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An armed anti-immunoglobulin light chain nanobody protects mice against influenza A and B infections

Xin Liu^{1, #}, Thomas Balligand^{1, #}, Claire Carpenet^{1, 2}, Hidde L. Ploegh^{1, *}

¹Program in Cellular and Molecular Medicine, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA.

²CBS2 University of Montpellier, 163 rue Auguste Broussonnet, 34090 Montpellier, France.

Abstract

The immune system eliminates pathogen intruders such as viruses and bacteria. To recruit immune effectors to virus-infected cells, we conjugated a small molecule, the influenza neuraminidase inhibitor zanamivir, to a nanobody that recognizes the kappa light chains of mouse immunoglobulins. This adduct was designed to achieve half-life extension of zanamivir through complex formation with the much larger immunoglobulins in the circulation. The zanamivir moiety targets the adduct to virus-infected cells, while the anti-kappa component simultaneously delivers polyclonal immunoglobulins of indeterminate specificity and all isotypes. Activation of antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity promoted elimination of influenza neuraminidase-positive cells. A single dose of the conjugate protected mice against influenza A or B viruses and was effective even when given several days after infection with a lethal dose of virus. In the absence of circulating immunoglobulins, we observed no *in vivo* protection from the adduct. The type of conjugates described here may thus find application for both anti-influenza prophylaxis and therapy.

One-Sentence Summary:

A nanobody-based composite recruits polyclonal immunoglobulins, attracts immune effectors and kills influenza virus-infected cells.

Introduction

Antibodies are immune effector molecules commonly used for various forms of immunotherapy. The genetic mechanisms by which antibody diversity is generated ensure wide coverage against a broad swath of immune challenges. The different immunoglobulin

*Corresponding author. hidde.ploegh@childrens.harvard.edu.

#Co-first authors

Author contributions: X.L., T.B. and H.L.P. conceived the idea. X.L., T.B., C.C., and H.L.P. designed the study. X.L. and T.B. synthesized compounds and prepared the nanobody conjugates. X.L. and C.C. tested the nanobody conjugates *in vitro* and *in vivo*. X.L., T.B., and C.C. performed the radio-imaging and pharmacokinetic studies. X.L., T.B., C.C., and H.L.P. interpreted the data. X.L., T.B., C.C. and H.L.P. wrote the manuscript.

Competing interests: HP serves as an advisor to and owns stock in Cerberus Therapeutics. H.P. and X.L. are inventors listed on a US patent application that covers the subject matter of this paper (application # 63/300,995). Cerberus Therapeutics has an exclusive license to the intellectual property covered by this patent application. HP serves as a consultant to Johnson and Johnson, Immatics Therapeutics, Cue Biopharma, Revela Therapeutics and Tiba Bio.

(Ig) isotypes not only recognize antigens through the variable regions of their heavy and light chains, they also execute different effector functions through the Fc portions of the various constant regions (1, 2). Fc receptor-bearing cells can engage in antibody-dependent cell-mediated cytotoxicity (ADCC) while fixation of complement results in complement-mediated cytotoxicity (CDC) and opsonization (3, 4). Both ADCC and CDC contribute to the elimination of virus-infected cells.

Most vertebrates produce the typical four-chain Igs, composed of two identical heavy chains and two identical light chains. Camelids are exceptions: in addition to conventional Igs, they produce heavy chain-only antibodies. The variable regions of the latter, when expressed recombinantly, are referred to as VHHs or nanobodies (5, 6). The small size of nanobodies imparts on them superior tissue penetration and a short circulatory half-life, but they lack effector functions (7, 8). Their small size and ease of production and modification allow configurations of nanobodies that would be more difficult to achieve for conventional Igs (9, 10). We asked whether polyclonal Igs of indeterminate specificity, when recruited to influenza virus-infected cells, would suffice to kill such targets via the effector functions carried in their Fc portions. We therefore produced an adduct composed of an anti-mouse kappa light chain nanobody (11) and the neuraminidase inhibitor zanamivir (12). Upon systemic administration, the former recruits polyclonal Igs and provides half-life extension, while the latter confers on the adduct the capacity to bind to influenza virus and virus-infected cells (13).

Zanamivir inhibits the neuraminidases of both type A and type B influenza viruses, and mutations that confer resistance to zanamivir are rare (12, 14). Intravenous (IV) zanamivir is used to treat influenza (15). Its small size, polarity and low binding to plasma proteins lead to rapid clearance of zanamivir from the circulation, with a half-life of about 10 minutes in mice and 2 hours in humans (16, 17). It therefore requires a high, frequent dose of IV zanamivir to achieve a therapeutic effect (15). New therapeutics capable of neutralizing influenza infections with broad activity against both A and B strains remain an important public health goal. Ideally, such therapeutics would confer not only an extended window of protection, not unlike seasonal influenza vaccines, but also offer immediate relief to a newly diagnosed virus-infected individual that did not receive the drug beforehand. Here, we show that a single dose of an adduct composed of an anti-mouse kappa light chain nanobody conjugated to zanamivir protects mice from a lethal challenge with influenza A or B viruses, even when administered as a single agent several days after infection and without prior immunization.

Results

Synthesis of the VHH_{kappa}-zanamivir conjugate

To extend the half-life of zanamivir and to create a composite capable of activating complement as well as attracting Fc-receptor-expressing cells to influenza virus-infected cells, we attached zanamivir to an anti-mouse kappa light chain nanobody (VHH_{kappa}) (Fig. 1A, fig. S1) with picomolar affinity for immunoglobulins (fig. S2). In mice, >95% of all circulating Igs carry the kappa light chain (18), and VHH_{kappa} binds to circulating kappa light chain-bearing Igs of all isotypes (11). Each Ig can bind two copies of this nanobody or

its derivatives, but its monovalent mode of binding avoids crosslinking of plasma Ig. Given the concentration of Igs in plasma and the amounts of VHH_{kappa}-zanamivir administered (2–200 µg/mouse; see below), only a minor fraction of circulating Igs, present in vast excess over the adduct, will bind VHH_{kappa}-zanamivir. Such zanamivir-nanobody adducts, if effective at delivering Igs to virus-infected cells (fig. S3), could recruit complement and attract Fc receptor-positive effector cells to provide wide coverage against influenza A and B strains. To produce the adduct, we first synthesized a triglycine-modified version of zanamivir. Zanamivir tolerates substitutions that allow the installation of a triglycine-modified PEG moiety (19, 20), affixed in a carbamate linkage to the C-7 hydroxyl group of zanamivir, in six steps (figs. S4 and S5). We then made use of a sortase reaction (21) to install this triglycine-modified zanamivir at the C-terminus of VHH_{kappa} to obtain the desired adduct (VHH_{kappa}-zanamivir) (Fig. 1B) in excellent yield and purity, as confirmed by SDS-PAGE and LC/MS (fig. S1).

VHH_{kappa}-zanamivir offers superior protection against influenza virus infection than zanamivir alone in mice.

To test the efficacy of VHH_{kappa}-zanamivir in protecting against influenza virus infection, we compared VHH_{kappa}-zanamivir to equivalent amounts of VHH_{kappa} and zanamivir administered as a simple mixture. A single intraperitoneal dose of 1 mg/kg of the adduct, equivalent to ~20 µg/kg of zanamivir, afforded complete protection against a lethal challenge (10 × LD₅₀) with a mouse-adapted influenza virus A strain (A/Puerto Rico/8/1934 (H1N1)) when given 1 hour prior to infection, whereas the mixture of VHH_{kappa} and zanamivir did not protect (Fig. 2A). Unlike most of the conventional broadly neutralizing anti-HA stem monoclonal antibodies that typically cover only influenza virus A strains, VHH_{kappa}-zanamivir also recognizes influenza virus B strains (fig. S3). We therefore examined the activity of VHH_{kappa}-zanamivir against different strains of influenza virus. We found it equally protective against a lethal challenge with a 2009 pandemic H1N1 strain, a H3N2 strain and a B strain of the virus (Fig. 2B).

