Published in final edited form as:

Sci Immunol. 2020 July 03; 5(49): . doi:10.1126/sciimmunol.aba4902.

Human Antibodies Neutralize Enterovirus D68 and Protect Against Infection and Paralytic Disease

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Abstract

Enterovirus D68 (EV-D68) causes outbreaks of respiratory illness, and there is increasing evidence that it causes outbreaks of acute flaccid myelitis (AFM). There are no licensed therapies to prevent or treat EV-D68 infection or AFM disease. We isolated a panel of EV-D68-reactive human monoclonal antibodies that recognize diverse antigenic variants from subjects with prior infection. One potently neutralizing cross-reactive antibody, EV68–228, protected mice from respiratory and neurologic disease when given either before or after infection. Cryo-electron microscopy studies

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Author contributions: M.R.V., J.F., R.B., M.G.R., R.J.K., and J.E.C. planned the studies. M.R.V., J.F., N.K., L.E.W., R.B., and T.K. conducted experiments. Y.A.B. and J.E.G. obtained human specimens. M.R.V., J.F., R.B., I.S., I.G., T.K., R.J.K., and J.E.C. interpreted the studies. M.R.V. and J.E.C. wrote the first draft of the paper. M.R.V., M.G.R., J.E.G. and J.E.C. obtained funding. All authors reviewed, edited and approved the paper.

Competing interests: J.E.C. is on the Scientific Advisory Boards of CompuVax and Meissa Vaccines and is a recipient of previous unrelated research grants from Moderna and Sanofi, and is founder of IDBiologics, Inc. J.E.G. serves as consultant for MedImmune, Regeneron, Ena Therapeutics, and Meissa Vaccines. Vanderbilt University has applied for a patent concerning antibodies discussed in this paper; M.R.V. and J.E.C. are cited as inventors. All other authors declared no competing interests.

Data and materials availability: The [EV-D68 virus + EV68–159 Fab] and the [EV-D68 virus + EV68–228 Fab] cryo-EM structures have been deposited to the Protein Data Bank (PDB) and Electron Microscopy Data Bank (EMDB) under accession numbers: PDB: 6WDS, EMD-21647 and PDB: 6WDT and EMD-21648, respectively. All relevant data are included within the manuscript. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, James E. Crowe, Jr. (james.crowe@vumc.org). Materials described in this paper are available for distribution under the Association of University Technology Managers (AUTM) Uniform Biological Material Transfer Agreement (UBMTA).

revealed that EV68–228 and another potentially neutralizing antibody (EV68–159) bound around the five-fold or three-fold axes of symmetry on virion particles, respectively. The structures suggest diverse mechanisms of action by these antibodies. The high potency and effectiveness observed *in vivo* suggest that antibodies are a mechanistic correlate of protection against AFM disease and are candidates for clinical use in humans with EV-D68 infection.

One Sentence Summary:

A human monoclonal antibody shows potential to prevent or treat acute flaccid myelitis associated with enterovirus D68 infection.

Introduction

Enterovirus D68 (EV-D68) is a re-emerging picornavirus. This family of single-stranded positive-sense RNA genome, non-enveloped viruses share a characteristic capsid. Sixty copies each of four viral proteins (VP1, VP2, VP3, and VP4) are arranged in pseudo $T=3$ icosahedral symmetry forming five-, three-, and two-fold axes of symmetry (1). EV-D68 has caused outbreaks of respiratory disease throughout the world (2–4). Clusters of acute flaccid myelitis (AFM), a poliomyelitis-like illness, were noted during a large outbreak of EV-D68-induced respiratory illness in the United States (U.S.) in 2014 (5). AFM outbreaks have occurred worldwide and have been well characterized in the U.S., with large peaks in incidence during the August to October months every other year since 2014 (6). These biennial AFM outbreaks have been associated with EV-D68 circulation in the population, and strong evidence of causality for enteroviruses and AFM has built over time (7–9). Understanding this causal association has increased the urgency of defining the molecular and cellular basis for EV-D68 pathogenesis and host response (10). Genetic diversification of EV-D68 has accelerated in recent years, resulting in the appearance of different viral clades with distinct genotypes designated A, B (with subclades B1, B2, and B3), C, and D (previously described as A2) (11). It remains unclear if the emergence of these new clades is associated with reduced immunity of the general population against EV-D68 infection or with increased occurrence of AFM. However, it is clear that EV-D68 has become a public health threat worldwide. Therefore, comprehensive studies of EV-D68 and the mechanisms by which the human immune system controls EV-D68 infection and AFM disease are needed.

The role of antibodies in protection against EV-D68 infection and disease is uncertain. Numerous seroepidemiology studies have shown nearly universal seroprevalence of neutralizing antibodies for EV-D68 in adult human sera (12). Two studies that more closely investigated EV-D68 seroepidemiology in children noted a nadir of population antibody prevalence around one year of age, with a gradual rise in the presence of EV-D68 neutralizing antibodies through childhood (13, 14). Polyclonal human immune globulin pooled for intravenous use (IVIG) contains EV-D68 neutralizing antibodies (15) and is capable of protecting mice from lethal experimental challenge causing AFM-like infections (16). While both children and adults are susceptible to infection of the respiratory mucosa with EV-D68, AFM occurs almost exclusively in children (6). Likely, the pathogenesis of EV-D68 infection associated with AFM mimics some of the mechanisms by which the

related picornavirus poliovirus causes infection of the nervous system during poliomyelitis. For poliovirus, vaccination induces serum neutralizing antibodies that are imperfect at blocking infection of the gastrointestinal mucosa but prevent progression to poliomyelitis (17). To determine if antibodies play a critical role in preventing EV-D68-associated AFM, we sought to characterize the different classes of antibodies made by humans in response to natural infection. Using human B cell hybridoma technology, we isolated a panel of naturally occurring human monoclonal antibodies (mAbs) that recognize EV-D68. After characterizing their neutralization and binding properties *in vitro*, we focused on two mAbs for structural studies to determine their epitopes on the virion and one mAb for subsequent *in vivo* studies of prevention and treatment of EV-D68 infection in mice.

