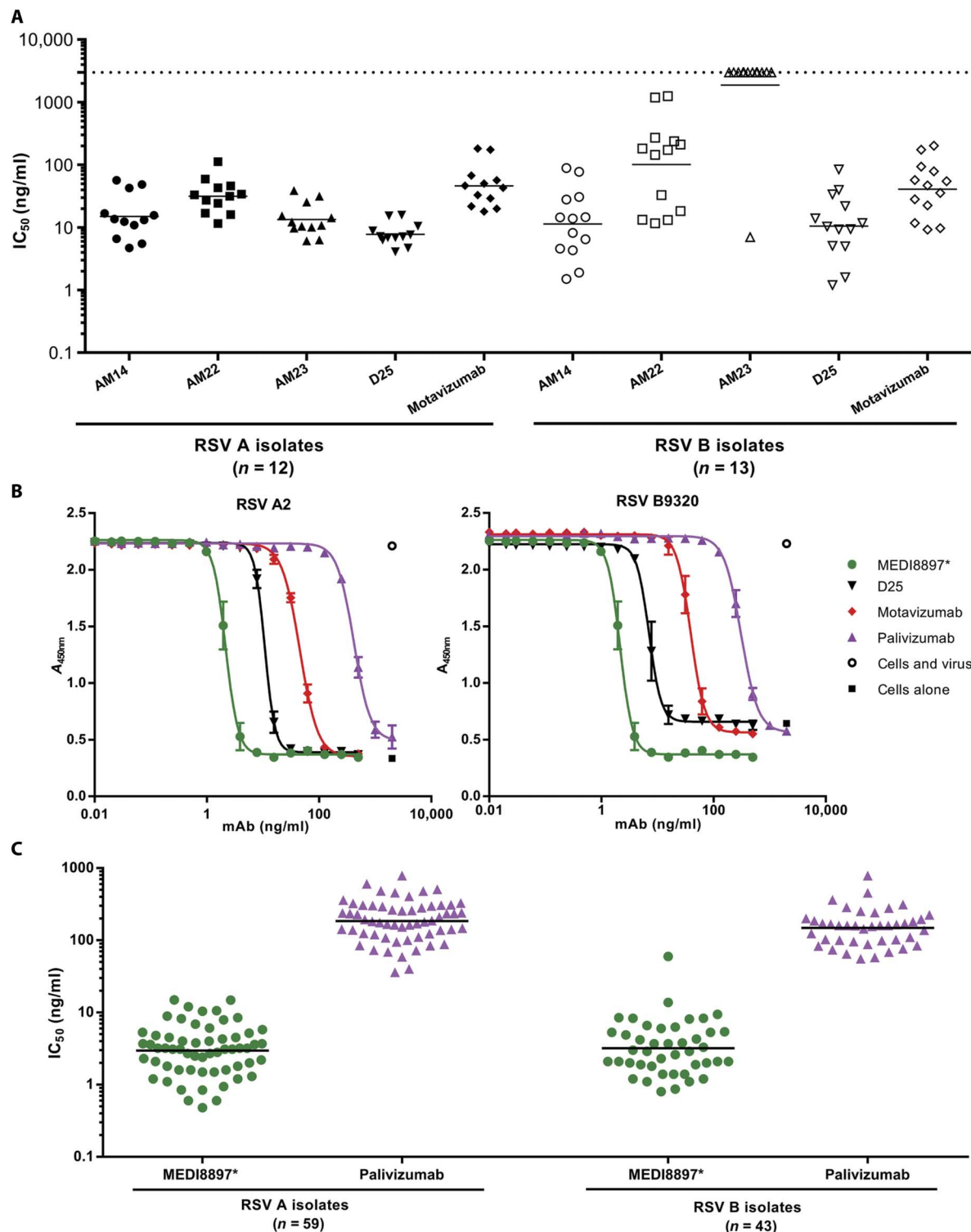
We thus selected D25 as the lead for in vitro optimization through parsimonious mutagenesis in which amino acids in the mAb complementarity-determining regions (CDRs) were systematically substituted either individually or in combination. In addition, non-germline residues in the D25 framework sequences that may elicit immune responses based on in silico prediction of human T cell epitopes were reverted to germline residues. The resulting mAbs were screened for improved activity in the microneutralization assay against laboratory strains of RSV A and B. The end result of interrogating more than 1500 individual mAb variants led to the selection of MEDI8897\*, which had five amino acid substitutions in the CDRs and four germline reversions in the framework sequences of the heavy chain (fig. S1).

The improvement in neutralizing activity exhibited by MEDI8897\* over D25 and the benchmark mAbs palivizumab and motavizumab was confirmed in microneutralization assays. MEDI8897\* exhibited mean IC<sub>50</sub> values of 2.2 and 1.8 ng/ml against laboratory strains RSV A2 and RSV B9320, respectively (Fig. 1B). In contrast, D25 exhibited mean IC<sub>50</sub> values of 10.8 ng/ml against RSV A2 and 7.1 ng/ml against RSV B9320, whereas the IC<sub>50</sub> values calculated for motavizumab and palivizumab were 45.4 and 416.8 ng/ml against RSV A2 and 39.2 and 309.3 ng/ml against RSV B9320, respectively. These data demonstrate that MEDI8897\* is about fourfold more potent than D25 and 20- and 150-fold more potent than motavizumab and palivizumab, respectively, against RSV A and B laboratory strains in vitro. The difference in RSV neutralization activity between MEDI8897\* and D25, although modest, was consistently observed in multiple experiments and was statistically significant ( $P < 0.0001$ ). To examine whether MEDI8897\* retained the neutralization breadth of D25 through the antibody optimization process, the antiviral activity of MEDI8897\* was further tested against an extended panel of 59 RSV A and 43 RSV B clinical isolates collected between 2003 and 2013 from various countries. MEDI8897\* neutralized all viruses tested, with calculated median IC<sub>50</sub> values of 3.1 ng/ml (range, 0.48 to 15 ng/ml) and 3.0 ng/ml (range, 0.8 to 59.7 ng/ml) against the RSV A and B isolates, respectively (Fig. 1C). Although palivizumab also neutralized all clinical isolates tested, MEDI8897\* was 50-fold more active against these same isolates. These studies demonstrated that MEDI8897\* exhibited highly potent and broad antiviral activity against a diverse panel of RSV A and B clinical isolates.

