Figure 2

Figure 3

Supplementary Table 1

| Animal	Group	Symptoms*	Outcome
NHP 1	1	Petechia (D6, T), fever** (D3, D6), viremic*** (D3, D6)	Euthanized on day 7
NHP 2	1	Petechia (D6, T), fever (T), viremic (D3, D6)	Euthanized on day 7
NHP 3	1	Petechia (D6), viremic (D3, D6); no fever	Euthanized on day 8
NHP 4	1	Petechia (D6), fever (D3, D6, T), viremic (D3, D6)	Euthanized on day 7
NHP 5	2	No petechia, fever, or detectable viremia	Survived
NHP 6	2	No petechia, fever, or detectable viremia	Survived
NHP 7	2	Fever (D6), no petechia or detectable viremia	Survived
NHP 8	2	Viremic (D3, D6), no petechia or fever	Survived
NHP 9	3	No petechia, fever, or detectable viremia	Survived
NHP 10	3	No petechia, fever, or detectable viremia	Survived
NHP 11	3	No petechia, fever, or detectable viremia	Survived
NHP 12	3	No petechia, fever, or detectable viremia	Survived
NHP 13	4	No petechia, fever, or detectable viremia	Survived
NHP 14	4	No petechia, fever, or detectable viremia	Survived
NHP 15	5	Petechia (D6, T), viremia (D3, D6); no fever	Euthanized on day 7
NHP 16	5	Fever (D6), petechia (D9), viremia (D3, D6)	Euthanized on day 9
NHP 17	5	Petechia (D6), viremia (D3, D6); no fever	Euthanized on day 6
NHP 18	5	Petechia (D6), fever (D6), viremia (D3, D6)	Euthanized on day 6
*Symptoms on day (D) after infection, or on day of euthanization (T) are indicated in parentheses. **Fever is defined as a temperature of more than 2.5°F above the temperature on the day of challenge. ***Viremia determined from whole blood samples.			

Supplementary Table 2

| Animal	Group	Serum Dilutions*	
		Day -14	Day 0
NHP 1	1	N.D.**	N.D.
NHP 2	1	N.D.	N.D.
NHP 3	1	N.D.	N.D.
NHP 4	1	N.D.	N.D.
NHP 5	2	≤ 10	10
NHP 6	2	10	10
NHP 7	2	10	10
NHP 8	2	≤ 10	≤ 10
NHP 9	3	20	20
NHP 10	3	40	40
NHP 11	3	40	40
NHP 12	3	20	20
NHP 13	4	≤ 10	10
NHP 14	4	10	10
NHP 15	5	N.D.	N.D.
NHP 16	5	N.D.	N.D.
NHP 17	5	N.D.	N.D.
NHP 18	5	N.D.	N.D.
*Reciprocal serum dilution that reduced the titer of VSV expressing EBOV GP by $\geq$ 50%, **N.D.; not detectable.			

Supplementary Table 3

	GP Antibody Endpoint Titers*		OD Reading at 405 nm**		T-cell Response***
	Day -14	Day 0	NP	VP40	
NHP 5	800	800	0.360	0.460	10
NHP 6	800	800	0.365	0.473	183
NHP 7	800	800	0.412	0.595	137
NHP 8	800	800	0.650	0.503	239

*Titers shown are the highest reciprocal dilution that resulted in an optical density ≥ 0.2; **Amount of IgG antibody against EBOV NP or VP40 expressed as mean absorbance (optical density [OD]) at 405 nm in the sera (diluted 1:400) on day -14; ***Expressed as number of interferon gamma (IFN-γ) secreting spot-forming cells (SFCs).
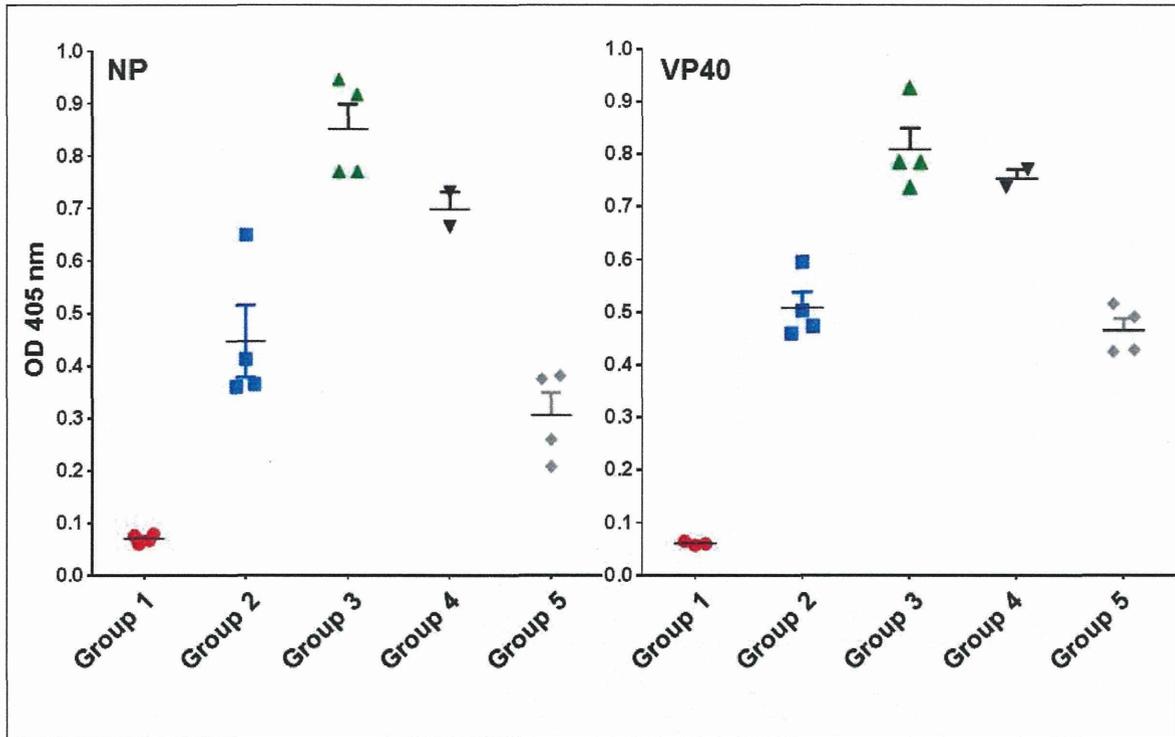
Supplementary Figure 1. The amount of IgG antibody against EBOV NP (left) and VP40 (right) in the sera of vaccinated nonhuman primates on day -14 as expressed as the mean absorbance (optical density [OD]) at 405 nm (± standard deviation) of sera diluted to 1:400.
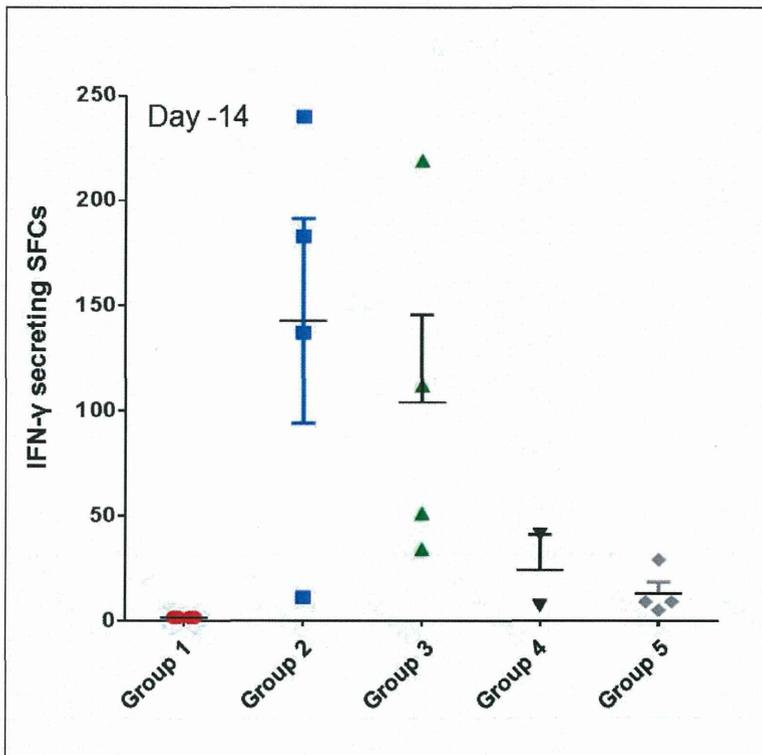
Supplementary Figure 2. Cellular immune responses on day -14 measured as the number of interferon gamma (IFN-γ) secreting spot-forming cells (SFCs).

