EBOLA VIRUS

Protective monotherapy against lethal Ebola virus infection by a potently neutralizing antibody

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Ebola virus disease in humans is highly lethal, with case fatality rates ranging from 25 to 90%. There is no licensed treatment or vaccine against the virus, underscoring the need for efficacious countermeasures. We ascertained that a human survivor of the 1995 Kikwit Ebola virus disease outbreak maintained circulating antibodies against the Ebola virus surface glycoprotein for more than a decade after infection. From this survivor we isolated monoclonal antibodies (mAbs) that neutralize recent and previous outbreak variants of Ebola virus and mediate antibody-dependent cell-mediated cytotoxicity in vitro. Strikingly, monotherapy with mAb114 protected macaques when given as late as 5 days after challenge. Treatment with a single human mAb suggests that a simplified therapeutic strategy for human Ebola infection may be possible.

Ebola virus disease (EVD) causes severe illness characterized by rapid onset of fever, vomiting, diarrhea, and bleeding diathesis (*1*, *2*). The challenges of a large outbreak and the failure of traditional quarantine and contact-tracing measures (*3*, *4*) to control the 2014 West Africa outbreak highlight the urgent need for therapies. The success in nonhuman primates (NHP) of ZMapp—a cocktail of three chimeric monoclonal antibodies (mAbs) derived from immunized mice (*5*–*7*)—illustrated the potential of mAb therapies against EVD, and ZMapp is currently being evaluated in human trials. To date, efforts in NHP to simplify the ZMapp regimen to contain fewer mAbs have not been successful (*7*). We sought to isolate mAbs from human EVD survivors, with the goal of identifying antibodies that confer clinical protection either as single or dual-combination agents.

We obtained blood from two survivors of the 1995 EVD outbreak in Kikwit, Democratic Republic of the Congo (*8*), 11 years after infection. To determine whether the subjects retained circulating antibodies against Ebola virus (EBOV) glycoprotein (GP), we assessed GP-specific antibodies by enzyme-linked immunosorbent assay (ELISA) (Fig. 1A) (*9*). The reciprocal 10% maximal binding EC_{90} titer (the reciprocal dilution at which there is a 90% decrease in antigen binding) for subject 1 (S1) was 2326, higher than control sera by more than a factor of 10. Moreover, serum from the more severely ill subject, S1, displayed potent virus-neutralizing activity (Fig. 1B); this finding indicates that S1 maintained serologic memory against EBOV GP more than a decade after infection and suggested the potential for cloning immunoglobulins with potent neutralizing activity from S1's memory B cells.

Therefore, we sorted memory B cells from S1's peripheral blood mononuclear cells and immortalized individual clones with Epstein-Barr virus (*10*). Forty clone supernatants displayed a range of GP binding (Fig. 1C); two of them, 100 and 114,

