

Cross-neutralization of four paramyxoviruses by a human monoclonal antibody

Davide Corti^{1,2}, Siro Bianchi¹, Fabrizia Vanzetta¹, Andrea Minola², Laurent Perez², Gloria Agatic¹, Barbara Guarino¹, Chiara Silacci², Jessica Marcandalli², Benjamin J. Marsland³, Antonio Piralla⁴, Elena Percivalle⁴, Federica Sallusto², Fausto Baldanti⁴ & Antonio Lanzavecchia^{2,5}

Broadly neutralizing antibodies reactive against most and even all variants of the same viral species have been described for influenza and HIV-1 (ref. 1). However, whether a neutralizing antibody could have the breadth of range to target different viral species was unknown. Human respiratory syncytial virus (HRSV) and human metapneumovirus (HMPV) are common pathogens that cause severe disease in premature newborns, hospitalized children^{2,3} and immune-compromised patients^{2,4,5}, and play a role in asthma exacerbations⁶. Although antisera generated against either HRSV or HMPV are not cross-neutralizing⁷, we speculated that, because of the repeated exposure to these viruses, cross-neutralizing antibodies may be selected in some individuals. Here we describe a human monoclonal antibody (MPE8) that potently cross-neutralizes HRSV and HMPV as well as two animal paramyxoviruses: bovine RSV (BRSV) and pneumonia virus of mice (PVM). In its germline configuration, MPE8 is HRSV-specific and its breadth is achieved by somatic mutations in the light chain variable region. MPE8 did not result in the selection of viral escape mutants that evaded antibody targeting and showed potent prophylactic efficacy in animal models of HRSV and HMPV infection, as well as prophylactic and therapeutic efficacy in the more relevant model of lethal PVM infection. The core epitope of MPE8 was mapped on two highly conserved anti-parallel β -strands on the pre-fusion viral F protein, which are rearranged in the post-fusion F protein conformation. Twenty-six out of the thirty HRSV-specific neutralizing antibodies isolated were also found to be specific for the pre-fusion F protein. Taken together, these results indicate that MPE8 might be used for the prophylaxis and therapy of severe HRSV and HMPV infections and identify the pre-fusion F protein as a candidate HRSV vaccine.

HRSV and HMPV are human paramyxoviruses that differ in their genomic organization and that share only 33% amino acid identity in the F protein, the primary target of neutralizing antibodies^{8,9}. Polyclonal antisera (RespiGam) were used to prevent HRSV infection in premature newborns or children at risk and were substituted in 1997 by the anti-F humanized monoclonal antibody palivizumab (Synagis), which is currently the only anti-infective monoclonal antibody approved for human use^{10,11}. However, palivizumab is not effective therapeutically¹² and has been shown to select escape mutants *in vivo*¹³. The limited efficacy of palivizumab, the lack of a treatment for HMPV and the lack of effective vaccines highlight the medical need for new therapies against these prominent human pathogens.

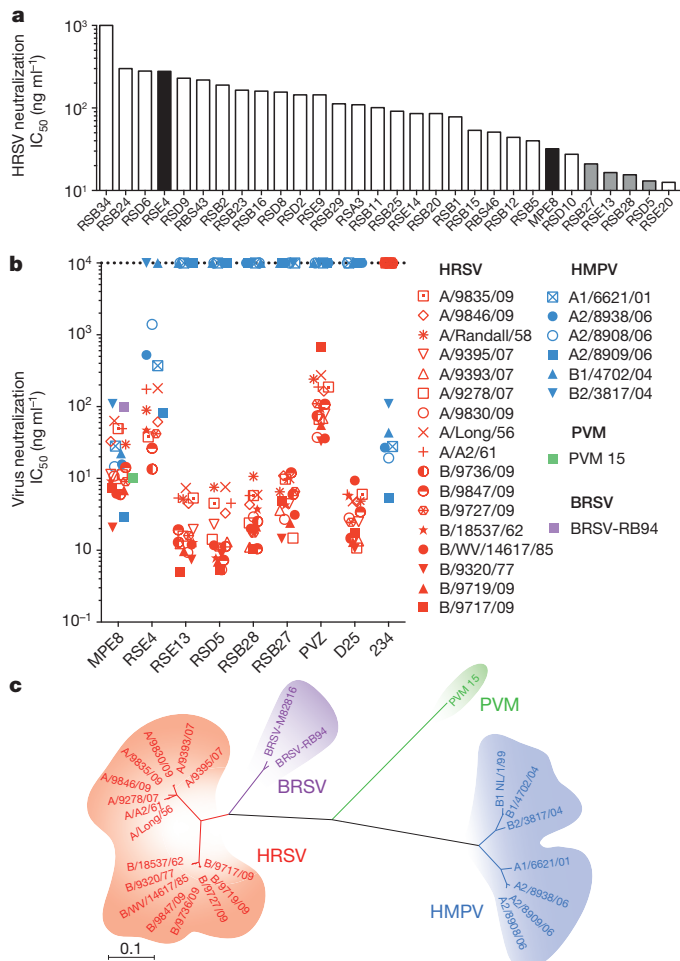
To search for a HRSV and HMPV cross-reactive neutralizing antibody, we screened a cohort of 200 blood donors and selected 7 donors with high serum neutralizing antibody titres against both viruses. A total of 114,000 IgG⁺ memory B cells were isolated and immortalized using Epstein-Barr Virus (EBV) and CpG, as previously described¹⁴. The B cell supernatants were analysed for their capacity to neutralize infection by HRSV A/A2/61 and subsequently rescreened

on HMPV A1/6621/01. Thirty HRSV-neutralizing antibodies were isolated and two of them, named MPE8 and RSE4, were found to cross-neutralize HMPV infection (Fig. 1a and Supplementary Table 1). MPE8, RSE4, as well as four HRSV-specific neutralizing antibodies (RSE13, RSD5, RSB27 and RSB28) selected for their high potency, were compared to palivizumab and two previously described antibodies, D25 (specific for HRSV)¹⁵ and 234 (specific for HMPV)¹⁶, for their capacity to neutralize a panel of HRSV and HMPV strains, as well as the two related animal paramyxoviruses PVM and BRSV (Fig. 1b, c). Strikingly, MPE8 neutralized many HRSV and HMPV isolates representative of all subgroups, as well as PVM and BRSV, with IC₅₀ (half-maximum inhibitory concentration) values ranging from 2 to 110 ng ml⁻¹ (mean 23 ng ml⁻¹). When compared to palivizumab, MPE8 was on average eightfold more potent in neutralizing HRSV (Fig. 1b and Supplementary Table 2). RSE4 showed a lower potency and breadth, as it failed to neutralize HMPV B strain viruses. The four HRSV-specific antibodies neutralized HRSV with IC₅₀ values 27- to 132-fold lower than palivizumab. The neutralizing antibodies were also tested for their capacity to prevent cell-to-cell viral spread, which has been reported to be a distinct property of anti-HRSV antibodies, independent of the neutralizing activity¹⁷. In this assay MPE8 showed a 50% spreading inhibiting capacity (IS₅₀) comparable to that of the most potent neutralizing antibodies RSD5, RSE13, RSB27 and RSB28 (Supplementary Figs 1 and 2). Taken together, these results indicate that MPE8 can potently neutralize four different paramyxoviruses and its mechanism of action involves both viral neutralization as well as inhibition of viral spread and syncytia formation.