別紙　参考文献

Generation of biologically contained Ebola viruses

Peter Halfmann*, Jin Hyun Kim*, Hideki Ebihara†‡, Takeshi Noda†, Gabriele Neumann*, Heinz Feldmann‡, and Yoshihiro Kawaoka*†§¶

*Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706; §Division of Virology, Department of Microbiology and Immunology, and †International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 113-0033, Japan; and ‡Special Pathogens Program, National Microbiology Laboratory, Public Health Agency of Canada and Department of Medical Microbiology, University of Manitoba, Winnipeg, MB, Canada R3E 3R2

Ebola virus (EBOV), a public health concern in Africa and a potential biological weapon, is classified as a biosafety level-4 agent because of its high mortality rate and the lack of approved vaccines and antivirals. Basic research into the mechanisms of EBOV pathogenicity and the development of effective countermeasures are restricted by the current biosafety classification of EBOVs. We therefore developed biologically contained EBOV that express a reporter gene instead of the VP30 gene, which encodes an essential transcription factor. A Vero cell line that stably expresses VP30 provides this essential protein in trans and biologically confines the virus to its complete replication cycle in this cell line. This complementation approach is highly efficient because biologically contained EBOVs lacking the VP30 gene grow to titers similar to those obtained with wild-type virus. Moreover, EBOVs lacking the VP30 gene are indistinguishable in their morphology from wild-type virus and are genetically stable, as determined by sequence analysis after seven serial passages in VP30-expressing Vero cells. We propose that this system provides a safe means to handle EBOV outside a biosafety level-4 facility and will stimulate critical studies on the EBOV life cycle as well as large-scale screening efforts for compounds with activity against this lethal virus.

antiviral screening | reverse genetics | vaccine development

Ebola viruses (EBOVs) cause hemorrhagic fevers in humans and nonhuman primates, with case fatality rates of 90% in some outbreaks (1). EBOVs and the closely related Marburg viruses belong to the *Filoviridae* family (2). Currently, there are no approved vaccines or antivirals for use against filoviruses, making biosafety level-4 (BSL-4) containment a mandatory requirement for work with these viruses. The lack of sufficient BSL-4 space and trained personnel and the rigors of working in BSL-4 laboratories have severely hampered basic research with EBOVs as well as the development of vaccines and large-scale screening for effective antiviral compounds. These limitations have prompted examination of various steps in the EBOV viral life cycle in the absence of infectious virus: (*i*) replication and transcription were studied by use of reporter gene assays that are based on the expression of necessary viral components from plasmids (3–7); (*ii*) entry and fusion processes were assessed with pseudotyping assays that rely on the use of recombinant vesicular stomatitis or retroviruses (8–11); and (*iii*) budding was examined by using virus-like particles that are generated from viral proteins provided by protein expression plasmids (12–16). However, several recent findings suggest that data obtained with these artificial systems may not always be reproducible with live, authentic EBOV (17). Thus, biologically contained EBOVs that resemble wild-type virus but can be handled outside BSL-4 containment are clearly needed.

EBOVs possess a negative-sense, nonsegmented RNA genome, ≈19 kb in length, that encodes seven structural proteins and at least one nonstructural protein (1). NP, viral protein (VP)35, VP30, and L, the RNA-dependent RNA polymerase, are components of the nucleocapsid and are essential for viral replication and transcription (5). VP40 is the matrix protein and is critical for viral budding (18, 19). VP24 is essential for the formation of nucleocapsids composed of NP, VP35, and viral RNA (20). The only viral surface glycoprotein, GP, plays a role in viral attachment and entry (21–24).

Reverse genetics systems for negative-sense RNA viruses (i.e., their artificial generation and modification from cloned cDNAs) are now established in the field (25, 26). We have exploited this technology to generate EBOVs that lack the essential VP30 gene and are maintained by—and biologically confined to—a cell line expressing VP30. The resultant viruses resemble wild-type virus in their life cycle, their morphology, and their growth properties but could be handled in a non-BSL-4 laboratory, opening new opportunities for study of the EBOV life cycle and for the identification of effective antiviral compounds. EBOVs lacking the VP30 gene are also candidates for vaccine development.

Results and Discussion

Generation and Passage of EbolaΔVP30-neo Virus. We previously established a full-length cDNA clone of the Zaire EBOV, Mayinga (26). Using a subgenomic fragment that encompasses nucleotides 6180–10942 of the viral genome (numbers refer to the positive-sense antigenome), we replaced the ORF for VP30 with that of neomycin (neo) using a series of overlapping PCR amplification steps. After confirmation of the authenticity of the PCR fragments by sequence analysis, the altered subgenomic fragment was inserted into the full-length EBOV cDNA construct via unique SalI and SacI restriction sites (Fig. 1), resulting in an EBOV cDNA genome deficient in the VP30 ORF. The artificial generation of EBOV from plasmids is afforded by flanking this viral cDNA with T7 RNA polymerase promoter and hepatitis delta virus ribozyme sequences (26).

To amplify VP30-deficient EBOVs, we established a stable Vero E6 cell line (designated VeroVP30) by cotransfecting Vero cells with two protein expression plasmids encoding VP30 (pCAG-VP30) and puromycin (pPur; Clontech) and selecting cell clones resistant to 5.0 μg/ml puromycin. VP30 expression in individual clones was determined by flow cytometry with antibodies to VP30. The clone with the highest percentage of VP30-expressing cells (>90% as measured by flow cytometry; data not shown) was used in further studies to amplify EbolaΔVP30 viruses.

EbolaΔVP30-neo virus was rescued under BSL-4 conditions as described previously for wild-type EBOV (26). All work involving infectious EboΔVP30 viruses and all steps before inactivation of biological material were performed under BSL-4 conditions at the National Microbiology Laboratory of the Public Health Agency of Canada.
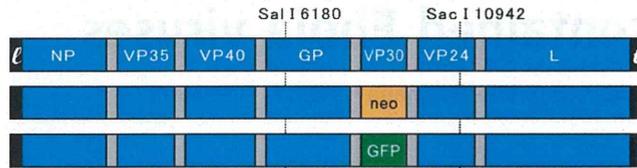
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Fig. 1. Schematic diagram of EbolaΔVP30 constructs. The top row shows a schematic diagram of the EBOV genome flanked by the leader sequence (l) and the trailer sequence (t) in positive-sense orientation. Two unique restriction sites for SalI and SacI (positions 6180 and 10942 of the viral antigenome, respectively) allowed the subcloning of a fragment that spans the VP30 gene. The subgenomic fragment was then used to replace the VP30 gene with genes encoding neo or eGFP, respectively. By using the unique restriction sites, the altered subgenomic fragments were cloned back into the full-length EBOV cDNA construct.

Briefly, human embryonic kidney (293T) cells were transfected with a plasmid for the transcription of the VP30-deficient EBOV RNA, with plasmids for the expression of the EBOV NP, VP30, VP35, and L proteins, and with a plasmid for the expression of T7 RNA polymerase. Five days after transfection, VeroVP30 cells were incubated with undiluted supernatant derived from plasmid-transfected cells. Seven days later, the supernatant was harvested, diluted 10-fold, and used to infect fresh VeroVP30 cells for the next passage. A total of seven passages were carried out, using the highest dilution of the inoculum that still produced replicating viruses for each passage. The presence of replicating virus was assessed by cytopathic effects and immunostaining of infected VeroVP30 cells with an antibody to VP40 (Fig. 2A Left). As a control, we also incubated the supernatants from each passage with wild-type Vero cells. As expected, we were unable to detect virus by cytopathic effects or viral antigens in wild-type Vero cells (Fig. 2A Right), demonstrating that replicating EbolaΔVP30-neo virus was confined to VeroVP30 cells.