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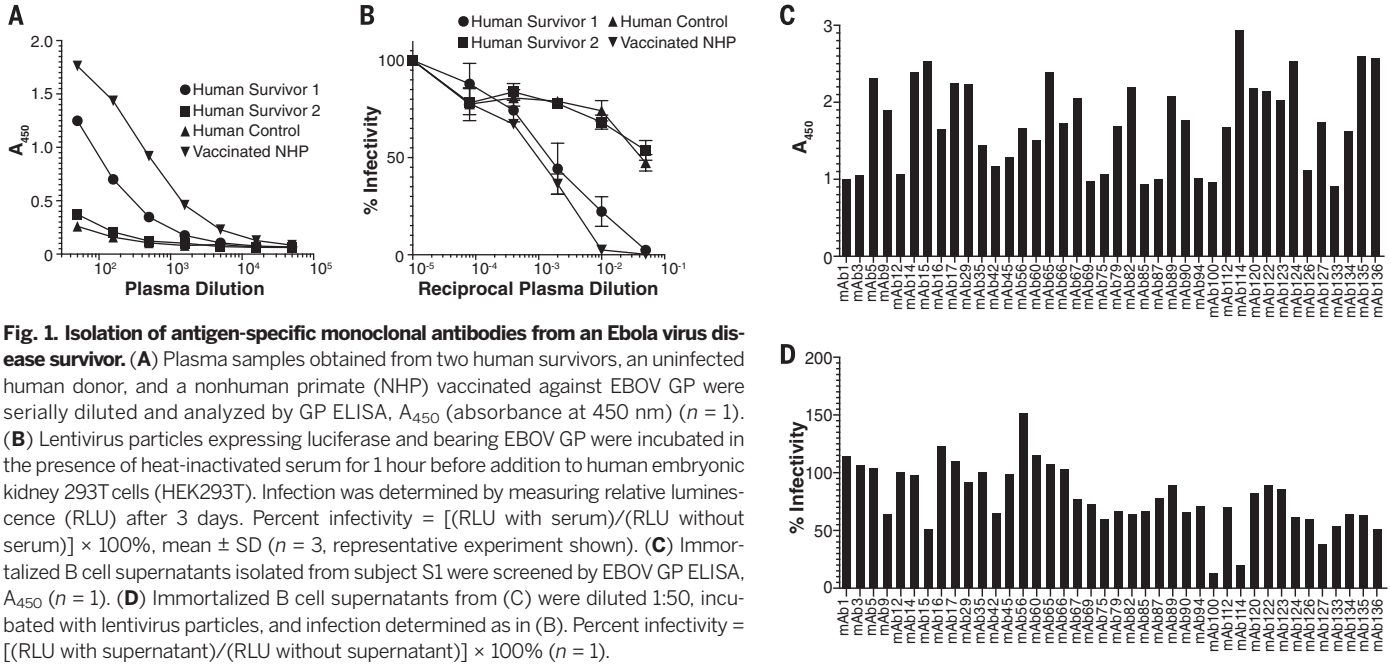


Fig. 1. Isolation of antigen-specific monoclonal antibodies from an Ebola virus disease survivor. (**A**) Plasma samples obtained from two human survivors, an uninfected human donor, and a nonhuman primate (NHP) vaccinated against EBOV GP were serially diluted and analyzed by GP ELISA, A_{450} (absorbance at 450 nm) (n = 1). (**B**) Lentivirus particles expressing luciferase and bearing EBOV GP were incubated in the presence of heat-inactivated serum for 1 hour before addition to human embryonic kidney 293T cells (HEK293T). Infection was determined by measuring relative luminescence (RLU) after 3 days. Percent infectivity = [(RLU with serum)/(RLU without serum)] × 100%, mean ± SD (n = 3, representative experiment shown). (**C**) Immortalized B cell supernatants isolated from subject S1 were screened by EBOV GP ELISA, A_{450} (n = 1). (**D**) Immortalized B cell supernatants from (C) were diluted 1:50, incubated with lentivirus particles, and infection determined as in (B). Percent infectivity = [(RLU with supernatant)/(RLU without supernatant)] × 100% (n = 1).

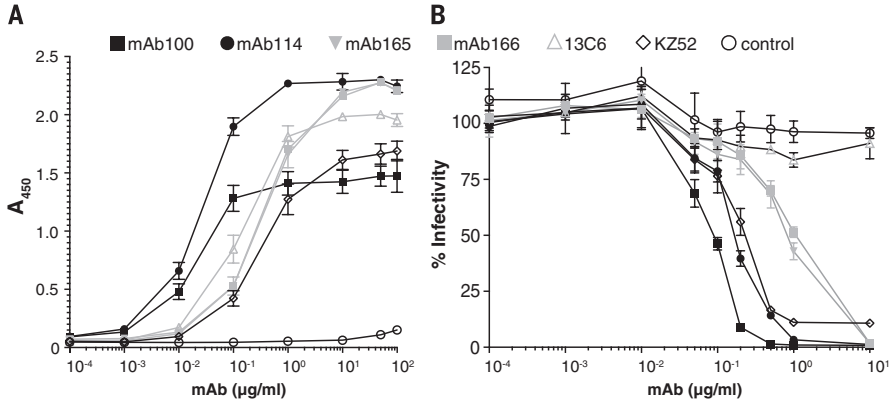
expressed antibodies with markedly higher neutralizing activity than all others (Fig. 1D). A second immortalization yielded 21 clones, from which the GP-specific clones 165 and 166 were rescued (fig. S1).

