A nonreplicating subunit vaccine protects mice against lethal Ebola virus challenge

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Ebola hemorrhagic fever is an acute and often deadly disease caused by Ebola virus (EBOV). The possible intentional use of this virus against human populations has led to design of vaccines that could be incorporated into a national stockpile for biological threat reduction. We have evaluated the immunogenicity and efficacy of an EBOV vaccine candidate in which the viral surface glycoprotein is biomanufactured as a fusion to a monoclonal antibody that recognizes an epitope in glycoprotein, resulting in the production of Ebola immune complexes (EICs). Although antigen–antibody immune complexes are known to be efficiently processed and presented to immune effector cells, we found that codelivery of the EIC with Toll-like receptor agonists elicited a more robust antibody response in mice than did EIC alone. Among the compounds tested, polyinosinic:polycytidylic acid (PIC, a Toll-like receptor 3 agonist) was highly effective as an adjuvant agent. After vaccinating mice with EIC plus PIC, 80% of the animals were protected against a lethal challenge with live EBOV (30,000 LD_{50} of mouse adapted virus). Surviving animals showed a mixed Th1/Th2 response to the antigen, suggesting this may be important for protection. Survival after vaccination with EIC plus PIC was statistically equivalent to that achieved with an alternative viral vector vaccine candidate reported in the literature. Because nonreplicating subunit vaccines offer the possibility of formulation for cost-effective, long-term storage in biothreat reduction repositories, EIC is an attractive option for public health defense measures.

Ebola vaccine | Ebola glycoprotein | protective antibody | antibody-antigen fusion | immunopotentiator

Ebola virus (EBOV) causes Ebola hemorrhagic fever (EHF), which is a severe and often deadly disease in humans and nonhuman primates, with a lethality rate as high as 90% (1). Transmission of EBOV can occur through direct contact with blood or bodily secretions from an infected individual, and evidence of aerosol transmission has been reported in laboratory conditions (2). The primary difficulty for patients with EBOV infection is the failure of the immune system to react to this fast-moving disease. Patients who die from EHF are unable to develop an adequate immune response as a result of immune dysregulation, which leads to uncontrolled virus replication and multiorgan infection and failure. In contrast, survivors of EBOV infection developed early and increasing levels of IgG antibody against EBOV, followed by viral antigen clearance and cytotoxic T-cell activation (3). In fatal cases, EBOV-specific IgG and T cell-related mRNA cannot be detected. This suggests that a combination of antibody and cell-mediated immune responses to an EBOV vaccine candidate are important for generating the appropriate and protective immune response (3, 4).

mAbs were shown to be effective postexposure therapeutics in a mouse model using lethal EBOV challenge (5). Two of these protective mAbs, designated as 13F6 and 6D8, recognized different linear epitopes in the C-terminal portion of Ebola glycoprotein (GP1). Biovation used the sequence for these two mAbs, via their proprietary peptide threading software, to remove T-cell epitopes to generate "deimmunized" (6) variable regions of the mAbs. These humanized 13F6 and 6D8 (h-13F6 and h-6D8) heavy-chain (HC) and light-chain (LC) variable regions were joined with human IgG1 and κ-chain constant regions that had been codon-optimized for expression in *N. benthamiana*. The h-6D8 mAb was found to express at levels as high as 0.5 mg/g of leaf tissue (7). The h-13F6 was also expressed in *N. benthamiana* to produce various glycoforms of the mAb; these were evaluated for efficacy in a lethal mouse EBOV challenge model (8). The pattern of glycosylation of the various mAbs was found to correlate to the level of protection of h-13F6, leading to the conclusion that mAbs manufactured with uniform glycosylation and a higher potency glycoform offer promise as biodefense therapeutic agents (8).