Administration of VHH_{kappa}-zanamivir could be delayed for at least three days after infection, when animals begin to show signs of weight loss, at which point it was still fully protective (Fig. 2C). This provides a significant improvement over administration of zanamivir alone, as free neuraminidase inhibitors must be given no later than 48h (for humans) or 24h (for mice) after infection for effective treatment (22). Finally, we administered 5 mg/kg of VHH_{kappa}-zanamivir intraperitoneally 7 days prior to infection as prophylaxis, and then challenged the recipients on day 0 with a lethal dose (10 × LD₅₀) of the virus. All mice challenged with virus survived, with no signs of weight loss (Fig. 2D).

Replacing zanamivir with the anti-HA nanobody SD36 yields a conjugate that is similarly protective.

To demonstrate the modularity of this approach, we replaced zanamivir with an influenza virus hemagglutinin (HA)-specific nanobody, SD36 (Fig. 1B and fig. S6) (23), as a molecularly distinct entity that recognizes an influenza virus-specific target unrelated to neuraminidase. SD36 recognizes a conserved region on the HA stem and binds to both group 1 and group 2 hemagglutinins of influenza A strains (23). VHH_{kappa}-SD36 binds

more weakly to H1 than H3 (23) (fig. S7), but it still protected against a lethal challenge ($10 \times \text{LD}_{50}$) with influenza virus A/Puerto Rico/8/1934 (H1N1) when administered just prior to infection (Fig. 3A). Treatment of influenza virus A/Hong Kong/8/1968 (H3N2)-infected mice with a single dose of 10 mg/kg VHH_{kappa}-SD36 could be delayed up to three days after infection while still achieving complete protection. A mixture of SD36 and VHH_{kappa} at the same dose failed to do so (Fig. 3B). A different covalent VHH_{kappa}-SD36 adduct was obtained by installation of dibenzocyclooctyne (DBCO) and azide click handles at the C-terminus of SD36 and VHH_{kappa}, respectively, and then mixing them (fig. S8 A to B) (24). This C-to-C-adduct was as potent as the genetic fusion version of VHH_{kappa}-SD36 (fig. S8C). Replacing SD36 with the irrelevant nanobody E11 (SARS-CoV-2 spike specific) (25) in the VHH_{kappa} adduct fails to protect mice from a lethal influenza virus infection (Fig. S9). We then compared the efficacy of VHH_{kappa}-zanamivir with an existing influenza therapy. Passive immunization at 1 mg/kg with MEDI8852, one of the most potent broadly neutralizing anti-HA stem monoclonal antibodies described thus far (26), was less effective than VHH_{kappa}-zanamivir in preventing infection-induced weight loss (Fig. S9).

⁸⁹Zr-labeled VHH_{kappa}-SD36 accumulated more than ⁸⁹Zr-labeled SD36 alone in the lungs of virus-infected mice

On the covalent fusion of SD36 and VHH_{kappa}, we installed a deferoxamine (DFO) moiety for labeling with ⁸⁹Zr and thus obtained a positron emission tomography (PET) imaging agent (Fig. 3C and fig. S10). The ⁸⁹Zr-labeled VHH_{kappa}-SD36 binds to circulating Igs and thus remains detectable in the circulation for as long its complex with Igs survives. At 48 hours post-injection of ⁸⁹Zr-labeled VHH_{kappa}-SD36, we saw a clear signal in uninfected mice that included the heart and highly perfused organs such as the liver (Fig. 3D and fig. S11). Retention of label in the kidneys, organs of elimination, is commonly seen when using ⁸⁹Zr-labeled nanobodies, regardless of their specificity (27). In influenza virus-infected mice, imaged at day 6 post-infection, we saw much stronger accumulation of label, especially in the lungs (Fig. 3D and fig. S11). The ⁸⁹Zr-labeled VHH_{kappa}-SD36 thus clearly distinguished between uninfected and virus-infected mice, as expected. SD36-DFO, the ⁸⁹Zr-labeled 'free' anti-HA nanobody, which is cleared from the circulation far more rapidly, showed accumulation mostly in the kidneys, yet with a detectable signal in the lung of infected mice at 24 hrs post-injection. No such signal was seen in uninfected mice (Fig. 3D).

VHH_{kappa} conjugates direct immunoglobulins to virus-infected cells and engage immune effector activities.

To explore the underlying mechanisms of protection conferred by VHH_{kappa}-zanamivir, we examined CDC on influenza virus-infected MDCK cells. Infected MDCK cells were incubated with VHH_{kappa}-zanamivir at 10 nM or with a mixture of its component parts at 10 nM, followed by incubation with polyclonal mouse IgGs and rabbit complement (Fig. 4A). Only the covalent adduct enabled robust CDC (Fig. 4B). The modest reduction in CDC seen in response to the zanamivir + VHH_{kappa} mixture might be due to the interference with non-specific complement recruitment. In the ADCC reporter assay, only VHH_{kappa}-zanamivir, but not the mixture of its components, induced strong luciferase signals (expressed as -fold induction) when incubated with virus-infected MDCK cells, polyclonal mouse IgGs, and

reporter Jurkat T cells (Fig. 4C). These T cells express a luciferase reporter under the control of an NFAT promoter that is activated downstream of the mouse Fc γ RIV receptor. Uninfected MDCK cells were not susceptible to either CDC or ADCC (Figs. 4B and 4C). The inhibitory activity of VHH $_{\text{kappa}}$ -zanamivir on viral neuraminidase is ~20- to 200-fold lower than free zanamivir (fig. S12). However, the adduct still binds to neuraminidase with low nanomolar affinity and subsequently recruits polyclonal Igs to the surface of MDCK cells infected with A or B strains (fig. S3). At 10 nM, VHH $_{\text{kappa}}$ -zanamivir induces CDC and ADCC on virus-infected cells (Figs. 4B and 4C). We repeated the CDC and ADCC assays for both genetic and C-to-C versions of VHH $_{\text{kappa}}$ -SD36 under comparable conditions. These conjugates induced strong ADCC but minimal CDC (Figs. 4D and 4E).

Recruitment of immunoglobulins to the targets of VHH $_{\text{kappa}}$ conjugates is crucial to the protection of virus-infected mice.

The contribution of Fc effector functions is often explored by comparing ADCC-disabled antibodies equipped with a mutant Fc region (e.g. the 'LALA' mutation) to their wild-type counterparts (28–30). Since this is not possible for the polyclonal Igs recruited by the VHH $_{\text{kappa}}$ fusions without germline engineering of mice for all Ig constant regions, we instead generated an adduct of zanamivir with an anti-albumin nanobody (ALB1) (fig. S13) (31). Albumin-targeted adducts achieve half-life extension by binding to serum albumin in the circulation (32, 33), but cannot recruit polyclonal Igs. In a head-to-head comparison with VHH $_{\text{kappa}}$ -zanamivir (Fig. 5A), 0.3 mg/kg of VHH $_{\text{kappa}}$ -zanamivir was fully protective and prevented all weight loss, but ALB1-zanamivir required a dose of 10 mg/kg to achieve 100% survival. Even at this dose, ALB1-zanamivir failed to prevent weight loss. We then compared the therapeutic potency of VHH $_{\text{kappa}}$ -SD36 with ALB1-SD36 (Fig. 5B) (see fig. S14 for its sequence). Because SD36 itself does not inhibit replication of influenza virus A/Puerto Rico/8/1934 (H1N1) (23), the difference between these two conjugates is meaningful: we increased the concentration of ALB1-SD36 up to 30 mg/kg (the highest attainable dose) yet failed to protect mice. In contrast, 3 mg/kg of VHH $_{\text{kappa}}$ -SD36 was sufficient to achieve 100% survival with minimal weight loss.

We measured the persistence in the circulation of VHH $_{\text{kappa}}$ and ALB1 by administering their ^{89}Zr -labeled equivalents (see fig. S15 for preparation of the imaging agents). For both VHH $_{\text{kappa}}$ and ALB1, a fraction of the injected dose remains in the circulation at 6 days (144h) after injection. In contrast, almost all the ^{89}Zr -labeled 'free' anti-HA nanobody SD36 was cleared from the circulation within an hour after injection (Fig. 5C). Half-life and area under the curve were slightly higher for the VHH $_{\text{kappa}}$ adduct than for the ALB1 adduct. However, we believe that the ~1.5-fold difference in area under the curve (total drug exposure over time) is unlikely to account for the >30-fold difference in therapeutic efficacy (Fig. 5A and 5B). Indeed, we observed that the lack of recruitment of Fc effector functions can be compensated for only in part by increasing the dose of the ALB1-zanamivir, indicating that half-life extension alone of zanamivir does not optimally protect against a lethal challenge with influenza virus.