mAb100, mAb114, mAb165, and mAb166 sequences were amplified by polymerase chain reaction (PCR) and antibodies produced by transient transfection. We assessed ELISA binding to EBOV GP and observed that mAb114, mAb165, and mAb166 displayed maximal binding nearly 50% higher than that of KZ52, a prototypic EBOV GP–specific human mAb (*11*), and 25% higher than that of 13C6, a component of the ZMapp cocktail (*6*, *7*) (Fig. 2A). The binding curve of mAb100 had a plateau similar to that of KZ52 (Fig. 2A). mAb100 and mAb114 achieved half-maximal binding (EC_{50}) at a concentration of 0.02 μg/ml, which is lower than the other mAbs by a factor of 7 to 19. The binding profiles of mAb165 and mAb166 were similar to each other, with EC_{50} values of 0.38 μg/ml and 0.35 μg/ml, respectively; EBOV control mAbs KZ52 and 13C6 had EC_{50} values of 0.33 μg/ml and 0.14 μg/ml, respectively (Fig. 2A).

To evaluate the four S1 mAbs' capacity for neutralization, we used lentiviral particles pseudotyped with EBOV GP, a method that has been demonstrated to recapitulate wild-type EBOV results without the need for high-level biocontainment procedures (*12*) (Fig. 2B and fig. S2A). mAb165 and mAb166 exhibited similar half-maximal inhibition (IC_{50}) concentrations of 1.77 and 0.86 μg/ml, respectively. mAb100 and mAb114 were the most potent, with IC_{50} values about one log unit lower (0.06 and 0.09 μg/ml, respectively) than those of mAb165 and mAb166. Notably, all four of the isolated mAbs inhibited 100% of the virus, unlike KZ52, which consistently displayed only 80 to 90% maximum inhibition, and 13C6, which neutralized <20% at 10 μg/ml. The S1 mAbs also potently neutralized the 2014 West African Makona variant (fig. S2B). Neutralization of wild-type EBOV particles by each of the isolated antibodies was confirmed by plaque reduction assay (fig. S3).

Sequence analysis revealed that the S1 mAbs displayed 85 to 95% germline identity for heavy chains and 89 to 97% germline identity for light chains (Fig. 2C). Analyses of germline gene usage and V(D)J recombination indicate that they originated from different B cell lineages. We analyzed the role of somatic hypermutations for the two most potent antibodies, mAb100 and mAb114, with the use of variants that were partially or completely reverted to the unmutated common ancestors (UCAs) (Fig. 2D and fig. S4, A and B). The fully reverted mAb100 (UCA/UCA), as well as a variant with a single change from the UCA VL (UCA/gL, Ala^{89} → Thr), recognized cells expressing GP, with binding weaker than that of the fully matured antibody by a factor of 2 to 4 (Fig. 2E and fig. S4, A and B). When three HCDR3 (heavy chain complementarity-determining region 3) mutations—Ala^{96} → Val, Val^{103} → Tyr, and Tyr^{114} → Ser—were introduced in the reverted germline antibody (gH/UCA), binding was comparable to the fully matured mAb100 (sH/sL); this result suggests that three mutations are sufficient to confer the binding observed for the fully matured mAb100.