Results

Isolation of human hybridomas secreting antibodies to EV-D68

After obtaining written informed consent, twelve subjects who had previous documented EV-D68 respiratory tract infections during the 2014 outbreak in the U.S. donated blood, from which we isolated peripheral blood mononuclear cells (PBMCs). The subjects were 12 to 15 years old when infected and 16 to 18 years old at time of blood collection. Each subject had a history of EV-D68-associated respiratory disease, and none had symptoms of AFM (Table S1). The collected PBMCs were transformed *in vitro* by inoculation with Epstein-Barr virus to generate memory B cell-derived lymphoblastoid cell lines (LCLs), which secrete antibodies. LCL culture supernatants were used in an indirect ELISA to screen for the presence of EV-D68-reactive IgGs. We selected cultures with antibodies that bound to laboratory-grown live virus preparations of EV-D68 generated from 2014 clinical isolates but that did not bind to a similarly prepared uninfected cell supernatant. After electrofusion of LCLs secreting EV-D68-specific antibodies with a non-secreting myeloma cell line, the resulting hybridoma cells were single-cell sorted to generate clonal hybridomas secreting fully human mAbs (Table S1).

Binding and neutralization properties of EV-D68-reactive monoclonal antibodies

We sought to determine how many major antigenic sites on the virus surface are bound by human mAbs made in response to natural infection. To identify groups of antibodies that recognized similar epitopes, we determined whether the mAbs could compete with the binding of each of the other mAbs to live virus in an indirect ELISA. For competition-binding experiments, virus was coated directly onto an ELISA plate and then incubated with high concentrations of one unlabeled mAb. Next, mAbs labeled by biotinylation were added at a lower concentration, and the ability of the second mAb to bind the virus in the presence of the first mAb was determined. We then used a Pearson correlation with the inhibition data to determine the relatedness of the antibody binding patterns to each other and identified four main competition-binding groups (Fig. 1), which we termed groups 1 to 4. We used each mAb to stain a Western blot of EV-D68 preparations and found that nearly all mAbs in competition-binding groups 2 and 3 bound to linear epitopes in the VP1 protein, whereas only a single other mAb bound to any protein in the virus preparation (Fig. S1).

During the 2014 EV-D68 outbreak in the U.S., nearly all viral isolates were of the newly emergent B1 clade, with fewer detections of virus from the closely related B2 or distantly related D clades (18). All but one of the subjects for this study were infected with B1 clade isolates (Table S1). Since 2014, B3 clade viruses have dominated, and B1 clade viruses are no longer circulating (19); in 2018 all EV-D68 isolates sequenced by the U.S. Centers for Disease Control and Prevention were from the B3 clade (20). We first measured the *in vitro* neutralization capability of each mAb in a 50% cell culture infectious dose (CCID₅₀) assay using a B1 clade EV-D68 isolate (Fig. 2A). Twenty-eight mAbs demonstrated neutralization with a half maximal inhibitory concentration (IC₅₀) below 50 µg/mL, with mAb EV68–159 exhibiting the strongest neutralization at an IC₅₀ value of 0.32 ng/mL (Fig. S2). We further tested the 21 most potently neutralizing mAbs against a D clade isolate and found that 11 mAbs neutralized that virus, with 7 of those exhibiting at least a ten-fold decrease in potency by IC₅₀ value for the heterologous virus. The Fermon strain is an isolate from 1962 and is so distantly related to modern EV-D68 isolates that it does not fit into the clade classification scheme (18). Nine mAbs neutralized the Fermon laboratory reference strain, but less potently than they inhibited the contemporary B1 clade virus.

Recognizing that neutralization assays may underestimate cross-reactivity, we used the same indirect ELISA approach described above to generate half-maximal effective concentrations (EC₅₀) of purified mAb for binding to representative EV-D68 isolates from the B1, B2, or D clades (Fig. 2, B and C, and Fig. S3). Of the mAbs with EC₅₀ values for binding of 1 µg/mL to B1 clade isolates, all bound to a B2 clade isolate, whereas about half also bound to a D clade isolate (Fig. 2B and Fig. S2). An additional class of mAbs was observed that bound weakly in general but cross-reacted to viruses from all clades tested.

Structural studies of two neutralizing anti-EV-D68 antibodies

To date, structural studies of antibody-EV-D68 interactions have been limited to murine mAbs (21). We selected two potently neutralizing human mAbs, the clade-specific mAb EV68–159 and the highly cross-reactive mAb EV68–228, to make immune complexes with antigen-binding fragments (Fabs) and a B1 clade EV-D68 isolate for cryo-electron microscopy (cryo-EM) studies. The final density maps attained a resolution of 2.9 Å (EV68–159) or 3.1 Å (EV68–228) (Fig. 3A, Fig. S4, Fig. S5, and Table S3). The structures revealed two distinct binding sites: EV68–159 attached around the three-fold axes of symmetry, whereas EV68–228 bound around the five-fold axes between depressions that form the canyon regions (Fig. 3, Fig. S6). Thus, for each Fab, a total of 60 copies bound to the virus particle. The Fab variable domains, which interacted with the viral surface, displayed strong densities similar to the viral capsid proteins, and an atomic model of each Fab was built together with the four viral capsid proteins. In contrast, the Fab constant domains, which are located further from the viral surface, displayed weaker densities and were excluded from atomic model building. The backbone of the polypeptide chains and the majority of amino acid side chains are well-ordered in the density maps, demonstrating the critical features of the binding interface between virus particle and Fab molecule.

For both models, the viral surface residues that were facing and within a 4 Å distance from the Fab were identified as the footprint (Fig. 3C, Fig. S7, and Table S4). The footprints show

that both Fab molecules sit within one protomer. In the EV-D68:Fab EV68–159 complex, each Fab masked a viral surface area around 990 Å². At the binding interface (Fig. 4), essential interactions were found between the EV68–159 light chain and three residues on the C-terminus of VP1: Glu271 and Arg272 (Fig. 5A) and Asp285 (Fig. 5B). Residues Glu271 and Arg272 formed hydrogen bonds with CDR3 and CDR1. Arg272 and Asp285 formed salt bridges with CDR3 and CDR2 residues, respectively. The heavy chain of EV68–159 contributed 77% of the masked surface areas. A series of hydrogen bonds was found between the heavy chain complementarity-determining region 2 (CDR2) and CDR3 and the VP3 N-terminal loop before the B-β strand (βB) (Fig. 5C).

In the EV-D68:Fab EV68–228 complex, each Fab masked approximately 1,170 Å² of the viral capsid surface. Similar to EV68–159, the heavy chain of the EV68–228 Fab dominated the interaction with the viral capsid by masking around 84% of the surface area. The binding interface (Fig. 4) was stabilized mainly by hydrogen bonds formed between the heavy chain CDRs and the VP1 βB as well as the VP3 C-terminus (Fig. 5D). The light chain CDR1 interacted with the VP2 EF loop. In addition, hydrogen bonds formed between the heavy chain framework region (FR) 3 and the VP1 DE loop. Furthermore, a salt bridge formed between the light chain CDR3 and the VP1 C-terminus. Overall, the EV68–228 Fab bound the viral surface around the five-fold axes and recognized the classical picornavirus neutralizing immunogenic sites (NIm)s NIm-IB (VP1 DE loop) and NIm-II (VP2 EF loop) (22).