Approximately 30 y after the first known EBOV outbreak, there is still no approved vaccine for human use. Recent reviews (9, 10) have summarized various candidate vaccine approaches that gave prophylactic protection in nonhuman primates, including vaccine antigens delivered by DNA, recombinant adenovirus serotype 5, recombinant human parainfluenza virus 3, and virus-like particles. One platform, recombinant vesicular stomatitis virus, has demonstrated prophylactic and postexposure protection in nonhuman primates. Many of these candidates have shown outstanding technical utility—especially the viral vectors (11–13). However, although highly active in controlled clinical settings, these candidate vaccines pose challenges for incorporation into a national biodefense stockpile, in which long-term vaccine stability with minimal cold chain requirements for storage and distribution are key factors in a successful program. The present study was conducted to determine if a subunit vaccine containing GP1, when formulated with or without an adjuvant agent, could induce protective immunity in the murine EBOV challenge model currently used by the United States Army Medical Research Institute of Infectious Diseases at Fort Detrick, Maryland. This model uses a mouse-adapted Zaire EBOV, as WT virus is not lethal in rodents. The model has been developed for first-stage evaluation of biothreat reduction vaccine candidates, and its use allowed us to correlate protection levels induced by Ebola immune complexes (EICs) to those achieved by other candidate vaccines as described earlier.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. HQ419193 and HQ407546).

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Results

Design of EICs with Murine or Human Antibody Sequences. The amino acid sequence for murine monoclonal antibody 6D8 (5) was used to design plant codon-optimized genes encoding the corresponding HC and LC variable regions; each gene was synthesized commercially, and fused to mouse γ-2a and κ-constant regions, respectively. When HC and LC were coexpressed in tobacco, we observed assembled murine 6D8 (m-6D8) (7). In addition, the sequences for H and L of the murine mAb were used by Biovation (Edinburgh, Scotland) to generate the variable regions of a "humanized" 6D8 (h-6D8) via proprietary peptide threading software on a fee-for-service basis. These deimmunized sequences (6) were joined with human IgG1 and λ-chain constant regions, and codon-optimized genes for h-6D8 H and L-chain expression in plants were synthesized commercially. When coexpressed in tobacco, they assembled h-6D8 (7). With the availability of expression vectors for both m-6D8 and h-6D8 in hand, we designed a plant-optimized DNA sequence encoding GP1, by using codons that are preferred in tobacco and removing spurious mRNA-processing signals (14). We created genetic fusions of the GP1 coding sequence to the C terminus of the HC (both murine and humanized forms), and coexpressed each with the gene for the corresponding LC in *N. benthamiana* (14). The resulting fusion protein complex isolated from these plants contained both light and heavy antibody chains, but with the latter extended by the protein encoded by the GP1 sequence. These molecules self-interacted to form an EIC in which the variable region of the antibody domain recognized its cognate epitope in the GP1 of another molecule. C1q, a protein component of the complement cascade of mammalian innate immune systems, bound to these complexes, indicating their association as immune complexes (14). We compared murine antibody fused to EIC GP1 (m-EIC) vs. humanized antibody fused to EIC GP1 (h-EIC) in anticipation that the human "scaffold protein" (the antibody domain of h-EIC) might influence the immunogenicity of the attached GP1. In general, we observed only minor differences, and thus most of the data generated by vaccination with m-EIC is presented in *SI Text*.

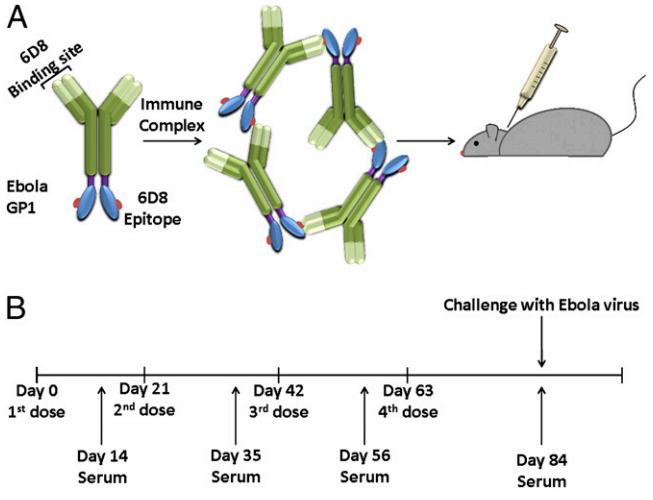

Fig. 1. Diagram illustrating the structure and the assembly of EIC. (*A*) EIC was designed and expressed by fusing Ebola GP1 to the C terminus of the H chain of 6D8 mAb (*Left*). The recombinant IgG can bind to Ebola GP1 and form an immune complex (*Center*). Female BALB/c mice were vaccinated with EIC s.c. with or without candidate adjuvant agents according to the designed vaccination schedule (*B*).

