Structural basis for antibody cross-neutralization of respiratory syncytial virus and human metapneumovirus

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Respiratory syncytial virus (RSV) and human metapneumovirus (HMPV) are two closely related viruses that cause bronchiolitis and pneumonia in infants and the elderly1, with a significant health burden2–6. There are no licensed vaccines or small-molecule antiviral treatments specific to these two viruses at present. A humanized murine monoclonal antibody (palivizumab) is approved to treat high-risk infants for RSV infection7,8, but other treatments, as well as vaccines, for both viruses are still in development. Recent epidemiological modelling suggests that cross-immunity between RSV, HMPV and human parainfluenzaviruses may contribute to their periodic outbreaks9, suggesting that a deeper understanding of host immunity to these viruses may lead to enhanced strategies for their control. Cross-reactive neutralizing antibodies to the RSV and HMPV fusion (F) proteins have been identified10,11.

Here, we examine the structural basis for cross-reactive antibody binding to RSV and HMPV F protein by two related, independently isolated antibodies, MPE8 and 25P13. We solved the structure of the MPE8 antibody bound to RSV F protein and identified the 25P13 antibody from an independent blood donor. Our results indicate that both antibodies use germline residues to interact with a conserved surface on F protein that could guide the emergence of cross-reactivity. The induction of similar cross-reactive neutralizing antibodies using structural vaccinology approaches could enhance intrinsic cross-immunity to these paramyxoviruses and approaches to controlling recurring outbreaks.

The respiratory syncytial virus/human metapneumovirus (RSV/HMPV) F protein is a class I viral fusion protein that refolds to drive membrane fusion and virus entry12–18, and it is the major target of the neutralizing antibody response. MPE8 was isolated from human B cells based on its ability to cross-neutralize a panel of RSV and HMPV strains10. We determined the crystal structure of an MPE8 variant in complex with a stabilized prefusion RSV F trimer (DS-CAV1)19–22. We generated single-chain Fv (scFv) constructs for MPE8 and predicted unmutated common ancestor (UCA) variants that retain RSV F binding and neutralization10 (Supplementary Figs 1 and 2). Complexes of MPE8 with DS-CAV1 (ref. 22) were prepared by co-transfection of 293-6E cells and purified using affinity and gel filtration chromatography (Supplementary Fig. 3). Optimized crystals of DS-CAV1 with the MPE8 scFv variant consisting of the predicted UCA heavy-chain variable domain (VHgl) and the fully mature light-chain variable domain (VLsm) were obtained, and the structure was solved by molecular replacement (Fig. 1, Methods and Supplementary Table 1).

MPE8 binds an epitope near the midsection of the RSV F ectodomain (Fig. 1), consistent with previous mapping studies10. The

Figure 1 | Overall structure of the RSV F:MPE8 complex. a,b, Structure of the complex of MPE8 scFv with prefusion RSV F. RSV F subunits are shown in surface format. Two subunits are coloured light grey. The third subunit is coloured by domain (DI, cyan; DII, green; DIII, magenta: heptad repeat B (HRB), blue). The MPE8 scFvs are shown in cartoon format with VH coloured pink and VL coloured yellow. The view in b is rotated 90° from a, and oriented down the three fold axis of the F trimer. MPE8 binds an epitope in the midsection of the RSV F ectodomain at the intersection of DI, DII and DIII domains from two subunits of the F trimer.
The three scFvs are positioned with VH and VL domains aligned nearly perpendicular to the long axis of the F trimer and parallel to the predicted plane of the viral membrane. When viewed along the three-fold axis of the trimer, the MPE8 V domains radiate horizontally outwards from three apices of the F subunits, engaging the widest section of the F head domain (Fig. 1). MPE8 binds preferentially to prefusion F. Its conformational specificity has three apparent structural determinants. First, MPE8 engages RSV F residues spanning two neighbouring subunits of the trimer (Figs. 1 and 2a), defining an intersubunit epitope present only in the prefusion conformation. Second, MPE8 contacts two β-strands of the prefusion heptad repeat A (HRA), which refold into a long helix and move away from the MPE8 interface in the postfusion form \(^{14,17}\) (Supplementary Fig. 4a,b). Third, docking of MPE8 onto the F structure indicates that steric clashes between F DII and the MPE8 VL domain could interfere with binding (Supplementary Fig. 4c,d).

