Antibodies/probes used were: rabbit anti-HA (Santa Cruz Biotechnology); Shank guinea-pig antibody 1123 (gift from E. Kim); bassoon mouse monoclonal (Stressgen); anti-GluR2 monoclonal (Chemicon International); anti-parvalbumin monoclonal (Sigma); Texas-red-conjugated phalloidin and Alexa-488 and Alexa-568 secondary antibodies (Molecular Probes).

**Fc–NTD fusion protein inhibition**

Amino acids 1–404 of GluR1 (NTD1) and 1–405 of GluR2 (NTD2) were amplified by PCR and subcloned in-frame, N-terminal of the Fe-cfragment of modified pFVE vector\(^1\). Fe-fusion proteins were expressed in HEK293T cells, purified from culture medium using Protein A Sepharose Flow chromatography (Pharmacia), eluted with 100 mM NaCl (pH 2.5), neutralized by adding Tri-HCl pH 8.5, and dialysed against 20 mM HEPES pH 7.4, 150 mM NaCl. Purified proteins were >90% pure as assessed by SDS–polyacrylamide gel electrophoresis. For the Fc–NTD fusion constructs, neuronal transfected neuron cultures (DIV14) were co-transfected with EGFP and Fc–NTD constructs. At DIV22, neurons were fixed and stained with anti-human Fe–Alexa-568 antibody. The Fe–NTD constructs were expressed using vector β-actin – 16-pl (β-actin promoter).

**GluR2 sRNA**

For plasmid-based RNA inhibition of GluR2, the following complementary oligonucleotides were inserted in the pSUPER vector (Oligo Engine\(^\text{TM}\)): 5'-GATCCCCGGACGCCTCTCAGTGTACGACTGACGAGTCTGTTCTG GCTCTTGTGAAA-3', and 5'-AGCTTCTTTCAAAAAGGACTCCTAGCTGTATCCTGTATGAACTGAGTGTCCGCG-3' (corresponding to nucleotides 400–418 of rat GluR2). The specificity and efficacy of this construct to interfere with GluR2 expression was first tested against heterologously expressed GluR in COS-7 cells.

**Image acquisition and quantification**

Labelled transfected neurons were chosen randomly for quantification from four coverslips from five independent experiments for each construct. Fluorescence images were acquired with a Biorad MRC1024 confocal microscope, using a Nikon \( \times 60 \) objective with sequential acquisition setting at \( 1,024 \times 1,024 \) pixel resolution. All morphometric measurements were made with Metamorph image analysis software (Universal Imaging Corporation). Fluorescence intensity of staining of endogenous or transfected Shank or GluR2 was determined with short exposures and requests for materials should be addressed to M.S. (msheng@mit.edu) or Correspondence and nucleoprotein (NP) vectors. Protection was highly effective against Fc–NTD effect on virus haemorrhagic fever in non-human primates

**Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates**

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**Containment of highly lethal Ebola virus outbreaks poses a serious public health challenge. Although an experimental vaccine has successfully protected non-human primates against disease\(^1\), more than six months was required to complete the immunizations, making it impractical to limit an acute epidemic. Here, we report the development of accelerated vaccination against Ebola virus in non-human primates. The antibody response to vaccination with an adeno-associated (ADV) vector encoding the Ebola glycoprotein (GP) was induced more rapidly than with DNA priming and ADV boosting, but it was of lower magnitude. To determine whether this earlier immune response could nonetheless protect against disease, cynomolgus macaques were challenged with Ebola virus after vaccination with ADV–GP and nucleoprotein (NP) vectors. Protection was highly effective and correlated with the generation of Ebola-specific CD8\(^+\) T-cell immunity.**
and antibody responses. Even when animals were immunized once with ADV–GP/NP and challenged 28 days later, they remained resistant to challenge with either low or high doses of virus. This accelerated vaccine provides an intervention that may help to limit the epidemic spread of Ebola, and is applicable to other viruses.

Mice were immunized with plasmid DNA encoding Ebola GP, the trimeric virion-associated glycoprotein involved in cellular pathogenicity, followed by boosting with ADV–GP, or with ADV–GP only. The antibody response, a surrogate for protection, was measured using an enzyme-linked immunosorbent assay (ELISA). After DNA vaccination, titres were modest but increased 100- to 1,000-fold with ADV–GP boosting (Fig. 1a). In contrast, vaccination with ADV–GP gave rise to a lower antibody titre, but it was generated more rapidly. To investigate whether immunization with adenoviral vectors alone might protect against Ebola virus infection, alternative immunization schedules in macaques were developed for comparison to the previous DNA/ADV protocol (Fig. 1b, middle and bottom panels compared with top panel).