MPE8 utilizes VH3-31*01 and VL1-40*01, has a 17 amino acid heavy chain complementarity determining region 3 (HCDR3), and carries 9 amino acid substitutions in VH and 4 in VL (Fig. 2a). To analyse the role of somatic mutations, we produced MPE8 variants that were completely or partially reverted to the germline sequence. The fully germlined version of MPE8 neutralized HRSV A and B strains, but failed to neutralize HMPV (Fig. 2b). HMPV binding required mutations in both heavy and light chains. In particular, the reversion of the somatic mutation at position 56 (D56G) in light chain complementarity determining region 2 (LCDR2) was sufficient to abolish neutralization of all HMPV strains, whereas reversion of the somatic mutation at position 65 in LCDR2 (N65S) and 109 in LCDR3 (R109S) had minor effects. Finally, reversion of the somatic mutation at position 113 (N113S), which introduced a glycosylation site in LCDR3, did not affect antibody breadth and potency (Fig. 2b and Supplementary Fig. 3). These findings indicate that the naive B cell that generated MPE8 was specific for HRSV and that the cross-reactivity to HMPV was acquired through somatic mutations, in particular a G to D mutation at position 56 in LCDR2.

All the 30 HRSV neutralizing antibodies isolated selectively stained 293F cells expressing the F, but not the G, protein of HRSV

¹Humabs BioMed SA, Via Mirasole 1, 6500 Bellinzona, Switzerland. ²Institute for Research in Biomedicine, Università della Svizzera Italiana, Via Vincenzo Vela 6, 6500 Bellinzona, Switzerland. ³University of Lausanne, Service de Pneumologie, CHUV, Rue du Bugnon 46, 1011 Lausanne, Switzerland. ⁴Policlinico San Matteo, IRCCS, Piazzale Camillo Golgi 1, 27100 Pavia, Italy. ⁵Institute of Microbiology, ETH Zurich, Wolfgang-Pauli-Strasse 10, 8093 Zurich, Switzerland.



(Supplementary Table 1), indicating that the F protein is the major target of human neutralizing antibodies. Because the HRSV pre-fusion F protein is very unstable, we engineered a stabilized soluble form by: 1)

Figure 1 | MPE8 cross-neutralizes four human and animal paramyxoviruses.

a, Thirty human HRSV neutralizing monoclonal antibodies were isolated from memory B cells. The histogram shows the antibodies ranked according to their IC_{50} values against the HRSV A/A2/61 strain. The six antibodies further characterized are highlighted in grey (HRSV specific) or in black (HRSV and HMPV specific). **b**, Neutralization of HRSV (red), HMPV (blue), PVM (green) and BRSV (purple) by the six selected antibodies and by palivizumab (PVZ), D25 and 234 as controls. IC_{50} values ($ng\ ml^{-1}$) were determined by immunofluorescence analysis and measured by nonlinear fitting. IC_{50} values above $10^4\ ng\ ml^{-1}$ were scored as negative. The data with complete viral strain designations and accession numbers are listed in Supplementary Table 2. **c**, Dendrogram showing the protein distance of the HRSV, HMPV, PVM and BRSV F proteins tested. The amino acid distance scale is indicated with a value of 10% distance. Experiments were performed in quadruplicates. Data are representative of at least two independent experiments. RSE20 was not further characterized because it failed to neutralize HRSV B strains.

introduction of inter-monomeric disulphide bridges¹⁸, 2) deletion of the fusion peptide region, and 3) substitution of the transmembrane region with a T4 fibrin motif ('foldon') to stabilize the trimeric structure (Supplementary Figs 4 and 5). We also produced the post-fusion F protein, which has been developed as a subunit HRSV vaccine¹⁹. Electron microscopy of HRSV post-fusion F protein showed a field of uniform 'crutch'-shaped molecule and rosette aggregates characteristic of post-fusion F proteins²⁰. Conversely, HRSV pre-fusion F protein showed a homogeneous arrangement of 'cone'-shaped molecules, characteristic of F proteins of other paramyxoviruses in the pre-fusion state²¹ (Supplementary Fig. 6). When tested by surface plasmon resonance (SPR), palivizumab bound to both pre- and post-fusion proteins with similar kinetics (Fig. 3a). In contrast, 26 out of the 30 neutralizing antibodies isolated (including MPE8, RSD5, RSE13, RSB27, RSB28 and D25), selectively bound to the pre-fusion F protein (Fig. 3a and Supplementary Table 1). Using cross-competition SPR experiments and the panel of available antibodies, we identified seven antigenic sites (S1 to S7) on the pre-fusion F protein, four of which were targeted by the potent neutralizing antibodies (Supplementary Fig. 7). MPE8 and palivizumab cross-competed for binding to the S1 site of the pre-fusion F protein, indicating that their cognate epitopes are in close proximity on the pre-fusion F protein, however, the epitope recognized by MPE8 is not accessible on the post-fusion F protein. This conclusion is consistent

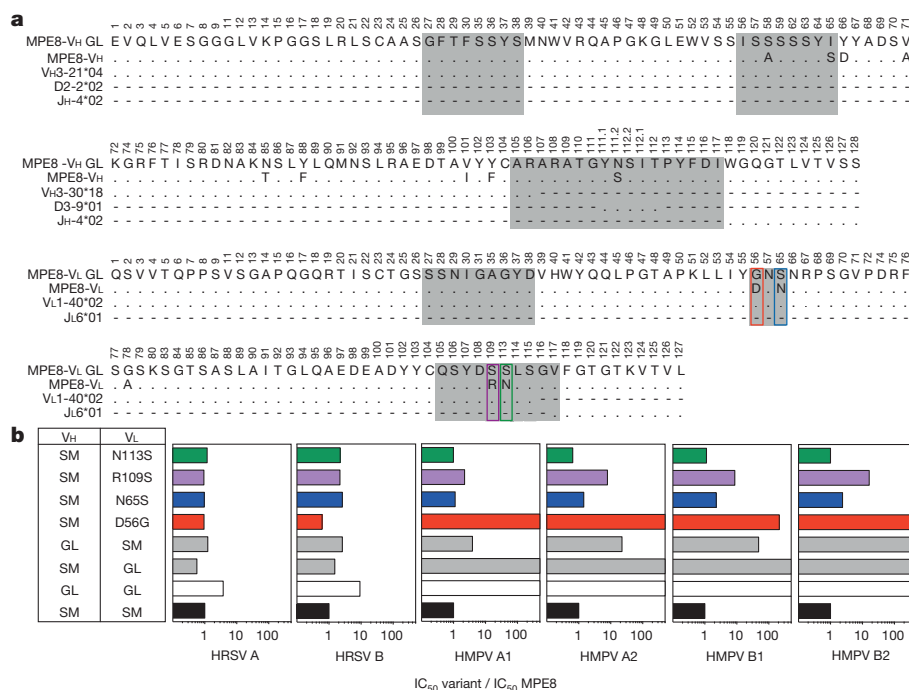


Figure 2 | Somatic mutations broadening the MPE8 specificity.

a, Alignment of V_H and V_L amino acid sequences of MPE8 with its germline configuration. Shown are V, D and J sequences determined using the IMGT database (<http://www.imgt.org/>). The CDR regions are highlighted in grey. Dots indicate identical residues; dashes indicate that residues are absent. **b**, Neutralization of HRSV and HMPV A and B strains by different versions of MPE8 in which all or individual somatic mutations in the V_H or V_L chain were reverted to the germline. Shown is the ratio between the IC_{50} values of the variants and IC_{50} values of MPE8. The mutations indicated represent reversions to the corresponding germline residues. GL, germlined V_H or V_L ; SM, somatically mutated V_H or V_L . The MPE8 (N113S) mutant was used in all further experiments. Coloured boxes in panel a reflect the colours used in the panel b bar charts.