PIC Codelivered with EIC Induced a Robust Anti-EBOV IgG Response Relative to Other Toll-Like Receptor Agonists. Vaccinating mice with EICs was conducted in combination with Toll-like receptor (TLR) agonists, polyinosinic:polycytidylic acid (PIC), and the experimental adjuvant agent CL097. Four doses of h-EIC (10 μg) were administered s.c. on days 0, 21, 42, and 63 (Fig. 1*B*), with or without PIC (20 μg), CL097 (20 μg), or a combination of PIC and CL097. Serum was assayed for EBOV-specific IgG by ELISA (Fig. 2). Recombinant replicons expressing the full-length GP gene and packaged into Venezuelan equine encephalitis virus (VEE) replicon particles (GP-VRP) (15) and PBS solution were used as a positive and negative vaccine control, respectively.

Increasing titers in the mice vaccinated with GP-VRP or with h-EIC codelivered with PIC could be detected after the second dose, which was earlier than in the mice vaccinated with h-EIC alone or h-EIC codelivered with CL097. The highest levels of antibodies were detected after the fourth dose in all groups. The h-EIC alone and h-EIC codelivered with CL097 resulted in the lowest anti-EBOV IgG titers (Fig. 2). In contrast, mice vaccinated with PIC codelivered with h-EIC produced significantly ($P < 0.05$) higher anti-EBOV IgG relative to h-EIC alone. The combination of PIC and CL097 did not result in a synergistic response with regard to antibody production (Fig. 2).

EIC Combined with PIC Protected Mice from Lethal EBOV Challenge. To evaluate whether EIC codelivered with PIC alone or with a combination of PIC and alum protected mice from a lethal EBOV challenge, groups of vaccinated mice ($n = 10$) were challenged with 1,000 pfu (30,000 LD_{50}) of mouse-adapted Zaire EBOV i.p. 21 d after the final dose. Survival and weight changes were recorded for 30 d and 14 d (Fig. 3), respectively. Four doses of h-EIC (10 μg) codelivered with PIC or codelivered with PIC and alum protected 80% of mice against lethal Ebola challenge (Fig. 3*A*). These experimental groups were protected similarly to GP-VRP–positive control group ($P > 0.05$). In contrast, 0% of mice vaccinated with four doses of h-EIC (10 μg) alone or h-EIC (10 μg) codelivered with alum survived a lethal viral challenge, similar to the PBS solution mock group (Fig. 3*A*). Three doses of h-EIC (10 μg or 25 μg) codelivered with PIC and alum protected only 20% and 50% of the mice from lethal challenge, respectively (Fig. 3*A*). Vaccination with mouse EIC (i.e., m-EIC)

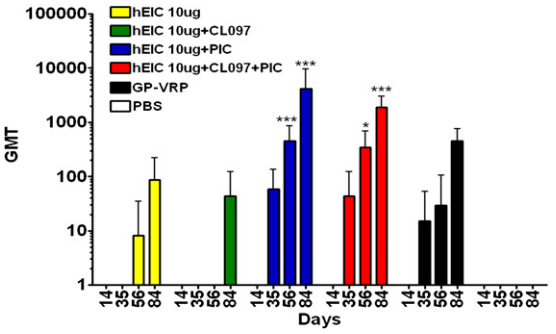

Fig. 2. Codelivery of PIC with h-EIC in mice induced the highest anti-Ebola IgG antibody response relative to other TLR agonist combinations. Each group was vaccinated s.c. with h-EIC alone, h-EIC with CL097, h-EIC with PIC, or h-EIC with CL097 plus PIC on days 0, 21, 42, and 63. Anti-Ebola IgG titers were measured by ELISA on days 0, 14, 35, 56, and 84. GMTs were defined as the highest serum dilution giving a positive reaction. The GMTs for PBS solution negative control group and preimmune (i.e., day 0) serum in all groups were no greater than 1. Error bars indicate SD. Data presented are representative of three independent studies. Statistically significant differences ($P < 0.05$) in the level of IgG anti-Ebola were determined by Kruskal–Wallis test. Comparisons were made between vaccination groups relative to PBS solution control (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

