A “Trojan horse” bispecific-antibody strategy for broad protection against ebolaviruses

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There is an urgent need for monoclonal antibody (mAb) therapies that broadly protect against Ebola virus and other filoviruses. The conserved, essential interaction between the filovirus glycoprotein, GP, and its entry receptor Niemann-Pick C1 (NPC1) provides an attractive target for such mAbs but is shielded by multiple mechanisms, including physical sequestration in late endosomes. Here, we describe a bispecific-antibody strategy to target this interaction, in which mAbs specific for NPC1 or the GP receptor–binding site are coupled to a mAb against a conserved, surface-exposed GP epitope. Bispecific antibodies, but not parent mAbs, neutralized all known ebolaviruses by coopting viral particles themselves for endosomal delivery and conferred postexposure protection against multiple ebolaviruses in mice. Such “Trojan horse” bispecific antibodies have potential as broad antifilovirus immunotherapeutics.

The development of therapeutics targeting Ebola virus (EBOV) and other filoviruses is a global health priority. The success of ZMapp—a cocktail of three monoclonal antibodies (mAbs) targeting the EBOV surface glycoprotein GP—in reversing Ebola virus disease in nonhuman primates (NHPs) has underscored the promise of antiviral immunotherapy (1). However, most available mAbs have a narrow antiviral spectrum, because they recognize variable surface-exposed GP epitopes (2). ZMapp protects against EBOV but not against other filoviruses with known epidemic potential, including the ebolaviruses Bundibugyo virus (BDBV) and Sudan virus (SUDV) and the more divergent marburgviruses. Given the scientific and logistical challenges inherent in developing a separate mAb cocktail for each filovirus, as well as the need for preparedness against newly emerging or engineered viral variants, broadly protective antifilovirus immunotherapies are highly desirable. A few mAbs have shown cross-neutralization and protection in rodents, indicating that cross-species protection by a single molecule is possible; however, such antibodies are rare (3–8).

An unusual feature of cell entry by filoviruses is the proteolytic cleavage of GP in endosomes to reveal “cryptic” epitopes (9, 10), including the receptor-binding site (RBS) that engages the critical intracellular receptor, Niemann-Pick C1 (NPC1) (fig. S1) (11–18). Engagement of NPC1’s second luminal domain, NPC2-C, by this highly conserved RBS in cleaved GP (GP$_{cleaved}$) is required for cell entry and infection by all filoviruses (11, 19–21). Consistent with this, MR72, an RBS-specific mAb isolated from a Marburg virus (MARV) disease survivor, blocked GP$_{C2}$-NPC1 interaction in vitro and broadly neutralized viruses bearing in vitro cleaved GP$_{C2}$ (15, 22, 23). However, MR72 failed to neutralize infection by uncleaved ebolaviruses, likely because it could not gain access to late endosomes, where the GP$_{C2}$ RBS becomes unmasked (24). Therefore, the development of broadly protective immunotherapies targeting the GP$_{C2}$-NPC1 interaction is challenged by the endosomal sequestration of this virus-receptor complex.

We envisioned a bispecific antibody (bsAb)–engineering strategy to block intracellular GP$_{C2}$-NPC1 interaction by a “Trojan horse” mechanism. We reasoned that, by coupling receptor or RBS-targeting mAbs to a delivery mAb directed against a broadly conserved epitope in uncleaved GP, virions themselves could be coopted to transport bsAbs to the appropriate endosomal compartments (Fig. 1, A and B). To block the filovirus-receptor interaction, we chose mAbs targeting both its viral and host facets: MR72, a human mAb that recognizes the GP$_{C2}$ RBS (above), and mAb-548, a novel murine mAb that engages human NPC1-C. mAb-548 bound with picomolar affinity to an NPC1-C epitope that overlaps the GP$_{C2}$-binding interface and blocked GP$_{C2}$-NPC1-C association in vitro at pH 5.5, the presumptive pH of late endosomes (figs. S1 and S2). mAb-548 resembled MR72 in its lack of neutralizing activity against uncleaved viruses (Fig. 2, A and B, and fig. S8), likely because NPC1 is absent from the cell surface (21, 24). To deliver mAb-548 and MR72 to endosomes, we selected the macaque mAb FVM09, which recognizes a conserved linear

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**SUPPLEMENTARY MATERIALS**

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**Material and Methods**

Figs. S1 to S4

Table S1 and S2

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epitope in the GP glycan cap of all known ebolaviruses (Fig. 1A and fig. S4) (8). FVM09 does not neutralize infection and confers limited in vivo protection against EBOV (8).