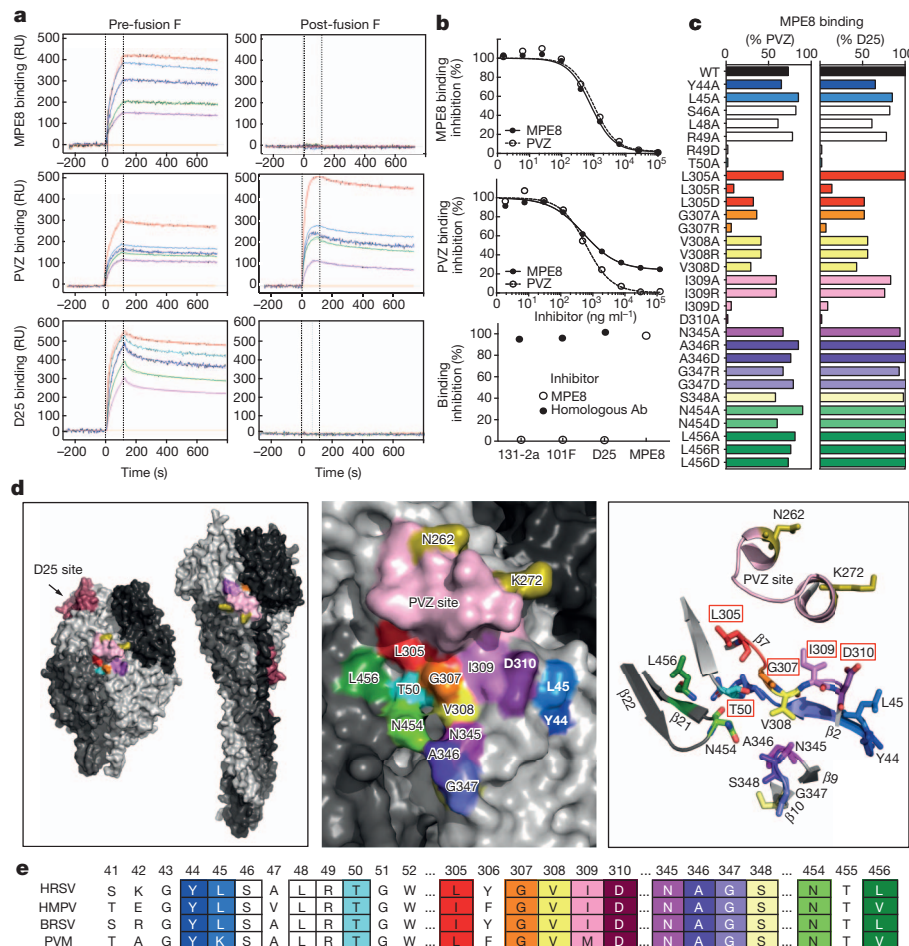


Figure 3 | MPE8 binds to a conserved site on the pre-fusion F protein.

a, Binding of MPE8, palivizumab (PVZ) and D25 to pre-fusion and post-fusion HRSV F proteins as measured by SPR. RU, resonance units. **b**, Cross-competition for binding to HRSV-infected cells. MPE8 binding is completely inhibited by PVZ (upper panel), whereas PVZ binding is only partially inhibited by MPE8 (middle panel); binding of antibodies 131-2a, 101F and D25 is not inhibited by MPE8. **c**, Binding of MPE8 to cells transfected with HRSV F genes carrying specific mutations. The results are expressed as percentage binding of PVZ and D25. The colour codes reflect the position of the mutated residues as shown in panel **d**. **d**, Left panel, model of the trimeric pre-fusion

(Protein Data Bank (PDB) code 4JHW) and post-fusion (PDB code 3RKI) HRSV F proteins showing the location of the D25 epitope (brown), the PVZ site (light pink) and the MPE8 site (as defined by residues T50, L305, G307, I309 and D310). The three monomers are depicted with different shades of grey. Middle and right panels show a close up of the MPE8 and PVZ sites on the pre-fusion F protein. The critical residues for MPE8 binding identified by mutagenesis are boxed in red. Shown in gold are the two residues N262 and K272 which are found mutated in the PVZ MARMs. **e**, Alignment of HRSV, HMPV, BRSV and PVM F protein residues which have been mutated.

with the finding that palivizumab completely inhibited binding of MPE8 to HRSV-infected cells (which display both pre- and post-fusion F proteins), whereas MPE8 only partially inhibited binding of palivizumab (Fig. 3b). In addition, MPE8 did not cross-compete with three antibodies that were mapped to distinct sites on the F protein^{22,23} (Fig. 3b).

MPE8 reacted in western blots with HRSV and HMPV F proteins under non-reducing conditions (Supplementary Fig. 8), suggesting that the epitope is at least transiently accessible in the presence of anionic detergents, allowing the antibody to 'lock-on'. We therefore screened a library of 7,095 structured peptides covering the sequence of HRSV F protein and identified a peptide in the amino-terminal region, spanning residues SAVSKGYLSALRTGWYTSVIT, which was bound by MPE8. The core sequence in this peptide (YLSALRTGW) identifies, on the surface of the recently described pre-fusion F protein structure²³, a patch of residues that are conserved in both HRSV and HMPV F proteins (Supplementary Fig. 9). To refine the MPE8 epitope mapping, we expressed in 293F cells HRSV F protein mutants located in the anti-parallel β 2 and β 7 strands (residues 44–50 and 305–310) and in two neighbouring loops, corresponding to residues 345–348 of the same monomer and 454–456 of the adjacent monomer (Fig. 3c–e). Complete loss of binding by MPE8 was observed with two alanine

mutants of the surface-exposed residues T50 and D310, as well as with the non-conservative aspartate substitution at R49, which lies below T50. Substantial loss of binding was also caused by mutations in surface residues L305R, G307R and I309D. Interestingly, the β -strands are reoriented in the post-fusion F protein structure and residues T50 and L305 are not surface exposed. All mutants tested were bound by a panel of antibodies, including palivizumab, D25, RSD5 and RSE13, indicating that the mutations were not disruptive to other epitopes. As a second approach for epitope mapping, we attempted to isolate monoclonal antibody resistant mutants (MARMs). HRSV MARMs were isolated at high frequency (1/200,000 TCID₅₀ (50% tissue culture infective dose)) in the presence of palivizumab and carried mutations at positions 262 and 272, consistent with previous findings^{24,25}. In contrast, neither HRSV nor HMPV MARMs were isolated in the presence of MPE8. Furthermore, all palivizumab MARMs were neutralized by MPE8, consistent with the two antibodies recognizing close, yet distinct, epitopes in the S1 site of the pre-fusion F protein (Supplementary Fig. 10 and Fig. 3d). Taken together, the above results indicate that MPE8 binds to a highly conserved epitope that is displayed on the surface of the pre-fusion, but not of the post-fusion, HRSV and HMPV F proteins. This epitope is close to, but distinct from, that recognized by