Most of the MPE8 epitope lies within one subunit of the F trimer, involving residues in DII and DIII, with a smaller contact area in DI of the neighbouring subunit (Fig. 2a). The DIII contacts overlap the helix-turn-helix motif of the palivizumab/motavizumab site A epitope (Fig. 2a,b). The VH domain lies distal to the F trimer interface, primarily contacting the F surface below and to one edge of site A (Fig. 2a–c). The heavy-chain complementarity-determining region 3 (HCDR3) loop extends underneath the site A motif in DIII to insert its tip into a pocket formed at the intersubunit interface (Fig. 2b,c), with residues Y100 and N100A forming contacts dependent on the prefusion F conformation. HCDR3 residues (100B–100G) interact with the turn residues within site A (Fig. 2b). HCDR2 also forms interactions with the second helix of site A and with residues in DI, while HCDR1 contacts F below site A in DI (Fig. 2c). RSV F mutations that have been shown previously to affect MPE8 binding also map to the structural interface (Supplementary Fig. 5). The mutation of D310A would disrupt interactions with HCDR2 and many of these previously identified mutations line the groove along which HCDR3 extends (L305R, G307R, R49D and T50A).

The VL domain is positioned near the trimer interface, with light-chain complementarity-determining region 2 (LCDR2) contacting...
DII of the adjacent subunit (Fig. 2c). LCDR2 interactions across the trimer, together with HCDR3, would contribute to preferential binding to prefusion F. LCDR1 and LCDR3 interact with the first helix of site A (Fig. 2). LCDR1 also contacts two β-strands of the DIII HRA motif (F residues 178, 180 and 184–187), present only in the prefusion conformation (Supplementary Fig. 4). The MPE8 paratope is a relatively flat surface composed of the six CDR loops, with the heavy-chain HCDR3 wrapping across the front of the VH domains and covering LCDR2 (Fig. 2d). HCDR1, HCDR2 and HCDR3 form an L-shaped surface that defines the majority of the MPE8 binding surface, with HCDR3 inserting into the F intersubunit groove.

The MPE8 epitope is only partially conserved in HMPV (Fig. 3a and Supplementary Table 2). HCDR1 and HCDR2 anchor the antibody to the largest contiguous surface of conserved residues (Fig. 3a). HCDR3 extends along a narrower conserved segment to contact additional, more fragmented patches of surface residue conservation. The light chain, particularly LCDR1, also contacts smaller, more isolated islands of conserved residues (Fig. 3a). MPE8 cross-reactivity appears to be achieved by the formation of a large interface with a mosaic patchwork of conservation rather than by a narrow focus on a single conserved site.