### Crystal structure analysis of MEDI8897\* Fab bound to RSV F

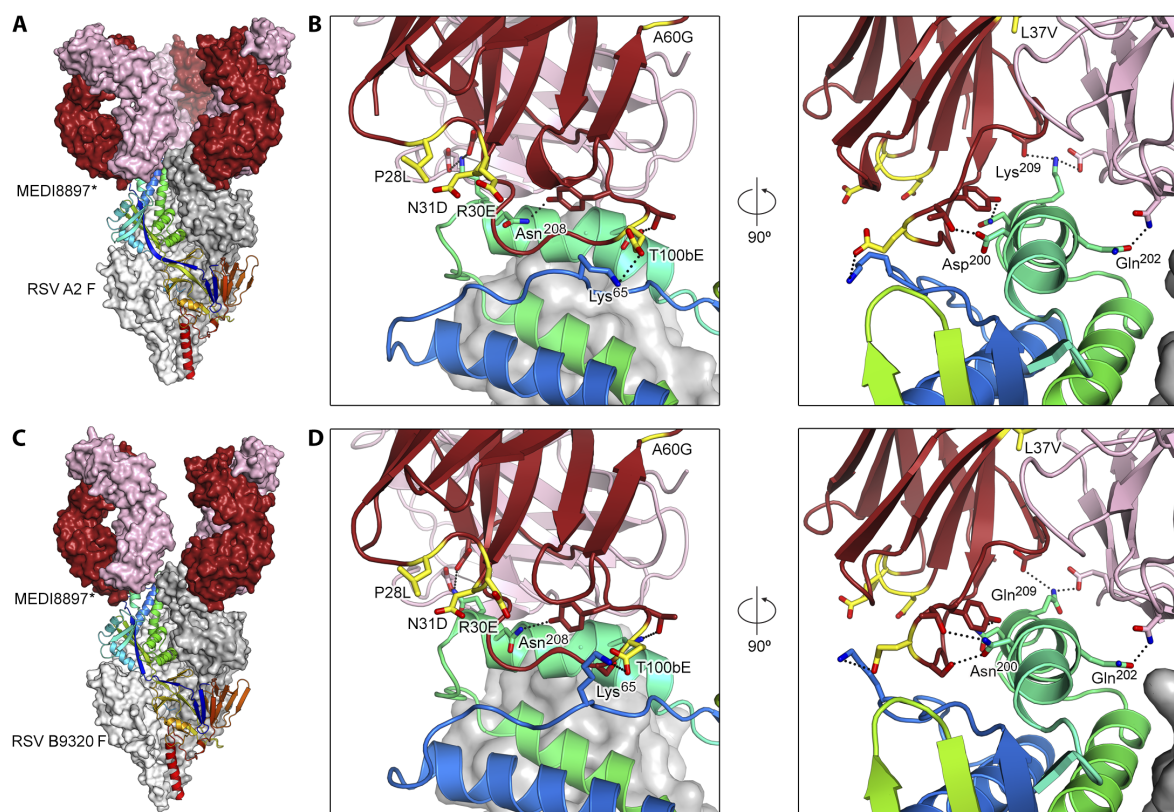
To define the epitope of MEDI8897\* and to explore the molecular basis for its increased neutralization potency as compared to D25, we determined the crystal structure of the MEDI8897\* Fab bound to a prefusion-stabilized RSV F protein (DS-Cav1) derived from the A2 strain (27). Crystals of the complex in space group  $P2_1$  diffracted x-rays to 3.45 Å, and after a molecular replacement solution was obtained, the structure was built and refined to an  $R_{\text{work}}$  and  $R_{\text{free}}$  of 17.5 and 23.2%, respectively (table S1). The asymmetric unit contained one prefusion RSV F trimer bound to three MEDI8897\* Fabs (Fig. 2A), consistent with the stoichiometry indicated by gel filtration (fig. S2) and the previous D25-RSV F A2 structure (28).

The heavy and light chains of MEDI8897\* make extensive interactions with the RSV F protein. As expected, D25 and MEDI8897\* bind similarly to RSV F and make specific interactions with RSV F residues 200, 202, 208, and 209 in the F1 subunit. The increased potency of MEDI8897\* is likely due to its interaction with the F2 subunit. Several of the residues altered in MEDI8897\* are in or near the CDRH1 and CDRH3, including substitutions P28L, R30E, N31D, and T100bE. The R30E and N31D substitutions increase the negative charge of the CDRH1, which is positioned close to the positively charged Lys<sup>68</sup> in the F2 subunit, although the electron density for the Lys<sup>68</sup> side chain is not well resolved at each interface observed in the structure. Similarly, the T100bE substitution increases the negative charge of the CDRH3 and creates a salt bridge with Lys<sup>65</sup> in the F2 subunit (Fig. 2B). To ascertain the effect of each substitution on the binding affinity of D25 for RSV F A2, we cloned, expressed, and purified five D25 variants, each containing a single amino acid substitution found in MEDI8897\* (P28L, R30E, N31D, A60G, and T100bE). The binding of each variant, as well as D25, to immobilized RSV F A2 DS-Cav1 was determined via surface plasmon resonance (SPR) experiments (fig. S3), but the picomolar affinity and extremely slow off-rate of D25 made it difficult to draw meaningful conclusions from the data.



**Fig. 1. RSV neutralization by mAbs.** The neutralization activity of different mAbs was determined by measuring the expression of RSV F protein in RSV-infected cells using enzyme-linked immunosorbent assay (ELISA). IC<sub>50</sub> values were calculated and represent the concentration of mAb required for a 50% reduction in absorbance measured at 450 nm ( $A_{450nm}$ ). **(A)** Neutralizing activity of AM14 (circle), AM22 (square), AM23 (triangle), D25 (inverted triangle), and motavizumab (diamond) against a panel of RSV A isolates (closed) and RSV B isolates (open). The dotted line represents the highest concentration of mAbs tested. **(B)** Neutralization activity curves of mAbs MEDI8897\* (green), D25 (black), motavizumab (red), and palivizumab (purple) against RSV A2 and B9320 laboratory strains. Data are representative of three independent experiments. **(C)** RSV neutralization IC<sub>50</sub> values of MEDI8897\* (green) and palivizumab (purple) against a large panel of RSV A and B isolates. The line represents geometric mean IC<sub>50</sub>.