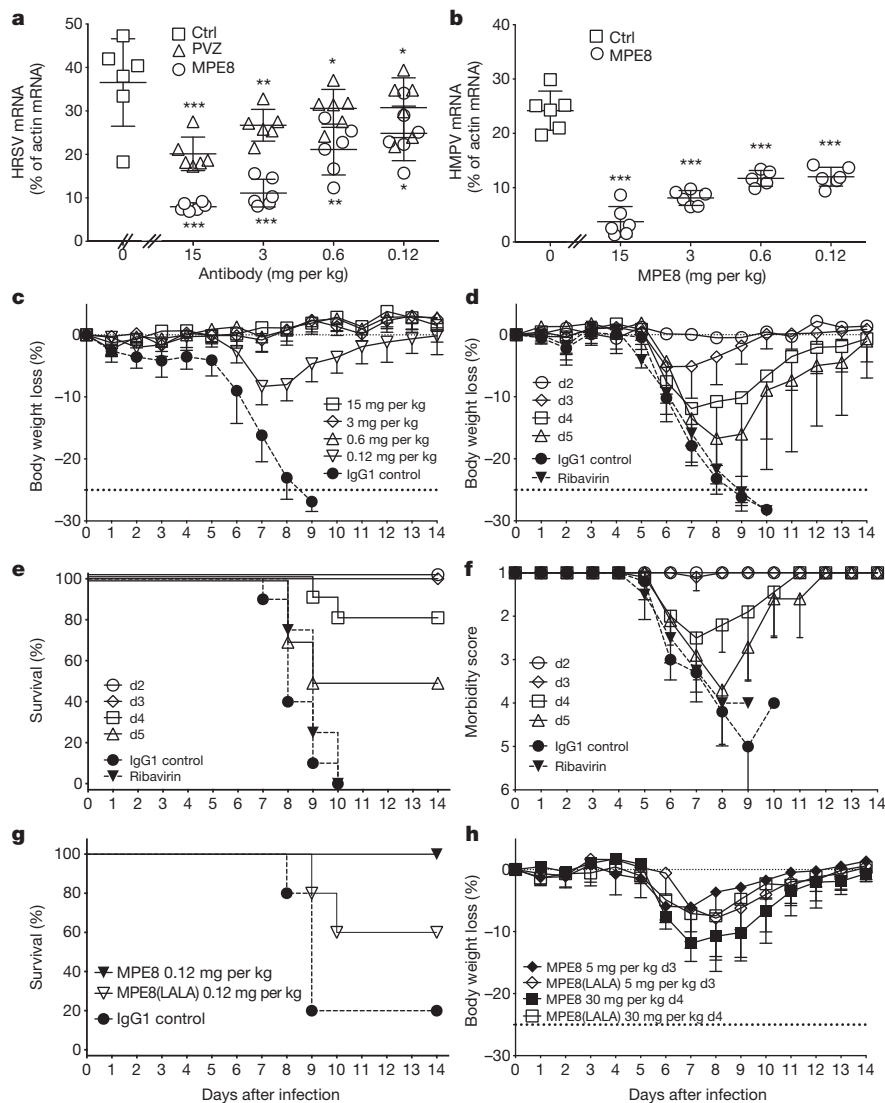


Figure 4 | Prophylactic and therapeutic efficacy of MPE8 against HRSV, HMPV and PVM.

a, b, HRSV and HMPV prophylaxis. BALB/c mice (*n* = 6) were injected with different doses of MPE8 (circles), palivizumab (PVZ, triangles) or the diluent as a control (Ctrl, squares) four hours before intranasal (i.n.) infection with 10^6 p.f.u. of HRSV A/A2/61 (**a**) or HMPV A/6621/04 (**b**). Mice were euthanized on day 5 and viral messenger RNA levels were determined by quantitative PCR (qPCR). Two-tailed *P* value was calculated with the unpaired Student's *t*-test. ****P* < 0.0001; ***P* = 0.001 to 0.01; **P* = 0.01 to 0.05. **c,** PVM prophylaxis. BALB/c mice (*n* = 5) were injected with different doses of MPE8 or control HJ16 anti-HIV antibody (Ctrl) and challenged (i.n.) after 4 h with $3 \times \text{MLD}_{50}$ (median lethal dose at 50%) of PVM 15. Shown is the percentage of body weight loss. **d–f,** PVM therapy. BALB/c mice (*n* = 10) were infected with PVM as above and injected with MPE8 at 30 mg per kg on day 2, 3, 4 or 5 after infection. Control mice were treated on day 0 with HJ16. A group of mice was treated with ribavirin intraperitoneal (i.p.) administration at 37.5 mg per kg, twice a day, from day 3 onwards. Shown is the percentage of body weight loss (**d**), per cent survival (**e**) and morbidity score (**f**). **g, h,** Role of antibody Fc in PVM prophylaxis and therapy. Comparison of MPE8 and MPE8(LALA) tested in a prophylactic setting at 0.12 mg per kg (**g**) or in a therapeutic setting at 5 mg per kg on day 3 or 30 mg per kg on day 4 after infection (**h**) (*n* = 5). All error bars indicate s.d.

palivizumab, is centred around the $\beta 2/\beta 7$ antiparallel strands and relies on residues T50, D305, G307, I309 and D310, which are highly conserved amongst 551 virus strains, comprising 364 HRSV, 162 HMPV, 8 BRSV and 5 PVM strains (Supplementary Fig. 11).

Finally, we set out to test the *in vivo* efficacy of MPE8 in prophylactic and therapeutic settings. In the HRSV mouse model, MPE8 was five- to tenfold more potent than palivizumab in reducing HRSV lung titres and was effective at concentrations as low as 0.12 mg per kg of body weight. Similar results were obtained in the HMPV mouse model (Fig. 4a, b). However, when tested at 15 mg per kg in a therapeutic setting in *Stat1*-deficient mice, which sustain higher HRSV replication²⁶, MPE8, RSE13 and palivizumab showed comparable activity in being effective only when administered on day 1 (Supplementary Fig. 12). These findings highlight the well-known limitations of the mouse model of HRSV infection, which requires a high level of virus challenge to achieve only a low and transient virus replication. We therefore tested MPE8 in the more relevant PVM mouse model, in which a small inoculum causes acute lower respiratory tract infection and lethality, recapitulating the features of severe HRSV and HMPV infection in humans²⁷. In a prophylactic setting, MPE8 fully protected mice from PVM lethality at 0.12 mg per kg and from body weight loss at 0.6 mg per kg (Fig. 4c). Furthermore, in a therapeutic setting MPE8 completely protected from lethality when administered up to 3 days after infection both at 30 and 5 mg per kg and conferred significant protection when given on day 4 or

5 at 30 mg per kg (Fig. 4d–f). In this system, ribavirin, which is the only approved standard of care for therapy in humans²⁸, was ineffective, as previously described²⁹.