For protection against infection by VHH $_{\text{kappa}}$ -zanamivir, we hypothesized that there is a strict requirement for the presence of Igs in the circulation. To demonstrate this dependency,

we infected RAG-1 knockout mice that either received PBS or polyclonal mouse IgG with H1N1 influenza virus. RAG-1 knockout mice are T- and B-cell deficient, but make normal amounts of complement proteins and have FcR-positive NK cells and macrophages (34). Only animals that received polyclonal mouse IgG were protected from a lethal virus infection by the VHH_{kappa}-zanamivir adduct (Fig. 5D). The dose of VHH_{kappa}-zanamivir adduct used (5 mg/kg) failed to protect mice that did not receive IgG, indicating that transfer of polyclonal Ig alone is not sufficient for protection and the Ig must be targeted to virus-infected cells.

Discussion

Here, we show that a VHH_{kappa}-zanamivir conjugate protects mice against a $10 \times \text{LD}_{50}$ of either influenza A or B strains. While this approach has yet to be applied in a clinical setting, we see no obvious obstacles to its translation. Anti-human kappa light chain VHHs have been described in the literature (35). Human immunoglobulins have a longer half-life *in vivo* (36) than those of mice, which would extend the duration of coverage by a VHH_{kappa}-zanamivir conjugate in a clinical setting. Nanobodies generally have a low immunogenicity risk profile, and simple substitutions in VHH framework regions further reduce immunogenicity (37). In a phase II clinical trial of a humanized VHH, caplacizumab, only 9% of patients dosed daily for 60 days with nanobody had antidrug antibodies (38), none of which were neutralizing or affected the pharmacokinetic and pharmacodynamic profiles of caplacizumab. Administration of VHHs under non-inflammatory conditions, even when they lack such framework mutations, does not typically elicit a strong immune response (39–41).

Zanamivir as a component of the VHH_{kappa}-zanamivir adduct serves not only to inhibit the viral neuraminidase itself, but also directs the conjugate to influenza virus particles and influenza virus-infected cells for destruction by CDC as well as ADCC. To explain the discrepancy between the ability of VHH_{kappa}-zanamivir and VHH_{kappa}-SD36 to induce CDC (where the latter lacks efficacy) (Fig. 4B and 4D), it is possible that the spatial requirements for complement activation, i.e. the sites of engagement of NANase as enabled by VHH_{kappa}-zanamivir, versus HA when recognized by VHH_{kappa}-SD36, are sufficiently distinct.

The VHH_{kappa}-zanamivir approach can perhaps serve as a template for the elimination of any cell population that can be selectively targeted *in vivo*. It requires a suitable ligand for a surface-exposed component, fused to a nanobody that recognizes immunoglobulin light chains. One ought to consider these types of fusions for nanobodies against blood stage parasites such as malaria or for nanobodies that recognize the envelope proteins of viral pathogens such as SARS-CoV2, HIV or Ebola virus. Although the nanobody component itself may lack neutralizing capacity, it can still contribute to prophylactic and therapeutic efficacy of the resulting fusion. Specific targeting of polyclonal immunoglobulins to virus-infected cells appears to be sufficient for protection *in vivo* against influenza virus. While small molecule drugs and antibodies can lead to pathogen resistance through mutation and selection, a set of nanobodies that direct the fusion product to the intended target, but each recognizing a distinct pathogen epitope, could be far more difficult to

evade. Distinct nanobody-VHH_{kappa} fusions could thus be cycled through, if and when resistance against any given nanobody were to emerge. Because nanobody-based drugs have significant manufacturing and cost advantages over traditional monoclonal antibodies, VHH_{kappa}-zanamivir or similar constructs could be easily and rapidly produced as need, for example in response to a flu pandemic or an Ebola outbreak.

Materials and Methods

Study design

The objective of this study was to develop an anti-immunoglobulin kappa light chain nanobody (VHH_{kappa})-based conjugate as a broad-spectrum anti-influenza immunotherapy, designed as follows. We site-specifically conjugated VHH_{kappa} either to the neuraminidase inhibitor zanamivir or to the anti-hemagglutinin stem nanobody SD36. We used influenza virus infected-MDCK cells and mice to test the ability of these nanobody conjugates to direct immunoglobulins to virus-infected targets and engage immune effector activities. The goal was to establish whether mice could be protected, both therapeutically and prophylactically, from a lethal influenza infection. To examine whether VHH_{kappa}-zanamivir has broad-spectrum inhibitory activity against multiple strains of influenza virus, we infected mice with influenza A and B viruses. The sample size for each experiment is specified in the figure legends. The number of independent experiments performed is stated in the figure legend if the experiment was performed more than once. For mouse protection studies, we used 5 mice per group, consistent with other influenza studies. The age- and weight-matched female mice were randomly assigned to each group. Experiments were conducted unblinded.

Expression of nanobodies and endotoxin removal

The relevant nanobody coding sequences (see figs. S5A, S6A, S13A, and S14A), extended with a C-terminal sortase recognition motif (LPETGGH₆) were cloned into pHEN6 expression vector by Gibson assembly and amplified using competent DH5α *E. coli*. The recombinant plasmids were transformed into competent WK6 *E. coli*. Cells were then grown at 37°C in Terrific Broth containing 100 µg/ml of ampicillin until the OD₆₀₀ reached to 0.6–0.8. To induce the VHH expression, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and incubation was continued overnight at 30 °C. Cells were harvested by centrifugation. VHHs were released by osmotic shock using TES buffer (200 mM Tris, 0.65 mM EDTA, 0.5 M sucrose, pH 8). The released VHHs were isolated on Ni-NTA beads (QIAGEN), and further purified by size exclusion chromatography using a Superdex 75 10/600 column. To deplete LPS, purified VHHs were reloaded on the HisTrap HP column (GE Healthcare) which was then washed with PBS solution (40 column volumes) containing 0.1% (V/V) TritonX-114. Elution was performed with endotoxin-free PBS (Teknova) containing 500 mM imidazole (2.5 column volumes). Imidazole was removed by desalting on a PD10 column using LPS-free PBS as the elution buffer.

Expression of MEDI8852

Two coding DNAs, one encoding the MEDI8852 heavy chain and the other the MEDI8852 light chain, were ordered and obtained as GeneBlocks™ (IDT). These were each ligated into

a custom-made pCDNA 3.1 (huCALR SP) plasmid vector by Gibson assembly. pCDNA 3.1 (huCALR SP) encodes the human calreticulin signal peptide so that the inserted cDNAs are expressed towards the secretory pathway of mammalian cells. DNA preps of both plasmids were verified by Sanger-sequencing. A total of 150 μ g of both heavy-chain and light-chain encoding plasmids were transfected into 450 million EXPI-293 mammalian cells (Thermo Fisher Scientific, cat. no. A14527) (at a starting concentration of 3 million cells per mL), using Expifectamine™ (ThermoFisher) at a 0.4:0.6 heavy:light chain cDNA ratio. Cells were kept growing for 5 days in EXPI expression medium (ThermoFisher). On the last day, the cells were pelleted at $2000 \times g$ for 45 minutes at 4°C and supernatant was kept. The supernatant was passed onto a Protein G-HiTrap™ 1 mL (Cytiva) purification column connected to a BioRad NGC FPLC system. The column was washed with 1X PBS before elution using 0.2 M Glycine pH 2.2. The pH of the eluate was immediately adjusted to pH 7.4 by addition of Tris 1M pH 9.1. The eluate was then further purified and buffer-exchanged for PBS by size-exclusion chromatography using an Akta© Pure system and Superdex® 200 Increase 10/300 GL column. Fractions containing MEDI8852 were pooled together and concentrated using a regenerated cellulose filter Amicon© tube with a cutoff of 50 kDa (Millipore Sigma). The total yield of MEDI 8852 for the production round was ~10 mg in PBS.