Cynomolgus macaques were immunized with ADV–GP and ADV–NP, followed by boosting 9 weeks later (Fig. 1b, middle panel). One week after the boost, animals were challenged with either a low (13 plaque-forming units (PFUs)) or high (1,500 PFUs) dose of a 1995 isolate of Ebola virus Zaire. These doses were uniformly fatal 6–12 days afterwards in saline-injected control animals. In contrast, the ADV–GP/NP immunized monkeys (n = 4) were completely protected, confirmed by viral load (Fig. 2). Analysis of the cell-mediated and humoral immune responses revealed significant increases in the CD8+ T-cell response to Ebola antigens by intracellular cytokine staining for interferon (IFN)–γ, seen before exposure to virus, in contrast to control animals where no response was seen (Fig. 3a). Similarly, antibody titres to the virus were stimulated in vaccinated animals, which minimally increased after the viral challenge (Fig. 3b). No substantial increases were observed in the numbers of Ebola-specific CD4+ T cells at this time (data not shown). Both CD8+ cellular and humoral immune responses therefore were associated with protection.

A second adenoviral immunization did not substantially increase the Ebola-specific immune responses (data not shown), raising the notion that the primary immunization was sufficient to confer protection. To address this possibility, a single immunization was given, and animals were challenged one month afterwards (Fig. 1b, bottom panel). Both at low and high viral challenge doses, animals were completely protected against infection (Fig. 4a). In this case, changes in the intracellular IFN–γ response in T lymphocytes were not consistently seen (data not shown); however, Ebola-specific T-cell responses were detected with intracellular tumour-necrosis factor (TNF)-α. CD8 responses were observed before challenge or were induced soon thereafter in five of eight animals, once again correlating with protection against infection (Fig. 4b, right). In contrast, CD4+ responses, not detectable before inoculation, increased after challenge (Fig. 4b, left). Immunoglobulin-γ (IgG) antibody titres, readily detected at the time of inoculation, were also associated with protection (Fig. 4c). These data demonstrated that a single ADV–GP/NP injection can accelerate vaccine protec-

Figure 1. Comparison of the Ebola-specific antibody responses by heterologous DNA/ADV primer–boost or ADV primer–boost vaccination in mice. a. The time course of Ebola-specific antibody responses by DNA prime and adenovirus boost compared with adenoviral immunization alone is shown (see Methods). Data represent the relative ELISA titre to Ebola GP after immunization with DNA/ADV–GP or ADV–GP/ADV–GP in BALB/c mice using a log scale. b. Immunization schedule for previously used heterologous prime–boost vaccine (top), adenoviral prime and boost (middle), and single adenoviral virus (bottom) immunizations. Challenge was performed with a 1995 isolate of Ebola virus (Zaire) at 32, 10 or 4 weeks after the initial immunization, respectively.

Figure 2. Protection against lethal challenge in non-human primates using adenoviral priming and boosting. Plasma viraemia in monkeys after infection with Ebola virus. Asterisks represent the time of death in control animals. The data represent the reciprocal endpoint dilution of serum for each monkey. Results are shown for four immunized animals challenged with Ebola Zaire at 13 PFUs (low dose; filled symbols, left), four immunized animals challenged at 1,500 PFUs (high dose; filled symbols, right), and five saline-injected control animals (open symbols).
tion and long-term survival against Ebola in non-human primates (Fig. 4d).

Ebola virus infection is characterized by its rapid onset, high person-to-person transmissibility, and significant mortality rate. The mainstay of treatment has been supportive therapy, and prevention has been dependent on containment using barrier precautions. Effective protection was achieved previously in primates with a heterologous DNA prime and adenoviral boost strategy. The prime–boost immunization relies on the ability of the adenoviral boost to expand the primary T-cell response induced by DNA vaccination. When animals are primed with ADV vectors alone, a robust Ebola-virus-specific cellular and humoral immune response is more rapidly achieved, although the response to a second ADV–GP/NP injection is blunted, probably because of anti-vector immunity. Here, we explored the possibility that this more rapid initial immune response may nonetheless confer protection and outweigh the stronger immune response that requires additional time. A single immunization with an adenoviral vector encoding Ebola virus proteins is sufficient to confer protection against lethal challenge within four weeks, and this response correlates with both cellular and humoral immune responses to the infection.