To address the role of effector mechanisms *in vivo*, we compared the IgG1 MPE8 with a mutant (L234A, L235A, referred to here as LALA) that lacks complement and Fc receptor binding (MPE8(LALA)). The two variants showed equivalent *in vitro* neutralizing activity (Supplementary Fig. 13). When administered in limiting amounts in a prophylactic setting (0.12 mg per kg), MPE8(LALA) showed a severely reduced efficacy, consistent with an important contribution of effector mechanisms *in vivo* (Fig. 4g). In contrast, when used in a therapeutic setting, MPE8(LALA) mutant was as effective as MPE8 at all doses tested (Fig. 4h). These findings suggest that the therapeutic efficacy of MPE8 in the PVM model may be due to a combination of factors, such as the potent neutralizing and spreading inhibition activity, the failure to select escape mutants, and the selective recognition of the pre-fusion F protein, which avoids the consumption of the antibody by the abundant post-fusion F protein acting as a decoy. Surprisingly, the therapeutic efficacy of MPE8 does not require effector functions, suggesting that the therapeutic activity *in vivo* relies primarily on viral neutralization and inhibition of viral spread. This is supported by the finding that MPE8 administered therapeutically on days 1, 2 and 3 after infection can efficiently block further increase in lung viral RNA (Supplementary Fig. 14).

In conclusion, MPE8 is the first example of an antibody that potently cross-neutralizes up to four different viral species and shows prophylactic and therapeutic efficacy *in vivo*. MPE8 appears to have been selected in a stepwise fashion by HRSV infection followed by HMPV infection in a donor who had high titres of neutralizing antibodies against both viruses. According to the original antigenic sin theory, the naive MPE8 B cell clone was initially primed by HRSV and generated a variant (carrying the G56D mutation), which was cross-reactive with HMPV and was therefore recalled by HMPV.

Given the large prevalence and overlapping winter epidemics of HRSV and HMPV infections, as well as the possibility of co-infections³⁰, there is need to develop an effective prophylactic and therapeutic approach against both viruses. Owing to its high potency and breadth, MPE8 could be developed for prophylaxis of both HRSV and HMPV infections not only in newborns, but also in immune-compromised adults. Furthermore, it would be important to assess the potential use of this antibody in a therapeutic setting, for instance to prevent the spreading of HRSV or HMPV from the upper to the lower respiratory tract in high-risk individuals, such as haematopoietic stem-cell transplant patients, or to treat patients with severe lower respiratory tract infections. Finally, the finding that most of the human neutralizing antibodies selectively recognize the pre-fusion F protein provides relevant insights for vaccine design. Our results suggest that a vaccine based on a stabilized pre-fusion F protein might be able to selectively elicit neutralizing antibodies, including MPE8-like antibodies, whose breadth is achievable through a very limited number of somatic mutations.

METHODS SUMMARY

Peripheral blood CD22⁺IgG⁺ B cells were sorted by flow cytometry, plated at 3–5 cells per well and immortalized with EBV and CpG on feeder cells. Culture supernatants of B cells were screened by microneutralization against HRSV A2 virus and the positive cultures were re-screened against HMPV. VH and VL genes from selected cultures were sequenced and evaluated for their potency and breadth against a panel of HRSV, HMPV, PVM and BRSV strains. MPE8 antibody variants in the VH, VL or Fc (LALA mutant) were generated by site-directed mutagenesis or gene synthesis and produced recombinantly in 293F cells. Epitope specificity was determined by scanning peptides, cross-competition studies and staining of 293F cells expressing HRSV F protein mutants. MPE8 prophylactic and therapeutic activity was evaluated in the mouse models of HRSV, HMPV and PVM as described in the Methods. Student's two-tailed paired *t*-test was used for statistical comparisons; *P* values of 0.05 or less were considered significant.

Full Methods and any associated references are available in the online version of the paper.

Received 25 March; accepted 8 July 2013.

Published online 18 August 2013.

1. Corti, D. & Lanzavecchia, A. Broadly neutralizing antiviral antibodies. *Annu. Rev. Immunol.* **31**, 705–742 (2013).
2. Hall, C. B. *et al.* The burden of respiratory syncytial virus infection in young children. *N. Engl. J. Med.* **360**, 588–598 (2009).
3. Edwards, K. M. *et al.* Burden of human metapneumovirus infection in young children. *N. Engl. J. Med.* **368**, 633–643 (2013).
4. Williams, J. V. *et al.* Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. *N. Engl. J. Med.* **350**, 443–450 (2004).
5. Falsey, A. R., Hennessey, P. A., Formica, M. A., Cox, C. & Walsh, E. E. Respiratory syncytial virus infection in elderly and high-risk adults. *N. Engl. J. Med.* **352**, 1749–1759 (2005).
6. Edwards, M. R., Bartlett, N. W., Huxsell, T., Openshaw, P. & Johnston, S. L. The microbiology of asthma. *Nature Rev. Immunol.* **10**, 459–471 (2012).
7. Wyde, P. R., Chetty, S. N., Jewell, A. M., Boivin, G. & Piedra, P. A. Comparison of the inhibition of human metapneumovirus and respiratory syncytial virus by ribavirin and immune serum globulin *in vitro*. *Antiviral Res.* **60**, 51–59 (2003).
8. Collins, P. L. & Crowe, J. E. Jr. in *Fields Virology* 6th edn. (eds Knipe D. M. & Howley P. M.) Respiratory syncytial virus and metapneumovirus (Lippincott Williams & Wilkins, 2013).
9. van den Hoogen, B. G. *et al.* A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nature Med.* **7**, 719–724 (2001).