Compound Synthesis

The synthesis of zanamivir-GGG and other triglycine-modified sortase-ready compounds is described in the Supplementary Materials.

Synthesis of nanobody-conjugates and hetero-bivalent nanobody adducts

The pentamutant sortase A was used to catalyze the addition of sortase-ready nucleophiles to the LPETG-containing VHHs. The sortase reactions were performed in 1 mL PBS containing 200 μ M VHH, 1000 μ M sortase-ready nucleophiles, 20 μ M sortase A, and 10 μ M CaCl_2 . After incubation overnight at 4°C, Ni-NTA beads were added to the reaction mixture. Incubation for 30 min at 4°C removed any unreacted VHHs that retained their His tag as well as His-tagged sortase A. The reaction mixture, including the Ni-NTA beads, was then loaded on a PD-10 desalting column to remove excess nucleophile. Fractions containing the modified VHH were combined and concentrated using 10-KDa cutoff Centrifugal Filters.

To prepare the VHH_{kappa}-SD36 adduct (C to C conjugation by click reaction), 400 μ M SD36-azide and 400 μ M VHH_{kappa}-DBCO in PBS were mixed and incubated overnight at 4°C. The final product was purified by size exclusion chromatography on a Superdex 75 10/300 column.

Affinity measurements of VHH_{kappa}-biotin and VHH_{kappa}-SD36-biotin for mouse immunoglobulins

96-well ELISA high binding plates were coated with 100 μ l of 5 μ g/ml a mouse Igs overnight at 4 °C (mouse IgG isotype control: Invitrogen, cat. no. 10400C, RRID: AB_2532980; mouse IgA isotype control: Invitrogen, cat. no. 14–4762-81, RRID: AB_470125; mouse IgM isotype control: BioLegend, cat. no. 401601, RRID: AB_2935847). Plates were washed 3x with the wash buffer (PBS supplemented with 0.1% (v/v) Tween-20) and incubated

with the blocking buffer (1% (w/v) BSA in PBS) at rt for 1h. After washing 4x with the wash buffer, each well was treated with 100 μ l of serial 4-fold dilutions of VHH_{kappa}-biotin, VHH_{kappa}-SD36-biotin, or SD36-biotin in blocking buffer. After incubation at room temperature for 2h, the plates were washed 4x with the wash buffer and incubated with Streptavidin-HRP (1:1000 dilution, Biolegend, cat. no. 405210) at room temperature for 1h. After washing 4x with the wash buffer, each well was incubated with 100 μ l of TMB substrate solution (Biolegend, cat. no. 421101) at room temperature for 10 min before addition of 100 μ l of 1N H₂SO₄ to terminate the enzymatic reaction. The optical density was then read at OD₄₅₀. The dissociation constant (K_d) was calculated from a plot of average absorbance values at 450 nm versus the concentration of VHHs, using the saturation binding equation in GraphPad Prism 7. (Saturation binding equations, One site -- Total).

Influenza viruses

Influenza virus A/Puerto Rico/8/1934 (H1N1) (NR-348) was obtained from BEI Resources and amplified in chicken embryonated eggs. Influenza virus A/Hong Kong/8/1968 (H3N2) (NR-346), A/WI/629-D00015/2009 (H1N1) (NR-19806), A/Hong Kong/1/68–1 Mouse-Adapted 12 (H3N2) (NR-28621), A/California/07/2009 (H1N1) (NR-13663), B/Florida/4/2006 (NR-41795), B/Brisbane/60/2008 (NR-42005), and A/NWS/33 (H1N1) (NR-2555) were obtained from BEI Resources and propagated in MDCK cells.

Affinity measurement of VHH_{kappa}-zanamivir for viral neuraminidases

MDCK cells (ATCC, cat. no. CCL-34) were seeded into 24-well plates and allowed to grow to confluence overnight. Infection of MDCK cells with influenza viruses was performed according to the *Manual for the laboratory diagnosis and virological surveillance of influenza* (World Health Organization – 2011).

The affinity of VHH_{kappa}-zanamivir adduct for viral neuraminidase on the surface of infected MDCK cells was determined using a saturation binding assay. Briefly, spent medium was aspirated from 24-well plates containing virus-infected MDCK cells and then replaced with 0.5 mL of fresh serum free medium containing various concentrations of VHH_{kappa}-zanamivir. After incubation for 1 h at 37 °C, virus-infected cells were rinsed with fresh medium (2 $\times$ 0.5 mL) to remove unbound VHH_{kappa}-zanamivir. To quantify the amount of VHH_{kappa}-zanamivir bound to NANase, a mouse IgG-Phycoerythrin (PE) (1:20 dilution, R&D Systems, cat. no. IC002P, RRID: AB_357242) in 0.25 mL fresh serum-free medium was added to each well. After incubation for 30 min at 37°C, virus-infected cells were rinsed with fresh medium (2 $\times$ 0.5 mL) again and then dissolved in 0.5 mL of 1% (w/v) sodium dodecyl sulfate (SDS). Cell-associated fluorescence was measured using an excitation wavelength of 560 nm and emission at 620 nm. The dissociation constant (K_d) was calculated from a plot of the cell-bound fluorescence intensity versus the concentration of VHHs using the saturation binding equation in GraphPad Prism 7. (Saturation binding equations, One site -- Total).

Mouse experiments

6–9-week-old female BALB/c (strain number: 00065) or Rag1 KO (strain number: 002216) mice were purchased from the Jackson Laboratory and housed in the animal facility at

Boston Children's Hospital (BCH). All the animal-related procedures were approved by the BCH Committee on Animal Care (protocol #: 19–12-4075R).

Mice were treated with a single intraperitoneal dose of VHH adducts or their components (100 μ L/mouse) at the indicated time points. Mice were anesthetized with isoflurane and inoculated intranasally with 50 μ L of 10 LD₅₀ of influenza virus. Mice were monitored daily. Body weights were recorded for the following 14 days. Mice that lost more than 25% of their body weight or that became moribund were euthanized by CO₂ asphyxiation.

Affinity measurement of VHH_{kappa}-SD36 for viral hemagglutinins

MDCK cells were seeded into 24-well plates and allowed to grow to confluence overnight. Infection of MDCK cells with influenza viruses (at 10 TCID₅₀) was performed according to the *Manual for the laboratory diagnosis and virological surveillance of influenza* (World Health Organization – 2011).

The affinity of VHHs for viral hemagglutinins on the surface of infected MDCK cells was determined using a saturation binding assay. Briefly, spent medium was aspirated from 24-well plates containing virus-infected MDCK cells and then replaced with 0.5 mL of fresh serum-free medium containing various concentrations of VHH_{kappa}-SD36. After incubation for 1 h at 37 °C, virus-infected cells were rinsed with fresh medium (2 $\times$ 0.5 mL) to remove unbound VHHs. To quantify the amount of VHHs bound to HAs, a mouse IgG-Phycoerythrin (PE) (1:20 dilution, R&D Systems, cat. no. IC002P, RRID: AB_357242) in 0.25 mL fresh serum-free medium was added to the VHH_{kappa}-SD36 containing wells. After incubation for 30 min at 37°C, virus-infected cells were again rinsed with fresh medium (2 $\times$ 0.5 mL) and then dissolved in 0.5 mL of 1% (w/v) sodium dodecyl sulfate (SDS). Cell-associated fluorescence was measured using an excitation wavelength of 560 nm and emission at 620 nm. The dissociation constant (K_d) was calculated from a plot of the cell bound fluorescence intensity versus the concentration of VHHs using the saturation binding equation in GraphPad Prism 7. (Saturation binding equations, One site -- Total).

Radio-PET imaging and pharmacokinetic studies

For radiolabeling of VHHs with zirconium-89 (⁸⁹Zr), the pH of the ⁸⁹Zr⁴⁺ stock solution (supplied in 1.0 M oxalic acid) was adjusted to 6.8–7.5 with 2.0 M Na₂CO₃. 1.0 mCi of ⁸⁹Zr⁴⁺ solution was then added to a chelexed PBS solution containing 100 μ g of VHH-DFO adduct. After incubation at room temperature for 1h, the reaction mixture was desalted on a PD10 column and eluted with chelexed PBS. The fractions containing ⁸⁹Zr-chelated VHHs were used directly for the injection of mice.