**Fig. 2. Crystal structures of human mAb MEDI8897\* Fab in complex with prefusion RSV F proteins.** (A) Crystal structure of MEDI8897\* bound to RSV A2 F. Molecular surfaces are shown for three MEDI8897\* Fabs (heavy chains are dark red and light chains are pink) as well as two of the RSV F protomers (white and gray). The third protomer is shown as ribbons colored as a rainbow from blue to red, N terminus of F2 to C terminus of F1, respectively. (B) Close-up of the interface between MEDI8897\* and RSV A2 F, colored as in (A) but with residues in MEDI8897\* that differ from D25 (colored yellow) and labeled with one-letter abbreviation. Residues involved in side chain–side chain interactions are shown as sticks, with oxygen atoms colored red and nitrogen atoms colored blue. Hydrogen bonds are depicted as black dotted lines. RSV F residues are labeled using three-letter abbreviation. (C) Crystal structure of MEDI8897\* bound to RSV B9320 F, depicted as in (A). (D) Close-up of the interface between MEDI8897\* and RSV B9320 F, depicted and labeled as in (B).

Nevertheless, the structural data indicate that the R30E, N31D, and T100bE substitutions improve the charge complementarity and recognition of the F2 subunit by MEDI8897\*.

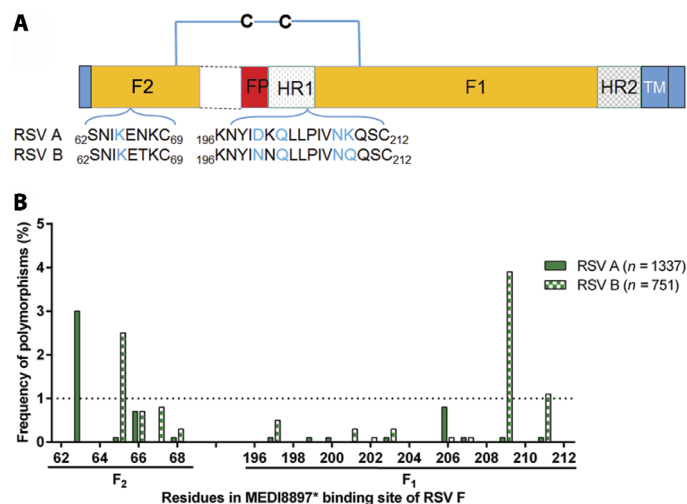
To understand the recognition of subtype B viruses by MEDI8897\*, we performed crystallization trials of the MEDI8897\* Fab in complex with a prefusion-stabilized RSV F protein (DS-Cav1) derived from RSV B9320. While attempting to crystallize the complex, we obtained crystals of the unbound B9320 DS-Cav1 protein that diffracted x-rays to 3.0 Å, and the structure was built and refined to an  $R_{\text{work}}$  and  $R_{\text{free}}$  of 19.3 and 23.4%, respectively. Comparison of the B9320 prefusion F structure to previously determined A2-derived structures revealed that the prefusion conformation of F is very similar between subtype A and B viruses, as expected, given the ~94% sequence identity in the mature ectodomain (fig. S4).

Ultimately, we succeeded in obtaining crystals of B9320 DS-Cav1 in complex with MEDI8897\* Fab, and these diffracted x-rays to 4.3 Å. The space group of the crystals was  $P3_221$ , and the asymmetric unit contained three RSV F trimers, each bound by only two MEDI8897\* Fabs (Fig. 2C). The stoichiometric binding does not appear to be a crystallographic artifact because the complex eluted from a gel filtration column with an apparent molecular weight lower than that of the A2-based complex (fig. S2). After molecular replacement, the structure was built and refined to an  $R_{\text{work}}$  and  $R_{\text{free}}$  of 23.7 and 29.5%, respectively. The binding of MEDI8897\* to RSV B9320 F is, overall, similar to the binding observed in the A2-derived structure described

above. The side chain of Lys<sup>68</sup> is not well ordered but would be in the vicinity of the R30E and N31D substitutions in the CDRH1. A salt bridge is formed between Lys<sup>65</sup> and Glu100b in the CDRH3, and RSV B9320 F residues 200, 202, 208, and 209 are also contacted by MEDI8897\* (Fig. 2D). Although residues 200 and 209 vary between the two subtypes, the changes are conservative (D200N and K209Q) and allow similar interactions to be made with MEDI8897\*.

### Conservation of the MEDI8897\* binding site and the impact of variations on neutralization

To gain more information about the MEDI8897\* binding surface, we identified the amino acids within the RSV F proteins that were adjacent to residues in contact with MEDI8897\* Fab in the crystal structures and had at least one atom within a 5 Å radius around any atom in the MEDI8897\* Fab. Overall, this analysis revealed a discontinuous binding site that was primarily composed of residues 62 to 69 in the F2 subunit and 196 to 212 in the F1 subunit (Fig. 3A). The sequence conservation of the MEDI8897\* binding site was evaluated by examination of 2088 F sequences derived from clinical and laboratory RSV isolates obtained from GenBank and MedImmune proprietary databases. The sequences analyzed were from viruses collected over a ~60-year period (1956 to 2014). The clinical isolates RSV A-NLD-13-005275 and RSV B-NLD-13-001273 were selected as references because their F protein sequences were closest to the consensus F sequence for RSV A and B, respectively.



**Fig. 3. Binding site of MEDI8897\* on RSV F and sequence conservation.** (A) Schematic of mature RSV F polyprotein indicating salient features: F2 and F1 subunits (the N- and C-terminal polypeptides, respectively, after proteolysis of F protein precursor); C, cysteine, FP, fusion peptide; HR1, heptad repeat 1; HR2, heptad repeat 2; TM, trans-membrane. The epitopes are indicated, and the amino acid residues with side chains that form hydrogen bonds or salt bridges with MEDI8897\* are colored blue. (B) Sequence conservation of F residues in the region recognized by MEDI8897\* was analyzed against 2088 F protein sequences from GenBank and an internal database.