Bulky side chains were found at the interface for both Fabs and act to stabilize the structures through hydrophobic interaction networks (Fig. S8). Furthermore, disulfide bonds also were detected around CDR1 and CDR3 in heavy and light chains. Another pair of cysteines, Cys101 and Cys106, was found within the CDR3 of the EV68–228 heavy chain and were at the correct distance and orientation to form a disulfide bond (Fig. 5D). Specifically, when the contour levels were reduced, the densities of the two cysteine side chains connected. As described above for the EV-D68:Fab EV68–228 complex, hydrogen bonds were observed between the heavy chain CDR3 and the VP3 C-terminal residues adjacent to the canyon involving Cys101, forming both a hydrogen bond and a disulfide bond. These cysteine residues play critical roles in stabilizing both Fab structure and the virus-Fab binding interface.

Prophylactic and therapeutic effects of neutralizing antibodies in mouse models

We next sought to determine if the potently neutralizing and highly cross-reactive human mAb EV68–228 could prevent or treat infection and disease in small animal models of EV-D68 infection. We tested for this antiviral activity *in vivo* using two different established models of infection causing either respiratory or AFM-like neurologic disease in AG129 strain mice that are deficient in receptors for interferon α/β and γ (23, 24). First, we tested whether antibodies could reduce viremia and lung virus replication in the respiratory model of infection. MAb EV68–228 administered systemically as prophylaxis a day before virus inoculation provided sterilizing immunity in the blood (Fig. 6A) and lungs (Fig. 6B) at each of the concentrations tested, whereas human IVIG only sterilized the blood. Induction of pro-inflammatory cytokine secretion was inhibited in the lungs of EV68–228 treated mice

(Fig. 7, A to C). When used as treatment given at increasing times after virus inoculation, again all treatments were highly effective at sterilizing the blood (Fig. 6C), but only EV68–228 had efficacy in the lungs (Fig. 6D). We similarly observed reduced pro-inflammatory cytokine levels in the lungs of EV68–228-treated mice (Fig. 7, D to F).

Next we assessed the effect of passive transfer of antibodies in a neurologic model of infection that mimics AFM disease. EV68–228 prophylaxis provided sterilizing immunity of the blood (Fig. 8A) and complete protection from death (Fig. 8B) or development of any neurologic disease (Fig. 8C), whereas IVIG treatment protected only partially. Given therapeutically, EV68–228 treatment sterilized the blood within 24 hours of administration (Fig. 8D) at each of the time points given. EV68–228 improved survival (Fig. 8E) and neurologic disease when given as late as 48 hours after infection; when given at 72 hours post-infection, the mouse that survived improved clinically (Fig. 8F and Table S5).

Discussion

These studies reveal diverse features of the human B cell response to EV-D68 infection. We attempted to be unbiased in our approach to isolating these mAbs, using live virus isolates as the screening antigen. The diversity in antibody phenotypes that we recovered may be a result of this strategy, as we observed a broad range of cross-reactivity among clades of EV-D68, both with binding and neutralization. Interestingly, strong binding to live virus particles did not necessarily predict high neutralizing potency. Of the competition-binding groups observed, only groups 2 and 3 exhibited uniformity in phenotype. MABs in both groups were cross-reactive, but group 2 mAbs neutralized virus whereas group 3 mAbs did not. Nearly all of the group 2 and 3 mAbs bound to VP1 in Western blot (Figs. S1 and S2), suggesting that they bind to linear epitopes. Notably, the competition-binding studies used full-length IgG molecules, so the competition seen is functional as would occur in human tissues and does not necessarily indicate that there are only four structurally distinct epitopes on the viral surface.

The lack of Western blot reactivity of EV68–159 and EV68–228 correlates with the findings in the structural studies that show both epitopes span all three major viral surface proteins. The conformation-dependent nature of the epitopes of these two potentially neutralizing mAbs is notable because recent diagnostic advances using peptide microarray (8) and phage library (9) technologies scanned for antibodies in the cerebrospinal fluid of patients with AFM that recognize linear epitopes. Detection of antibodies recognizing linear epitopes currently can be used in valuable diagnostic tools, however our studies reveal these tests are at best only partially informative about the quality of antibody response these patients make in response to enterovirus infection. The structures also suggest the molecular basis for antibody-mediated neutralization. By contacting all three structural proteins within a protomer, both mAbs appear capable of inhibiting dynamic structural transitions necessary for infection, which are poorly understood.

The disulfide bond in the CDR3 of EV68–228 heavy chain is a structural moiety we have now observed in broadly neutralizing antiviral human mAbs for a number of viruses, including both hepatitis C (25) and influenza A virus (26). The intervening four to five

amino acids between cysteines forms a smaller structured loop at the most distal tip of the full CDR3 loop, stabilizing the CDR3 in a preconfigured state optimal for binding the viral antigen. For EV68–228 specifically, the Cys101 also directly interacts with VP3 via a hydrogen bond, so the cysteine participates in both CDR3 loop stabilization and interaction with target.

The three VP1 residues that interact with EV68–159 light chain (Glu271, Arg272 and Asp285) and Glu59 on the N-terminal loop of VP3, which interacts with EV68–159 heavy chain, are adjacent to the sialic acid receptor binding site (27), suggesting that the EV68–159 Fab may block virus from binding sialic acid receptors. In particular, these three VP1 residues are located on a 22-amino acid VP1 C-terminal peptide that is bound by antibodies found in the cerebrospinal fluid of patients with AFM (8, 9). Furthermore, the interaction of the EV68–159 Fab heavy chain with the VP3 N-terminal loop may prevent the virus from uncoating, since the N-termini of the four VPs contribute to capsid stability (28). EV68–228 may prevent the virus from uncoating by binding VP1 β B, inhibiting the externalization of the N-terminus of VP1 that is required for entry. In addition, the antibody footprint includes residues on the C-terminus of VP3, which is not part of a classical NIm. These residues are adjacent to the canyon receptor binding site, suggesting that mAb EV68–228 also may block virus binding to receptors.