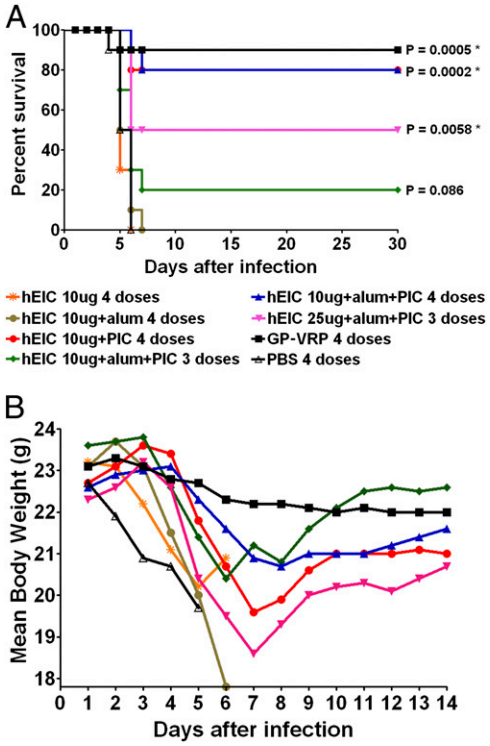

Fig. 3. h-EIC codelivered with PIC significantly protected mice from Ebola lethal challenge. Vaccination with three or four doses of h-EIC with or without candidate adjuvant agents was performed in mice (n = 10 per group). After vaccination, mice were challenged i.p. with 1,000 pfu (30,000 LD_{50}) of mouse-adapted EBOV on day 84. (*A*) Survival was recorded for 30 d. (*B*) Percent body weight change was recorded for 14 d. Because body weight values are means from surviving mice, the loss of weight in the first week of the experiment for EIC-treated mice primarily corresponds to lack of eating of animals that would not survive challenge. Statistically significant differences ($P < 0.05$) in survival were determined by log-rank test and comparisons were made between vaccination groups relative to PBS solution control as indicated by asterisks.

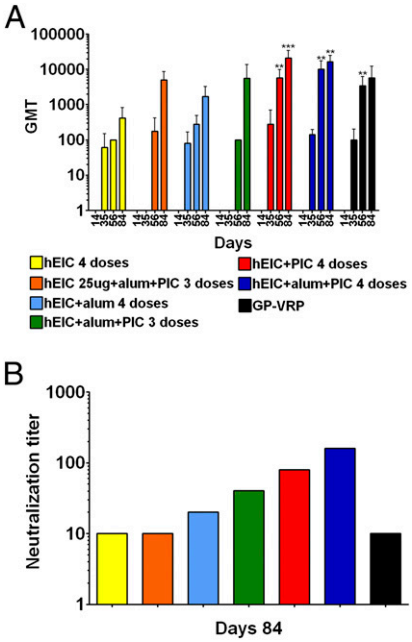

Fig. 4. Anti-Ebola IgG levels correlate with the level of protection afforded against Ebola challenge. Mice vaccinated as described for the challenge study were bled 2 wk after each dose and 3 wk after the last vaccination. Serum was analyzed for (*A*) total IgG and (*B*) neutralizing antibody specific to EBOV (n = 5 per group). (*A*) Individual serum samples for each mouse in each vaccination group were analyzed for total anti-Ebola IgG by ELISA on days 0, 14, 35, 56, and 84. The GMT for preimmune (i.e., day 0) serum in all groups were no greater than 1. Error bars indicate SD. Statistically significant differences ($P < 0.05$) in the level of IgG anti-Ebola were determined by Kruskal–Wallis test. Comparisons were made between vaccination groups relative to PBS solution control (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). (*B*) Virus neutralization assays were performed on serum collected on day 84. Pooled sera from groups of five mice were assayed. Serial dilutions of serum were mixed with EBOV and applied to Vero cell monolayers. Neutralization titers were determined to be the last dilution of serum that reduced the number of plaques by 80% compared with control wells.

codelivered with PIC and alum protected 70% of the mice from lethal challenge (Fig. S1*A*), which is comparable to h-EIC codelivered with PIC and alum. There was no significant difference ($P > 0.05$) in antibody production or survival between respective vaccine regimens with h-EIC and m-EIC. The P values comparing survival between each respective vaccination group are shown in Table S1.