In the case of mAb114, the fully reverted version (UCA/UCA) demonstrated negligible binding



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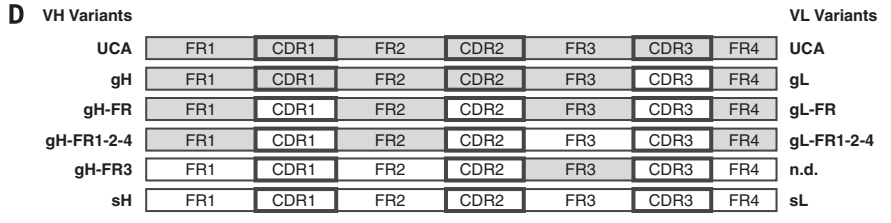
	VH	Identity %	D	JH	VL	Identity %	JL	HCDR3 length	IgG subclass
mAb100	V4-59*01	94.74%	D3-16*02	J4*02	VL3-1*01	92.11%	J2*01	19	IgG3
mAb114	V3-13*01	87.37%	D6-19*01	J4*02	VK1-27*01	92.83%	J4*01	13	IgG1
mAb165	V3-7*01	89.93%	D2-2*02	J4*02	VK1-39*01	89.96%	J1*01	21	IgG1
mAb166	V1-69*04	92.01%	D3-22*01	J6*03	VK3-20*01	97.16%	J3*01	16	IgG3



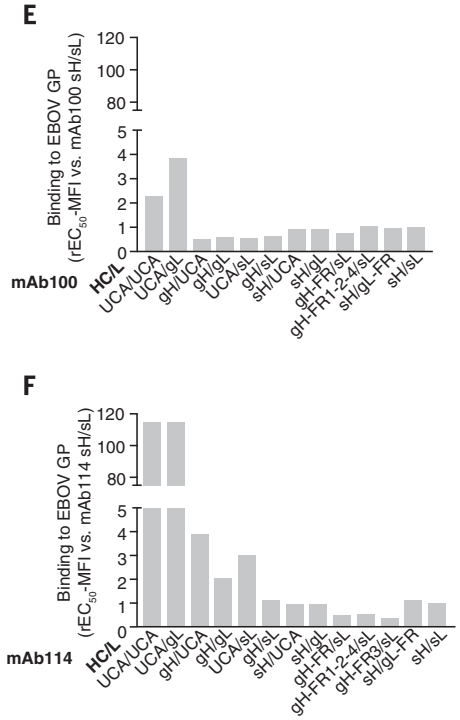


Fig. 2. Characterization of purified EBOV GP monoclonal antibodies. (**A**) EBOV GP ELISA in the presence of purified mAbs as indicated: A_{450}, mean ± SD (n = 3, representative experiment shown). (**B**) Pseudotyped EBOV GP lentivirus particles were incubated with increasing amounts of purified mAbs and infection of HEK293T cells determined as in Fig. 1B. Percent infectivity = [(RLU with antibody)/(RLU without antibody)] × 100%, mean ± SD (n = 3, representative experiment shown). (**C**) V-gene usage, sequence analysis, and immunoglobulin G (IgG) subclass of antibodies from S1. (**D**) Schematic of mAb100 and mAb114 unmutated common ancestor (UCA) and variants created for investigation of the binding requirements of these mAbs. Shaded areas represent sequence from UCA; light regions are from the somatic, mature antibody. Wild-type, somatically mutated heavy (sH), or somatically mutated light (sL) chains; gH or gL, germline V-gene revertants of sH or sL in which the HCDR or LCDR3 (light chain complementarity-determining region 3) are mature; gH-FR or gL-FR, germline V-gene revertants of sH or sL in which the HCDRs or LCDRs are mature; gH-FR1-2-4, germline V-gene revertants of sH in which the HCDRs and HFR3 (framework region 3 of heavy chain) are mature; gH-FR3, germline V-gene revertants of sH in which the HCDRs and HFR1, HFR2, and HFR4 are mature. (**E** and **F**) Binding to EBOV GP expressed on the surface of Madin-Darby canine kidney-2,6-sialtransferase (MDCK-SIAT) cells by versions of mAb100 (E) and mAb114 (F) in which all or subsets of somatic mutations were reverted to the germline sequence. Shown is the ratio between the EC_{50} values of the variants and the wild type (sH/sL). Ratio values above 100 indicate a lack of detectable binding (n = 1).