Although the manifestation of cytopathic effects by virus in infected VeroVP30 cells suggested the formation of infectious (but biologically contained) EBOVs, we sought further evidence for the presence of virions in cell culture supernatant derived from infected VeroVP30 cells. Briefly, 5 days after VeroVP30 cells were infected with EbolaΔVP30-neo virus, supernatant was collected and partially purified over 20% sucrose. The pellet was suspended in PBS and separated on a 4–20% polyacrylamide gel. Western blot analyses were carried out with antibodies specific to the respective EBOV protein. All viral proteins (with the exception of L, for which no antibody was available) were detected (Fig. 2B, + lanes). Note that VP30 protein in virions originates from VeroVP30 cells whereas the remaining proteins are encoded by EbolaΔVP30-neo virus. By contrast, no viral proteins were detected in a control sample derived from wild-type Vero cells infected with EbolaΔVP30-neo virus (Fig. 2B, − lanes).

Genetic Stability of EbolaΔVP30-neo Virus. A major concern with the use of VP30-deficient EBOVs is the potential recombination with VP30 sequences integrated into the genome of the VeroVP30 helper cell line. Thus, to assess the genomic stability of EbolaΔVP30-neo virus, we performed three independent passage experiments (seven passages each). Whereas EbolaΔVP30-neo virus replicated in VeroVP30 cells, viral replication was not observed in wild-type Vero cells. Total viral RNA was isolated from the cell culture supernatant of infected VeroVP30 cells after the seventh passage. A viral genomic fragment spanning the neo gene was amplified by RT-PCR, cloned, and sequenced. A total of 20 clones were sequenced, and the sequences were identical to that of the EbolaΔVP30 cDNA construct used for virus generation. Hence, there was no evidence of recombination in any of three
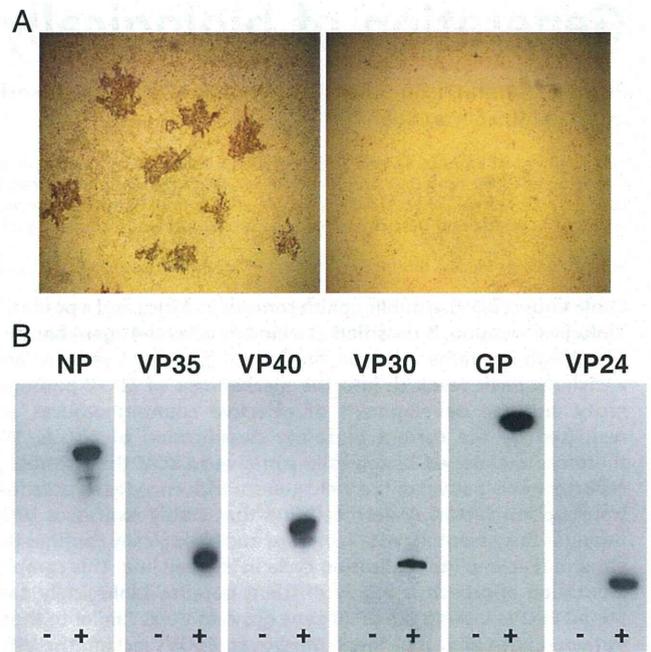

Fig. 2. Characterization of EbolaΔVP30-neo virus. (A) Expression of EBOV antigens by infected VeroVP30 cells. Confluent VeroVP30 cells (Left) or wild-type Vero cells (Right) were infected with EbolaΔVP30-neo for 60 min, washed, and overlaid with propagation medium with 1.5% methylcellulose. Seven days later, cells were fixed with 10% buffered formaldehyde and an immunostaining assay with an antibody to EBOV VP40 protein was performed as described in Materials and Methods. The formation of plaques in the VeroVP30 cell monolayer (Left), but not in monolayers of wild-type Vero cells (Right), illustrates that EbolaΔVP30-neo virus is biologically contained. (B) Detection of EbolaΔVP30-neo viral proteins. Supernatants derived from infected VeroVP30 (labeled +) or wild-type Vero (labeled −) cells were collected 5 days after infection and partially purified over 20% sucrose. Protein pellets were suspended in PBS and separated on polyacrylamide gels, transferred to membranes, and probed with specific antibodies to EBOV proteins.

independent passage experiments, attesting to the genomic stability of the EbolaΔVP30-neo viral genome.

To further demonstrate the biosafety of EbolaΔVP30-neo virus, we collected EbolaΔVP30-neo virus after seven consecutive passages in VeroVP30 cells and used this virus for three consecutive "blind" passages in wild-type Vero cells. Briefly, Vero cells were infected at a multiplicity of infection (m.o.i.) of 5 with EbolaΔVP30-neo virus (passage 7). Six days later, supernatant was used for the next blind passage as well as for Western blot analysis. No viral NP protein was detected after any of the blind passages (data not shown). After three consecutive blind passages, plaque assays and immunostaining were carried out in wild-type Vero cells to confirm the absence of replicating EBOV. As expected, we did not detect any replicating virus (data not shown). Collectively, these data further attest to the biosafety of the EbolaΔVP30 system.

Growth Kinetics of EbolaΔVP30-neo Virus. One of the major concerns raised by providing viral proteins in trans is that their amounts, expression kinetics, or both may not match those found in cells infected with wild-type virus, leading to reduced virus titers and/or aberrant virion morphology. To address this potential pitfall, we first compared the growth kinetics of EbolaΔVP30-neo virus (Fig. 3 Upper) with that of wild-type EBOV (Fig. 3, open circles). VeroVP30 cells (Fig. 3 Upper) or wild-type Vero cells (Fig. 3 Lower) were infected at a high m.o.i. of 1.0 or a low m.o.i. of 0.01, and supernatant was
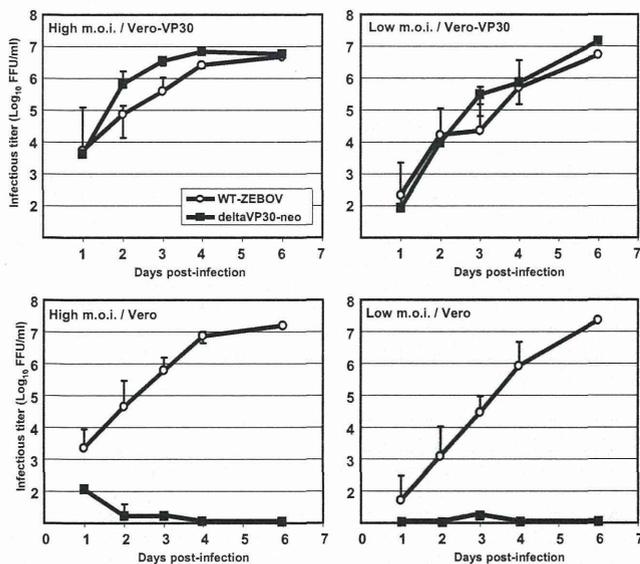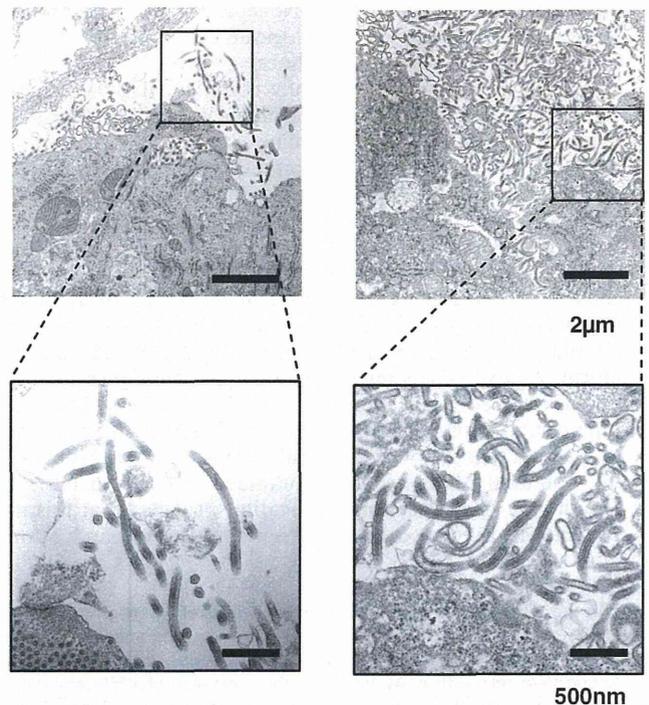
Fig. 3. Replication kinetics of wild-type EBOV and EbolaΔVP30-neo virus. VeroVP30 cells (*Upper*) and wild-type Vero cells (*Lower*) were infected with EBOV or EbolaΔVP30-neo at a high m.o.i. of 1.0 (*Left*) or a low m.o.i. of 0.01 (*Right*). Supernatants were harvested every 24 h after infection for 6 days. Viral titers of the respective viruses were determined by infecting confluent VeroVP30 cells or wild-type Vero cells with 10-fold dilutions of the supernatants and subsequent immunostaining as described in *Materials and Methods*. Virus titers for EbolaΔVP30-neo virus (filled squares) and wild-type EBOV (open circles) were comparable in VeroVP30 cells (*Upper*). In wild-type Vero cells (*Lower*), no replication was detected for EbolaΔVP30-neo virus (filled squares).