The percent conservation of each amino acid within the binding region was calculated for the different subtypes (1337 RSV A sequences and 751 RSV B sequences). Sequence analysis revealed that most of the amino acids in the MEDI8897\* binding regions were highly conserved (>99%) at 24 of the 25 positions in RSV A and at 22 of the 25 positions in RSV B. The remaining four positions (one in RSV A and three in RSV B) showed >96% sequence conservation (Fig. 3B and table S2).

To further this analysis, we evaluated the impact of polymorphisms within the MEDI8897\* binding site in microneutralization assays. We focused on the assessment of binding site polymorphisms that were detected in at least two or more F sequences in the database. Whenever possible, a clinical isolate encoding a polymorphism was evaluated, and when a viable clinical isolate containing a sequence variation was not available, recombinant RSV A2 or RSV B9320 viruses engineered to express the polymorphism were used. Ten of the 13 RSV variants containing amino acid polymorphic changes in the MEDI8897\* binding site were efficiently neutralized by MEDI8897\* with  $IC_{50}$  values that were within threefold of the reference viruses (N63T, N63S, E66K, D200N, and I206V in RSV A and E66D, T67I, N197S, Q209K and N201S/Q209K in RSV B) (Table 1). Recombinant RSV B viruses containing K65Q or K65T and a clinical RSV B isolate encoding substitutions K65Q and S211N were neutralized by MEDI8897\* but with a modest shift in susceptibility (about 7- to 20-fold change compared to the wild-type strains). In vivo, the quantity of antibody required to almost completely block RSV replication with 3-log reduction in the lungs of cotton rats was only threefold higher for RSV B encoding K65Q and S211N compared with wild-type virus (table S3). SPR experiments revealed that the affinity ( $K_d$ ) of MEDI8897\* Fab for B9320-derived RSV F was reduced from 0.03 nM for the wild-type protein to 258 nM and to 326 nM for the K65T single variant and the K65Q/S211N double variant, respectively (fig. S5). The variation at Lys<sup>65</sup> would disrupt the salt bridge formed by the T100bE substitution in the CDRH3, providing a structural basis for the reduced potency of MEDI8897\* for these viral isolates and supporting the hypothesis that

the T100bE substitution was partly responsible for the increased potency of MEDI8897\* over D25. However, the frequency with which the single K65Q or K65T variants or the K65Q/S211N double variant appeared among RSV B sequences was low at 1.3, 0.3, and 0.8%, respectively. Overall, our data indicate that the MEDI8897\* binding site is highly conserved among RSV A and B strains, and all viruses with binding site polymorphisms that were tested were effectively neutralized by MEDI8897\*.

### Prophylactic efficacy of MEDI8897\* and EC<sub>90</sub> determination in cotton rats

RSV infects both the upper and lower respiratory tract of cotton rats (*Sigmodon hispidus*), with peak pulmonary replication seen on day 4. The serum level of antibody that correlates with 50% protection ( $EC_{50}$ ) or a 2-log reduction in viral load in the lungs of RSV-infected cotton rats was used for dose selection of palivizumab that was subsequently demonstrated to be clinically efficacious (20, 29, 30). To assess whether the enhanced potency against RSV exhibited by MEDI8897\* in vitro translated to greater antiviral activity in vivo, we administered weight-based doses of MEDI8897\* and palivizumab by intramuscular injection to cotton rats and then challenged the animals with RSV A2 or RSV B9320 1 day later. Lungs and noses were harvested 4 days after challenge, and RSV titers were determined. Both MEDI8897\* and palivizumab exhibited dose-dependent antiviral activity in preventing RSV replication in the lungs of cotton rats infected with RSV A or B (Fig. 4, A and B); however, MEDI8897\* was significantly ( $P < 0.001$ ) more potent at inhibiting viral replication. Viral titers were >1 to 2 logs lower in the lungs of cotton rats that received MEDI8897\* than in those treated with palivizumab at the same or similar doses (1 to 2 mg/kg for RSV A2 and 0.75 to 2 mg/kg for RSV B9320). In addition, MEDI8897\* was highly active in reducing viral titers in the noses of infected cotton rats, whereas palivizumab failed to inhibit viral replication in the upper airways even when tested at high doses (8 mg/kg).

To further assess the potential of MEDI8897\* as a passive vaccine candidate against RSV, we compared the serum concentrations of MEDI8897\* and palivizumab that correlated with a >3-log mean reduction of RSV titers in the lungs of infected cotton rats. The effective serum concentration yielding a 90% protection ( $EC_{90}$ ) or 3-log mean reduction in lung viral titer compared with control was calculated. MEDI8897\* was found to exhibit mean  $EC_{90}$  values of 6.8  $\mu$ g/ml against RSV A2 and 5.6  $\mu$ g/ml against RSV B9320, which were significantly lower than the respective  $EC_{90}$  values of 76.4 and 51.1  $\mu$ g/ml determined for palivizumab (Fig. 4, C and D). These results indicated that at similar serum concentrations, MEDI8897\* was about ninefold more potent than palivizumab at reducing pulmonary viral loads by >3 logs in RSV A- and RSV B-infected cotton rats.

### PK of MEDI8897 with extended half-life in monkeys and PK simulations in human infants

We conducted a modeling exercise based on the PK of palivizumab in infants and children to assess whether the highly potent MEDI8897\* could protect infants from RSV disease throughout the 5-month RSV season with a single intramuscular administration. Given that both MEDI8897\* and palivizumab contain human IgG1 Fc regions, and that neither is known to bind to any endogenous human target, we reasoned that the PK of these antibodies in infants would be similar. Figure 5A shows the predicted median and 5th to 95th percentile (dark green line and green shaded area, respectively) serum concentrations after a single injection of MEDI8897\* (15 mg/kg) based on published PK of palivizumab in infants and children (26). On the basis of the calculated  $EC_{90}$  for

**Table 1. The effect of natural polymorphisms in MEDI8897\* binding site of RSV F protein on neutralization.** WT, wild-type; r, recombinant.