10. Groothuis, J. R., Simoes, E. A., Hemming, V. G. & Respiratory Syncytial Virus Immune Globulin Study Group. Respiratory syncytial virus (RSV) infection in preterm infants and the protective effects of RSV immune globulin (RSVIG). *Pediatrics* **95**, 463–467 (1995).
11. The-IMPact-RSV Study Group. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. *Pediatrics* **102**, 531–537 (1998).
12. de Fontbrune, F. S. *et al.* Palivizumab treatment of respiratory syncytial virus infection after allogeneic hematopoietic stem cell transplantation. *Clin. Infect. Dis.* **45**, 1019–1024 (2007).
13. Adams, O. *et al.* Palivizumab-resistant human respiratory syncytial virus infection in infancy. *Clin. Infect. Dis.* **51**, 185–188 (2010).
14. Traggiai, E. *et al.* An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nature Med.* **10**, 871–875 (2004).
15. Kwakkenbos, M. J. *et al.* Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming. *Nature Med.* **16**, 123–128 (2010).
16. Ulbrandt, N. D. *et al.* Isolation and characterization of monoclonal antibodies which neutralize human metapneumovirus *in vitro* and *in vivo*. *J. Virol.* **80**, 7799–7806 (2006).
17. Beeler, J. A. & van Wyke Coelingh, K. Neutralization epitopes of the F glycoprotein of respiratory syncytial virus: effect of mutation upon fusion function. *J. Virol.* **63**, 2941–2950 (1989).
18. Magro, M. *et al.* Neutralizing antibodies against the preactive form of respiratory syncytial virus fusion protein offer unique possibilities for clinical intervention. *Proc. Natl Acad. Sci. USA* **109**, 3089–3094 (2012).
19. Swanson, K. A. *et al.* Structural basis for immunization with postfusion respiratory syncytial virus fusion F glycoprotein (RSV F) to elicit high neutralizing antibody titers. *Proc. Natl Acad. Sci. USA* **108**, 9619–9624 (2011).
20. Calder, L. J. Electron microscopy of the human respiratory syncytial virus fusion protein and complexes that it forms with monoclonal antibodies. *Virology* **271**, 122–131 (2000).
21. Connolly, S. A., Leser, G. P., Yin, H.-S., Jardetzky, T. S. & Lamb, R. A. Refolding of a paramyxovirus F protein from prefusion to postfusion conformations observed by liposome binding and electron microscopy. *Proc. Natl Acad. Sci. USA* **103**, 17903–17908 (2006).
22. McLellan, J. S. *et al.* Structure of a major antigenic site on the respiratory syncytial virus fusion glycoprotein in complex with neutralizing antibody 101F. *J. Virol.* **84**, 12236–12244 (2010).
23. McLellan, J. S. *et al.* Structure of RSV fusion glycoprotein trimer bound to a prefusion-specific neutralizing antibody. *Science* **340**, 1113–1117 (2013).
24. Zhu, Q. *et al.* Natural polymorphisms and resistance-associated mutations in the fusion protein of respiratory syncytial virus (RSV): effects on RSV susceptibility to palivizumab. *J. Infect. Dis.* **205**, 635–638 (2012).
25. Zhao, X. Respiratory syncytial virus escape mutant derived *in vitro* resists palivizumab prophylaxis in cotton rats. *Virology* **318**, 608–612 (2004).
26. Durbin, J. E. *et al.* The role of IFN in respiratory syncytial virus pathogenesis. *J. Immunol.* **168**, 2944–2952 (2002).
27. Domachowske, J. B., Bonville, C. A. & Rosenberg, H. F. Animal models for studying respiratory syncytial virus infection and its long term effects on lung function. *Pediatr. Infect. Dis. J.* **23**, S228–S234 (2004).
28. Shah, J. N. & Chemaly, R. F. Management of RSV infections in adult recipients of hematopoietic stem cell transplantation. *Blood* **117**, 2755–2763 (2011).
29. Bonville, C. A. *et al.* Functional antagonism of chemokine receptor CCR1 reduces mortality in acute pneumovirus infection *in vivo*. *J. Virol.* **78**, 7984–7989 (2004).
30. Greensill, J. *et al.* Human metapneumovirus in severe respiratory syncytial virus bronchiolitis. *Emerg. Infect. Dis.* **9**, 372–375 (2003).

Supplementary Information is available in the online version of the paper.

Acknowledgements We would like to thank M. Nussenzweig and H. Wardemann for providing reagents for antibody cloning and expression, I. Capua, B. Zecchin and L. Selli for experiments involving BRSV and R. Wepf for electron microscopy. This work was supported by the European Research Council (grant no. 250348 IMMUNExplore) and the Swiss National Science Foundation (grant no. 141254). A.L. is supported by the Helmut Horten Foundation.

Author Contributions D.C. designed experiments, analysed the data and wrote the manuscript; S.B., F.V., C.S., B.G. performed and analysed *in vitro* experiments; A.M., G.A., B.J.M. performed and analysed *in vivo* experiments; L.P. and J.M. prepared pre- and post-fusion F proteins and performed SPR experiments; F.B., E.P. and A.P. isolated and sequenced HRSV and HMPV strains; F.S. wrote the manuscript; A.L. provided overall supervision, analysed the data and wrote the manuscript.

Author Information The GenBank accession numbers for MPE8 antibody VH and VL are KF314816 and KF314817. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.L. (lanzavecchia@irb.usi.ch) or D.C. (davide.corti@humabs.ch).

METHODS

Viruses. The HRSV viruses used in this study were A/A2/61 (ACO83301), A/Randall/58, A/Long/56 (ACO83302), B/18537/62 (BAA00240), B/WV/14617/85 (ATCC VR-1400), A/9320/77 (AAR14266), A/9846/09 (JX171066), A/9835/09 (JX171067), A/9278/07 (KC618407), A/9393/07 (KC618408), A/9395/07 (KC618409), A/9830/09 (KC618410), B/9719/09 (JX171070), B/9736/09 (JX171068), B/9847/09 (JX171073), B/9717/09 (JX171071), B/9727/09 (JX171069). The HMPV viruses used in this study were A1/6621/01 (KC588902), A2/8938/06 (KC588905), A2/8909/06 (KC588906), A2/8908/06 (KC588907), B1/4702/04 (KC588903) and B2/3817/04 (KC588904). The PVM strain used in this study was obtained from ATCC (catalogue no. VR-25; AAW02840) and expanded by continuous passage in mice. The BRSV strain used in this study was RB93 (BAA00798).

Sequencing of F proteins from HRSV and HMPV viral isolates. Viral RNA was extracted from isolates by the automated extraction kit NucliSENS easyMAG (BioMerieux). The HRSV F gene was amplified by the SuperScriptIII One-Step RT-PCR amplification kit (Invitrogen) and specific primers for RSV-A and RSV-B: RSV-A-F1 5'-ATGGAGTTGCCAATCCTCA-3', RSV-A-R1670 5'-CATTGT AAGAAYATGATTARGTRCT-3', RSV-B-F1 5'-ATGGAGYTGCTGATCCAYA GRTYAAGT-3', RSV-B-R1740 5'-CAGGTGGTTTTTTGTCTATTGCTGAAT GC-3'. The RT-PCR conditions for both HRSV types were: incubation at 56 °C for 30 min, denaturation at 95 °C for 2 min, followed by 45 cycles at 94 °C for 30 s, 56 °C for 30 s and 68 °C for 2 min. The HMPV F gene was amplified using specific primers: MPV-F1 5'-ATGTCTTGAAAGTGRGTATYA-3', HMPV-R1680 5'-TTGCAATGGAGCYTTRCGAGAC-3' and the following conditions: 56 °C for 30 min, denaturation at 95 °C for 2 min, followed by 45 cycles at 94 °C for 30 s, 56 °C for 30 s and 68 °C for 2 min. HRSV purified PCR products were directly sequenced using the following primers: HRSV-A-F1, HRSV-A-F513 5'-ACT ATCCACAAACAAGGCGTAGTCAGC-3', HRSV-A-F1013 5'-ACAGAGGAT GGTACTGTGACAATGCAGG-3', HRSV-A-R1670, HRSV-A-R1229 5'-TGCCA TAGCATGACACAATGGCTCCTA-3', HRSV-B-F1, HRSV-B-F529 5'-GCTGTA GTCAGTCTATCAATGGGGTGC-3', HRSV-B-F1225 5'-TCTCTWGGAGCTA TAGTGTGCATGC-3', HRSV-B-R1740, HRSV-B-R1135 5'-TGTCAGTGTACAA AGGCTGAC-3', HRSV-B-R667 5'-CAATCTGCTGTTCTTCTGCTGGAA-3'. HMPV purified PCR products were sequenced using the following primers: HMPV-F1; HMPV-F574 5'-AGCTTCAGTCAATTCACAGAAG-3'; HMPV-F1075 5'-TACCCATGCAAAGTYAGCACAGG-3', HMPV-R1680, HMPV-R1593 5'-KGTRACACCAATCAGCTCTGGAG-3' and HMPV-R1103 5'-TGTC TTCTGTGCTRACTTTTC-3'. Cycle sequencing was performed using the BigDye Terminator Cycle-Sequencing kit (Applied Biosystems) in ABI Prism 3100 DNA sequencer (Applied Biosystems).