The heavy- and light-chain variable domains (VH and VL, respectively) of FVM09 were fused to mAb-548 and MR72 by using the dual–variable domain immunoglobulin (DVD-Ig) design strategy (25). The DVD-Ig format was chosen as a test case because it allows bivalent binding of both combining sites but does not use long polypeptide linkers that may be susceptible to proteolysis or immunogenic presentation. The FVM09–548 and FVM09–MR72 DVD-Igs could be readily isolated from transiently transfected human embryonic kidney 293 (HEK293) cells (fig. S3A). Size-exclusion chromatography–multichannel light scattering indicated a monodisperse population of monomers, with some higher aggregate present (fig. S3, B and C). Each DVD-Ig could bind to EBOV GP via the FVM09 “outer” variable domains, with no loss of affinity relative to the parent FVM09 immunoglobulin G (IgG), as determined by biolayer interferometry (BLI) (Fig. 1C and table S1). FVM09–548 could recognize human NPC1-C, by means of its “inner” variable domains, with a subpicomolar equilibrium dissociation constant (Kd). The MR72 variable domains also retained subnanomolar affinity toward GPCL in the DVD-Ig format. Two-phase binding studies, in which each DVD-Ig was first exposed to EBOV GP and then to NPC1-C or GPCL (Fig. 1D), indicated that there were no steric restrictions to engagement of both combining sites.

We tested the DVD-Igs for their capacity to neutralize infection in human cells by recombinant vesicular stomatitis viruses bearing EBOV GP (rVSV–EBOV GP) or control nonfilovirus glycoproteins derived from VSV and Andes hantavirus (Fig. 2, A and B, and fig. S5) (26). Both FVM09–548 and FVM09–MR72 specifically and potently neutralized rVSV–EBOV GP, whereas the parental mAbs FVM09, mAb-548, and MR72 had little or no neutralizing activity. Equimolar mixtures of the “delivery” IgG, FVM09, with each receptor-RBS-targeting IgG (mAb-548 or MR72) also did not neutralize infection (Fig. 2, A and B); this indicated that DVD-Ig antiviral activity requires the physical linkage of delivery and receptor-RBS-binding specificities. Overall, the DVD-Ig half-maximal inhibitory concentration (IC50) values were in the nanomolar range, similar to the measured Kd values for the FVM09–GP complex but higher than those of the mAb-548–NPC1-C and MR72–GPCL complexes.

The GPCL–NPC1 interaction is conserved among filoviruses (21, 25, 27, 28), and thus, we postulated that the DVD-Igs would exhibit broad neutralizing activity. rVSVs bearing GP proteins from the four other ebolaviruses were sensitive to neutralization by both DVD-Igs, whereas rVSV–MARV GP was resistant (Fig. 2, C, D, and G), consistent with the known specificity of FVM09 toward ebolaviruses (8). We next tested the DVD-Igs against authentic EBOV, BDBV, and SUDV (Fig. 2, E and F). Each ebolavirus was neutralized by both receptor- and RBS-targeting DVD-Igs but not by the individual parent IgGs (Fig. 2G and fig. S6).

The success of antibody therapeutics has fueled the development of a panoply of optimized bsAb architectures, several of which (including the DVD-Ig) are in clinical trials (29). To explore the generality of our strategy to other formats, we generated an FVM09MR72 “asymmetric IgG” using the DuetO bridge platform (figs. S7 and S8) (30). FVM09MR72 broadly neutralized rVSVs bearing ebolavirus GPs, albeit with reduced potency against SUDV GP, possibly because of its loss of bivalent recognition of GP and/or GPCL (31). Nonetheless, these results illustrate that endosomal targeting of the ebolavirus-receptor interaction is amenable to other bispecific-antibody formats.

Our observation that bsAbs combining two nonneutralizing antibodies could confer potent neutralization implied critical roles for both binding specificities. This hypothesis is supported by three pieces of evidence. First, the activity of the DVD-Igs against rVSV–EBOV GP particles containing two point mutations in the FVMO9 epitope was greatly reduced (Fig. 3A and fig. S9).
**Fig. 2.** DVD-Igs, but not parent IgGs or their mixtures, have broad neutralizing activity against ebolaviruses. (A to D) Neutralization of rSVs encoding enhanced green fluorescent protein (eGFP) and bearing filovirus GP proteins in human U2OS osteosarcoma cells. Virions were preincubated with increasing concentrations of each parent IgG, DVD-Ig, or equimolar mixtures of parent IgGs (e.g., FVM09 + mAb-548) (1:1 mixture) and then exposed to cells for 12 to 14 hours at 37°C. Infection was measured by automated counting of eGFP+ cells and normalized to infection obtained in the absence of Ab. TAFV, Taï forest virus; RESTV, Reston virus; MARV, Marburg virus. (E to F) Neutralization of authentic filoviruses in human U2OS osteosarcoma cells, measured in microneutralization assays. Infected cells were immunostained for viral antigen at 48 hours postinfection and enumerated by automated fluorescence microscopy. (A) to (F) Averages ± SD for four to six technical replicates pooled from two or three independent experiments. (G) Data in (A) to (F) were subjected to nonlinear regression analysis to derive Ab concentrations at half-maximal neutralization (IC50 ± 95% confidence intervals for nonlinear curve fit). *IC50 values derived from curves that did not reach 90% neutralization at the highest concentration tested in the experiments are shown.