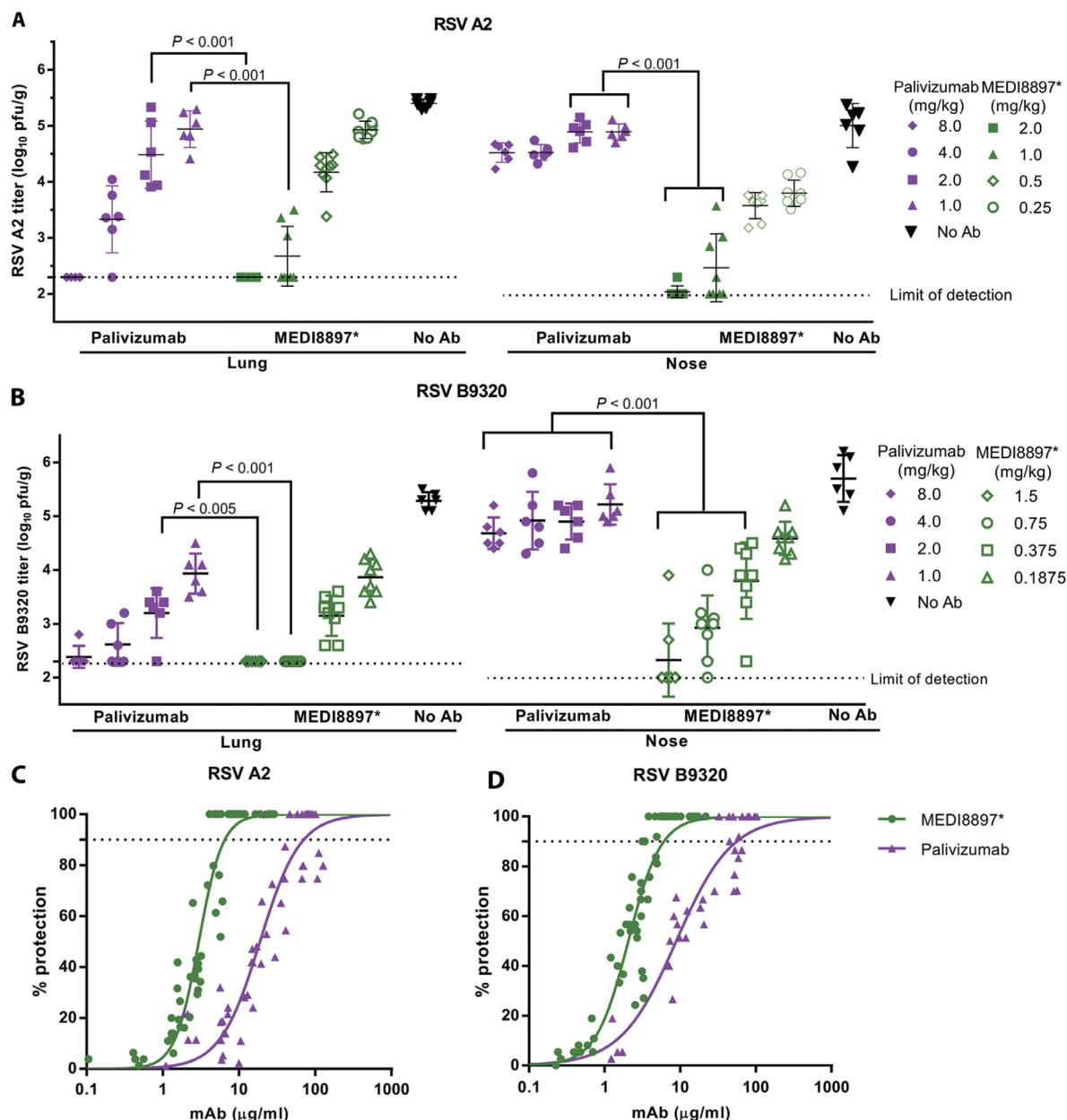
RSV subtype	Amino acid changes	Location in F protein subunit	Frequency of polymorphism (%)	MEDI8897* IC <sub>50</sub> (ng/ml)	Fold change to wild-type virus*
RSV A (n = 1337)	N63T	F2	0.4	7.9	2.1
	N63S	F2	2.6	4.8 <sup>†</sup>	1.5
	E66K <sup>‡</sup>	F2	0.7	3.7	1
	D200N	F1	0.1	3.4 <sup>†</sup>	1.1
	I206V	F1	0.7	3.2 <sup>†</sup>	1.1
	WT rRSV A2	—	—	3.7	1
	A_NLD_13_005275	—	—	4.9 <sup>†</sup>	1.6
	WT RSVA isolates (range)	—	—	3.1 <sup>§</sup>	—
				(0.5 to 15)	
RSV B (n = 751)	K65Q	F2	1.3	26.1	6.5
	K65T	F2	0.3	32.5	8.1
	E66D	F2	0.7	1.1 <sup>†</sup>	0.4
	T67I	F2	0.8	5.3	1.3
	N197S <sup>‡</sup>	F1	0.5	4	1
	Q209K	F1	3.6	4.7 <sup>†</sup>	1.6
	N201S, Q209K	F1	0.3	2.8 <sup>†</sup>	0.9
	K65Q, S211N	F2, F1	0.8	59.8 <sup>†</sup>	20
	WT rRSV B9320	—	—	4	1
	B_NLD_13_001273	—	—	7.5 <sup>†</sup>	2.5
	WT RSV B isolates (range)	—	—	3.0 <sup>  </sup>	—
				(0.8 to 59.7)	

\*Fold change calculated by dividing the IC<sub>50</sub> values of recombinant or clinical variants with IC<sub>50</sub> values of parental recombinant virus or with median IC<sub>50</sub> values of a panel of RSV clinical isolates, respectively. <sup>†</sup>IC<sub>50</sub> determined using clinical isolate containing indicated polymorphism. <sup>‡</sup>The E66K and N197S polymorphisms occur in the F protein of rRSV A2 and B9320 reference viruses, respectively. <sup>§</sup>Median IC<sub>50</sub> from 59 RSV A isolates. <sup>||</sup>Median IC<sub>50</sub> from 43 RSV B isolates.

MEDI8897\* against RSV A2 and RSV B9320 in the cotton rat model of RSV disease, a target serum concentration of 6.8 µg/ml would need to be maintained over the 5-month RSV season to ensure optimal protection against RSV. However, the predicted PK profile for MEDI8897\* after a single intramuscular dose indicates that serum concentrations of antibody would not be sustained at this level for more than about 2 months, thus providing insufficient coverage.