Fig. 4. Morphology of EBOVs budding from infected cells. Vero cells infected with wild-type EBOV (*Left*) and VeroVP30 cells infected with EbolaΔVP30-neo virus (*Right*) were processed for TEM 3 days after infection as described in *Materials and Methods*. The pictures show virus budding from infected cells. No significant differences in morphology or budding efficiencies were observed for wild-type EBOV and EbolaΔVP30-neo virus. [Magnification: ×6,000 (*Upper*) and ×20,000 (*Lower*, boxed area from *Upper*).]

harvested every 24 h. Virus titers of EbolaΔVP30-neo were determined in VeroVP30 cells, whereas virus titers of wild-type EBOV were determined in wild-type Vero cells. To determine virus titers, cells were overlaid with 1.5% methylcellulose and, 7 days later, assayed for VP40 expression by using an immunostaining assay. EbolaΔVP30-neo virus replicated efficiently in VeroVP30 cells at both conditions tested, reaching 10^7 focal-forming units per milliliter on day 6 after infection (Fig. 3 *Upper*, filled squares). No replication of EbolaΔVP30-neo was detected in wild-type Vero cells (Fig. 3 *Lower*, filled squares); the low titers that were detected for up to 3 days after infection likely reflect input virus. Together, these findings attest to the biological confinement of the EbolaΔVP30 system. The replication kinetics of EbolaΔVP30-neo in VeroVP30 cells are similar to those of wild-type EBOV in either VeroVP30 cells (Fig. 3 *Upper*, open circles) or wild-type Vero cells (Fig. 3 *Lower*, open circles), establishing the described approach as a highly efficient method for generating biologically contained EBOVs.

Morphology of EbolaΔVP30-neo Virus. Next we assessed the morphology of EbolaΔVP30-neo virus by transmission electron microscopy (TEM). VeroVP30 cells were infected with EbolaΔVP30-neo virus and fixed 36 h later. Samples were processed for TEM as described (14). As shown in Fig. 4 *Right*, the particles budding from VeroVP30 cells infected with EbolaΔVP30-neo virus were indistinguishable in their size and shape from wild-type EBOVs (Fig. 4 *Left*). Thus, providing VP30 protein *in trans* does not have a discernable effect on virion morphology, suggesting that the described system would be suitable for studies of virion formation and budding, for example.

Taken together, the above results demonstrate that the EbolaΔVP30-neo virus is biologically contained, replicates to

high titers in a helper cell line, is genetically stable, and is morphologically indistinguishable from wild-type virions. Having provided proof-of-concept for the generation of biologically contained EBOVs, we next sought to assess the utility of this strategy in basic research and drug screening applications.

Generation of an EbolaΔVP30-eGFP Virus and Its Usefulness for Basic Research Applications. We first generated an EbolaΔVP30 virus encoding eGFP instead of VP30 (Fig. 1; designated EbolaΔVP30-eGFP) using the same procedures described earlier for EbolaΔVP30-neo virus. Analogous to EbolaΔVP30-neo virus, the eGFP variant replicated efficiently with virus titers reaching 8.0 × 10^7 focal-forming units per milliliter. Expression of eGFP was observed as early as 10 h after infection (data not shown).

Takada *et al.* (27) used replication-competent vesicular stomatitis virus pseudotyped with EBOV GP and two neutralizing mAb, 133/3.16 and 226/8.1, to map EBOV GP epitopes and to generate escape mutants. To confirm with authentic EBOV virions the findings of Takada *et al.* (27) based on a vesicular stomatitis virus-pseudotyping system, we also generated escape mutants by amplifying EbolaΔVP30-eGFP virus in the presence of mAb 133/3.16 or 226/8.1. Each of eight escape mutants to mAb 133/3.16 possessed a histidine-to-arginine substitution at position 549 (H549R) in GP, reported by Takada *et al.* (27). Using mAb 226/8.1, we isolated 12 escape mutants that all contained an arginine-to-tryptophan substitution at position 134 (R134W), a mutation identical to one identified by Takada *et al.* (27). We, however, were unable to detect the remaining two escape mutations described by Takada *et al.* (27). Whether this discrepancy in escape mutants reflects differences between the biological systems used or random

mutations is presently unclear. Nonetheless, these experiments illustrated one of the ways that biologically contained EBOVs could be used in basic research applications.

We concluded that biologically contained EBOVs lacking the VP30 gene afford a safe, alternative way to study authentic EBOV, to develop EBOV vaccines, and to screen chemical libraries for compounds that interfere with the EBOV life cycle. Indeed, each of the two different biologically contained viruses we generated (encoding neomycin or eGFP instead of VP30) was biologically contained, as demonstrated by their ability to replicate in VeroVP30 cells but not in wild-type Vero cells. Moreover, virus titers were in the range of 10^7 focal-forming units per milliliter and hence comparable to those obtained for wild-type EBOV (Fig. 3) (25, 26, 28) whereas morphological, biochemical, and virological analyses indicated that the tested properties of EbolaΔVP30 viruses are indistinguishable from those of wild-type EBOV. These physical properties, together with the results of pilot studies to illustrate the potential of biologically contained EBOVs in basic research and drug screening applications, suggest that this new system will greatly accelerate current filovirus research efforts.

Materials and Methods

Cells and Cell Lines. Vero cells (green monkey kidney cells) were grown in Eagle's MEM supplemented with 10% FCS, L-glutamine, vitamins, nonessential amino acid solution, and antibiotics. The VeroVP30 cell line was established by cotransfecting Vero cells with pCAG-VP30 (for the expression of VP30) and pPur, a protein expression plasmid for the puromycin resistance gene (Clontech), using the transfection reagent TransIT LT-1 (Mirus). Two days after transfection, puromycin-resistant cells were selected with 5 μg/ml puromycin (Sigma). Individual cell clones were screened for VP30 expression by flow cytometry with a polyclonal peptide antibody to VP30.

Human embryonic kidney 293T cells were grown in high-glucose Dulbecco's modified Eagle's medium containing 10% FCS, L-glutamine, and antibiotics. All cells were maintained at 37°C and 5% CO_2.

Flow Cytometry. Cells were detached in PBS containing 0.02% EDTA and then washed once with cold PBS supplemented with 2% FCS and 0.1% sodium azide (wash buffer). Cells were incubated with a VP30 antibody on ice for 20 min. After washing in buffer, the cells were further incubated with a secondary antibody labeled with fluorescent isothiocyanate (Zymed). They were then washed with buffer and analyzed by FACSCalibur with CellQuest software (Becton Dickinson).

Generation of EbolaΔVP30 Viruses. The plasmid pTM-T7G-Ebo-Rib, containing the full-length EBOV cDNA flanked by T7 RNA polymerase promoter and ribozyme sequences, is described in a previous publication (26). First, a fragment encompassing nucleotides 6180–10942 (numbers refers to the positive-sense antigenome) was subcloned into a kanamycin-resistant cloning vector. Next, the VP30 ORF was replaced with those encoding neo or eGFP, respectively, by a series of overlapping PCR amplification steps using Pfu Turbo (Stratagene). The altered subgenomic fragments were transferred back into the full-length EBOV cDNA plasmid using two unique restriction sites, SalI and SacI (Fig. 1). The resultant plasmids, designated pTM-EbolaΔVP30-neo or pTM-EbolaΔVP30-eGFP, were sequenced to verify the replacement of the VP30 ORF and the lack of any unwanted mutations.