Finally, at a time when poliovirus types 2 and 3 have been eradicated, AFM is on the rise, and the role of EV-D68 in causing epidemics of this paralytic disease is increasingly evident. Given how well prophylaxis with human mAb EV68–228 works *in vivo*, these data suggest that an effective EV-D68 vaccine might prevent AFM disease. Indeed, recent studies indicate that virus-like particle (29, 30) and inactivated EV-D68 (31) vaccine candidates are immunogenic and protective against infection in mice. However, a study of cotton rats vaccinated with inactivated EV-D68 suggested that they may have suffered worse respiratory disease upon subsequent EV-D68 infection (32). While this finding could suggest the possibility of antibody-dependent enhancement (ADE) of EV-D68 infection, in mice we did not observe ADE caused by polyclonal or monoclonal antibodies, within the range of antibody concentrations we tested. Also, the prospect of using mAb EV68–228 as a therapy early during EV-D68 infection is appealing, especially since this antibody potently neutralizes a diverse set of viral isolates without obvious autoreactive binding to human cell materials (Fig. 2C). Even though IVIG protected mice from AFM-like disease due to EV-D68 in prior *in vivo* studies (16), so far IVIG has not been shown to confer benefit for humans with AFM (33). However, IVIG is a complex mixture of polyclonal antibodies with only a small fraction that recognize EV-D68. MAb prophylaxis or therapy for EV-D68 associated AFM is more promising than IVIG because of the high specificity, high potency, and lower antibody dose that can be used. It is possible, however, that a cocktail of mAbs directed at multiple epitopes may be more protective than mAb monotherapy. A mAb cocktail theoretically would provide a higher barrier to emergence of mAb resistant virus, but we did not observe resistance *in vivo* (Fig. 6 and 8). Even under conditions optimized for selecting EV68–228 resistant viruses *in vitro*, we could only identify virus genomes with mutations of unclear significance (Table S6). In the absence of a reverse genetics system for making recombinant viruses with these mutations, we were unable to verify specifically if these mutations caused escape from neutralization. Therefore, we find emergence of

resistance during potential therapeutic use unlikely. These experiments also provide hope for therapeutic efficacy in patients with severe respiratory disease due to EV-D68, which is the clinical syndrome that brought the 2014 EV-D68 outbreak to the attention of public health authorities prior to recognition of the association with AFM (3). Overall, the studies we present here show that natural EV-D68 infection of humans induces B cells encoding broad and potentially neutralizing antibodies that can prevent or treat infection and disease in both the respiratory tract and the nervous system.

Materials and Methods:

Study Design

We designed this study to try to identify any antibodies that humans can make in response to EV-D68 infection. Therefore, we used live virus isolates in an indirect ELISA screen to identify B cells secreting EV-D68-binding antibodies, and then electrofused those B cells with myeloma cells to create monoclonal antibody secreting hybridomas. We then characterized the neutralization and binding properties of these individual mAbs *in vitro* using CCID₅₀, ELISA, and cryo-EM based techniques. We pursued *in vivo* experiments to generate pre-clinical data supporting the development of mAb EV68–228 as a prophylactic and/or therapeutic agent in humans. For this purpose, we studied the effectiveness of mAb EV68–228 at protecting mice from EV-D68 infection as compared to human IVIG, which is widely used to treat humans with AFM based on theoretical benefit, but this IVIG treatment so far has not been proven to be effective. An advantage of the AG129 murine model of infection is that we could measure the effect of antibody treatment in both respiratory and neurologic models of infection.

Cell lines

RD cells (human, female origin) were obtained from the American Type Culture Collection (ATCC CCL-136). RD cells were cultured in 5% CO₂ at 37°C in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS; HyClone), 1 mM sodium pyruvate and 1% penicillin-streptomycin-amphotericin B (ThermoFisher Scientific). For structural studies, RD cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% HI-FBS (Sigma-Aldrich) and nonessential amino acids (NEAA, Life Technologies). The ExpiCHO (hamster, female origin) cell line was purchased from ThermoFisher Scientific and cultured according to the manufacturer's protocol. The HMMA2.5 line is a non-secreting mouse-human heteromyeloma cell line (sex information is not available) that was generated by fusing a murine myeloma cell line with a human myeloma cell line (34). This cell line was cultured as described previously (35). All cell lines were tested on a monthly basis for *Mycoplasma* and found to be negative in all cases.

Viruses

See Table S7 for a list of the virus isolates used in this study. EV-D68 isolates were propagated for two generations in RD cell monolayer cultures for use in the ELISAs described below. RD cell monolayers were inoculated with a given virus isolate and monitored until 70 to 90% cell death was observed. This cell culture flask then was frozen to

–80°C, thawed, and the contents scraped and collected into a 50 mL conical tube. This preparation was sonicated three times for 20 s in an inverted cup sonicator at maximum power settings (Fisherbrand), vortexed for 30 s, and then sonicated two more times for 20 s. Cell debris was pelleted, and the virus-containing supernatant was spun over a 30% sucrose in PBS (w/v) cushion at 10°C for four hrs at 100,000 x *g*. Supernatant was discarded, and the pellet allowed to soak in 0.01% (w/v) bovine serum albumin (BSA) in NTE buffer (20 mM Tris, 120 mM NaCl, 1 mM EDTA pH 8.0) overnight at 4°C. The resuspended pellet then was clarified further by centrifugation at 10,000 x *g* for 10 min, before storage of virus aliquots at –80°C until ready for use.

For structural studies the US/MO/14–18947 isolate was used. Virus was passaged in RD cells and stored at –80°C before large scale propagation. RD cells were grown to 80% confluency and were infected with EV-D68 at a multiplicity of infection of 0.01. Two days post infection, the cells were collected together with the supernatant and spun down. The cell pellets were collected and after multiple freeze/thaw cycles spun down to remove cell debris. All supernatants were combined and pelleted at 210,000 x *g* for 2 hours. The pellets were incubated and resuspended in 250 mM HEPES (pH=7.5), 250 mM NaCl buffer, then supplemented with final concentrations of 5 mM MgCl₂, 0.01mg/mL DNase (Sigma-Aldrich), 0.8 mg/mL trypsin, 15 mM EDTA and 1% (w/v) n-lauryl-sarcosine. The sample was then pelleted at 210,000 x *g* for 2 hours, resuspended, and loaded onto a potassium tartrate gradient (10 to 40%, w/v) for the last round of ultracentrifugation at 160,000 x *g* for 2 hours. The purified virus sample, which was observed as a blue band in the middle of the tube, was extracted and buffered exchanged into 20mM Tris, 120mM NaCl, 1mM EDTA (pH=8.0) buffer to remove potassium tartrate.