**Fig. 3.** Roles of delivery and endosomal receptor–RBS–targeting specificities in ebolavirus neutralization by DVD-Igs. (A) Neutralizing activity of DVD-Igs against rSVs bearing WT GP or a GP(E288D/W292R) mutant, in which Asp replaces Glu288 and Arg replaces Trp292 (see fig. S9). (B) Neutralizing activity of mutant DVD-Igs bearing mAb-548 or MR72 combining sites with mutations in the third VH complementarity determining region (FVM09~548Mut and FVM09~MR72Mut, respectively). (C) Neutralizing activity of DVD-Igs against rSV–EBOV GP in U2OS cells bearing endogenous levels of NPC1 (NPC1WT) or ectopically overexpressing NPC1 (NPC1high). (A) to (C) Averages ± SD for six technical replicates pooled from two independent experiments. (D) Internalization of labeled Abs into cells in the absence or presence of viral particles. A schematic of the experiment is shown at the left. Parent IgGs and DVD-Igs covalently labeled with the acid-dependent fluorophore pHrodo Red were incubated with rSV–EBOV GP particles and exposed to cells. Virus–Ab+ and virus+ Ab+ populations were measured by flow cytometry. Averages ± SD for four technical replicates pooled from two independent experiments are shown. Group means for the percentage of Ab+ cells were compared by two-factor analysis of variance (ANOVA) (see fig. S11). Šídák’s post hoc test was used to compare the capacity of each Ab to internalize into virus– versus virus+ cell populations (**P < 0.001; ns, not significant). Dunnnett’s post hoc test was used to compare the internalization of each Ab to that of the “no Ab” control in virus+ cell populations (****P < 0.0001; all other Ab versus no Ab comparisons were not significant). (E) Delivery of Abs to NPC1+ endosomes. FVM09–548 was incubated with rSV–EBOV GP particles and exposed to cells expressing an NPC1–enhanced blue fluorescent protein–2 fusion protein. Viral particles, Ab, and NPC1 were visualized by fluorescence microscopy (also see figs. S12 and S13). Representative images from two independent experiments are shown. Scale bar, 20 μm.
Second, DVD-Igs bearing mAb-548 and MR72 variable domains with mutations that abolish binding (FVM09-548mut and FVM09-MR72mut) lacked neutralizing activity (Fig. 3B). Third, FVM09-548 could not neutralize rVSV–EBOV GP infection in a cell line bearing supraphysiological levels of NPC1, likely because NPC1 overexpression saturates available mAb-548 combining sites (Fig. 3C). Viral neutralization by FVM09-MR72 was unaffected in NPC1-overexpressing cells, consistent with the higher affinity of the GPCL-MR72 complex (55 pM) (table S1), relative to the GPCL-NPC1-C complex (150 μM) (16).

We postulated that the bsAbs harness extra-cellular virions for their delivery to endosomal sites of filovirus-receptor interaction in the context of natural infection. Accordingly, we evaluated the internalization of DVD-Igs and their parent IgGs into cellular endosomes (Fig. 3D and figs. S10 and S11). Each Ab was covalently labeled with the acid-dependent fluorescent probe pHrodo Red and exposed to cells, either alone or following preincubation with fluorescent rVSV–EBOV GP particles (19, 26). Cells were measured for both virus- and Ab-associated fluorescence by flow cytometry. Virus-negative cells displayed little Ab signal; this indicated that neither the DVD-Igs nor their parent IgGs could internalize into cells with virions. By contrast, virus-positive cells were strongly positive for the DVD-Igs but not for the parent mAb-548 and MR72 IgGs. Concordantly, only FVM09 and the DVD-Igs could efficiently colocalize with virions (Fig. 3E and fig. S12) or Ebola virus–like particles (VLPs) (fig. S13) in NPC1– late endosomes, where viral membrane fusion takes place (19, 31). These results, together with the capacity of FVM09 to bind EBOV GP with high affinity between pH 5.5 and 7.5 (fig. S14 and table S1), suggest that virion-bsAb complexes remain associated in early endosomes and traffic together to late endosomes, where proteolytic removal of the GP glycan cap dislodges FVM09, and where mAb-548 and MR72 can engage their respective cellular and viral targets. Collectively, our findings support a two-step “deliver-and-block” mechanism for bsAb neutralization.