Mice in each of the vaccination regimes lost weight over the course of the 14-d observation period. Mice were weighed in groups and the daily mean for live animals is reported (Fig. 3*B*). Mice receiving the VRP or EIC vaccines had a delay in mean weight loss relative to the PBS solution controls [approximately 5% loss for those vaccinated with VRP and as much as approximately 12% for those with EIC (10 μg) codelivered with PIC or with PIC and alum]. Initially, groups of mice receiving EIC vaccine lost more weight relative to the VRP group; however, the mice that survived challenge gained weight over the observation period, resulting in a similar mean body weight values at 14 d between the VRP and EIC plus PIC groups.

Levels of IgG and Neutralizing Antibody Specific to EBOV Correlated with Protection. Anti-EBOV IgG was determined by ELISA, and neutralizing antibody specific to EBOV was determined by viral neutralization assay (Fig. 4). Mice vaccinated with four doses of h-EIC codelivered with PIC or h-EIC codelivered with PIC and alum had significantly higher ($P < 0.05$) anti-EBOV IgG production compared with h-EIC alone (Fig. 4*A*). These two vaccination regimens resulted in the highest level of neutralizing antibody titer (Fig. 4*B*). In addition, these two vaccine groups were significantly protected against lethal viral challenge, similar to GP-VRP vaccinated mice (Fig. 3*A*).

EIC Codelivered with PIC Induced Mixed Th1/Th2 Response That Correlated with Protection Against Lethal EBOV Challenge. The isotype of IgG may vary with the type of immunogen and adjuvant in a vaccine formulation. In general, IgG2a and IgG2b isotypes are associated with a Th1 immune response, and IgG1 and IgG3 isotypes are associated with a Th2 immune response (16). We used an ELISA to analyze and compare the level of IgG isotypes induced by h-EIC codelivered with different adjuvant agents. The h-EIC alone predominantly induced IgG1, low levels of IgG2b and IgG3, and undetectable levels IgG2a specific for EBOV (Fig. 5). However, h-EIC codelivered with PIC alone or with the combination of PIC and alum induced high levels of IgG2a production and also enhanced the levels of IgG1, IgG2b, and IgG3, similar to vaccination with GP-VRP (Fig. 5).

The titers of anti-EBOV IgG1, IgG2a, IgG2b, and IgG3 after each vaccination are shown in Fig. S2. From the IgG subtype profile (Fig. S2), IgG1 was produced in all groups receiving h-EIC antigen, whereas IgG2a was produced only in the mice vaccinated with h-EIC codelivered with PIC. Alum codelivered with h-EIC induced low levels of IgG2a (Fig. S2).

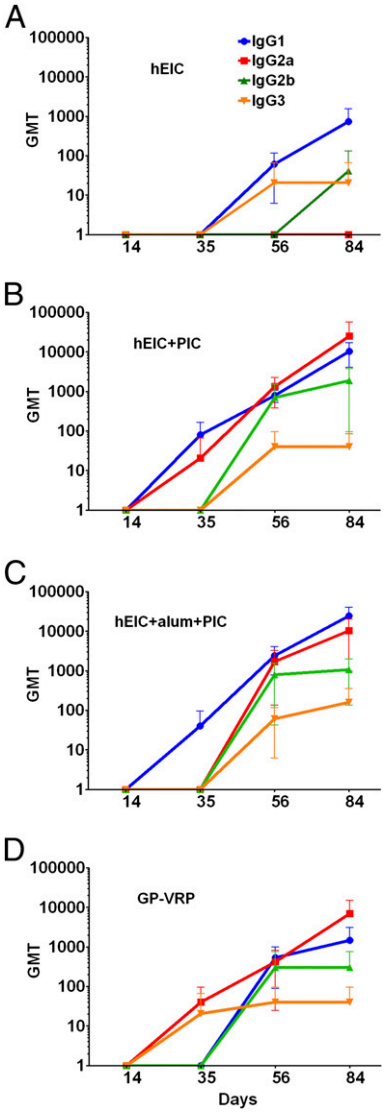

Fig. 5. PIC codelivered with h-EIC induced a mixed Th1/Th2 response that correlated with the level of protection against lethal EBOV challenge. Mice vaccinated as described for the challenge study were bled 2 wk after each vaccination (days 14, 35, and 56) and 3 wk after the last vaccination (day 84). Anti-Ebola antibody isotypes were measured by ELISA. IgG1 (blue), IgG2a (red), IgG2b (green), and IgG3 (orange) levels are presented for mice vaccinated with (*A*) h-EIC, (*B*) h-EIC codelivered with PIC, (*C*) h-EIC codelivered with alum and PIC, and (*D*) GP-VRP.