The predicted MPE8 UCA selectively neutralizes RSV, but not HMPV\(^{10}\), indicating that cross-reactivity emerges as a result of somatic hypermutation that broadens its reactivity to HMPV. Fourteen residues in MPE8, nine in VH and five in VL are mutated in mature MPE8. VH residues play a major role in HMPV cross-reactivity\(^{10}\). In the MPE8 structure, three of the five VL residues (D50, N52 and R93) are located in or near the RSV F interface (Fig. 3b,c). For VH, only three of the nine somatic mutations are within the RSV interface (residues S52A, Y58 and N100A in VH\(_{\text{F}}\); Fig. 3b,c). Vl D50 is a major determinant of cross-reactivity to HMPV F (ref. 10). Mutation of D50 to glycine, representing a reversion to the UCA, ablates HMPV F binding. D50 is located behind HCDR3 and does not directly contact F, but forms a hydrogen bond to HCDR3 residue T98 (Fig. 3d). D50 may therefore indirectly stabilize an HCDR3 conformation that can bind both RSV and HMPV F, similar to somatic mutations observed in other antibodies. Mutation of Vl R93S in LCDR3 reduces MPE8 binding to HMPV B strains\(^{10}\). R93 lies above antigenic site A and forms a hydrogen bond to MPE8 residue T98, which could impact HCDR3 conformation and HMPV cross-reactivity.
Figure 4 | 25P13 is an MPE8-related cross-reactive neutralizing antibody. a, 25P13 binds both RSV and HMPV F proteins and neutralizes both viruses. Top panel: 25P13 and motavizumab antibody binding to RSV and HMPV F proteins, measured by ELISA, and with influenza virus haemagglutinin (H5 A/Vietnam HA) as a negative control. Bottom panel: RSV and HMPV neutralization by 25P13, with anti-Dengue virus antibody (2D22) as a negative control. For ELISA curves, data points are the average of four technical replicates from one of at least two independent experiments. For neutralization experiments, data points are the average of four technical replicates from one of at least two independent experiments. All error bars represent 95% confidence intervals. OD₄₀₅ nm, optical density at 450 nm. b, Epitope binning using the OctetRed system with anti-penta-HIS biosensors. Sensors were coated with RSV A2 fusion protein (DS-CAV1, prefusion conformation). mAbs on the y-axis were loaded first, followed by mAbs on the x-axis. mAbs were judged to compete for the same site if maximum binding of the competing mAb was reduced to <33% of its uncompeted binding (black boxes with white numbers). mAbs were considered non-competing if maximum binding of the competing mAb was >66% of its uncompeted binding (white boxes with black numbers). Dark grey boxes with black numbers indicate an intermediate phenotype (between 33% and 66% of uncompeted binding). c, Epitope binning using OctetRed system with anti-penta-HIS biosensors. Sensors were coated with RSV A2 fusion protein (post-fusion conformation, fusion peptide removed). mAbs on the y-axis were loaded first, followed by mAbs on the x-axis. mAbs were judged to compete for the same site if maximum binding of the competing mAb was reduced to <33% of its uncompeted binding (black boxes with white numbers). mAbs were considered non-competing if maximum binding of the competing mAb was >66% of its uncompeted binding (white boxes with black numbers). Dark grey boxes with black numbers indicate an intermediate phenotype (between 33% and 66% of uncompeted binding). d, 25P13 uses the same VH and VL segments as MPE8 and shows homology in the HCDR3 and LCDR3 sequences. Many conserved amino acids of the 25P13 LCDR3 correspond to the F-contacting residues in MPE8 (indicated above the sequences with asterisks), suggesting a similar mode of F engagement. DH, antibody heavy-chain diversity segment; JH, antibody heavy-chain joining segment; JL, antibody-light chain joining segment. e, ELISA assays measuring the binding of 25P13 and MPE8 antibodies to RSV F mutants that disrupt MPE8 binding and lie within the MPE8 epitope (left panel: L305R; middle panel: G307R; right panel: D310A). Data points are the average of four technical replicates from one of at least two independent experiments. Error bars represent 95% confidence intervals.
bond with the main chain of RSV F residue 263 (Fig. 3b,c). To a lesser extent, somatically mutated residues in V\text{\textit{HV}} have also been shown to influence MPE8 cross-reactivity\textsuperscript{19}. The structure indicates that these effects are probably due to V\text{\textit{HV}} residues Y58 and N100A in HCDR2 and HCDR3, respectively. Y58, the predicted germline residue, forms contacts to RSV F D310, an F residue important for MPE8 binding. N100A is at the tip of HCDR3 and is mutated to serine in the mature MPE8 sequence (Fig. 2b,c).

To further explore the diversity of cross-reactive antibodies to RSV and HMPV, we identified a novel antibody (25P13) from a blood donor that also binds and neutralizes both RSV and HMPV F (Fig. 4a). 25P13 binding is stronger to a chimaeric HMPV F blood donor that also binds and neutralizes both RSV and HMPV F (Fig. 4a). Cross-competition assays with a panel of RSV-specific antibodies show that 25P13 competes with MPE8, as well as motavizumab and palivizumab (Fig. 4b), while partially competing with 101F and 54G10 antibodies. The similar cross-competition pattern for MPE8 and 25P13 indicates that both antibodies overlap antigenic sites A and IV. However, in contrast to MPE8, 25P13 binds more readily to postfusion RSV F (Fig. 4c).