Finally, we evaluated the protective efficacy of the DVD-Igs in two murine models of lethal ebolavirus challenge. Because our prior experiments were conducted in human cells, we first tested DVD-Ig neutralization activity in murine NIH/3T3 cells (fig. S15). Whereas FVM09-MR72 retained full activity, FVM09-548 exhibited poor neutralization in murine cells (fig. S15, A and B). This could be readily explained by FVM09-548’s reduced binding affinity for the murine NPC1 ortholog (fig. S15C) and consequent reduced capacity to block the GPCL–NPC1 interaction (fig. S15E). The discrepancy between binding of mAb-548 to human and mouse NPC1 likely arises from species-dependent amino acid sequence differences in the mAb-548–binding region of NPC1-C (fig. S16). By contrast, this region in human NPC1-C is identical to those of rhesus macaques and crab-eating (cynomolgus) macaques (fig. S16), which provide the two NHP models of filovirus challenge currently in use. mAb-548 bound strongly to, and inhibited GPCL interaction with, an NHP NPC1 ortholog derived from the mantled guereza, which also shares an identical mAb-548–binding region (fig. S15, D and F). Therefore, although host-species-specific differences in NPC1 binding may affect FVM09-548’s efficacy in rodents, they are unlikely to do so in NHPs and humans.

Both DVD-Igs were tested for their capacity to protect BALB/c mice when administered 2 days after a lethal challenge with EBOV-MA (Fig. 4A) (29). FVM09–MR72 afforded a high level of protection (70%) relative to the untreated group, whereas no significant survival was recorded for FVM09–548 and parent IgG mixtures. We also evaluated the DVD-Igs for postexposure protection against a lethal human SUDV isolate in the immunocompromised, type 1 interferon α/β receptor–deficient (IFNARβ−/−) mouse model (Fig. 4B) (30, 33). FVM09–MR72 was fully protective, and FVM09–548 provided partial protection, relative to the untreated group. The limited in vivo efficacy of FVM09–548 was consistent with its reduced capacity to inhibit the GPCL–murine NPC1 interaction (fig. S15). These findings provide evidence that a bsAb targeting the critical intracellular virus-receptor interaction can confer broad protection against lethal ebolavirus challenge, even under stringent conditions of postexposure treatment.

Recent antibody discovery efforts have demonstrated the existence of conserved GP surface epitopes that can elicit broadly reactive mAbs with cross-protective potential (3–7, 34). Herein, we describe a complementary strategy to generate broadly protective Abs that target highly conserved epitopes at the intracellular filovirus-receptor interface, which are normally shielded from GP-specific mAbs. Because the cryptic epitope–targeting components of the bsAbs engineered in this study block endosomal receptor binding by all known filoviruses (15) (this study), next-generation molecules combining them with appropriate delivery mAbs of viral or cellular origin may afford coverage against all filoviruses, including newly emerging and engineered variants. This Trojan horse bsAb-specific-antibody approach may also find utility against other viral pathogens known to use intracellular receptors [e.g., Lassa virus (35)], or more generally, to target entry-related virus structural rearrangements that occur only in the endosomosomal pathway.
A large fraction of HLA class I ligands are proteasome-generated spliced peptides

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The proteasome generates the epitopes presented on human leukocyte antigen (HLA) class I molecules that elicit CD8+ T cell responses. Reports of proteasome-generated spliced epitopes exist, but they have been regarded as rare events. Here, however, we show that the proteasome-generated spliced peptide pool accounts for one-third of the entire HLA class I immunopeptidome in terms of diversity and one-fourth in terms of abundance. This pool also represents a unique set of antigens, possessing particular and distinguishing features. We validated this observation using a range of complementary experimental and bioinformatics approaches, as well as multiple cell types. The widespread appearance and abundance of proteasome-catalyzed peptide splicing events has implications for immunobiology and autoimmunity theories and may provide a previously untapped source of epitopes for use in vaccines and cancer immunotherapy.

The presentation of epitopes on the cell surface is a key mechanism by which organisms identify the presence of pathogens, metabolic malfunctioning, or tumors. The HLA class I (HLA-I) immunopeptidome—the set of epitopes allocated onto the HLA-I molecules—impinges on the CD8+ T cell repertoire and the cell-mediated immune response (2). HLA-I immunopeptidomes are usually investigated by sequence

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SUPPLEMENTARY MATERIALS
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Materials and Methods
Figs. S1 to S16
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References (36–48)
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