To enable once-per-season dosing for all infants entering their first RSV season, we considered the impact of antibody half-life extension on PK. We previously demonstrated that the three amino acid substitution M252Y/S254T/T256E (referred to as YTE) in the Fc domain of antibody increased IgG affinity for the human FcRn at lower pH, allowing for recirculation of the mAb. This resulted in an extended serum half-life (*t*<sub>1/2</sub>) of 70 to 100 days, which is a two- to fourfold increase compared with the parental antibody in both cynomolgus monkeys and healthy human adults (23, 25). Therefore, the YTE substitutions were engineered into the Fc region of MEDI8897\*, and the resulting antibody, referred to as MEDI8897, was tested in vitro to confirm its anti-RSV activity.

As expected, MEDI8897 gave IC<sub>50</sub> values against laboratory strains of RSV A and B that were similar to those determined for MEDI8897\* (table S4). MEDI8897 was then tested in a Good Laboratory Practice (GLP)-compliant nonclinical repeat-dose toxicology study in which cynomolgus monkeys received five once-weekly intramuscular (300 mg, fixed dose) or intravenous (300 mg/kg, body weight-scaled) administrations of mAb. Results showed no local or systemic adverse effects through day 31 (terminal necropsy) nor through day 169 after a 5-month dose-free recovery phase. The concentrations of MEDI8897 in monkey serum samples were determined for PK assessment. MEDI8897 exhibited linear and dose-proportional PK, with a mean *t*<sub>1/2</sub> calculated to be 40.5 or 39.9 days after intravenous or intramuscular dosing, respectively (Fig. 5B). As expected, the serum *t*<sub>1/2</sub> for MEDI8897 in cynomolgus monkeys was similar to what was observed previously for motavizumab-YTE, which was three- to fourfold longer than that determined for palivizumab or motavizumab without Fc modification. A similar magnitude of half-life extension for motavizumab-YTE was subsequently demonstrated in humans (23, 25).



**Fig. 4. Prophylactic efficacy of MEDI8897\* and determination of  $EC_{90}$  in RSV-infected cotton rats.** (A and B) Animals were administered with MEDI8897\* or palivizumab by intramuscular injection at the indicated concentrations 1 day before challenge with RSV. The viral titers in the lungs and nose were determined by plaque assay on day 4 after infection with (A) RSV A2 or (B) RSV B9320. Error bars indicate mean values with SD ( $n = 6$  to 8). The dotted line in (A) and (B) indicates the limit of detection. Serum concentrations of human IgG were measured by ELISA. (C and D) The serum level of MEDI8897\* that correlates with  $EC_{90}$  or  $>3$ -log reductions compared to control in RSV viral titers in the lungs of infected rats was calculated using a nonlinear regression analysis for (C) RSV A2 and (D) RSV B9320. The dotted line in (C) and (D) represents  $EC_{90}$  for viral reduction. Data are representative of two to three independent experiments (A and B) or were from the combined two to three experiments (C and D). Significance is determined by Mann-Whitney test (A and B).

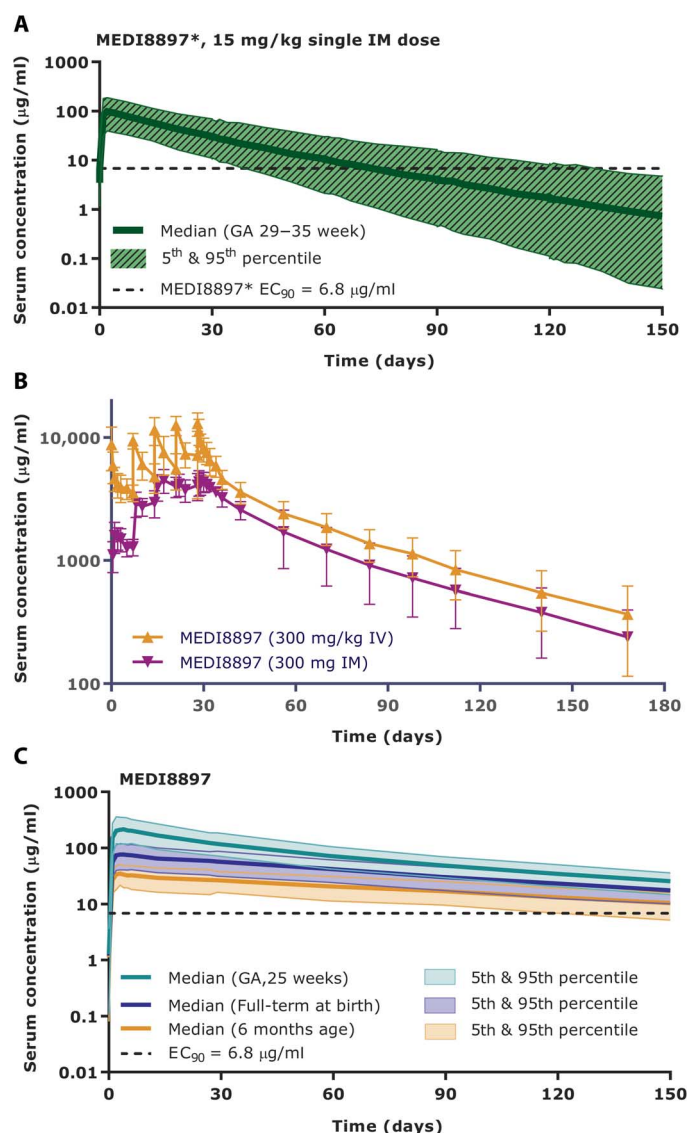
The population PK model developed for palivizumab incorporating the effect of body weight and age on clearance to adjust for growth and maturation of infants was used to predict concentration of MEDI8897 after a single intramuscular dose in infants from birth to 6 months of age, entering their first RSV season (Fig. 5C). The simulated PK profiles of MEDI8897 indicated that targeted serum levels  $\geq 6.8 \mu\text{g/ml}$  could be maintained for the duration of the 5-month RSV season with a single fixed 50-mg dose of MEDI8897 for all infants. A similar sustained PK exposure with palivizumab is obtained only by administration of five

monthly doses to infants (fig. S6) (31). On the basis of improved in vivo potency and extended half-life, PK simulations predict that MEDI8897 should provide protection against RSV with once-per-season dosing for all infants in their first RSV season.