We sequenced the 25P13 heavy- and light-chain variable regions (Fig. 4d and Supplementary Fig. 6). 25P13 uses the same V\text{\textit{HV}} gene segment (3–21*01) as MPE8 and a closely related or identical V\text{\textit{L}} segment (Fig. 4d). Sequences of the HCDR and LCDR loops show significant homology. In particular, residues in MPE8 HCDR1 and HCDR2 are >80% conserved, indicating that 25P13 could engage the same conserved surface patch of residues on F similar to MPE8 (Fig. 3a). Indeed, 25P13 and MPE8 both lose binding to RSV F DS-CAV1 with the D310A mutation (Fig. 4e and Supplementary Fig. 5). LCRD3 is also well conserved (seven out of ten residues), including two key residues (Y107 and R93) that are buried in the MPE8 complex with RSV F. HCDR3 exhibits the largest differences (~50% identity) and is shorter by two residues, indicating that the 25P13 HCDR3 would not insert as deeply into the intersubunit crevice in F and may not engage two subunits of F as observed for MPE8 (Fig. 2). A G307R mutation was previously shown to disrupt MPE8 binding\textsuperscript{10}. This mutation significantly reduces but does not eliminate 25P13 binding, consistent with the interaction differences of its shorter HCDR3 loop (Fig. 4e and Supplementary Fig. 5). Together, these data indicate that related cross-reactive neutralizing antibodies can be readily identified that use germline HCDR1 and HCDR2 sequences to anchor interactions with F through a conserved surface determinant present in RSV and HMPV F proteins.

The MPE8 epitope is distinct from other structurally characterized anti-F antibodies (Supplementary Fig. 7). D25 (ref. 14) and AM14 (ref. 24) are two neutralizing antibodies specific for the prefusion F conformation, while motavizumab can bind both pre- and post-fusion RSV F. D25 binds an epitope in the top of DIII that does not have any overlap with MPE8 (ref. 14). Low-resolution structures of AM14 and motavizumab with RSV F have been determined\textsuperscript{24}, and binding of both of these antibodies would be sterically blocked by MPE8. The epitopes of AM14 and motavizumab partially overlap that of MPE8. However, only MPE8 engages the larger patch of conserved residues lying below the site A epitope that provides its nascent cross-reactivity (Fig. 3a).

Broadly neutralizing antibodies to HIV Env and to influenza virus HA have been shown in several cases to involve a focusing of the antibody interaction on highly conserved elements of an indispensable functional site\textsuperscript{25–28}. MPE8 and 25P13 appear to achieve their cross-reactivity through an anchoring interaction mediated by germline HCDR1 and HCDR2 loops with a conserved F surface within D1, while fine-tuning interactions across a patchwork of conserved and non-conserved epitope residues. Specific recognition of the prefusion F conformation through this cross-reactive site is not required for neutralization, as shown by our observations with 25P13, although the conformational specificity of MPE8 could contribute to its greater potency of neutralization. Other potentially cross-reactive antibodies to RSV and HMPV, which bear the same V\text{\textit{HV}} domain as MPE8 and 25P13, may be present as nascent variants within the antibody repertoire, providing an attractive target for structure-based vaccine design.

Methods

Expression and purification of proteins. The gene encoding the stabilized prefusion DS-CAV1 RSV F trimer was synthesized (GeneWiz) and cloned into the PTT5 expression vector (National Research Council (NRC), Canada) with a T4-fibrin trimerization domain in-frame with the heptad repeat of the C-terminal HRB and with a thrombin cleavage site. The wt MPE8 single-chain Fv (scFv) gene was synthesized by Life Technologies as a GeneArt Strings DNA fragment, was cloned into the PCEP4 expression vector (Invitrogen) and contains a C-terminal TEV cleavage site and His6 tag. The RSV F and scFv expression plasmids were prepared using the Plasmid Mega Kit (Qiagen) and transfected into suspension 293-SE cells (NRC) at a density of 1.8 to 2.0 million cells per ml using 25 kDa linear polyethylenimine (PEI, Polysciences), following 293-SE cell protocols. Supernatants were collected five days post-transfection by centrifugation (20 min at 8,000 g at room temperature), filtered through 0.45 µm filters and dialysed against 200 mM NaCl, 50 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 7.4. The RSV F protein was purified by Co\textsuperscript{2+} affinity chromatography (TALON Resin, BD Biosciences) and size exclusion chromatography using Superdex-200 column equilibrated in 25 mM sodium phosphate, pH 7.4, and 100 mM NaCl, 100 mM imidazole. The purified protein was concentrated with Amicon Ultra centrifugal filters with a 10 kDa molecular weight cutoff (Millipore). RSV F mutants L305R, G307R and D310A were prepared from the DS-CAV1 RSV F construct (Bioult - Mutagenesis). Additional MPE8 variants were synthesized and expressed into the PTT5 expression vector corresponding to the PTT5 expression vector encoding HgL-BSM, hS-ISM-DS50G and HgL-IGL G56D. The MPE8 mutant proteins were expressed similarly to wt MPE8 in 293-SE cells, with supernatants collected five days post-transfection. The MPE8 proteins were purified by Co\textsuperscript{2+} affinity chromatography and size exclusion chromatography and concentrated as with wt MPE8. RSV F DS-CAV1 mutants L305R, G307R and D310A were expressed in explCHO cells following the manufacturer’s protocol, collected after six days, and purified by HiTrap TALON crude columns (GE Healthcare). cDNAs encoding the published variable gene sequences encoding the mAbs motavizumab, 101F and D25 were synthesized (Genescript), and heavy and light chain sequences were cloned into expression vectors using lambda or kappa light-chain constant regions, respectively. Plasmids encoding the heavy and light chains of mAb 54G10 were a gift from D. Burton (Scripps Research Institute). Mab 131-2a protein was obtained from Sigma (MAB8599). Commercial preparations of palivizumab (Medimmune) were obtained from the pharmacy at Vanderbilt University Medical Center. Recombinant mAbs were expressed in HEK293F cells for five days following the manufacturer’s protocol and purified by HiTrap MabSelectSure columns (GE Healthcare).