to GP (Fig. 2F and fig. S4, C and D). Introduction of two mutations (Ala^{96} → Val and Tyr^{108} → Ser) in the HCDR3 of the mAb114 germline (gH/UCA) was sufficient to confer an increase in binding, although still not to the degree seen with the fully matured mAb. Because these mutations are located at the base of the HCDR3 loop, they likely do not make direct contact with GP and thus may have a stabilizing effect on the whole HCDR3. The fully matured light chain and the two HCDR3 mutations (gH/sL) were sufficient to confer binding equivalent to the fully matured mAb (sH/sL). The fully mutated light chain gene (UCA/sL) could partially compensate for a lack of somatic mutation in the heavy chain (Fig. 2F and fig. S4, C and D). The presence of additional mutations on either VH or VL is required to achieve the level of the fully matured mAb114 binding. These results suggest a rapid pathway of mAb114 affinity maturation through one or two somatic mutations, which became redundant as further mutations accumulated—a finding that is reminiscent of what was recently observed for the generation of broadly neutralizing influenza antibodies (*13*).

Because mAb100 and mAb114 were the most potently neutralizing antibodies, they were considered optimal candidates for further evaluation in NHP. To assess the potential for combination therapy, we first sought to rule out antagonistic binding to GP. We found that each antibody bound to GP in the presence of the other, which suggests that they recognize distinct regions on GP (Fig. 3A) and therefore could be used as combination immunotherapy to maximize efficacy (*14*). To define the regions targeted by mAb100 and mAb114, we used biolayer interferometry to assess GP binding in competition with mAbs KZ52 and 13C6, which have epitopes in the GP base and glycan cap, respectively (*15*, *16*). We found that mAb100 competes with KZ52 for binding at the base of GP, whereas mAb114 recognizes (at least in part) the glycan cap region, as demonstrated by competition with 13C6 (Fig. 3, B and C).

Because some EBOV GP antibodies have been suggested to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) (*17*), we determined the ADCC activity of mAb100 and mAb114 in a flow cytometric assay (Fig. 3D). We found that mAb100 and mAb114 mediated ADCC, with maximal activity observed at a mAb concentration of 0.03 μg/ml. Target cell killing was mediated through Fc receptors, because mAbs containing Fc LALA mutations [Leu^{234} → Ala, Leu^{235} → Ala (*18*)] abrogated ADCC activity. Therefore, these mAbs have the potential to induce direct killing of infected cells in vivo, a key viral clearance mechanism.

The presence of potent neutralizing and ADCC activity and the absence of cross-competition supported testing whether mAb100 and mAb114 offered in vivo protective efficacy. We challenged four rhesus macaques with a lethal dose of EBOV. One day after challenge, the treatment group (n = 3) received an intravenous injection with a mixture of mAb100 and mAb114 at a combined dose of 50 mg/kg, and the treatment was repeated twice at 24-hour intervals. Circulating GP-specific antibody titers in mAb recipients peaked after the second injection, and reciprocal ELISA titers remained above 10^5 throughout the study, suggesting minimal clearance of the mAbs (Fig. 4A). The untreated macaque succumbed to EVD on day 10 with a circulating viral load exceeding 10^8 genome equivalents per milliliter (ge/ml) (Fig. 4, B and C). In contrast, all three mAb-treated macaques survived challenge without detectable viremia. Consistent with historical controls, the untreated animal displayed hallmark indicators of EVD including thrombocytopenia and elevations in alanine transaminase and creatinine from day 6 through the time of death (Fig. 4D and figs. S5 to S8). In contrast, the treatment group remained within normal ranges for these parameters and remained free of all EVD symptoms.