To artificially generate EBOV, we transfected 5 × 10^5 293T cells with 1.0 μg of pTM-EbolaΔVP30, 2.0 μg of pCAG-L, 1.0 μg of pCAG-NP, 0.5 μg of pCAG-VP35, 0.5 μg of pCAG-VP30, and 1.0 μg of pCAG-T7 pol using TransIT LT1 (Mirus) in BSL-4 containment (26). Five days after transfection, the supernatant was harvested, cellular debris was removed by low-speed centrifugation, and the virus was amplified in VeroVP30 cells at 37°C and 5% CO_2 with propagation medium containing 2% FCS in MEM supplemented with

L-glutamine, vitamins, nonessential amino acid solution, and antibiotics without puromycin.

Plaque Assay and Immunostaining Assay. To determine the titers of wild-type EBOV or EbolaΔVP30 viruses, we absorbed 10-fold dilutions of the viruses to confluent VeroVP30 or wild-type Vero cells for 1 h at 37°C, after which any unbound virus was removed by washing cells with propagation medium. The cells were then overlaid with propagation medium containing 1.5% methyl cellulose (Sigma). Seven days after infection, cells were fixed with 10% buffered formaldehyde, taken out of BSL-4, permeabilized with 0.25% Triton X-100 in PBS for 10 min, and blocked with 4% goat serum and 1% BSA in PBS for 60 min. Cells were then incubated for 60 min with a 1:1,000 dilution of a mouse anti-VP40 mAb, washed with PBS, and incubated for 60 min with a 1:1,000 dilution of an anti-mouse IgG-peroxidase-conjugated secondary antibody (Kirkegaard & Perry Laboratories). After washing with PBS, cells were incubated with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in PBS. The reaction was stopped by rinsing cells with water.

Western Blotting. Partially purified virus resuspended in lysis buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.5% Triton X-100/0.1% SDS) containing protease inhibitors [complete protease inhibitor cocktails (Roche)] was incubated at 100°C for 5 min, taken out of BSL-4, and separated on 4–20% polyacrylamide gels. Resolved proteins were transferred to Western polyvinylidine difluoride membranes (Schleicher & Schuell) and blocked overnight at 4°C with 5% skim milk in PBST [0.05% Tween 20 (Sigma) in PBS]. Blots were incubated with primary antibodies (a mouse anti-NP antibody, a rabbit anti-VP35 antibody, a rabbit anti-VP40 antibody, a mouse anti-GP antibody, a rabbit anti-VP30 antibody, or a mouse anti-VP24 antibody) for 60 min at room temperature, washed three times with PBST, incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Zymed) for 60 min, and finally washed three times with PBST. Blots were then incubated in Lumi-Light Western blotting substrate (Roche) and exposed to x-ray film (Kodak).

RNA Isolation and RT-PCR. Cell culture supernatant from virus-infected VeroVP30 cells was inactivated with guanidinium isothiocyanate buffer and taken out of BSL-4. Viral RNA was isolated with the RNeasy Mini kit (Qiagen). RT-PCR was carried out with the RobusT One-Step RT-PCR kit (Finnzyme) using 1 μg of isolated RNA and EBOV-specific primers. The resultant PCR products were cloned into pT7Blue (Novagen) and sequenced.

TEM. Ultrathin-section electron microscopy was performed as described previously (14). Briefly, at 36 h after infection, VeroVP30 cells infected with EbolaΔVP30-neo virus were fixed and inactivated with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, taken out of BSL-4, and postfixed with 2% osmium tetroxide in the same buffer. Cells were then dehydrated with a series of ethanol gradients followed by propylene oxide before being embedded in Epon 812 Resin mixture (TAAB Laboratories Equipment). Thin sections were stained with 2% uranyl acetate and Raynold's lead and examined under a Hitachi H-7500 electron microscope at 80 kV.

Selection of Escape Mutants. EbolaΔVP30-eGFP was diluted 10-fold (10^{-1} to 10^{-6}) and incubated with the indicated mAbs at a concentration of 250–500 μg of mAb per milliliter at 37°C for 60 min. The virus/mAb mixtures were inoculated onto VeroVP30 cells for 60 min. Viruses were amplified for 5 days in the presence of antibodies. Then, viruses that grew in the presence of mAbs (as determined by GFP expression) were harvested at the highest virus-positive dilution and passaged for a total of three to six times in the presence of antibodies. Viral RNA was isolated and RT-PCR-amplified, and the GP sequence was determined by sequence analysis.

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Replication-Deficient Ebolavirus as a Vaccine Candidate[▽]

Peter Halfmann,[1] Hideki Ebihara,[2,3]† Andrea Marzi,[3]† Yasuko Hatta,[1] Shinji Watanabe,[1] M. Suresh,[1]
Gabriele Neumann,[1] Heinz Feldmann,[3,4]† and Yoshihiro Kawaoka[1,2,5]*

*Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison,
Wisconsin[1]; Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science,
University of Tokyo, Tokyo, Japan[2]; Special Pathogens Program, National Microbiology Laboratory, Public Health Agency of
Canada[3], and Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada[4]; and
Institute of Medical Science, University of Tokyo, Tokyo, Japan[5]*

Ebolavirus causes severe hemorrhagic fever, with case fatality rates as high as 90%. Currently, no licensed vaccine is available against Ebolavirus. We previously generated a replication-deficient, biologically contained Ebolavirus, EbolaΔVP30, which lacks the essential VP30 gene, grows only in cells stably expressing this gene product, and is genetically stable. Here, we evaluated the vaccine potential of EbolaΔVP30. First, we demonstrated its safety in STAT-1-knockout mice, a susceptible animal model for Ebolavirus infection. We then tested its protective efficacy in two animal models, mice and guinea pigs. Mice immunized twice with EbolaΔVP30 were protected from a lethal infection of mouse-adapted Ebolavirus. Virus titers in the serum of vaccinated mice were significantly lower than those in nonvaccinated mice. Protection of mice immunized with EbolaΔVP30 was associated with a high antibody response to the Ebolavirus glycoprotein and the generation of an Ebolavirus NP-specific CD8+ T-cell response. Guinea pigs immunized twice with EbolaΔVP30 were also protected from a lethal infection of guinea pig-adapted Ebolavirus. Our study demonstrates the potential of the EbolaΔVP30 virus as a new vaccine platform.

Ebolaviruses (EBOVs; family *Filoviridae*) cause severe hemorrhagic fever in humans and nonhuman primates (NHPs), with case fatality rates up to 90% (26). Since the first recorded outbreak in 1976 of EBOV in the Democratic Republic of the Congo, increasing numbers of outbreaks in Central Africa have occurred over the last decade (23). Transmission of the virus results from direct contact of infected humans and animals with body fluids; however, lethal transmission by aerosol droplets can also occur (13, 14). Due to its high pathogenicity and potential for aerosol transmission, EBOV and its sister virus, Marburgvirus (MARV), are classified as category A bioterrorism threats.

Currently, there are no licensed vaccines or antivirals available against EBOV. A vaccine against EBOV would be invaluable to those closely affected during an outbreak along with health care personnel, laboratory workers, and military personnel. Several vaccine candidates have been shown to protect NHPs: a replication-incompetent adenovirus expressing the EBOV glycoprotein (GP) (29–31), a replication-competent vesicular stomatitis virus (VSV) expressing GP (7, 15), a recombinant paramyxovirus expressing GP (4), and virus-like particles (VLPs) (38, 41). However, questions regarding acceptable vaccine doses, prior immunity to the vaccine vector, effective-

ness in humans, and safety considerations still linger with these vaccine candidates (10, 25, 42). Therefore, alternative EBOV vaccine candidates continue to be sought.