Although antibody titres correlated here with the protective response, previous studies in non-human primates have suggested that the passive transfer of antibody is insufficient to provide long-lasting protection against Ebola virus8. In rodent studies with adapted Ebola virus, passive transfer of antibodies9,10 or adoptive transfer of cytotoxic T cells11 showed protection when given before infection. A more sensitive but less quantitative CD4 lymphoproliferative response correlated with protection in the previous DNA/ADV prime–boost study, in which CD8 responses were not measured1. In addition to the antibody response induced by the
Intracellular cytokine analysis

Peripheral blood mononuclear cells were isolated from cynomolgus macaque whole-blood samples by separation over Ficoll. Approximately 1×10^7 cells were stimulated in 200 μl RPMI medium (GIBCO) for 6 h at 37°C with anti-CD3ε and anti-CD49d antibodies and either DMSO or a pool of 15-nucleotide peptides spanning the Ebola GP Zaire (Mayinga strain) open reading frame. The peptides were 15 nucleotides overlapping by 11 spanning the entire Ebola glycoprotein at a final concentration of 2 μg ml^-1. Cells were fixed and permeabilized with FACS lye (Becton Dickinson) supplemented with Tween-20, and stained with a mixture of antibodies against lineage markers (CD3-PE, CD4-PerCP, CD8-FITC) and either TNF-α-APC or IFN-γ-APC. Samples were run on a FACS Calibur and analysed using the software FlowJo. Positive gating for lymphocytes using forward versus side scatter was followed by CD3^+CD8^- and CD3^+CD4^- gating, and specific populations were further defined by anti-CD4 and anti-CD8 positivity, respectively. Cytokine-positive cells were defined as a percentage within these individual lymphocyte subsets, and at least 200,000 events were analysed for each sample.

Macaque immunization

In conducting this research, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Cynomolgus macaques were injected intramuscularly at the indicated times (Fig. 1b) with an equal mixture of 2×10^10 particles of ADV-GP and ADV-NP. Viral challenge was performed by inoculation of animals in the left or right caudal thigh with 0.5 ml of viral stock that contained a target dose of either 10 or about 1,000 PFUs of Ebola virus (Zaire species) at ten weeks (Fig. 2) or four weeks (Fig. 4) after the initial immunization, and actual titre was confirmed by plating. No adverse effects of the adenovirus vaccination were observed acutely. The Ebola virus used in this study was originally obtained from a fatally infected human from the former Zaire in 1995 (ref. 17). Collection of serum and blood for viral load and ELISA titres was performed as previously described.

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Methods

Vector construction

ADV-GP and ADV–NP were prepared as described previously14. The recombinant adenoviral vector was made according to previously published methods14. A dose of 1×10^8 (mice) or 1×10^9 (non-human primates) adenoviral vector particles for each component was administered to each animal without adverse effects.

Animal study and safety

Twenty cynomolgus macaques (Macaca fascicularis), 3 yr old and weighing 2–3 kg, obtained from Covance, were used for immunization and challenge experiments. The monkeys, housed singly, were anaesthetized with ketamine to obtain blood specimens and to administer vaccines. They received regular enrichment according to the Guide for the Care and Use of Laboratory Animals (DHEW number NIH 86-23). Before Ebola virus challenge and to the end of each experiment, the animals were maintained in the Maximum Containment Laboratory (BSL-4) and fed and checked daily.

Mouse immunization

DNA and adenoviral vectors expressing Ebola Zaire glycoprotein (Mayinga strain) were constructed as described previously15–17 with gene expression under control of the cytomegalovirus enhancer and promoter in the plasmid. Mice (n = 10 per group) were immunized intramuscularly with 100 μg DNA (pGP) and/or 1×10^10 particles of adenovirus (ADV–GP). DNA vaccination was performed on days 0, 14 and 24 with adenoviral infection on days 0 and 42, and samples were collected for ELISA titres at the indicated times. ELISA IgG titres were determined using 96-well plates as previously described14, and specific antigen binding was detected using a goat anti-human IgG (H + L)-horseradish conjugate and ABTS/peroxide (substrate/indicator).

ELISA

Polyvinyl chloride ELISA plates (Dynatech) were coated with 50 μl antigen per well and incubated overnight at 4°C. All further incubations were carried out at room temperature. The antigen used was purified Ebola virus (about 1 mg ml^-1 total protein) inactivated by gamma irradiation. Plates were then washed five times with PBS containing Tween-20. Test sera were diluted in half-log concentrations from 1:31.6 to 1:100,000 and allowed to react with the antigen-coated wells for 60 min. After washing plates five times, goat anti-mouse IgG (whole molecule; ICN Biomedicals) conjugated to horseradish peroxidase was used as a detection antibody. Bound IgG was detected by 2.2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt and the optical density was determined. A panel of normal serum was run each time the assay was performed. A cut-off value for a positive result was calculated as the mean optical density (at a 1:100 dilution) for the normal sera plus 3 standard deviations.