Crystalization and structure determination of the MPE8:RSV F complex. Complexes of MPE8 scFvs with the DS-CAV1 RSV F variant were prepared by covalent cross-linking of 293-SE cells, followed by purification chromatography and gel filtration chromatography. Complexes of MPE8 with the DS-CAV1 RSV F were observed using size exclusion chromatography with a Superdex-200 column (Supplementary Fig. 3) and verified by SDS–PAGE. Complexes were dialysed into 25 mM Tris-HCl and 125 mM NaCl pH 7.0 and concentrated to 6–8 mg ml\textsuperscript{-1}. Crystals were grown from hanging drops with a well solution containing 0.1 M potassium nitrate, 0.1 M citrate phosphate pH 4.2, 1% tacsimate pH 7.0, 14% PEG 6000. Crystals were observed after 3–10 days.

Native crystals were transferred to a cryoprotectant solution of 0.1 M potassium nitrate, 0.1 M citrate phosphate pH 4.2, 1% tacsimate pH 7.0, 14% PEG 6000 and 15% glycerol, followed by flash-cooling in liquid nitrogen. Data were collected at LS-CAT 21-ID-F beamline at the Advanced Photon Source (APS), Argonne National Laboratory. The crystals belonged to space group P3121 and exhibited significant diffraction anisotropy. The native data were initially processed to 3.1 Å with X-ray Detector Software (XDS) and then submitted to the Diffraction Analysis Server, which truncated the data to 3.1 Å along the c* axis and 3.6 Å along the a*/b* axes (Supplementary Table 1).

Molecular replacement searches were conducted with the program Phaser (ref. 29) using the CCP4 suite of programs\textsuperscript{30}. The PCK9 Fab model (3H42) and prefusion RSV F model (4HUW) yielded clear molecular replacement solutions. Initial refinement provided an R\textsubscript{free} of 39.9% and R\textsubscript{work} of 41.4%. Automated model building to improve early models was performed using Phenix AutoBuild\textsuperscript{31}. The complex structure was refined using Phenix Refine\textsuperscript{32}, followed by manual rebuilding with the program COOT (ref. 33). The F model starts with residue 25 and ends with residue 509 (of 562 in the ectodomain construct). The Fv V\text{\textit{H}} model starts with residue 113 and ends with residue 135. The Fab heavy chain was truncated to 3.1 Å along the c* axis and 3.6 Å along the a*/b* axes (Supplementary Table 1).