We recently generated a replication-deficient, biologically contained EBOV via reverse genetics, a technique that allows the generation of infectious EBOV from cloned cDNA (21, 37). This technique provides the opportunity to introduce mutations into the EBOV genome and observe the resulting viral phenotypes (6, 11, 20, 21, 36, 37). Specifically, we generated a replication-deficient virus (EbolaΔVP30) (9) by deleting the entire open reading frame of the VP30 gene, which encodes the essential transcription factor VP30 (18, 27). EbolaΔVP30 is thus replication deficient and biologically contained unless VP30 protein is provided in *trans*. To allow for viral amplification, we therefore generated a Vero cell line that stably expresses VP30, designated VeroVP30 (9). EbolaΔVP30 undergoes multiple cycles of replication in VeroVP30 cells but not in parental Vero cells. More importantly, the EbolaΔVP30 genome is genetically stable over sequential passages in VeroVP30 cells (i.e., recombination between the EbolaΔVP30 genome and the VP30 cDNA present in VeroVP30 cells does not occur), thereby partially alleviating safety concerns (9). Hence, the EbolaΔVP30 virus can be grown to reasonably high titers in helper cells, expresses all viral antigens/proteins, is genetically stable, and is safe. Here, we evaluate the safety of EbolaΔVP30 as a vaccine in STAT-1-knockout (KO) mice and assess its protective efficacy in two rodent models, mice and guinea pigs.

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* Corresponding author. Mailing address: Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, 2015 Linden Drive, Madison, WI 53706. Phone: (608) 265-4925. Fax: (608) 265-5622. E-mail: kawaokay@svm.vetmed.wisc.edu.

† Present address: Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rocky Mountain Laboratories, 903 South 4th Street, Hamilton, MT 59840.

MATERIALS AND METHODS

Cells. VeroVP30 cells were established as previously described (9) and grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf
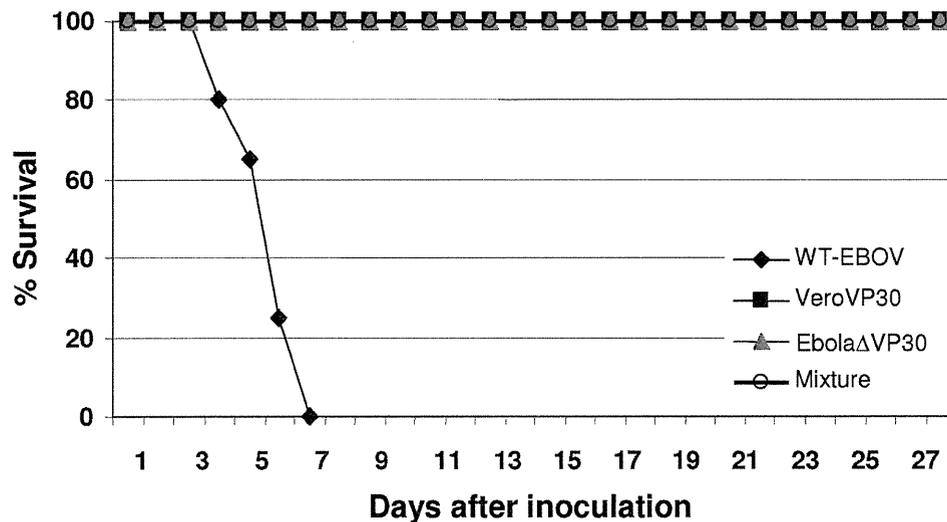
FIG. 1. EbolaΔVP30 virus is not virulent in STAT-1-KO mice. Survival curves of STAT-1-KO mice inoculated i.p. with (i) 1,000 MLD_{50}/mouse of wild-type (WT) EBOV (n = 3), (ii) 10^6 cells/mouse of VeroVP30 cells (n = 5), (iii) 2 × 10^6 FFU/mouse of EbolaΔVP30 virus (n = 5), or (iv) a mixture of EbolaΔVP30 virus and VeroVP30 (n = 5). Animals were observed for 28 days after inoculation.

serum (FCS), L-glutamine, vitamins, nonessential amino acid solution, and 5 μg/ml puromycin (Sigma, St. Louis, MO).

Viruses. The EbolaΔVP30 virus was generated as previously described (9) and propagated in VeroVP30 cells in MEM as described above but supplemented with 2% FCS. The virus was harvested 6 days after infection of the cells at a multiplicity of infection of 0.1. Harvested virus was partially purified by ultracentrifugation at 27,000 rpm for 2 h over 20% sucrose. The viral pellet was resuspended in sterile phosphate-buffered saline (PBS) and stored at −80°C. Viral titers were determined by use of a focus-forming assay (visualized with antibody to the viral VP40 protein) in confluent VeroVP30 cells overlaid with MEM containing 2% FCS and 1.5% methylcellulose (Sigma).

For challenge studies in mice or guinea pigs, we used a mouse-adapted EBOV (MA-EBOV) or guinea pig-adapted EBOV (GP-EBOV), subtype Zaire, generated as previously described (3, 5). All studies involving live EBOV were carried out under biosafety level 4 conditions at the National Microbiology Laboratory of the Public Health Agency of Canada in Winnipeg, Canada.

Antibody titers. The levels of EBOV GP-specific immunoglobulin G (IgG) antibodies in vaccinated mice were examined by using an enzyme-linked immunosorbent assay (ELISA). Briefly, wells of Immulon 2HB plates (Thermon Labsystems, Franklin, MA) were coated with purified EBOV GP (34) and blocked with PBS containing 10 mg/ml bovine serum albumin. EBOV GP-coated wells were incubated with mouse serum from nonvaccinated and vaccinated mice, and bound antibodies were detected with goat anti-mouse IgG conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) by use of an ELISA plate reader at an absorbance of 405 nm.

Intracellular staining and flow cytometry. The number of cytokine-producing $CD8^+$ T cells was determined by intracellular staining as described previously (19). Briefly, splenocytes were stimulated with the control peptide PB1$_{703-711}$ (SSYRRPVGI, derived from influenza virus PB1) (1) or the EBOV peptide NP$_{279-288}$ (SFKAALSSLA, derived from the nucleoprotein NP) (22, 28), VP40$_{171-180}$ (YFTFDLTALK, derived from the matrix protein VP40), or GP$_{161-169}$ (LYDRLASTV, derived from GP) (22, 39) for 5 h in the presence of brefeldin A and interleukin-2. Following activation, cells were stained for cell surface CD8 and intracellular gamma interferon (IFN-γ) by using the Cytofix/Cytoperm kit from BD Biosciences (San Jose, CA). The number of cytokine-producing $CD8^+$ T cells was determined by using a FACSCalibur flow cytometer (BD Biosciences).

Inoculation of STAT-1-KO mice. Six-week-old female STAT-1-KO mice (Charles River) were anesthetized with isoflurane and intraperitoneally (i.p.) inoculated with (i) wild-type EBOV (1,000 mouse 50% lethal doses [MLD_{50}]/mouse), (ii) VeroVP30 cells (10^6 cells/mouse), (iii) EbolaΔVP30 virus (2 × 10^6 focus-forming units [FFU]/mouse), or (iv) a mixture of EbolaΔVP30 virus and VeroVP30 (10^6 cells and 2 × 10^6 FFU/mouse). Animals were observed for at least 28 days after inoculation.

Challenge studies. Four-week-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were anesthetized with isoflurane and i.p. immunized twice at 3-week intervals with 10^6 FFU of EbolaΔVP30 virus; nonvaccinated mice were immunized with PBS. Eight weeks after the second vaccination, mice were challenged i.p. with 1,000 MLD_{50} of MA-EBOV (3). Four days after challenge, viral titers were determined in the serum of three vaccinated and three nonvaccinated mice. The remaining mice were monitored for survival for 28 days. Female guinea pigs (Charles River) between 200 and 250 g in body weight were anesthetized with isoflurane and i.p. immunized twice at 3-week intervals with 10^7 FFU of EbolaΔVP30 virus; nonvaccinated guinea pigs were inoculated with PBS. Three weeks after the second vaccination, guinea pigs were challenged i.p. with 1,000 LD_{50} of GP-EBOV (5) and observed for 16 days.

All animal experiments were performed in accordance with approved animal use protocols and according to the guidelines set forth by the Canadian Council of Animal Care and the University of Wisconsin—Madison.