Detection of virus load by CCID₅₀ assay

Titration of virus stocks or virus in murine blood or lung samples was performed by CCID₅₀ assay in RD cell culture monolayers. Briefly, increasing 10-fold dilutions of the samples were applied to RD cell monolayers in triplicate wells (50 µL) of a 96 well plate, incubated for five days in 5% CO₂ at 33°C, and then fixed with 1% paraformaldehyde and stained with crystal violet. Wells with any cytopathic effect were scored as positive for virus, and titers were determined using a formula based on the Spearman-Kärber equation (36); the limit of detection was 136 CCID₅₀/mL.

Virus neutralization assay

Virus neutralization assays were performed in a CCID₅₀ format using the indicated viruses, essentially as described previously for poliovirus (37). Virus was incubated with increasing concentrations of mAb in duplicate for one hr at 33°C, then each suspension was added to a monolayer of RD cells in technical quadruplicate wells (50 µL) of a 96-well plate. After five days incubation in 5% CO₂ at 33°C, cells were fixed with 1% paraformaldehyde and stained with crystal violet. Wells with any cytopathic effect were scored as positive for virus, and half maximal inhibitory concentrations (IC₅₀) were determined using a formula based on the Spearman-Kärber equation (36); the limits of detection were 57 µg/mL to 4.8 pg/mL.

Mouse models

Ten-day old (neurologic model) or four-week old (respiratory model) male and female AG129 mice (deficient in receptors for interferon α/β and γ) were obtained from a specific-pathogen-free colony maintained at the Utah Science Technology and Research (USTAR) building at Utah State University. The mice were bred and maintained on irradiated Teklad Rodent Diet (Harlan Teklad) and autoclaved tap water at the USTAR building of Utah State University. This study was conducted in accordance with the approval of the Institutional Animal Care and Use Committee of Utah State University dated March 2, 2019 (expires March 1, 2022). The work was done in the AAALAC-accredited Laboratory Animal Research Center of Utah State University. The U. S. Government (National Institutes of Health) approval was renewed March 9, 2018 (PHS Assurance No. D16-00468[A3801-01]) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Revision; 2011).

Antibody and control treatments were diluted in PBS and administered by intraperitoneal injection at indicated time points before or after EV-D68 inoculation. Guanidine HCl (Sigma-Aldrich) served as a positive control for treatment (24), started 4 hours post-infection and continued twice daily for 5 days. A suspension of mouse adapted EV-D68 was administered by intraperitoneal injection (neurologic model; $10^{6.5}$ CCID₅₀ in a 100 μ L volume of MEM) or intranasal instillation (respiratory model; $10^{4.5}$ CCID₅₀ in a 90 μ L volume of MEM). Mice were weighed prior to treatment and daily thereafter. Mice were euthanized humanely at indicated time points post-infection for measurement of lung virus titers, blood virus titers, or lung cytokine concentrations, as indicated. For the neurologic model, all mice were observed daily for morbidity, mortality, and neurological scores through day 21. Neurological scores (NS) were recorded as follows: NS0 – no observable paralysis, NS1 – abnormal splay of hindlimb but normal or slightly slower gait, NS2 – hindlimb partially collapsed and foot drags during use for forward motion, NS3 – rigid paralysis of hindlimb and hindlimb is not used for forward motion, NS4 – rigid paralysis in hindlimbs and no forward motion. Any animals observed with a score of NS4 were euthanized humanely.

Lung cytokine/chemokine evaluations

Each sample of lung homogenate was tested for cytokines and chemokines using quantitative chemiluminescent ELISA-based assays according to the manufacturer's instructions (Quansys Biosciences Q-Plex™ Array, Logan, UT). The Quansys multiplex ELISA is a quantitative test in which 16 distinct capture antibodies are applied to each well of a 96-well plate in a defined array.

Generation of human monoclonal antibodies (mAbs)

Subjects were identified from the Childhood Onset of Asthma (COAST) birth cohort (38) who had laboratory documented EV-D68 upper respiratory tract infections (39). After written informed consent was obtained, peripheral blood was collected and stored at room temperature until peripheral blood mononuclear cells (PBMCs) could be purified using SepMate tubes (Stemcell Technologies), per the manufacturer's protocol, and then cryopreserved in 10% (v/v) dimethyl sulfoxide (DMSO) in fetal bovine serum (FBS) and

stored in the vapor phase of liquid nitrogen. Lymphoblastoid cell lines (LCLs) were generated as described previously (40) from memory B cells within the PBMCs by mixing with Epstein-Barr virus, cell cycle checkpoint kinase 2 (chk2) inhibitor (Sigma-Aldrich), CpG (Sigma-Aldrich), and cyclosporin A (Sigma-Aldrich) in Medium A (STEMCELL Technologies). One week later, LCLs were counted and then expanded on a feeder layer of gamma-irradiated, human PBMCs from an unrelated donor. In one more week, LCL supernatants were screened for the presence of EV-D68-reactive IgG by indirect ELISA using live EV-D68 virus as the antigen, comprising cell culture grown EV-D68 virus generated from a 2014 clinical isolate. LCLs from wells containing virus-reactive antibodies were fused to HMMA2.5 myeloma cells by electrofusion, as previously described (35). After the fusion reaction, hybridoma lines were cultured in a selection medium containing HAT media supplements (Sigma-Aldrich) and ouabain (Sigma-Aldrich) in 384-well plates before screening of supernatants for antibody production. Two weeks later, supernatants from the resulting hybridoma cell lines were screened by indirect ELISA with live virus as antigen, and cell lines from wells with EV-D68-reactive antibodies were expanded in culture and then cloned by single-cell flow cytometric sorting into 384-well cell culture plates. These cloned cells were expanded in Medium E until about 50% confluent in 12-well tissue culture treated plates (Corning) and their supernatants screened for virus binding by ELISA. Wells with the highest signal in ELISA were selected as the mAb-producing hybridoma cell lines for further use.