Discussion

Antigen–antibody immune complex is an attractive vaccine strategy because the complexes target antigen-presenting cells and activate the processing and presentation of the antigen more efficiently than soluble antigen (17). In addition, these complexes have been shown to induce robust humoral and cell-mediated immune responses (18–20). In one example, doses of serum albumin administered bound to syngeneic antibodies were fivefold more immunogenic than free antigen (measured as antigen-specific T-cell proliferation) (20). In our previous study of EIC expression in plants (14), we did find that vaccination with the isolated complex resulted in production of EBOV-specific antibodies.

TLR agonists act as immunomodulators and have been studied as potential adjuvant agents for a variety of vaccines: influenza virus (21), HSV (22), hepatitis B virus (23), and different types of cancer (24, 25). After an initial screen of different TLR agonists, based on strongest immunomodulation, we selected PIC, a TLR3 agonist, and CL097, a TLR7/8 agonist, to test in combination with an EIC vaccine. PIC and CL097 can mimic viral double-stranded and single-stranded RNA, respectively, which are present in EBOV-infected cells. Both TLR agonists induce the activation of NF-κB, leading to increased production of type I IFN that signal surrounding cells to activate antiviral defenses (26).

Vaccination with EICs in combination with PIC (as a single adjuvant agent or in combination with other adjuvant agents) resulted in the most robust antibody responses. PIC shifted the Th2-dominant response observed with EIC alone toward a more Th1 phenotype. This led us to conduct challenge trials using mouse-adapted Zaire EBOV. We have found that EIC vaccination s.c. with PIC protected mice from a lethal EBOV challenge, with 30,000 LD_{50} or 1,000 pfu of live EBOV (27). In our challenge studies, EIC codelivered with PIC protected 80% of mice from a lethal amount of live EBOV; this level of protection (as measured by survival) is statistically the same as the protection against lethality observed with recombinant replicons expressing the full-length GP gene and packaged into VEE replicon particles (GP-VRP) (15). It is clear, however, that the groups differ with respect to animal weight loss. This is a reflection of lethality; for example, those groups in which animals were destined to die had greater mean weight loss in the day 4 to 8 range because some animals had stopped eating and drinking. However, among survivors, there was a very similar mean weight.

We observed that protection from challenge corresponded not only to the amplitude of the antibody response, but also with the IgG subtype profile, and in particular with the amount of IgG2a (which is associated with a stronger Th1 response). Whereas IgG1 was produced in all groups that received h-EIC antigen, IgG2a was only produced in the mice vaccinated with h-EIC codelivered with PIC, and this corresponded to greater protection. In previous studies, passive treatment with IgG2a isotype has been shown to afford protection against EBOV challenge (5). Taken together with our data, the results support the concept that the IgG2a isotype and a mixed Th1/Th2 response generate a broadly reactive immune response that can protect against a lethal EBOV challenge.

Alum, a classical FDA-approved adjuvant considered safe (28), was codelivered with h-EIC and found to induce low levels of IgG2a (Fig. S2). However, alum codelivered with EIC failed to provide protection against a lethal EBOV challenge in mice and cannot be considered as an effective adjuvant agent for the EIC vaccine candidate. To address a potential synergistic response with PIC, we codelivered alum and PIC with EIC, but found no difference between EBOV-specific antibody production and EBOV protection of mice relative to PIC alone codelivered with EIC.

In this study, we have used the Zaire EBOV mouse challenge model, which is well accepted as the first indication of the efficacy of an Ebola vaccine candidate and a first step before moving a candidate into nonhuman primate trials; we demonstrate that EICs have activity equivalent to the best of the current viral vaccine candidates (measured as animal survival). Although the potential value of a GP1-based subunit vaccine has been long appreciated, the commercial-scale manufacture of this antigen has been a technical obstacle. We have overcome this hurdle by fusing GP1 to a carrier protein that stabilizes the GP1 in the host production cell, but also provides a scaffold that allows easy purification of the antibody backbone with standard pharmaceutical protocols (protein A column as a primary step) (14).