In PET imaging experiments, mice (n = 3) were infected intranasally with 50 μ L of influenza virus A/Hong Kong/8/1968 (H3N2) (=10 LD₅₀). Mice were retro-orbitally injected with a single dose of 50 μ Ci of VHH_{kappa}-SD36-DFO (⁸⁹Zr chelated) or SD36-DFO (⁸⁹Zr chelated) at day 4 post-infection. At 48 hours after the injection of the imaging agents, mice were scanned for 10 minutes by a G8 PET-CT small-animal scanner (PerkinElmer). Images were processed through Vivoquant using same intensity settings.

To determine the pharmacokinetics of systemically administered VHHs, mice were retro-orbitally injected with 250 μ Ci of either VHH_{kappa}-DFO (⁸⁹Zr chelated, n=3), anti-Albumin VHH (ALB1)-DFO (⁸⁹Zr chelated, n=4) or SD36-DFO (⁸⁹Zr chelated, n=3). This is equivalent to a dosage of 1 mg/kg of VHH. 10 μ L of whole blood was drawn by a slight incision of the tail vein at 10 minutes, 1h, 24h, 48h, 72h, 96h and 144 hours after injection of the radiolabeled VHHs. After the final blood draw, all samples were subjected to gamma-counting using a gamma-counter (PacKard Instrument Company) to eliminate having to account for isotope decay.

CDC assay

MDCK cells at 10000 cells/well were seeded in a 96-well plate and incubated with 100 TCID₅₀ of influenza virus A/NWS/33 (H1N1) for 24 hours. Spent medium was aspirated from 96-well plates containing virus-infected MDCK cells and then treated with 50 μ L of VHH_{kappa}-zanamivir (or VHH_{kappa}-SD36) or a mixture of VHH_{kappa} and zanamivir (or SD36) (final concentration: 10 nM). After incubation at room temperature for 30 min, 50 μ L of fresh serum-free medium containing 40 μ g/mL normal mouse IgG isotype control (Invitrogen, cat. no. 10400C, RRID: AB_2532980) and 40% (v/v) rabbit complement serum (Sigma-Aldrich, cat. no. S7764) was added to the cells. The plate was then incubated for 2.5h at 37°C. Cell viability was measured by CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega, cat. no. G7572). Maximal cell killing was achieved by treating cells with 5% H₂O₂. The percent cytotoxicity induced by VHH_{kappa}-zanamivir was calculated as:

$$\% \text{ Cytotoxicity} = \frac{\text{luminescence}_{\text{no VHH}} - \text{luminescence}_{\text{expt}}}{\text{luminescence}_{\text{no VHH}} - \text{luminescence}_{\text{maxkilling}}} \times 100$$

ADCC assay

MDCK cells at 10000 cells/well were seeded in a 96-well plate and incubated with 100 TCID₅₀ of influenza virus A/NWS/33 (H1N1) for 24 hours. Spent medium was aspirated from 96-well plates containing virus-infected MDCK cells and then treated with 25 μ L of VHH_{kappa}-zanamivir (or VHH_{kappa}-SD36) or a mixture of VHH_{kappa} and zanamivir (or SD36) (final concentration: 10 nM), followed by addition of 25 μ L of 40 μ g/mL normal mouse IgG isotype control (Invitrogen, cat. no. 10400C, RRID: AB_2532980). After incubation at room temperature for 30 min, 25 μ L of ADCC reporter cells (Promega, cat. no. 10400C) were added at 75,000 cells/well and incubated for 6h at 37°C. To measure luciferase generated by the ADCC reporter cells, 75 μ L of Bio-Glo[™] Reagent (Promega, cat. no. 10400C) were added to each well and the luminescence intensity was measured by the plate reader (SpectraMax[®] iD5, Molecular Devices).

Neuraminidase inhibition assays

The neuraminidase inhibition activities of VHH_{kappa}-zanamivir, ALB1-zanamivir, zanamivir, and VHH_{kappa} were measured by the NA-Star[™] Influenza Neuraminidase Inhibitor Resistance Detection Kit (Invitrogen, cat. no. 4374422). The influenza strains indicated in Figure. S12 were used as the neuraminidase source. All the viruses were diluted to a signal:noise ratio of 40:1 (luminescence intensity of the virus containing wells : NA-Star

assay buffer containing wells). In brief, a series dilutions of the tested molecules (25 μ L) were incubated with 25 μ L of virus in the NA-StarTM detection microplates for 20 min at 37 °C. 10 μ L of NA-Star substrate was then added to each well and incubated for 30 min at rt. Finally, 60 μ L of NA-Star accelerator solution was added to all wells and their luminescent intensity were immediately read by the plate reader (SpectraMax[®] iD5, Molecular Devices). The half maximal inhibitory concentration (IC50) values were calculated by GraphPad Prism 7.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9. A two-way ANOVA with Bonferroni's multiple comparisons test was used to analyze whether a significant difference occurred between the different treatment groups in the mouse therapy studies (for weight loss) and in the CDC and ADCC assays. The log-rank (Mantel-Cox) test was used to compare the difference in survival between the different treatment groups. The data were presented as mean $\pm$ standard deviation. The comparison was considered statistically different if $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials availability:

All data needed to support the conclusions of the paper are available in the main text or the supplementary materials. Reagents and materials described in this paper are available from the authors upon request, for which a material transfer agreement is to be executed with Boston Children's Hospital. Requests should be addressed to H.L.P. Cerberus Therapeutics has an exclusive license for commercial applications of the type of adducts described.

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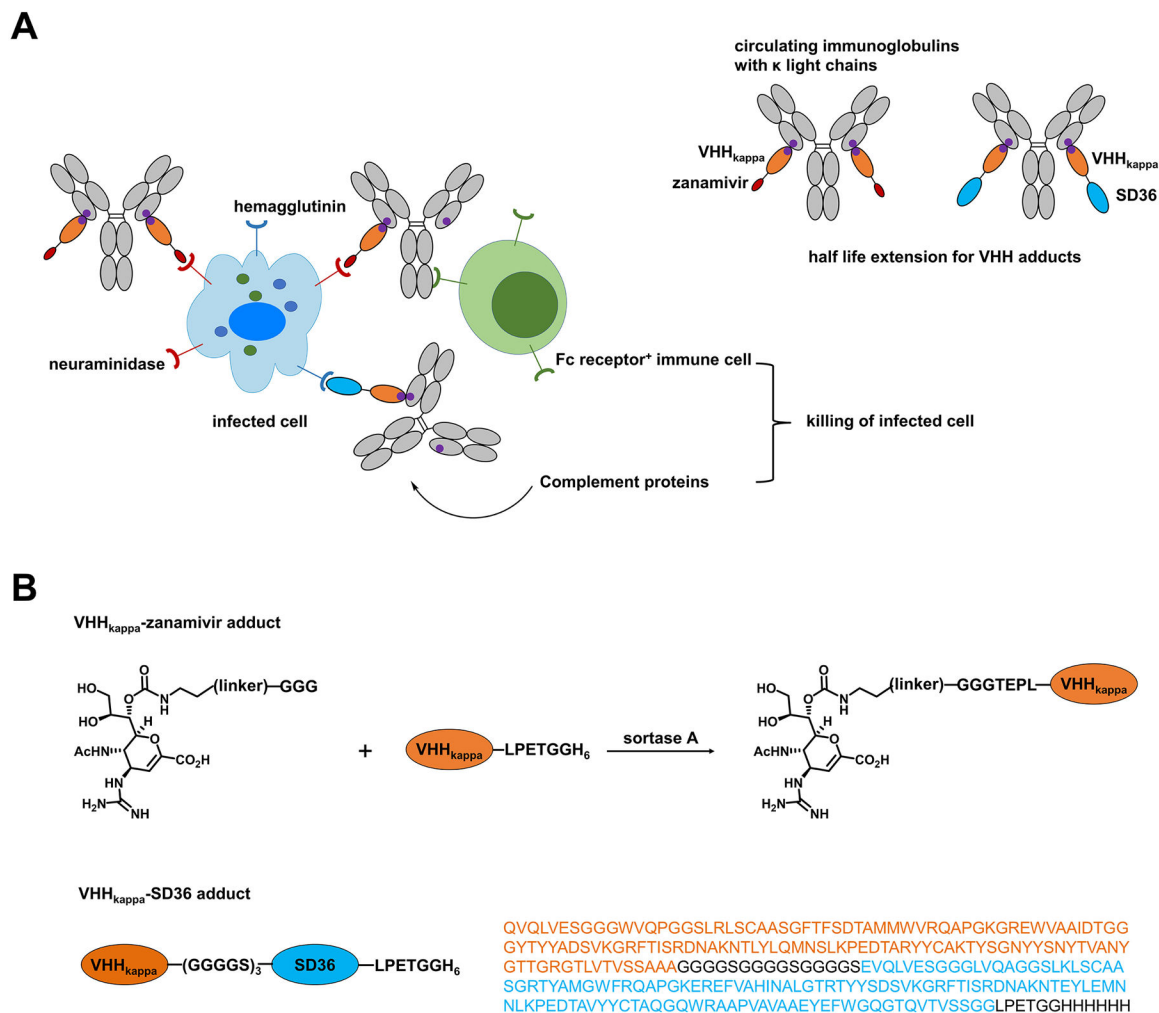