MAB isotype and gene sequence analysis

The isotype and subclass of secreted antibodies were determined using mouse anti-human IgG1, IgG2, IgG3 or IgG4 antibodies conjugated with horseradish peroxidase (Southern Biotech). Antibody heavy- and light-chain variable region genes were sequenced from RNA obtained from hybridoma lines that had been cloned biologically by flow cytometric sorting. Total RNA was extracted using the RNeasy Mini kit (Qiagen). A modified 5' RACE (Rapid Amplification of cDNA Ends) approach was used (41). Briefly, 5 μ L total RNA was mixed with cDNA synthesis primer mix (10 μ M each) and incubated for 2 min at 70°C followed by a decrease in the incubation temperature to 42°C to anneal the synthesis primers (1 to 3 min). After incubation, a mixture containing 5 $\times$ first-strand buffer (Clontech), DTT (20 mM), 5' template switch oligo (10 μ M), dNTP solution (10 mM each) and 10 $\times$ SMARTScribe Reverse Transcriptase (Clontech) was added to the primer-annealed total RNA reaction and incubated for 60 min at 42°C. The first-strand synthesis reaction was purified using the Ampure Size Select Magnetic Bead Kit at a ratio of 1.8 $\times$ (Beckman Coulter). Following purification, a single PCR amplification reaction containing 5 μ L first-strand cDNA, 2 $\times$ Q5 High Fidelity Mastermix (NEB), dNTP (10 mM each), forward universal primer (10 μ M) and reverse primer mix (0.2 μ M each in heavy-chain mix, 0.2 μ M each in light-chain mix) was subjected to thermal cycling with the following conditions: initial denaturation for 90 s followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 40 s, followed by a final extension step at 72°C for 4 min. All primer sequences used in this protocol were described previously (41). The first PCR reaction was purified using the AMPure Size Select Magnetic Bead Kit at a ratio of 0.6 $\times$ (Beckman Coulter). Amplicon libraries were then prepared according to the Multiplex SMRT Sequencing protocol (Pacific Biosciences) and sequenced on a Sequel

platform instrument (Pacific Biosciences). Raw sequencing data was demultiplexed and circular consensus sequences (CCS) were determined using the SMRT Analysis tool suite (Pacific Biosciences). The identities of gene segments, complementarity-determining regions (CDRs), and mutations from germline genes were determined by alignment using the ImMunoGeneTics database (42).

Antibody production and purification

For hybridoma-derived mAb, hybridoma cells were grown to exhaustion in Hybridoma SFM (1X) serum free medium (Gibco). For recombinant mAb production, cDNA encoding the genes of heavy and light chains were synthesized and cloned into a DNA plasmid expression vector encoding a full-length IgG1 protein (43) and transformed into *E. coli* cells. MAb proteins were produced after transient transfection of ExpiCHO cells following the manufacturer's protocol. The resulting secreted IgGs were purified from filtered culture supernatants by fast protein liquid chromatography (FPLC) on an ÄKTA instrument using a Protein G column (GE Healthcare Life Sciences). Purified mAbs were buffer exchanged into PBS, filtered using sterile 0.45-µm pore size filter devices (Millipore), concentrated, and stored in aliquots at -80°C until use. An aliquot of each mAb also was biotinylated directly in 96-well format using the EZ-Link NHS-PEG₄-biotin kit (ThermoFisher Scientific) with a 20-fold molar excess of biotin to mAb, followed by buffer exchange back to PBS using a desalting plate (Zeba, 7 kDa cutoff). Hybridoma-derived mAbs were used in *in vitro* experiments, and recombinant mAbs were used in *in vivo* experiments. Pooled human immunoglobulin was purchased as intravenous immunoglobulin (IVIG, Carimune, CSL Behring, King of Prussia, PA). RSV90 is a recombinant human IgG1 mAb produced in our laboratory that was used as a negative control, placebo mAb in mouse experiments. Polyclonal anti-VP1, -VP2, and -VP3 antibodies used in Western blot were purchased from Genetex.

Fab fragment production

Fab fragments were generated and purified via Pierce Fab Preparation Kit (ThermoFisher Scientific). The Immobilized Papain vial spin column and Zeba Spin Desalting Column were equilibrated with digestion buffer (35 mg cysteine·HCl per 10 mL of supplied Fab Digestion Buffer, pH ~7.0) before use. The NAb Protein A Plus Spin Column was equilibrated with PBS buffer before use. The original IgG samples were passed through the Zeba Spin Desalting Column, and 0.5 mL of the prepared IgG samples were applied on the Immobilized Papain vial and incubated at 37°C for 5 hours Fab digestion. Then the final Fab fragments were buffer exchanged to PBS and stored at 4°C.

EV-D68-specific ELISA

Wells of medium binding, black fluorescent immunoassay microtiter plates (Greiner Bio-One) were coated with virus stocks diluted in 100 mM bicarbonate/carbonate buffer, pH 9.6 and incubated at 4°C overnight. Plates were blocked with 2% BSA in Dulbecco's phosphate-buffered saline (DPBS) containing 0.05% Tween-20 (DPBS-T) for 1 hr. For mAb screening assays, hybridoma culture supernatants were added to the wells and incubated for 2 hr at ambient temperature. The bound antibodies were detected using Fc-specific goat anti-human IgG conjugated with HRP (Southern Biotech) and QuantaBlu fluorogenic peroxidase

substrate (ThermoFisher Scientific). After 20 min 100 mM glycine, pH 10.5 was added to quench the reaction, and the emission was measured at 420 nm after excitement at 325 nm using a Synergy H1 microplate reader (Biotek). For dose-response and cross-reactivity assays, serial dilutions of purified mAbs were applied to the wells in duplicate technical replicates and mAb binding was detected as above; the experiments were performed at least three times. For the competition ELISA, microtiter plates were first coated with virus, and then a purified mAb was added at 100 $\mu\text{g}/\text{mL}$ and allowed to incubate at 33°C for 3 hr. Then, a biotinylated mAb was spiked into this mixture at a final concentration of 5 $\mu\text{g}/\text{mL}$ and allowed to incubate at ambient temperature for 1.5 hr. After a wash and 30 min of incubation with avidin-peroxidase (ThermoFisher Scientific), biotinylated mAb binding was detected as above.

Western blot

B1 clade virus preparation was mixed with denaturing and reducing loading buffer, boiled at 100°C for 5 min, and then run on an SDS-PAGE gel along with Novex sharp pre-stained protein standard (ThermoFisher Scientific). Protein was transferred to a membrane, blocked in blocking buffer (Li-Cor), and then cut into strips so that individual lanes could be stained with purified mAb in blocking buffer. An IRDye 800CW-conjugated goat anti-human secondary antibody (Li-Cor) was used to detect mAb binding. Strips were reassembled to visualize molecular weight and imaged on an Odyssey CLx near infrared imager (Li-Cor).