Sample collection. Peripheral blood mononuclear cell (PBMC) and plasma samples were obtained from healthy donors. Memory B cells were isolated from cryopreserved PBMC. All donors gave written informed consent for research use of blood samples, following approval by the Cantonal Ethical Committee of Cantone Ticino.

Isolation of monoclonal antibodies. IgG⁺ memory B cells were isolated from cryopreserved PBMC using CD22 microbeads (Miltenyi Biotec), followed by depletion of cells carrying IgM, IgD and IgA by cell sorting, and immortalized with EBV and CpG in multiple replicate wells as previously described¹⁴. Culture supernatants were tested for their ability to neutralize HRSV A2 infection in a micro-neutralization assay. Positive cultures were collected and expanded. From positive cultures the V_H and V_L sequences were retrieved by RT-PCR and cloned into human IgG1 and Igk or Igλ expression vectors (provided by M. Nussenzweig) essentially as described³¹. Monoclonal antibodies were produced by transient transfection of 293 Freestyle cells (Invitrogen). Supernatants from transfected cells were collected after 7 days and IgG were affinity purified by protein A chromatography (GE Healthcare) and desalted against PBS.

Inhibition of binding assay on HRSV infected cells. Palivizumab and 101F were biotinylated using the EZ-Link NHS-PEO solid phase biotinylation kit (Pierce). Labelled antibodies were tested for binding to HRSV infected Hep-2 cells to determine the optimal concentration of each antibody to achieve 70–80% maximal binding. The biotin-labelled antibodies were then used as probes to assess, by flow cytometry, whether their binding (measured using fluorophore-conjugated streptavidin) was inhibited by pre-incubation of HRSV-A2-infected cells with a 50-fold excess of homologous or heterologous unlabelled antibodies.

Virus neutralization. Culture supernatants were analysed for the presence of neutralizing antibodies using a microneutralization assay based on infection of Hep-2 cells by HRSV strain A2 or of LLC-MK2 cells by HMPV strain A1/6621/01. Neat supernatants were incubated with 50–100 TCID₅₀ of viruses for 1 h at room temperature before addition of Hep-2 or LLC-MK2 target cells which were incubated for 6 or 8 days, respectively. Viable cells were detected using the WST-1 reagent (Roche). A secondary screening was performed to test whether the isolated monoclonal antibodies were able to neutralize both HMPV and HRSV. The IC₅₀

was determined using the microneutralization assay described above with 100 TCID₅₀ of virus and viral infection was measured on day 3 or 4 (depending on the strain) by indirect immunofluorescence using an automated Pathway 855 analyser (BD). IC₅₀ values were calculated by interpolation of neutralization curves fitted with a 4-parameter nonlinear regression with a variable slope. Neutralization of PVM was measured by incubating serial dilutions of the antibodies with 1,000 TCID₅₀ that were then added on BHK cells. Three days after the cells were detached, fixed, stained with labelled anti-PVM antibodies and analysed by flow cytometry. IC₅₀ titres were measured as above.

Antibody variants. Germline sequences of the isolated antibodies were determined with reference to the IMGT database³². Antibody variants in which single or multiple mutations were reverted to the germline sequence were produced either by gene synthesis (GenScript) or by site-directed mutagenesis (Promega) and confirmed by sequencing. Palivizumab, 101F, 234 and D25 V_H and V_L sequences were synthesized by GenScript and the corresponding antibodies were expressed by transient transfection. Expression vectors for human IgG1 were engineered to introduce the L234A, L235A mutations (LALA variant lacking FcR and C1q binding).

Transient expression of recombinant HRSV and HMPV F protein. The full length F genes of HRSV A2 strain and HMPV NL/1/99 (AAS92882) were codon-optimized for eukaryotic cell expression and cloned into the pHCMV1 vector (Genlantis). F protein mutants were generated by site-directed mutagenesis (Promega) and tested in transiently transfected 293T cells. Two days after transfection, cells were collected, fixed and permeabilized with saponin for immunostaining with MPE8 or palivizumab. Binding of antibodies to transfected cells was analysed using a Becton Dickinson FACSCanto2 (BD Biosciences) with FlowJo software (TreeStar).

Construction of the F protein amino acid sequence dendrogram. Sequences were aligned using the ClustalW program integrated in the MEGA version 5.0 package³³. The best-fitting nucleotide substitution model was estimated by means of a hierarchical likelihood ratio test implemented in MEGA. The phylogenetic tree was inferred using the neighbour-joining distance method, with genetic distances calculated under the Tamura-Nei model with gamma-distributed rates among sites (T93+Γ). The reliability of the neighbour-joining tree was estimated using 1,000 bootstrap replications.

Western blotting. Hep-2 cells were infected with HRSV and LLC-MK2 cells with MPV, lysed with CHAPS and separated on SDS-PAGE gel under reducing or non-reducing conditions. Proteins were then transferred on a PVDF (polyvinylidene difluoride) membrane, which was then incubated with either MPE8, palivizumab or 234 antibodies. Antibody binding was detected with an anti-human HRP-conjugated antibody in combination with the enhanced chemiluminescence (ECL) western blotting detection reagent (GE Healthcare) and LAS4000 charge-coupled device (CCD) camera system.