Fig. 1. Immunoglobulin kappa light chain-specific nanobody adducts as anti-influenza therapy. (A) Schematic overview of the mode of action of the viral neuraminidase-targeted VHH_{kappa}-zanamivir adduct and the viral hemagglutinin-targeted VHH_{kappa}-SD36 adduct. Conjugation with VHH_{kappa} was performed to extend the circulatory half-life of zanamivir and SD36 and enable them to kill virus-infected cells by attracting immune effectors. (B) Structures of VHH_{kappa}-zanamivir and VHH_{kappa}-SD36. VHH_{kappa}-zanamivir is prepared by a sortase-mediated conjugation of triglycine modified zanamivir to VHH_{kappa}. VHH_{kappa}-SD36 is expressed as a genetically fused hetero-bivalent nanobody with a C-terminal sortase recognition motif (LPETG).

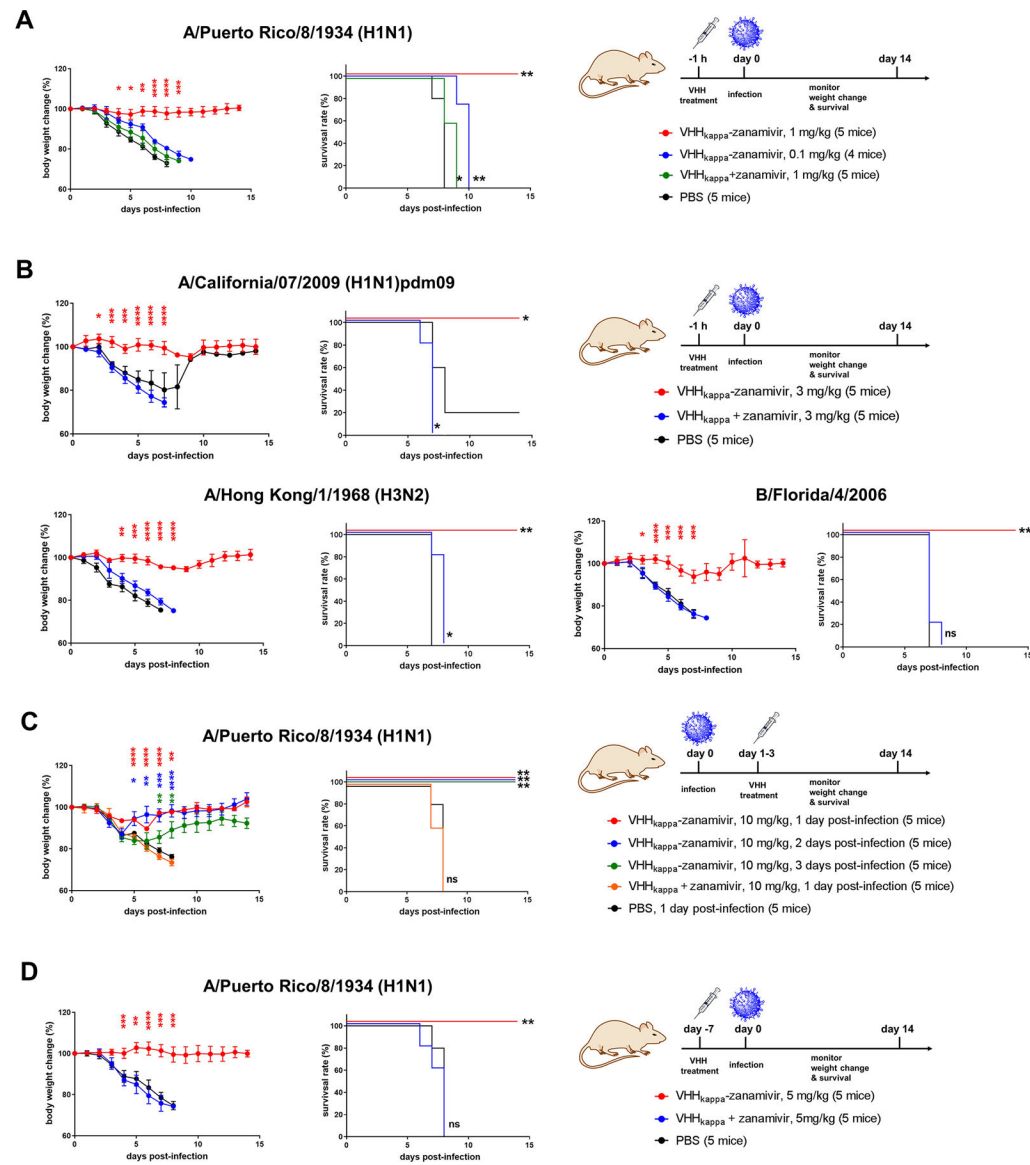


Fig. 2. VHH_{kappa}-zanamivir protects mice from lethal influenza virus infections. 6–9-week-old female BALB/c mice were infected with 10 LD₅₀ of influenza virus as indicated. Mice were treated with equivalent amounts of VHH_{kappa}-zanamivir, a mixture of VHH_{kappa} and zanamivir, or with an equal volume of PBS by intraperitoneal injection. Mice were euthanized when they lost 25% of their body weight or became moribund. Weight loss curves (left) and survival curves (right) are shown. **(A)** Dose range experiment using either 0.1 or 1.0 mg/kg of VHH_{kappa}-zanamivir or a mixture of VHH_{kappa} and zanamivir. **(B)** Efficacy of VHH_{kappa}-zanamivir against the different strains of influenza virus indicated. **(C)** Delayed addition of VHH_{kappa}-zanamivir on day 1, 2 or 3 post-infection. **(D)** Infection of mice with influenza A/Puerto Rico /8/1934 (H1N1) 7 days after a single dose of VHH_{kappa}-zanamivir. For weight loss curves, % body weight change represents the mean $\pm$ standard deviation. A two-way ANOVA with Bonferroni's multiple comparisons test was used to analyze whether a significant difference in weight loss occurred between the

VHH_{kappa}-zanamivir treated groups and a mixture of VHH_{kappa} + zanamivir-treat group (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, see P values in Data_file_S1). For survival curves, statistical differences between the indicated group and the PBS-treated group were calculated by Log-rank (Mantel-Cox) test (*P < 0.05, **P < 0.01, see P values in Data_file_S1).