RESULTS

EbolaΔVP30 virus is not pathogenic in STAT-1-KO mice. To evaluate the safety of EbolaΔVP30 virus, STAT-1-KO mice, which are susceptible to wild-type EBOV infection (2), were inoculated i.p. with wild-type EBOV (1,000 MLD_{50}/ mouse), VeroVP30 cells (10^6 cells/mouse), EbolaΔVP30 virus (2 × 10^6 FFU/mouse), or a mixture of EbolaΔVP30 virus and VeroVP30 (10^6 cells and 2 × 10^6 FFU/mouse). STAT-1-KO mice inoculated with EbolaΔVP30 virus, VeroVP30 cells, or a mixture of both showed no signs of illness and survived for the entire observation period (28 days) after inoculation (Fig. 1). In contrast, 3 days after inoculation with wild-type EBOV, STAT-1-KO mice showed signs of disease, with ruffled fur and weight loss (data not shown), and by day 6, all mice had succumbed to infection (Fig. 1). These data demonstrate the safety and lack of virulence of the EbolaΔVP30 vaccine candidate.

Antibody response of mice immunized with EbolaΔVP30 virus. To assess EbolaΔVP30 virus as a potential vaccine candidate, we first determined its immunogenicity in mice by vaccinating mice three times at 3-week intervals. When serum samples, collected 2 weeks after each vaccination, were tested
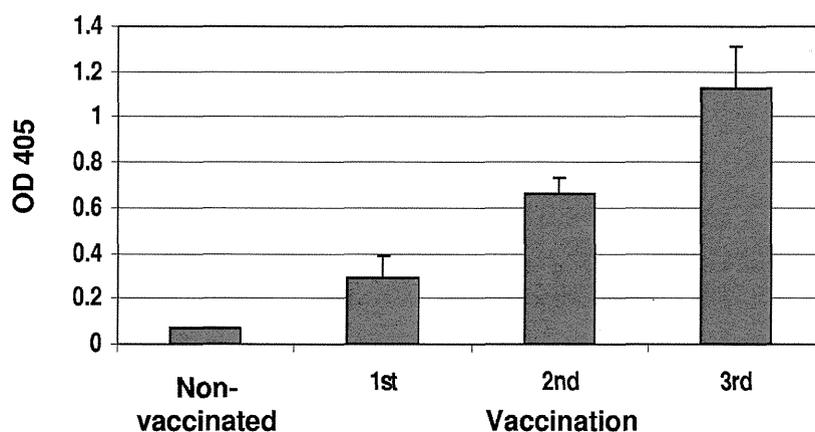
FIG. 2. EbolaΔVP30 virus induces an antibody response to the EBOV glycoprotein GP. Mice (n = 4) were vaccinated with EbolaΔVP30 virus three times at 3-week intervals, whereas nonvaccinated mice (n = 4) were inoculated with PBS. Serum samples were collected 2 weeks after each vaccination, and the amounts of IgG against purified EBOV GP were determined by ELISA, as described in Materials and Methods. Results are expressed as the mean absorbance (optical density [OD]) at 405 nm (± standard deviation) of samples diluted to 1:100.

for IgG antibodies by ELISA with purified GP (Fig. 2), vaccinated animals showed elevated antibody titers against GP after the first vaccination relative to those in nonvaccinated mice (Fig. 2); these antibody titers further increased after the second and third vaccinations This finding demonstrates the ability of the biologically contained EbolaΔVP30 virus to elicit antibodies to GP.

CD8$^+$ T-cell responses in vaccinated mice. We next examined cellular immune responses to vaccination in mice. Since two vaccinations resulted in high antibody titers in mice (see above), we performed only two vaccinations for all further experiments. Eight days after the second immunization, four vaccinated and two nonvaccinated mice were euthanized and their spleens were removed. Splenocytes were isolated and stimulated with the EBOV peptide NP$_{279-288}$ (SFKAALSS LA), VP40$_{171-180}$ (YFTFDLTALK), or GP$_{161-169}$ (LYDRLA

STV), or with the influenza virus peptide PB1$_{703-711}$ (SSYRR PVGI), for 5 h in the presence of brefeldin A and interleukin-2.

In vaccinated animals, stimulation with the EBOV peptide NP$_{279-288}$ resulted in IFN-γ-positive CD8$^+$ cells in the range of 0.11% to 1.26% of the total CD8$^+$ cell population (Fig. 3). For the two nonvaccinated mice, the numbers of IFN-γ-positive CD8$^+$ cells were appreciably lower, i.e., 0.056% and 0.032% (Fig. 3). Stimulation of cells with the influenza virus peptide PB1$_{703-711}$ did not elicit a significant response in vaccinated or nonvaccinated mice (data not shown). No IFN-γ-positive CD8$^+$ cells were detected for cells stimulated with EBOV peptide VP40$_{171-180}$ or GP$_{161-169}$ (data not shown).

Protective efficacy of EbolaΔVP30 virus in mice. To assess the protective efficacy of EbolaΔVP30 virus, we immunized 18 4-week-old mice twice (with a 3-week interval) with 10^6 FFU of EbolaΔVP30 virus; 13 control mice were similarly inoculated

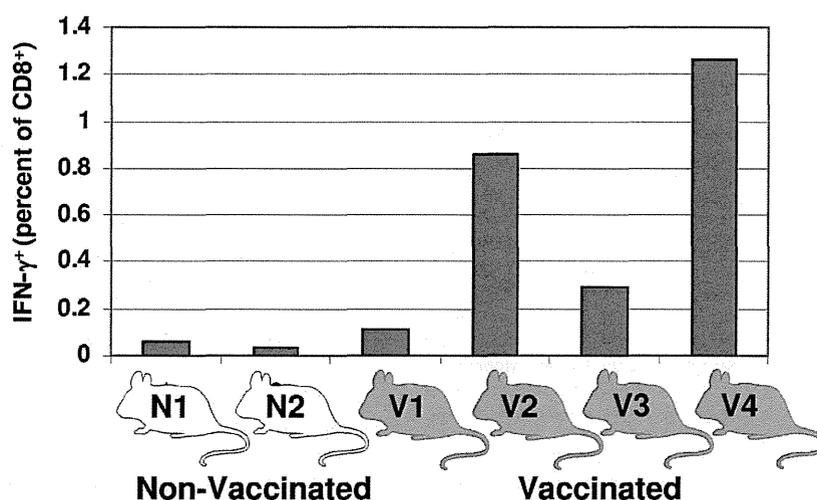FIG. 3. Cellular immune response in EbolaΔVP30-vaccinated mice. Mice (n = 4) were immunized twice at 3-week intervals with EbolaΔVP30; nonvaccinated mice (n = 2) were simultaneously inoculated with PBS. Splenocytes were collected 8 days after the second vaccination and stimulated with an EBOV NP peptide. Cells were stained for the cell surface antigen CD8$^+$ and for intracellular IFN-γ. The number of cytokine-producing CD8$^+$ T cells was determined by using a FACSCalibur flow cytometer (BD Biosciences). N, nonvaccinated; V, vaccinated.
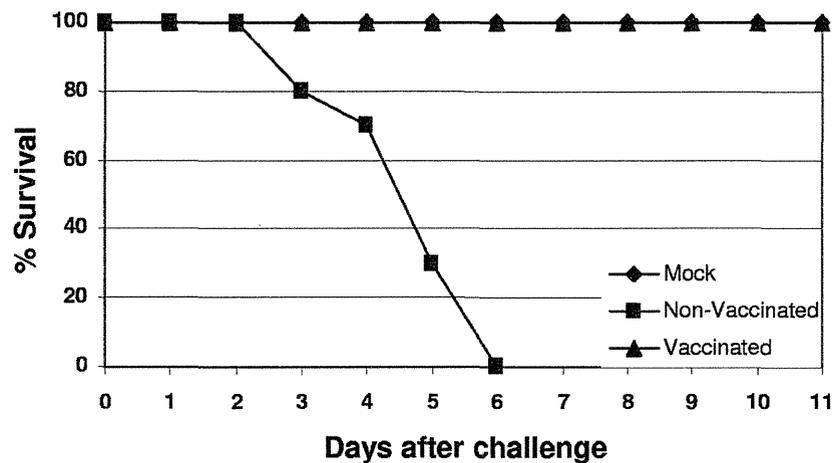
FIG. 4. Protective efficacy of EbolaΔVP30 virus in mice. Mice ($n = 15$) were immunized twice with EbolaΔVP30 virus; nonvaccinated mice ($n = 10$) were inoculated with PBS. Eight weeks after the second vaccination, mice from the vaccinated and nonvaccinated groups were challenged with 1,000 MLD_{50} of MA-EBOV. A control group (Mock, $n = 3$) remained nonvaccinated and nonchallenged. Survival curves are shown.

with PBS. Mice were challenged 8 weeks after the second immunization with 1,000 MLD_{50} of MA-EBOV (3). After challenge, no signs of illness were seen in mice vaccinated with EbolaΔVP30 virus, whereas nonvaccinated mice began to show signs of sickness (e.g., ruffled fur) and weight loss on day 3 postchallenge (data not shown). By day 6 postchallenge, all nonvaccinated mice had succumbed to infection, whereas the vaccinated mice were fully protected (Fig. 4). All animals were observed for 21 days after challenge. On day 4 postchallenge, three vaccinated and three nonvaccinated mice were sacrificed to determine virus titers in their sera. Vaccinated mice had significantly lower titers than did nonvaccinated mice (Fig. 5), with one mouse showing no viremia by day 4. Taken together, these data demonstrate that EbolaΔVP30 virus efficiently protects mice against challenge with a lethal dose of MA-EBOV.