We recognize that further research needs to be conducted to optimize formulation of EIC vaccines, and safety studies are needed that compare fusion protein immune complexes (e.g., EICs) to large immune complexes formed in vivo (that can have adverse effects such as autoimmune disease). However, the data

presented herein demonstrate early potential to create an EIC vaccine for filoviruses that could be highly relevant to a national biothreat reduction stockpile. Combined with the many emerging technologies for stabilization of subunit vaccines in long-term storage, even at ambient temperatures, EICs offer an attractive approach for filovirus vaccine manufacture.

Materials and Methods

Construction of Expression Vectors for EICs. The constructs used to produce h-EIC were previously described (14). For the mouse EIC, plant-optimized DNA sequences encoding HC and LC variable regions of mouse 6D8 mAb were designed for mouse EIC by using codons that are preferred in tobacco and removing spurious mRNA processing signal (29), and deposited in GenBank (accessions nos. HQ419193 and HQ407546). For HC-GP1 fusion, the constant region was amplified from the DNA encoding the HC of mAb 278.02 (18) using primer NheImCHFor (5′-TCAGCTAGCACAACAGCCCCATCGGTCTA), which added NheI restriction site at the 5′ end, and primer BamHImCHBack (5′-GATGGATCCACCTCCGCCTGAACCGCCTGAACCGCCTCC), which added BamHI at the 3′ end. The PCR product was digested with NheI-BamHI. pBYH2GP1kdel (14) was digested separately with BamHI-KpnI, SbfI-KpnI, and SbfI-NcoI, and the resulting three fragments were joined together with the PCR (NheI-BamHI) fragment and the synthetic variable region (NcoI-NheI) in a five-piece ligation, to make pBYHGP1kdel. For LC, the gene encoding the constant region of the LC of mAb 278.02 (18) was amplified using primer XhoImCLFor (5′-CTGCTCGAGATCAAACGGGCTGATGCTGC), which added a XhoI site at the 5′ end, and primer KpnImCLBack (5′-CTGGGTACCCTAACACTACTTCCTGTTGA), which added a KpnI site at the 3′ end, and the resulting product was digested with XhoI-KpnI. pBYK3R (14) was digested separately with SbfI-KpnI and SbfI-NcoI, and the resulting two fragments were joined together with the PCR (XhoI-KpnI) and the synthetic variable region (NcoI-XhoI) in a four-piece ligation to make pBYK1R.

Plant Inoculation, Protein Expression, and Protein Purification. *N. benthamiana* leaves were vacuum-infiltrated with pBYRH2GP1kdK3 (14) and pPSp19 (30) for h-EIC. For m-EIC, the leaves were infiltrated with pBYHGP1kdel, pBYK1R, and pPSp19. The final optical density at a wavelength of 600 nm of *Agrobacterium* was 0.25. The infiltrated leaves were harvested on day 4 after infiltration and extracted with the extraction buffer (400 mM Tris-HCl, 160 mM ascorbic acid, 4 mM EDTA, pH 7.5). The crude extract pH was adjusted to 4.9 with phosphoric acid and pH was neutralized to 7.9 with Tris-base. After centrifugation, the supernatant was concentrated by using a 50-kDa cutoff filter of a Pall TFF system (Pall). The extract was filtered with a 0.2-μm filter before loading into protein G or protein A column (GE Healthcare) for h-EIC or m-EIC, respectively. The column was washed with PBS solution, pH 7.5, and eluted with 50 mM citric acid, pH 2.5. After the protein was eluted from the column and 1M Tris-base was added to neutralize to final pH 7.5. The purified protein was filtered through a 0.2-μm filter and concentrated with Amicon Ultra-4 Centrifugal Filter Units (30 kDa; Millipore).