We next asked whether monotherapy is sufficient for protection, focusing on mAb114 because it showed higher maximal binding than mAb100. We exposed four macaques to a lethal dose of EBOV and administered mAb114 (50 mg/kg; n = 3) to the treatment group after a 1-day delay, followed by two doses at 24-hour intervals. All treated macaques survived, whereas the control animal succumbed to EVD on day 6 with a peak viral load of 10^{10} ge/ml (Fig. 4, E to G). In contrast to the previous experiment, transient viremia was observed in the treated animals (Fig. 4F) but

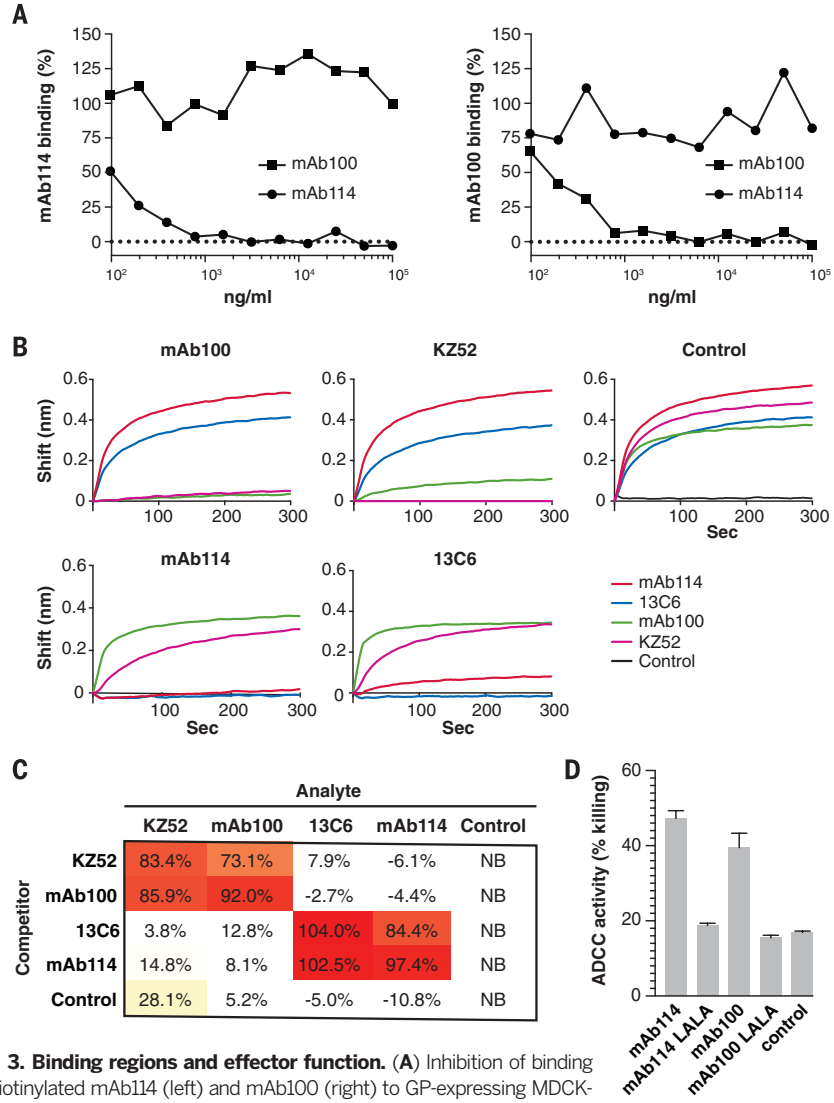


Fig. 3. Binding regions and effector function. (**A**) Inhibition of binding of biotinylated mAb114 (left) and mAb100 (right) to GP-expressing MDCK-SIAT cells by pre-incubation with increasing amounts of homologous or heterologous unlabeled antibodies. Shown is the percentage binding of biotinylated antibody (n = 1). (**B** and **C**) Biolayer interferometry competitive binding assay to soluble EBOV GP using mAb100, mAb114, KZ52, 13C6, and isotype negative control. Biosensors were preloaded with GP followed by the competitor and analyte antibodies as indicated. Analyte binding curves (B) and quantitated percent inhibition (C) are reported (n = 3, representative experiment shown). (**D**) Antibody-dependent cell-mediated cytotoxicity (ADCC) assay was determined for mAb100, mAb114 (n = 3, representative experiment shown), control antibody, or derivative antibodies with LALA mutations that abrogate Fc-mediated killing of HEK293T cells (n = 1), all at 31.6 ng/ml. ADCC activity is shown as mean ± SD.