Reference
of the second mAb was second mAb alone. MAbs were considered non-competing if maximum binding

Isolation of the 25P13 antibody. 25P13 was isolated from the peripheral blood mononuclear cells (PBMCs) of a Nashville Red Cross donor. PBMCs were isolated from human donor blood samples using Ficoll-Histopaque density gradient centrifugation. PBMCs were transformed with Epstein–Barr virus as described previously34. Cells were screened by ELISA for binding to post-fusion RSV F, and with Opti-MEM I+GlutaMAX to remove serum, and then incubated with HMPV:

RSV and HMPV neutralization experiments. 25P13 isolated from hybridoma supernatants were incubated 1:1 with a suspension of infectious RSV strain A2 for 1 h. Following this, confluent HEP-2 cells, maintained in Opti-MEM 1+GlutaMAX (Fisher) supplemented with 2% fetal bovine serum at 37 °C in a CO 2 incubator, in pig serum (or a mouse mAb targeting the HMPV nucleoprotein, Meridian Life Technologies). Antibody production was accomplished by expanding the hybridoma to four 225 cm2 cell culture flasks in serum-free medium (Hybridoma-SFM, Gibco). After 21 days, supernatants were sterile-filtered using 0.45 µm pore size filter devices. For antibody purification, HiTrap MabSelectSure columns (GE Healthcare Life Sciences) were used to purify antibodies using the manufacturer’s protocol.

Neutralization experiments with hMPV used strain TN/96-12, which was obtained originally from a nasopharyngeal-wash specimen from a young child with upper respiratory tract illness in 1996 in Nashville, Tennessee. Virus was grown in Vero cells with Opti-MEM 1+ GlutaMAX medium (Fisher) 37 °C in a CO2 incubator. The medium for cell propagation was supplemented with 2% fetal bovine serum. Confluent Vero cell monolayer cultures in 24-well plates were washed once with Opti-MEM 1+GlutaMAX to remove serum, and then incubated with HMPV: mAb mixtures for 1 h. Following this, the cells were overlaid with 0.75% methocellulose in Opti-MEM 1+GlutaMAX containing 5 µg ml−1 trypsin. Cells were incubated for four days before being fixed with neutral buffered formalin. Plaques were visualized by immunoperoxidase staining, where anti-HMPV F guinea pig serum (or a mouse mAb targeting the HMPV nucleoprotein, Meridian Life Sciences) was used at 1:1000 dilution in milk to overlay the cells for 1 h, followed by incubation with 1:100 diluted goat anti-guinea pig IgG or anti-mouse IgG coupled to horseradish peroxidase (Meridian Life Science) for 1 h. TrueBlue peroxidase substrate (Kirkegaard and Perry) was then added to visualize plaques. Plaques were counted and compared to the virus control. Data were analysed with Prism software (GraphPad) to obtain half-maximum inhibitory concentration (IC50) values.

Competition binding. Competition binding was conducted on an Octet Red system (Fortebio) using anti-penta-HIS biosensors (Fortebio) and kinetics buffer (Fortebio). After obtaining an initial baseline in kinetics buffer, 10 µg ml−1 of his-tagged RSV F postfusion or prefusion (DSCAV1) protein was immobilized onto anti-penta-HIS biosensor tips for 120 s. The baseline signal was measured again for 60 s before biosensor tips were immersed into wells containing 100 µg ml−1 primary antibody for 300 s. Following this, biosensors were immersed into wells containing 100 µg ml−1 of a second mAb for 300 s. Percent binding of a second mAb in the presence of the first mAb was determined by comparing the maximal signal of the second mAb after the first mAb was added to the maximum signal of the second mAb alone. MABs were considered non-competing if maximum binding of the second mAb was ≥66% of its uncompetitive binding. A level between 33% and 66% of its uncompetitive binding was considered intermediate competition and ≤33% was considered competing.

Data availability. The DS-CAV1:MPe8 complex crystal structure and reflection data have been deposited in the Protein Data Bank under accession code 5U68. Other data supporting the findings of this study are available on request.

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Author contributions
X.W., J.J.M., J.T.B., R.A.L., J.E.C. and T.S.J. conceived and designed the experiments. X.W., J.J.M. and J.T.B. performed the experiments. X.W., J.J.M., J.T.B., R.A.L., J.E.C. and T.S.J. analysed the data. X.W., J.J.M. and J.T.B. contributed reagents, materials and analysis tools. X.W., J.J.M., J.T.B., R.A.L., J.E.C. and T.S.J. wrote and edited the manuscript.

Additional information
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Competing interests
One of the authors (J.E.C.) declares a competing interest. J.E.C. is a consultant to Compuvax and a member of the Scientific Advisory Board of Meissa Vaccines. The remaining authors declare no competing interests.