Vaccination. All animals were housed in Association for Assessment and Accreditation for Laboratory Animal Care-approved quarters, provided unlimited access to food and water, and handled in accordance with the Animal Welfare Act and Arizona State University IACUC. Before initiating any treatment, inbred female BALB/c mice (Charles River Laboratories), 5 wk old, were randomly distributed among cages and allowed to acclimate for at least 1 wk before vaccination. Female BALB/C mice were vaccinated s.c. with h-EIC (10 μg) alone or h-EIC (10 μg) with candidate adjuvant agents that included 20 μg of PIC (InvivoGen), 20 μg of CL097 (InvivoGen), or PIC (20 μg) and CL097 (20 μg) on days 0, 21, 42, and 63 (n = 7). Control mice were vaccinated with PBS solution alone as negative control or GP-VRP (1.6×10^8 replicon) (15) as positive control. Blood was collected from mice on days 0, 14, 35, and 56 by submandibular vein bleed in accordance with the Arizona State University Institutional Animal Care and Use Committee. On day 84, mice were humanely euthanized, and blood was collected by cardiac venipuncture in accordance with the Arizona State University IACUC.

EBOV Challenge. Female BALB/C mice (n = 15 per group) were vaccinated s.c. with h-EIC (10 μg) or m-EIC (10 μg) alone, h-EIC (10 μg) plus Imject alum adjuvant (Sigma-Aldrich), h-EIC (10 μg) plus PIC (20 μg), or h-EIC (10 μg) plus alum and PIC on days 0, 21, 42, and 63. Two groups of mice (n = 15 per group) were s.c. vaccinated with h-EIC (10 μg) plus PIC (20 μg) or h-EIC (25 μg) plus PIC and alum on days 21, 42, and 63. Mice were vaccinated with PBS solution (negative control) or 1.6×10^8 GP-VRP (positive control) as previously described (15) on days 0, 21, 42, and 63. Five mice from each group of 15 mice were humanely euthanized on day 84 by cardiac puncture, and the serum was analyzed for anti-Ebola IgG, each IgG subtype, and neutralizing antibody.

The remaining 10 mice in each group were shipped to the United States Army Medical Research Institute for infectious diseases on day 70, allowed to acclimate for 2 wk, and then challenged with a lethal dose of live EBOV on day 84. For the challenge study, mouse-adapted EBOV was obtained from Mike Bray (National Institutes of Health, Bethesda, MD) (31). Research was conducted under an IACUC-approved protocol in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals, and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The facility in which this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. For EBOV challenge, mice were inoculated i.p. with 1,000 pfu (30,000 LD_{50}) of mouse-adapted EBOV in a biosafety level 4 laboratory on day 84. Morbidity and mortality was recorded for 28 d after challenge. The average weight of the mice in each group that survived was measured for 14 d.

Anti-EBOV Antibody Analysis by ELISA. Levels of EBOV-specific antibody were determined, as previously described (32). Briefly, the wells were coated with irradiated EBOV overnight at 4 °C. EBOV-specific antibodies were detected by using the following antibodies: HRP-conjugated antibodies to measure total IgG, IgG1, IgG2a, IgG2b, or IgG3 (SouthernBiotech) diluted 1:4,000 in 1% dry milk in PBST. The plates were developed with TMB substrate (Pierce). Antibody titers were defined as the reciprocal of the highest dilution showing a net optical density of at least 0.02.

Neutralizing Antibody Measurement. Four 10-fold serial dilutions of pooled serum from each group were mixed with 100 pfu Ebola Zaire at 37 °C for 1 h and transferred to VERO cells monolayers. Cells were overlaid with agarose, and a second overlay containing 5% neutral red was added 7 d later. Plaques were counted the next day. Neutralization titers were determined to be the last dilution of serum that reduced the number of plaques by 80% compared with control wells.

Statistical Analysis. Prism software (GraphPad) was used to graph and make statistical comparisons of all data. EBOV-specific IgG titers are expressed as geometric mean titers (GMTs) for each vaccination group at each time point. All responders and nonresponders were included in the computation of the GMT. Negative samples were assigned a value of 1.0 for the purpose of calculating the GMT. Groups were compared using the Kruskal–Wallis one-way ANOVA, followed by a Dunn posttest at each time point. All statistical comparisons displayed graphically were made between individual treatment groups versus the PBS solution-alone group. For the survival data, groups were compared by using the log-rank test. Statistical significance was considered to require a P value lower than 0.05.

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