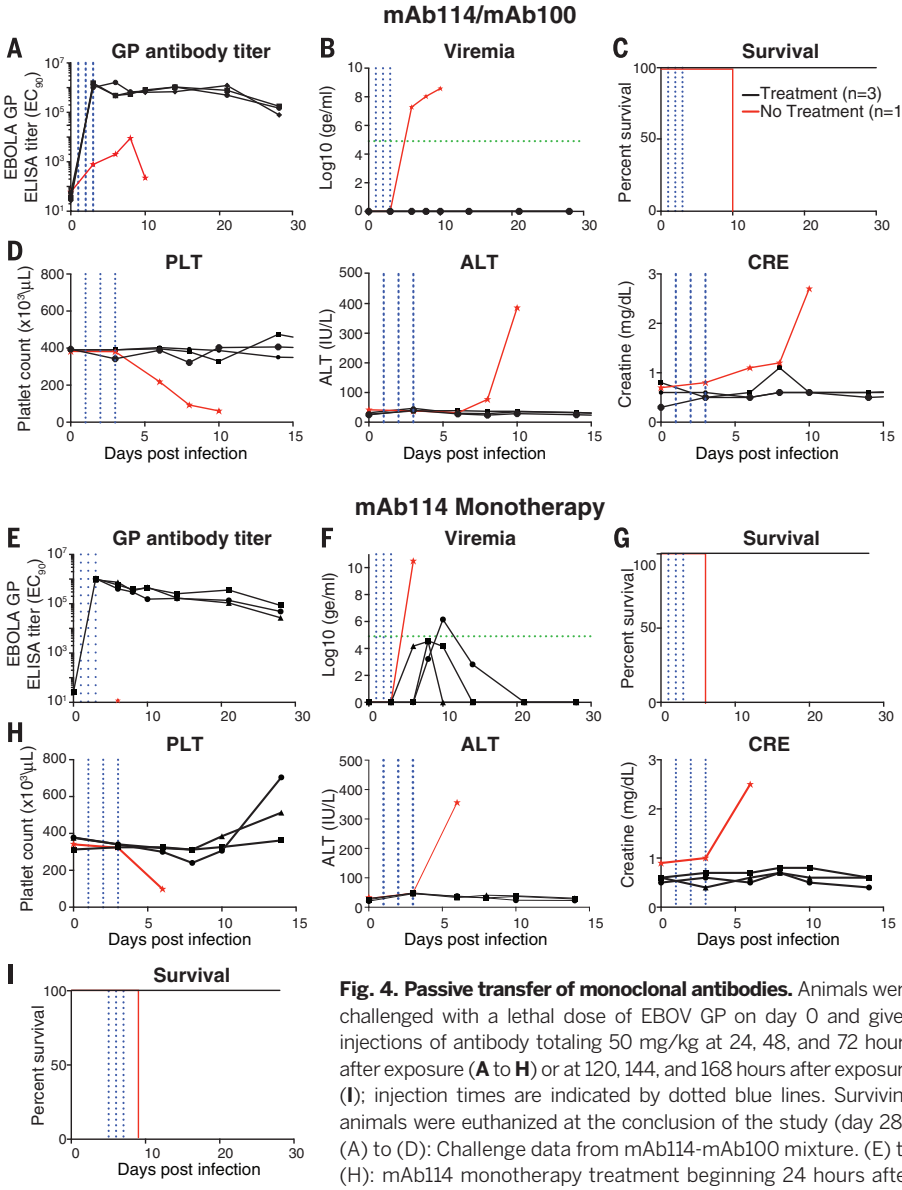


Fig. 4. Passive transfer of monoclonal antibodies. Animals were challenged with a lethal dose of EBOV GP on day 0 and given injections of antibody totaling 50 mg/kg at 24, 48, and 72 hours after exposure (**A** to **H**) or at 120, 144, and 168 hours after exposure (**I**); injection times are indicated by dotted blue lines. Surviving animals were euthanized at the conclusion of the study (day 28). (A) to (D): Challenge data from mAb114-mAb100 mixture. (E) to (H): mAb114 monotherapy treatment beginning 24 hours after exposure. (I): The same treatment beginning 120 hours after exposure. Black, treatment animals; red, untreated controls. (A) and (E): Ebola GP–specific ELISA titer (reciprocal EC_{90}). (B) and (F): Viremia in blood, as assessed by quantitative reverse transcription PCR (qRT-PCR). The dotted green lines denote the lower limit of quantitation of the qRT-PCR assay. (C), (G), and (I): Survival. (D) and (H): Selected hematologic and chemistry data. PLT, platelets; ALT, alanine transaminase; CRE, creatinine.

remained at levels less than 0.1% of the untreated control animal. Despite transient viremia, treated animals remained free of clinical and laboratory abnormalities (Fig. 4H and figs. S9 to S12).

Because a delay in treatment is a distinct possibility in an outbreak setting, we evaluated the therapeutic potential for mAb114 when treatment was delayed until 5 days after lethal EBOV challenge. All three animals in the treatment group survived, while the control animal succumbed to EVD on day 9. Moreover, animals in the treatment group remained symptom-free and protected against thrombocytopenia, transaminitis, and renal dysfunction (Fig. 4I and figs. S13 to S16).

mAb114 has several characteristics that may contribute to protection as a monotherapy when compared to KZ52 and 13C6, which were nonprotective in NHPs (*7*, *19*). First, although KZ52 and mAb114 potently neutralize EBOV in vitro, only mAb114 completely neutralizes input virus. Second, mAb114 does not require complement for neutralizing