Evaluation of the prophylactic and therapeutic efficacy in mice infected with HRSV, HMPV or PVM. Groups of 6–8-week-old female BALB/c mice or 129S6/svEv-Stat1-deficient mice (Taconic) were injected intravenously with purified antibodies at concentrations varying from 0.12 to 30 mg per kg. In both prophylactic and therapeutic experiments with HRSV or HMPV, mice were deeply anaesthetized with isoflurane and four hours later challenged intranasally (i.n.) with HRSV A/A2/61 or HMPV A/6621/01 containing 0.7×10^6 – 1×10^6 p.f.u. In the therapeutic setting, mice received the antibodies 1, 2, or 3 days after infection. On day 5, mice were killed, lungs were isolated and homogenized for viral RNA extraction. RT-PCR on viral RNA was performed using a SYBR Green RT-PCR kit (QuantiFast). qPCR was performed on samples with β-actin and HRSV or HMPV specific primers using a CFX96 Real Time PCR Detection System (Bio-Rad). Lung homogenates were also analysed for the presence of mouse inflammatory cytokines TNF, IL6 and IFN-γ using a multiplex bead array system (CBA Flex Sets, BD). In the lethal model of PVM infection BALB/c mice were infected (i.n.) as above with $3 \times \text{MLD}_{50}$ of PVM strain 15 (2,000 p.f.u.) and treated prophylactically or therapeutically at the indicated time points. Mice were monitored daily for survival and weight loss until day 14 post-infection. Animals that lost more than 25% of their initial body weight were euthanized in accordance with our animal study protocol. In addition, morbidity score (1, healthy; 2, ruffled fur at neck; 3, piloerection and difficulty breathing, less alert; 4, lethargic with laboured breathing; 5, pre-morbid, with emaciation and cyanosis; or 6, death) was obtained daily. To measure viral replication in lungs, PVM-challenged mice were killed on days 3, 4, 5 or 7 days to collect lung tissues. PVM lung titres were determined by qPCR using PVM specific primers as above. Animal experiments were approved by the Cantonal Authorities of Cantone Ticino.

Selection of escape mutants. A fixed amount of HRSV A/Long/56 or HMPV A/6621/01 virus containing 16×10^6 p.f.u. was incubated with a constant amount (0.5 mg ml^{-1}) of antibody MPE8 or palivizumab for 1 h at 37 °C. The incubated mixture was then adsorbed by Hep-2 or LLC-MK2 cells for 4 h at $10,000 \text{ p.f.u.}$ per well in 96-microwell plates. Infected cells were then washed twice with PBS and

replenished with fresh medium containing $50 \mu\text{g ml}^{-1}$ of selecting antibodies. The cytopathic effect (CPE) on infected cells was monitored for up to 6 days after infection. Supernatants from CPE-positive wells were collected for a subsequent round of infection. The sequences of the F proteins from monoclonal antibody resistant mutants (MARMS) were determined as above.

Epitope mapping using structured peptides. Overlapping linear 15-mer and cyclized 15-mer peptides based on the F protein of HRSV strain A2 (residues 26–516) were synthesized on polypropylene support (minicards) and were tested for reactivity with MPE8 as described^{34,35}. Peptide binding analysis was carried out by an enzyme-linked immunosorbent assay (ELISA)-based format in which the coloured substrate was quantified with a CCD camera and an image processing system. The values mostly ranged from 0 to 3,000, a log scale similar to 1 to 3 of a standard 96-well plate ELISA-reader.

Cloning, expression and purification of recombinant HRSV F protein. A DNA construct encoding the F ectodomain (GenBank accession code FJ614814) (HRSV F residues 1 to 513, with fusion peptide residues 137 to 146 deleted) with a C-terminal 'foldon', tobacco etch virus (TEV) site, His tag and StreptagII was codon-optimized for mammalian expression (GenScript) and cloned into the pcDNA3.1 vector (Invitrogen). The stabilized pre-fusion form of HRSV F protein was designed by substitution of the residues Leu 481, Asp 489, Ser 509 and Asp 510 by cysteines as described¹⁸ in the presence of the stabilizing bacteriophage T4 fibrin 'foldon' sequence. Both proteins were expressed by transient transfection of 293F cells. Cell supernatants were loaded on affinity column Ni^{2+} -nitrilotriacetic acid (NTA) resin (GE Healthcare). After NTA purification, all samples were run on StrepTactin II chromatography (Qiagen). Finally, proteins were further purified by size-exclusion chromatography (Superdex 200 from GE Healthcare) with PBS as a running buffer.

Surface plasmon resonance. SPR experiments were performed on a ProteOn-XPR36 instrument (Bio-Rad). Antibodies were immobilized on a ProteOn GLC sensor chip surface through amine coupling at 2,000 response units (RU) and a blank surface with no antibody was created under identical coupling conditions for use as a reference. HRSV F protein was injected at a flow rate of $100 \mu\text{l min}^{-1}$, at concentrations of 200, 100, 75, 50 and 25 nM in different sensor channels. The data were processed using Proteon software and double referenced by subtraction of the blank surface and buffer-only injection before local fitting of the data. Binding curves were locally fitted to the two states model, to perform a K_d calculation. For competition experiment, antibodies were covalently coupled to a GLC sensor chip

at 2,000 RU. HRSV F protein was injected at 200 nM and the competing antibody was injected after 20 s of dissociation time.

HPLC and SDS-PAGE analysis. The proteins were applied onto a TSK G3000 column equilibrated with PBS. The elution volume was determined by monitoring the ultraviolet absorption at 280 nm. The apparent molecular weight of the different proteins was determined from a calibration curve with protein standards (GE Healthcare). All HRSV F proteins eluted as a single peak with an apparent mass of 180 kilodaltons, consistent with a trimeric structure. SDS-PAGE was performed on NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen) and the proteins were visualized using Imperial Blue (Thermo Scientific).

CD spectropolarimetry. Circular dichroism (CD) spectra from F proteins (0.5 mg ml^{-1} in 10 mM NaPO_4 , pH 7.4) were recorded on a Chirascan spectropolarimeter (Applied Photophysics) over the wavelength range of 195 to 260 nm at a band width of 1 nm, step size of 0.5 nm and 1 s per step. The spectra in the far-ultraviolet region required an average of five scans and were subtracted from blank spectra performed with buffer. Thermal denaturation was monitored by performing scans at intervals of 5°C , after equilibration for 2 min at each temperature. Data were fitted to a simple first order curve. The values of $\Delta\text{A}222$ are represented on the y axis as the percentage of the values recorded at 20°C .

Electron microscopy. HRSV F protein sample ($100 \mu\text{g ml}^{-1}$ in PBS) was absorbed onto a 400-mesh carbon-coated grid (and stained with 0.75% uranyl formate). A Philips CM 12 microscope, operated at 80 kV, was used to analyse the samples. Micrographs were taken at $\times 65,000$ magnification.

Statistics. Student's two-tailed paired *t*-tests were used for statistical comparisons; *P* values of 0.05 or less were considered significant.

31. Tiller, T. *et al.* Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J. Immunol. Methods* **329**, 112–124 (2008).
32. Lefranc, M.-P. *et al.* IMGT, the international ImMunoGeneTics information system. *Nucleic Acids Res.* **37**, D1006–D1012 (2009).
33. Tamura, K. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739 (2011).
34. Timmerman, P., Puijk, W. C. & Melen, R. H. Functional reconstruction and synthetic mimicry of a conformational epitope using CLIPS technology. *J. Mol. Recognit.* **20**, 283–299 (2007).
35. Slootstra, J. W., Puijk, W. C., Ligtoet, G. J., Langeveld, J. P. & Melen, R. H. Structural aspects of antibody-antigen interaction revealed through small random peptide libraries. *Mol. Divers.* **1**, 87–96 (1996).