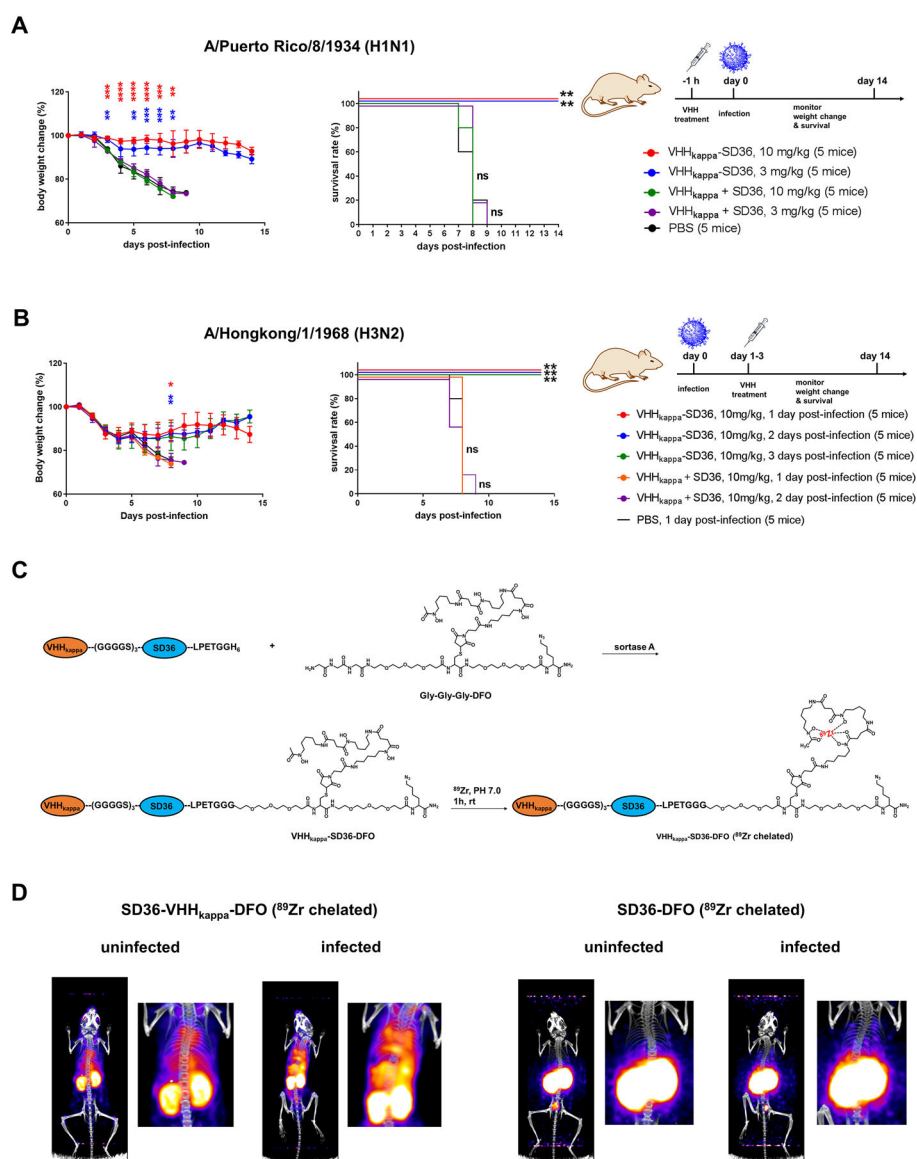


Fig. 3. VHH_{kappa}-SD36 protects mice from lethal influenza virus infections. 6–9-week-old female BALB/c mice were infected with 10 LD₅₀ of influenza virus. Mice were treated at the indicated doses with VHH_{kappa}-SD36, a mixture of VHH_{kappa} and SD36, or with an equal volume of PBS by intraperitoneal injection. Mice were euthanized when they lost 25% of their body weight or became moribund. Weight loss curves (left) and survival curves (right) are shown. **(A)** Dose range experiment using either 3.0 or 10.0 mg/kg of VHH_{kappa}-SD36. **(B)** Delayed addition of VHH_{kappa}-SD36 at 1, 2 or 3 days post-infection. **(C)** Preparation of ⁸⁹Zr labeled VHH_{kappa}-SD36-DFO. VHH_{kappa}-SD36 was modified with a triglycine-DFO moiety in a sortase-catalyzed reaction and labeled with ⁸⁹Zr. **(D)** PET imaging with ⁸⁹Zr-chelated VHH_{kappa}-SD36-DFO (left) and ⁸⁹Zr-chelated SD36-DFO (right) for control and influenza virus A/Hong Kong/8/1968 (H3N2)-infected mice (n = 3). 50 μCi of each imaging agent was administered by retro-orbital injection on day 4 post-infection. The images were acquired 48 hours post-injection. For weight loss curves,

% body weight change represents the mean $\pm$ standard deviation. A two-way ANOVA with Bonferroni's multiple comparisons test was used to analyze whether a significant difference in weight loss occurred between the VHH_{kappa}-SD36 treated groups and 10 mg/kg of mixture of VHH_{kappa} +SD36 (panel **A**) or 10 mg/kg of mixture of VHH_{kappa} + SD36 treated at day 1 post-infection (panel **B**) (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, see P values in Data_file_S1). For survival curves, statistical differences between the indicated group and the PBS-treated group were calculated by Log-rank (Mantel-Cox) test (*P < 0.05, **P < 0.01, see the more comprehensive comparisons and P values in Data_file_S1).

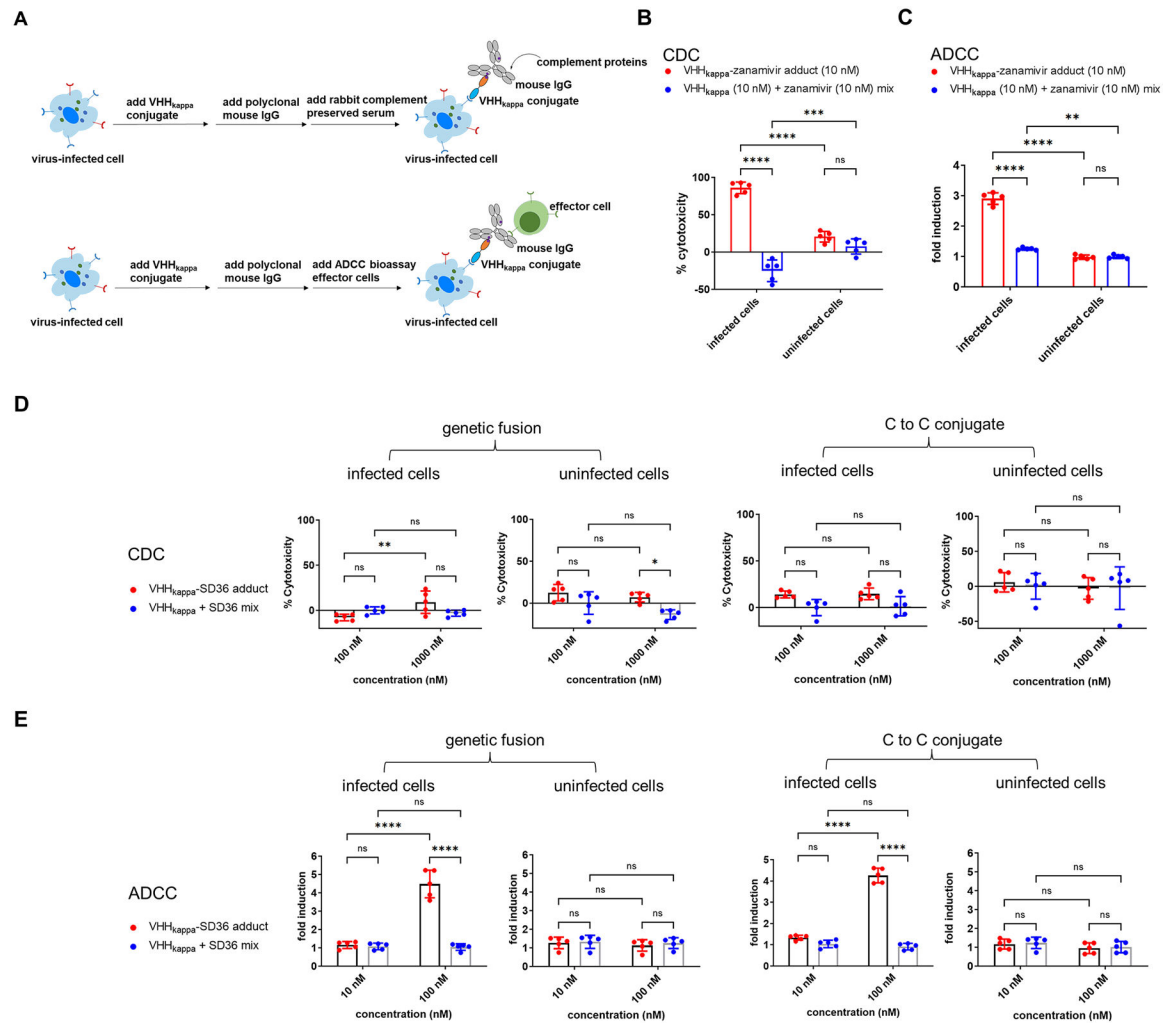


Fig. 4. VHH_{kappa} conjugates direct immunoglobulins to virus-infected cells and engage immune effector activities.