Protective efficacy of EbolaΔVP30 virus in guinea pigs. To further demonstrate the protective efficacy of EbolaΔVP30 virus, we immunized six guinea pigs twice (with a 3-week interval) with 10^7 FFU of EbolaΔVP30 virus; six guinea pigs were similarly inoculated with PBS. Guinea pigs were chal-
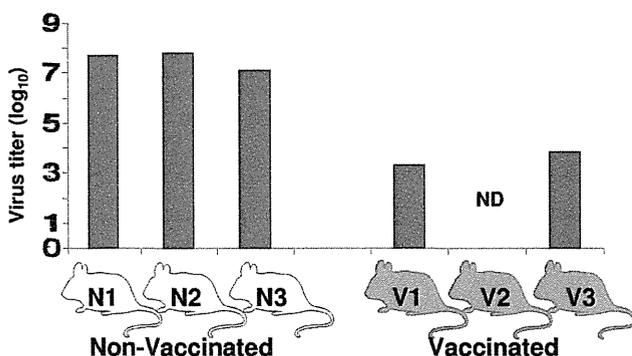

FIG. 5. Virus titers in immunized mice. Vaccinated and nonvaccinated mice (three per group) were euthanized on day 4 postchallenge. Virus titers in the serum were determined by use of a focus-forming assay as described in Materials and Methods. N, nonvaccinated; V, vaccinated; ND, not detectable.

lenged 6 weeks after the second vaccination with 1,000 LD_{50} of GP-EBOV (5). After challenge, no signs of sickness were seen in the vaccinated guinea pigs, whereas nonvaccinated animals began loosing weight on day 3 postchallenge (data not shown). By day 7 postchallenge, all nonvaccinated guinea pigs had succumbed to infection, while all vaccinated guinea pigs showed no signs of disease and were fully protected (Fig. 6). All animals were observed for 16 days after challenge.

DISCUSSION

Here, we assessed the potential of EbolaΔVP30 as a vaccine candidate. We found that EbolaΔVP30 is safe and does not cause disease or death in STAT-1-KO mice, in contrast to wild-type EBOV. Moreover, we found that mice and guinea pigs vaccinated with EbolaΔVP30 virus are protected against lethal challenge with MA- or GP-EBOV, respectively. In vaccinated mice, virus titers in sera were significantly lower than those in nonvaccinated mice.

Protection against EBOV infection likely requires both humoral and cellular responses. The humoral response to EBOV infection is important, as demonstrated by protection from a lethal challenge in rodent models by passive transfer of antibodies to the viral glycoprotein GP (8, 33). However, the ability of a vaccine to elicit an antibody response does not in itself correlate with protection. For example, classic vaccine approaches, such as gamma-irradiated EBOV and MARV, or GP expressed by baculovirus, elicit a moderate antibody response but fail to protect mice against lethal challenge (12, 16, 17). In contrast, EBOV and MARV VLPs that express GP and VP40 elicit both humoral and CD8$^+$ T-cell responses and protect mice against a lethal challenge of EBOV and MARV (38–40), highlighting the importance of cellular responses for protection. Here, we demonstrate that EbolaΔVP30 virus induces both humoral and CD8$^+$ T-cell responses (specific for an EBOV NP epitope). The next logical step is to test whether the immune responses elicited by EbolaΔVP30 are sufficient to protect NHPs from a lethal challenge with EBOV.
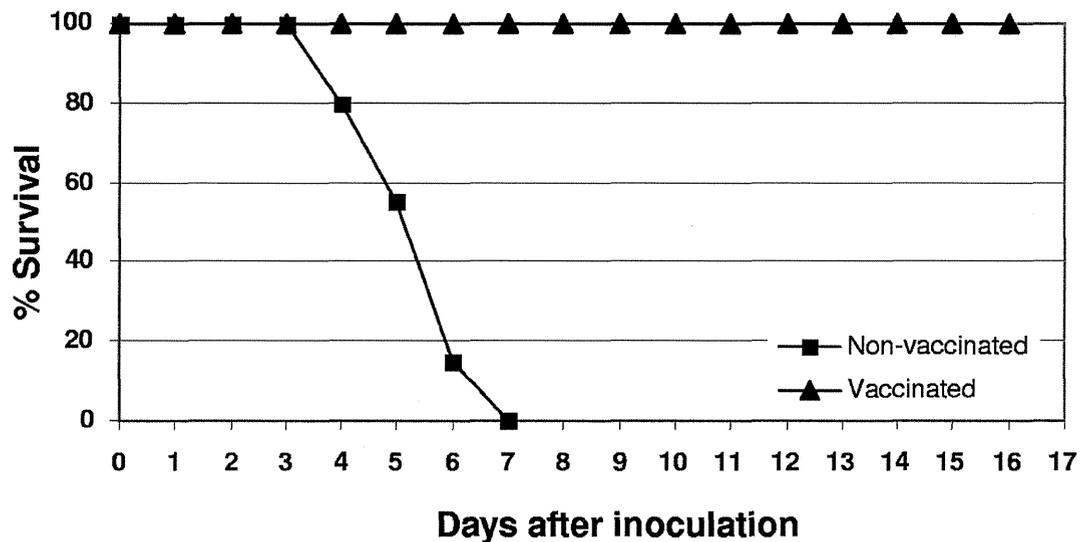
Three vaccine candidates, based on recombinant viral vec-

FIG. 6. Protective efficacy of EbolaΔVP30 virus in guinea pigs. Survival curves of guinea pigs vaccinated twice with EbolaΔVP30 virus or inoculated with PBS (n = 6 per group). Six weeks after the last vaccination, guinea pigs were challenged with 1,000 LD_{50} of GP-EBOV. Animals were observed for 16 days after challenge.

tors expressing EBOV GP, are protective in an NHP model: adenovirus (29–31), VSV (7, 15), and paramyxovirus (4). However, none of these candidates is practical in its current form. The paramyxovirus-based vaccine does not confer full protection in the NHP model (4). The adenovirus-based vector, a replication-incompetent vector, is based on a human pathogen (adenovirus type 5), raising concerns of preexisting immunity to the vector. In addition, a large dose of this virus (10^{10} particles) is needed to confer protection in NHPs (29, 32, 35). A vaccine based on the VSV vector protects NHPs at reasonable doses; however, this vector is replication competent and safety is, therefore, a concern, particularly the potential for infection of the human central nervous system (24). EBOV and MARV VLPs have been shown to protect various animal models from a lethal challenge with these viruses (38, 40, 41). While VLPs are safe and preexisting immunity is not a concern for VLP vaccines, large-scale production of pure VLPs in cell culture can be an obstacle.

Here, we present a vaccine approach that is safe, efficacious, and highly efficient since EbolaΔVP30 virus grows to high titers in Vero VP30 cells (9). As with other EBOV vaccine candidates, our vaccine would be of value to health care personnel, laboratory workers, and military personnel, as well as to those at risk during outbreaks. Further studies are needed to establish the protective efficacy of EbolaΔVP30 in NHPs and to assess its potential for postexposure treatment.

ACKNOWLEDGMENTS

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