Cryo-EM sample preparation and data collection

For both EV-D68:Fab EV68–159 and EV-D68:Fab EV68–228 complexes, purified EV-D68 viruses and Fabs were mixed at a molar ratio of 1:200. After incubating at room temperature for 45 to 60 minutes, 3.5 μL of virus-Fab mixture sample were added to a glow-discharged 400 mesh lacey carbon film copper grid (Ted Pella Inc.). Grids were plunge frozen (Cryoplunge 3 system, Gatan) in liquid ethane after being blotted for 3.5s in 75 to 80% humidity. Cryo-EM datasets were collected on a 300 kV Titan Krios Microscope (Thermo Fisher Scientific). For the EV-D68:Fab EV68–228 dataset, movies were collected using the program Leginon (44) with a K3 Direct Detection Camera (Gatan) at a magnification of 64,000X, resulting in a super resolution pixel size of 0.662 Å, with a defocus range from 0.7 to 2 μm . A total electron dose of 44.2 electrons/Å² over 2.6 seconds of exposure was recorded over 50 frames. The EV-D68:Fab EV68–159 dataset was acquired with a K2 Summit direct electron detector (Gatan) at a nominal magnification of 81,000X, resulting in a super resolution pixel size of 0.874 Å, a defocus range from 0.7 to 3.5 μm . A total electron dose of 31.4 electrons/Å² over 12 seconds of exposure was split into 60 frames. Overall, 462 movies and 732 movies were acquired for the EV-D68:Fab EV68–228 and EV-D68:Fab EV68–159 datasets, respectively.

Image processing

For both datasets, motion correction was performed on the raw movie frames via MotionCor2 (45) as implemented in Appion (46) during data collection. The contrast transfer function (CTF) was estimated on the aligned frames with CTFFIND4 (47). Particle-picking templates were generated using the Appion Manual Picker (46) and templates for auto picking were obtained through 2-dimensional (2D) classification in XMIPP (48). These

templates were then used for auto-picking in FindEM (49) and particles were extracted using RELION. These particles were then subjected to multiple rounds of 2D and 3D classifications in RELION (50). This resulted in 20,194 and 30,554 particles for the EV-D68:Fab EV68–228 and EV-D68:Fab EV68–159 datasets which were selected for final 3D icosahedral reconstructions using the program JSPR following the gold-standard refinement method (51). The final resolutions for both maps were estimated based on a gold-standard Fourier shell correlation cutoff of 0.143 (52). Map sharpening was done in RELION (50) post-processing. Data collection parameters and related items are summarized in Table S3.

Model building, refinement and analysis

The same methods were used for the atomic structures of both EV-D68:Fab EV68–159 and EV-D68:Fab EV68–228. The X-ray crystallography model of the EV-D68 Fermon strain (PDB code: 4WM8) was selected as a starting reference for model building and was manually fitted into the density maps using the program Chimera (53). Using the initial fitting as a basis, the models were rebuilt in Coot (54) and refined using real-space-refinement in PHENIX (55) to correct for outliers and poorly fitted rotamers. Chimera (53), Coot (54) and CCP4i2-PISA (56) were used to determine the binding interface residues. The final atomic models were validated in MolProbity (57). Refinement statistics are described in Table S3.

Selection of neutralization escape mutant virus

A clade B1 EV-D68 isolate was passaged under selection with increasing amounts of purified mAb in RD cells. After incubating mAb and virus for 1 hr at 33 °C, this mixture was added to a cell monolayer for 2 hr at 33 °C. The monolayer then was rinsed thrice, and mAb containing medium was added back. This culture was incubated at 33 °C until at least 70% cytopathic effect was observed (cells lifted off of plate), at which point the cells and supernatant together were collected and frozen to –80 °C. This sample was thawed and sonicated in the same microfuge tube in an inverted cup sonicator at maximum power 3×20 s, vortexed for 30 s at maximum power, and sonicated again 2×20 s. Cellular debris was clarified for 10 min at 10,000 x g. Then the virus-containing supernatant was mixed 1:1 with fresh medium containing mAb at higher concentration. Over three passages, mAb concentration was increased from 5 to 50 to 500 ng/mL. Viral RNA was harvested using TRI Reagent and Direct-zol RNA MiniPrep kit (Zymo Research). In triplicate we generated cDNA templates, from which a 3,080 bp amplicon covering the P1 region of the viral genome was generated with the PrimeScript One Step RT-PCR Kit Ver. 2 (Takara) and primers 5′ - CCTCCGGCCCCCTGAAT (Fwd) and 5′ - CCATTGAATCCCTGGGCCTT (Rev). Then we used a Pacific Biosciences (PacBio) next generation sequencing platform to generate sequences of each of the three replicates. 2,000 reads of each sequencing run were used to quantitate the percentage of reads in which each mutation was observed. Mutations were determined as compared to a wild-type consensus sequence of all of the reads from the negative control selection mAb selection.

Quantification and statistical analysis

Technical and biological replicates are indicated in the methods and figure legends. Statistical analyses were performed using Prism v8 (GraphPad).

Competition-binding assay.

ELISA fluorescence values were normalized to a percentage of maximal binding determined from a control well without an irrelevant prior competing mAb added. The Pearson correlation of each biotinylated mAb to each other biotinylated mAb was calculated using the median inhibition percentage from three different experiments using the *corr* method of the *Pandas* Python package (58). Hierarchical clustering was then performed on these Pearson correlations using the *clustermap* method of the *Seaborn* Python package. The clustering information was exported in newick format and imported into Interactive Tree of Life v4 (59), which was used to display the hierarchically-clustered heatmap before importation into Excel (Microsoft) for final display.

Antibody ELISA binding experiments.

EC₅₀ values for mAb binding were determined after log transformation of antibody concentration using four-parameter sigmoidal dose-response nonlinear regression analysis constrained to a bottom value of zero and top value less than the maximal fluorescent value of the mAb with the highest saturation fluorescence value.

Virus assays.

MAB IC₅₀ values were calculated using a formula based on the Spearman-Kärber equation (36). Viral titers in murine plasma and lungs were compared using a one-way analysis of variance (ANOVA) and Dunnett's multiple comparisons test, with a single pooled variance. A value of $p < 0.05$ was considered significant.

Lung cytokine/chemokine evaluations.

For each cytokine/chemokine, the concentrations from treated mice were compared to placebo-treated mice using a Brown-Forsythe one-way ANOVA test and Dunnett's T3 multiple comparisons test, with individual variances computed for each comparison. This analysis was chosen because we did not assume equal standard deviations for each measurement.

In vivo protection studies.