activity (Fig. 2B), in contrast to 13C6 (*6*). One hypothesis that might be derived from these observations is that protective monotherapy may require both potent binding and complete complement-independent neutralization. In addition, mAb114's specific mechanism of neutralization [which targets an essential step in virus entry (*20*)] and observed in vitro ADCC activity may contribute to mAb114's ability to protect against lethal EVD in macaques.

Our results show that antibodies as well as memory B cells specific to EBOV are maintained in a survivor more than a decade after infection. For the mAbs isolated from this survivor, a potential role for antibody-dependent killing is suggested by in vitro ADCC activity that in vivo may be mediated by multiple effector cells such as natural killer cells, macrophages, or neutrophils. In addition, these mAbs showed potent neutralizing activity against Ebola GP variants that have evolved over a 40-year period. Together, these data demonstrate the therapeutic potential of these mAbs as dual-combination therapy and/or monotherapy and contribute to understanding the mechanisms of antibody-mediated protection against Ebola virus disease.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S16
References (*21*–*25*)

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Antibodies block Ebola virus entry

The recent Ebola virus outbreak in West Africa illustrates the need for both an effective vaccine and therapies to treat infected individuals. Corti *et al.* isolated two monoclonal antibodies from a survivor of the 1995 Kikwit outbreak and demonstrated their therapeutic efficacy in Ebola virus–infected macaques. In fact, one antibody protected macaques when it was given up to 5 days after infection. Misasi *et al.* solved the crystal structures of fragments of the two antibodies bound to the Ebola virus glycoprotein (GP), which mediates viral cell entry. The two antibodies targeted different regions of GP, but in both cases blocked steps required for viral entry.

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