(A) Experimental design schematics of the CDC and ADCC assays. (B and D) Influenza virus-infected MDCK cells were killed by VHH_{kappa}-zanamivir (B) but not VHH_{kappa}-SD36 (D) in the presence of rabbit complement and mouse polyclonal IgG. Differences in the % cytotoxicity between the different treatment groups were analyzed by a two-way ANOVA with Bonferroni's multiple comparisons test (Data represent mean $\pm$ standard deviation, $n = 5$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, see P values in Data_file_S1). (C and E) Virus-infected MDCK cells induced expression of luciferase in reporter cells that express luciferase upon engagement of mouse Fc γ RIV receptor in the presence of VHH_{kappa}-zanamivir (C) or VHH_{kappa}-SD36 (E) and mouse polyclonal IgG. Induction of ADCC was calculated by dividing the luminescence intensity of the indicated samples by the mean of control samples containing virus-infected cells and reporter cells with no VHs. Differences in -fold induction between the different treatment groups were analyzed by a two-way ANOVA with Bonferroni's multiple comparisons test (Data represent mean $\pm$ standard deviation, $n = 5$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, see P

values in Data_file_S1). The results represent at least two independent experiments for the CDC and ADCC assays.

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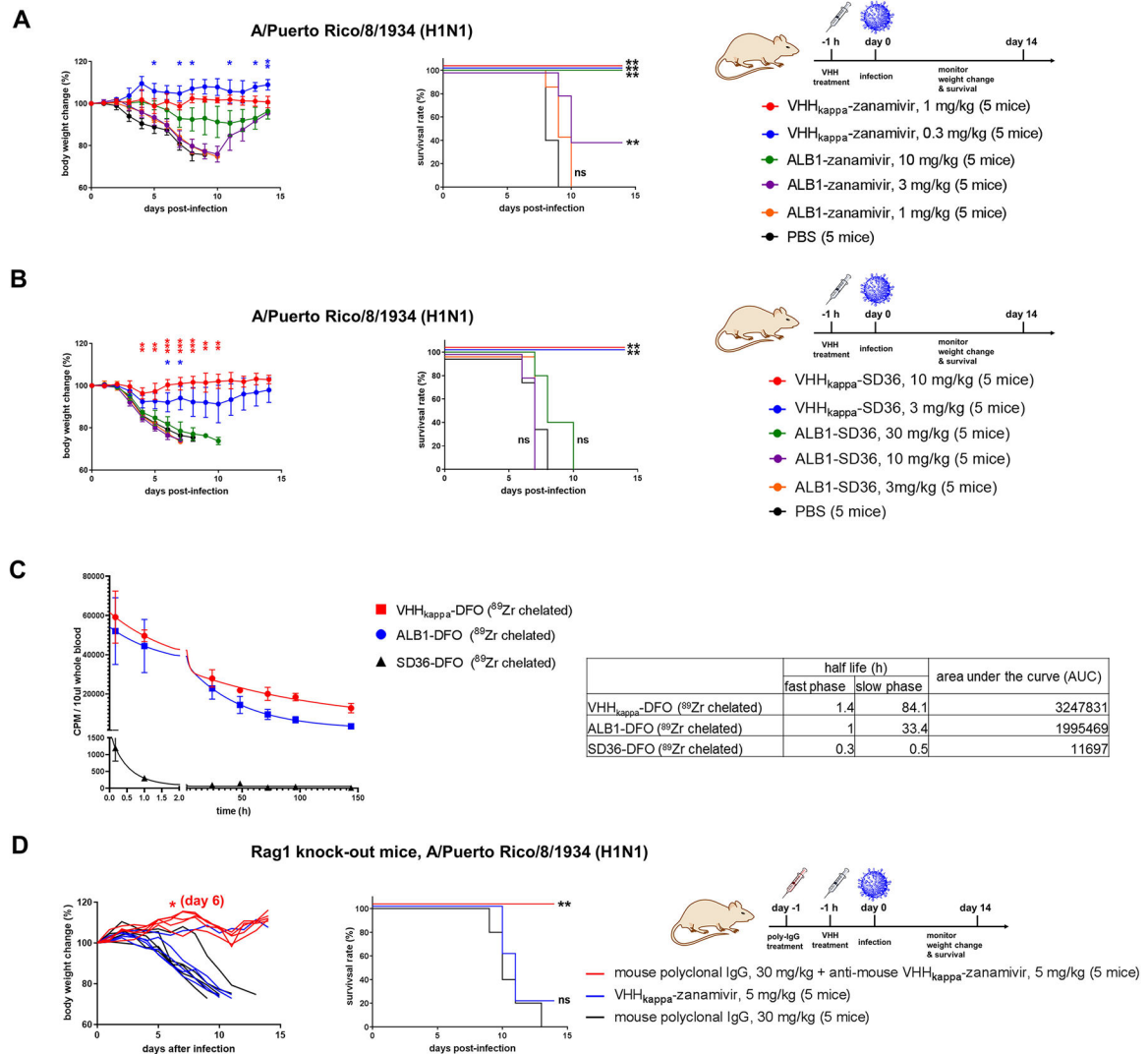


Fig. 5. Recruitment of immunoglobulins to the targets of VHH_{kappa} conjugates is crucial to the protection of virus-infected mice.

(A) Weight loss curve (left) and survival curve (right) for the comparison of efficacy between VHH_{kappa}-zanamivir and ALB1-zanamivir. (B) Weight loss curve (left) and survival curve (right) for the comparison of efficacy between VHH_{kappa}-SD36 and ALB1-SD36. (C) Measure of the clearance rate of nanobodies after retro-orbital injection of their ⁸⁹Zr-labelled constructs. Each individual measurement of the ⁸⁹Zr disintegration rate as counts per minute (CPM) from 10 µL of whole blood (y-axis) is shown as a blue square (VHH_{kappa}-DFO-⁸⁹Zr, n = 3), red circle (ALB1-DFO-⁸⁹Zr, n = 4) or black triangle (SD36-DFO-⁸⁹Zr, n = 3) for each blood draw timepoint after initial injection of the construct (x-axis: 10 min, 1h, 24h, 48h, 72h, 96h, and 144h). All mice received an initial dose of 250µCi of ⁸⁹Zr-labelled VHH (equals to 1 mg/kg VHH). Each data point represent mean ± standard deviation. The half-life (fast phase and slow phase) for each VHH was estimated using a two-phase decay model. Total VHH exposure across the first 144h post-injection was calculated by integrating the concentration of VHH in blood over time. It is expressed as 'area under the curve' (AUC). (D) Weight loss curve (left) and survival curve (right) for the

virus-infected Rag1 knockout mice having received anti-mouse VHH_{kappa}-zanamivir plus mouse polyclonal IgG. For panel (A), (B) and (D), % body weight change represents mean $\pm$ standard deviation in panel (A) and (B), while the body weight change curves for individual mice are shown in panel (D). A two-way ANOVA with Bonferroni's multiple comparisons test was used to analyze whether a significant difference in weight loss occurred between the VHH_{kappa} conjugate-treated groups and 10 mg/kg of ALB1-zanamivir (A) or 30 mg/kg ALB1-SD36 (B)-treated group (*P < 0.05, **P < 0.01, ***P < 0.001, see P values in Data_file_S1). For panel (D), body weight loss between the VHH_{kappa}-zanamivir + mouse polyclonal IgG-treated group and VHH_{kappa}-zanamivir alone-treated group was compared. For survival curves, statistical differences between the indicated group and PBS-treated group were calculated by Log-rank (Mantel-Cox) test (*P < 0.05, **P < 0.01, see P values in Data_file_S1).