Survival curves were generated using the Kaplan-Meier method and curves compared using the log rank test (Mantel-Cox). Neurologic scores were compared using a chi-square test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

We thank Mattie Jensen, Rachel Sutton, Rachel Nargi, Erica Armstrong, Matthew Goff, Walter Reichard, Leyn Shakhmourad, Tracy Cooper, Matthew Jenkins, Pavlo Gilchuk, and Robert Carnahan for laboratory support in generation or characterization of antibodies. Meghan Schildts and Tany Tan assisted with data about virus isolates and sequences. Suman Das contributed helpful discussion and provided the Fermon virus isolate. We thank Christopher Tisler, Daniel J. Jackson, and Robert F. Lemanske Jr. for coordinating human subjects and sample acquisition. We also thank the Purdue Cryo-EM Facility. Vanderbilt University Medical Center used the non-clinical and pre-clinical services program offered by the National Institute of Allergy and Infectious Diseases,

coordinated by Eunchung Park. Contract murine studies supported by NIAID were performed by Brett Hurst and Bart Tarbet at Utah State University.

Funding: M.R.V. was supported by a PIDS-St. Jude Research Hospital's Fellow Program in Basic & Translational Research fellowship. This work was supported by U.S. N.I.H. grant T32 HL069765 (to L.E.W.), U19 AI117905 (to J.E.C.), P01 HL070831 and U19 AI104317 (to J.E.G.), and R01 AI011219 and CSGID contract HHSN272201700060C (to R.J.K.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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Fig. 1. Competition binding groups of mAbs from EV-D68-immune human subjects. Relatedness scores were generated from competition-binding ELISAs with a B1 clade EV-D68 isolate and used to cluster mAbs into four competition-binding groups designated 1 to 4. Clone numbers listed in red or blue are potentially neutralizing mAbs, with blue clone names indicating the two mAbs studied in detail in later figures.

Fig. 2. Neutralization potency and binding capacity of human mAbs.

(A) MAbs were ranked within competition-binding group (Comp. group, group 5 indicates the residual collection of singletons) by IC₅₀ value in a CCID₅₀ neutralization assay using a B1 clade isolate. We also tested neutralization of a D clade and Fermon (Fer.) isolate for the 21 most potently neutralizing mAbs. ">" denotes neutralization was not detected when tested in concentrations up to 50 µg/mL. Blank cells indicate not tested. Clone numbers listed in red or blue are potently neutralizing mAbs, with blue clone names indicating the two mAbs studied in detail in later figures. (B) Binding strength to live virus isolates or a

mock virus preparation is denoted using EC₅₀ values generated by using (C) indirect ELISA with purified mAb dilutions to fit sigmoidal dose response curves. “>” indicates EC₅₀ value exceeds the maximum concentration tested of 100 µg/mL, suggesting poor or no binding.

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Fig. 3. Structural feature comparison between two immune complexes.

(A) Radially colored cryo-EM maps of EV-D68:Fab EV68–159 (left) or EV-D68:Fab EV68–228 (right). Each map is projected down a two-fold axis of symmetry. The five-, three-, and two-fold axes of each asymmetric unit are depicted using a triangle outline labeled with one pentagon, two small triangles, and one oval, respectively. (B) Binding position comparison on an asymmetric unit. Viral proteins are colored in blue (VP1), green (VP2) and red (VP3). Fab molecules are colored in grey (EV68–159) or purple (EV68–228), and the heavy or light chains are shown in the same colors with dark or light intensities, respectively. (C) Footprints of EV68–159 Fab (left) or EV68–228 Fab (right). Radially colored 2D projections of the viral surface were created with RIVEM. Virus surface residues facing any atoms from the Fab molecules within a distance of 4 Å are outlined in light blue (VP1), light green (VP2) and light red (VP3). The canyon region is outlined in yellow. Scale bars in (A) and (C) indicate radial distance measured in Å.

Fig. 4. Close-up view of the binding interfaces of EV68–159 and EV68–228.

The viral capsid is shown as surface and the Fab is shown in a cartoon representation. VP1, VP2 and VP3 are colored in white, dark grey and silver, respectively. Heavy or light chains are colored in orange or yellow, respectively. Viral residues making interactions are colored based on the heavy and light chains, and the color intensities vary based on which of the VPs. The heavy and light chain complementarity-determining regions (HCDR and LCDR, respectively) involved in the binding interfaces are shown with arrows.

Fig. 5. Molecular detail of virion-Fab interactions.

Representative interactions at the binding interface of EV-D68:Fab EV68–159 (**A, B, and C**) and EV-D68:Fab EV68–228 (**D**). Hydrogen bonds are colored in cyan and salt bridges are colored in magenta.

Fig. 6. MAb EV68-228 protects mice from EV-D68-induced respiratory disease, when used as either prophylaxis or therapy.

Four-week-old AG129 strain mice ($n = 4$ per time point) were inoculated with mouse-adapted B1 clade EV-D68 intranasally; antibody was administered intraperitoneally; and viral titers for indicated tissue were measured by a CCID₅₀ assay. (A, B) Mice were inoculated with virus 24 hours after indicated dose of antibody, then viral titers were measured at indicated time points. (C, D) Mice were inoculated with virus followed by 10 mg/kg (except where indicated) of antibody 4, 24, or 48 hours later, then viral titers were measured.

Fig. 7. MAb EV68–228 decreases lung inflammation in EV-D68 infected mice.

Four-week-old AG129 mice ($n = 4$ per time point) (A, B, C) were inoculated with virus intranasally 24 hours after indicated dose of antibody or (D, E, F) were inoculated with virus intranasally followed by 10 mg/kg (except where indicated) of antibody 4, 24, or 48 hours later, then cytokines were measured at indicated time points. Cytokines were quantified from lung homogenates using an ELISA. Values from the treatment groups were compared to the placebo group for each time point using a one-way ANOVA with Dunnett's T3 multiple comparisons test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). IL - interleukin; MCP - monocyte chemoattractant protein

Fig. 8. MAb EV68-228 protects mice from EV-D68-induced neurologic disease, when used as either prophylaxis or therapy.

Ten-day-old mice were inoculated with mouse-adapted B1 clade EV-D68 intraperitoneally; antibody was administered intraperitoneally; and viral titers for indicated tissue were measured by a CCID₅₀ assay. (A, B, C) Mice were inoculated with virus 24 hours after indicated dose of antibody, then (A) viral titers were measured (n = 3 per time point), (B) survival was monitored, and (C) neurologic scores (n = 6 per time point) were recorded at indicated time points. Higher scores indicate more severe motor impairment. (D, E, F) Mice were inoculated with virus followed by a 10 mg/kg dose of antibody, then (D) viral titers were measured (n = 3 per time point), (E) survival was monitored, and (F) neurologic scores (n = 6 per time point, except n = 9 for 120 hr post) were recorded. Colored vertical arrows indicate time of treatment.