## DISCUSSION

A paradigm shift from active to passive vaccination may enable a safe and cost-effective solution to the problem of serious RSV lower respiratory





**Fig. 5. PK predictions and profiles.** (A) MEDI8897\* serum PK simulations in infants [gestational age (GA), 29 to 35 weeks] after a single 15-mg/kg intramuscular (IM) dose. Simulations were based on the published PK of palivizumab in infants and children. (B) Observed MEDI8897 serum PK profile in cynomolgus monkeys ( $n = 6$ ) after five weekly 300 mg/kg intravenous (IV) or 300 mg fixed intramuscular doses. (C) Predicted MEDI8897 serum PK profile in infants (GA of 25 weeks, full-term at birth, and 6 months) after a single fixed intramuscular dose (50 mg). Dashed line in (A) and (C) represents  $EC_{90}$ , effective concentration yielding a 90% protection. Data and error bars in (B) represent means  $\pm$  SD.

tract disease in infants. The objective of this study was to develop a candidate prophylactic agent that could be evaluated in human clinical studies against RSV and was suitable for administration to all infants. Although maternal vaccination is also being explored as a means to prevent RSV, and circumvents the challenges inherent in active vaccination of infants (32), it is not known whether the maternal immunity induced by such vaccines will translate into sufficient protection of babies. In contrast, passive vaccination of infants with a neutralizing mAb has been clinically validated to protect at-risk populations from severe RSV diseases. For passive vaccination to be applied beyond the narrow population currently being addressed by palivizumab, it was necessary to

identify an antibody with exceptional potency and breadth of coverage against RSV. We selected the human mAb D25 based on its high potency and neutralization breadth and then further optimized it for improved activity and reduced potential immunogenicity to generate MEDI8897\*. Although the potency improvement of MEDI8897\* over D25 was modest (about fourfold), we believe that this small improvement in activity is important because it could help keep the required dose of the antibody and the accompanying cost-per-dose relatively low.

We conducted a structural analysis of MEDI8897\* bound to prefusion forms of RSV A and B F protein and found the structurally defined contact residues to be highly conserved. Our study is also the first to report the cocrystal structure of an RSV antibody complexed with RSV B–derived F protein. The complex contained only two MEDI8897\* Fabs bound to each RSV B F protein trimer. Although there is no room in the crystal lattice for a third Fab to bind the RSV F trimer because of crystal packing, it is not obvious from the structural data why all three sites are not occupied in solution. Whether this occurs on the surface of a virus or for all subtype B proteins is unknown. Similar substoichiometric complexes have also been observed for other viral glycoproteins, such as HIV-1 envelope glycoprotein and influenza hemagglutinin (33, 34).

We investigated the level of conservation of the MEDI8897\* binding site by examining F protein sequences from clinical and laboratory isolates obtained from GenBank and MedImmune databases. This analysis revealed that most of the amino acids within the binding site are >99% conserved. Thus, for the RSV F protein, even regions of relative sequence variation are quite stable. Previously, in describing the crystal structure of RSV F bound to D25, McLellan *et al.* noted that the D25 binding region, named antigenic site  $\emptyset$ , is among the least conserved on the F glycoprotein (28). It was subsequently shown that this site is the target of most of the neutralizing antibodies found in human serum in response to natural RSV infection (35). The breadth of MEDI8897\* activity was investigated using a panel of clinical RSV isolates and recombinant viruses encoding sequence polymorphisms identified in the MEDI8897\* binding site. MEDI8897\* neutralized all RSV A strains tested within a threefold range of  $IC_{50}$  values. The range of  $IC_{50}$  values for MEDI8897\* against RSV B was broader. RSV B strains encoding changes at F amino acid 65 (K65Q and K65T) or K65Q in combination with S211N were effectively neutralized by MEDI8897 but with a 7- to 20-fold shift in  $IC_{50}$  in vitro. However, only about threefold change in  $EC_{90}$  in vivo was observed. This difference between in vitro  $IC_{50}$  and in vivo  $EC_{90}$  may reflect antibody-mediated mechanisms beyond direct virus neutralization, such as viral clearance and killing of infected cells that may contribute to in vivo protection but are not measured in an in vitro microneutralization assay. A recent study in the cotton rat model suggests that antibody-dependent cell-mediated toxicity may play an important role in protection against RSV challenge (36).

Overall, our results indicate that the sequence conservation in the MEDI8897\* binding region is high, and the breadth of coverage against RSV is anticipated to be very good because MEDI8897\* is able to effectively neutralize all RSV F protein sequence variants tested. The impact that broad use of MEDI8897 will have on emergence of resistant variants and onward transmission of resistant variants is unknown, although the selective pressure that MEDI8897 will exert is likely to be similar to the selective pressure already exerted by a subset of the polyclonal mAb repertoire against the antigenic site  $\emptyset$  of humans who are repeatedly exposed to RSV. In addition, it is important to understand whether the widespread use of MEDI8897 could mask the site  $\emptyset$  epitope and induce an altered immune response during natural RSV infection.



To enable RSV protection throughout the winter season with a single dose of antibody, MEDI8897 was engineered to carry the YTE mutations in the heavy-chain CH2 constant region of the Fc domain. MEDI8897 was found to have an acceptable preclinical safety profile and extended half-life in cynomolgus monkeys consistent with that observed for other YTE-modified mAbs. In addition, recent results in adults confirmed that the half-life of MEDI8897 was about threefold greater than that of other anti-RSV mAbs (palivizumab and motavizumab) (ClinicalTrials.gov: NCT02114268) (37).

A modeling exercise based on the known PK of palivizumab in infants suggests that a single administration of MEDI8897 at an appropriate dose will result in serum levels that correlate with near-complete protection against RSV in the cotton rat model. Whereas it is very well known that results in rodents often do not translate to humans, our past history of using data in the cotton rat model of RSV to predict an effective dose of palivizumab provides us with a significant level of confidence that a single intramuscular dose of MEDI8897 can provide most babies with protection against RSV throughout the entire season, even taking into account a minority of RSV sequence variants with reduced susceptibility to this mAb.

## MATERIALS AND METHODS

### Study design

This study was designed to assess the potential to develop a once-per-season, RSV-neutralizing antibody as passive prophylaxis for RSV disease in all infants. We tested a panel of RSV-neutralizing antibodies isolated directly from human B cells in *in vitro* functional activity screens, and the selected antibody with highest activity was further optimized for the improved activity and reduced immunogenicity based on *in silico* analysis (EpiVax) and validated in the functional microneutralization assay against RSV laboratory strains as well as a diverse panel of RSV A and B clinical isolates. The resulting antibody MEDI8897\* exhibited the improved potency and breadth of neutralization and was selected as the lead candidate and further evaluated in the RSV A and B infection cotton rat model. To define the epitope and explore the molecular basis of broad neutralization of this antibody, we determined the crystal structure of the MEDI8897\* Fab bound to a prefusion-stabilized RSV F protein (DS-Cav1) derived from the A2 and B9320 strains. In addition, we introduced triple amino acid substitutions into the Fc constant region to generate antibody MEDI8897 with extended serum half-life, which was further confirmed in cynomolgus monkeys. The potential of MEDI8897 as a passive RSV prophylactic agent for all infants with one dose per RSV season was then predicted through population PK simulation based on the model of palivizumab validated in the clinic.

All *in vivo* research experiments were designed in compliance with good statistical practices. All *in vivo* research study protocols were approved by MedImmune's Institutional Animal Care and Use Committee and performed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International-accredited facility. Sample size estimation was performed using nQuery Advisor 7.0 software (Statsols). The hypothesized effect size and dose groups for each comparison were derived from historical data or pilot study data. All samples were included in all the experiments. All *in vitro* and *in vivo* research experiments were repeated at least two times, unless otherwise stated within the figure legends.

The repeat-dose toxicology study in cynomolgus monkeys was conducted in compliance with GLP at an AAALAC-accredited contract research laboratory (MPI Research Inc.) under the study protocol

approved by the Animal Care and Use Committee of the facility. The objective of the study was to evaluate the potential toxicity of MEDI8897 when administered once weekly (five total doses) via intravenous infusion (30 min) or via intramuscular injection compared to vehicle treated controls. Group sizes were based on standard study designs of nonhuman primate toxicology studies typically acceptable to regulatory health authorities (38). Primary data are located in table S5.

### Microneutralization assays

Microneutralization assays were performed as described previously with modifications (39). Briefly, assays were performed in 384-well microtiter plates in HEP-2 cell culture medium. Plates containing 15  $\mu$ l per well of virus with a tissue culture half-maximal infectious dose (TCID<sub>50</sub>) of 25 to 1000 and 15  $\mu$ l per well of serially diluted antibodies were incubated for 1 to 1.5 hours at 37°C with 5% CO<sub>2</sub>. HEP-2 cells were then added at  $2.5 \times 10^5$  cells/ml in 30  $\mu$ l to each well, and the plates were incubated at 37°C for 3 to 4 days. Cells were fixed with ice-cold 80% acetone/20% phosphate-buffered saline for 15 min at 4°C. Viral replication was measured by ELISA using a horseradish peroxidase-conjugated anti-RSV F mAb targeting the C site of RSV F (1331H) and was quantified by monitoring absorbance at 450 nm using a microplate reader. IC<sub>50</sub> values were calculated using a nonlinear fit algorithm in GraphPad Prism version 5 (GraphPad Software).

### Prophylaxis in cotton rat model of RSV infection

Six- to 8-week-old cotton rats (Envigo) were administered intramuscularly with antibodies with the dose determined on the basis of the body weight of individual animals measured 2 days before dosing. A nonrelevant IgG1 mAb, R347, or virus only without antibody treatment was used as negative control. Animals were anesthetized 24 hours later using an isoflurane chamber and infected intranasally with  $1 \times 10^6$  plaque forming units (PFU) per animal of RSV A2 or  $2.7 \times 10^5$  PFU per animal of RSV B9320. Four days later, animals were euthanized; their lungs and noses were harvested. Blood was collected by cardiac puncture at the time of sacrifice to measure serum mAb concentrations. To assess virus load, the lungs (bisected or whole) or noses were homogenized and titrated on HEP-2 cells in a plaque assay on 24-well plates, as described previously (40). The limit of detection of virus for the bisected lungs was 200 PFU/g of tissue and 100 PFU/g for the whole lungs and nose samples. The human IgG concentration in the serum was measured using a standard human IgG-specific sandwich ELISA, as described previously (41).

### Statistical analyses

Lines present in the graphs represent the mean as indicated. The Mann-Whitney test was used for lung and nose titers in MEDI8897-treated group compared with palivizumab. We considered  $P < 0.05$  (two-sided) as statistically significant. All statistical analyses were performed using the GraphPad Prism software for the groups receiving comparable doses of antibodies.

## SUPPLEMENTARY MATERIALS

[www.sciencetranslationalmedicine.org/cgi/content/full/9/388/eaaj1928/DC1](http://www.sciencetranslationalmedicine.org/cgi/content/full/9/388/eaaj1928/DC1)

Materials and Methods

Fig. S1. Alignment of V<sub>H</sub> amino acid sequences of D25 and MEDI8897\* with their unmutated common ancestor sequences.

Fig. S2. Gel filtration of soluble prefusion RSV F with MEDI8897\* Fab.

Fig. S3. Binding of D25 variants to RSV F A2 determined by SPR.

Fig. S4. Comparison of prefusion-stabilized F derived from subtypes A and B.

Fig. S5. Binding of MEDI8897\* to RSV F B9320 variants determined by SPR.

Fig. S6. Simulated palivizumab serum PK profile in infants (gestational age, 29 to 35 weeks) after five monthly 15 mg/kg intramuscular doses.

Table S1. Crystallographic data collection and refinement statistics.

Table S2. The frequency of conserved amino acid residues in the F protein of RSV A and B viruses within the MEDI8897\* binding site.

Table S3. Comparison of the antiviral activities of MEDI8897\* and palivizumab in the cotton rats infected with RSV B isolate containing K65Q and S211N.

Table S4. Comparison of the antiviral activities of MEDI8897\* and MEDI8897 in vitro.

Table S5. Primary data.

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