

Chimpanzee adenovirus vaccine generates acute and durable protective immunity against ebolavirus challenge

Daphne A Stanley¹, Anna N Honko^{2,7}, Clement Asiedu^{1,8}, John C Trefry², Annie W Lau-Kilby¹, Joshua C Johnson^{2,7}, Lisa Hensley^{2,7}, Virginia Ammendola³, Adele Abbate³, Fabiana Grazioli³, Kathryn E Foulds¹, Cheng Cheng¹, Lingshu Wang¹, Mitzi M Donaldson¹, Stefano Colloca³, Antonella Folgore³, Mario Roederer¹, Gary J Nabel^{1,7}, John Mascola¹, Alfredo Nicosia³⁻⁵, Riccardo Cortese⁶, Richard A Koup¹ & Nancy J Sullivan¹

Ebolavirus disease causes high mortality, and the current outbreak has spread unabated through West Africa. Human adenovirus type 5 vectors (rAd5) encoding ebolavirus glycoprotein (GP) generate protective immunity against acute lethal Zaire ebolavirus (EBOV) challenge in macaques, but fail to protect animals immune to Ad5, suggesting natural Ad5 exposure may limit vaccine efficacy in humans. Here we show that a chimpanzee-derived replication-defective adenovirus (ChAd) vaccine also rapidly induced uniform protection against acute lethal EBOV challenge in macaques. Because protection waned over several months, we boosted ChAd3 with modified vaccinia Ankara (MVA) and generated, for the first time, durable protection against lethal EBOV challenge.

Human-derived rAd vectors have undergone extensive development in preclinical and human trials as vaccines for multiple pathogens¹⁻⁶. For ebolavirus, rAd vaccines that confer protection against acute EBOV challenge generate effector CD8⁺ T cell responses within 3 weeks of immunization⁷ and therefore have the potential to provide rapid immunity in an acute human outbreak setting. rAd5 EBOV vaccines protect against acute EBOV challenge (4 weeks after vaccination), whereas EBOV vaccines using alternative human adenoviruses do not⁸, perhaps owing to differences in antigen expression⁶ or target cell receptor preference⁹.

However, human-derived rAd vectors are limited by preexisting immunity to the vectors. Adenoviruses isolated from nonhuman sources

including chimpanzees and apes¹⁰ may overcome this limitation, and they have shown promise for EBOV protection, but only against a modified virus in mice¹¹. ChAds have been evaluated as vaccine vectors in mice^{6,10}, nonhuman primates (NHPs)¹² and human clinical trials^{5,13,14}, have low worldwide seroprevalance^{6,10} and are not cross-neutralized by human anti-adenovirus sera⁶. Here we evaluated monovalent (EBOV GP) or bivalent (EBOV plus Sudan ebolavirus (SUDV) GPs) ChAd- and MVA-vectored ebolavirus vaccines for immunogenicity, protection against acute EBOV challenge and durable protection using single-inoculation and prime-boost approaches.

We identified ChAd3 and ChAd63 as potential candidate vaccine vectors on the basis of their low neutralization by human sera, genetic distance from poor-efficacy alternative vectors⁸ and lack of replication in the vaccine recipient (a potential drawback of replicating vaccine vectors such as vesicular stomatitis virus (VSV))¹⁵, as well as the protection conferred by a ChAd63 vaccine against malaria¹². We evaluated a replication-defective ChAd3-vectored vaccine for protection against EBOV at vaccine doses previously shown to be uniformly protective using rAd5 (ref. 16). We immunized groups of four cynomolgous macaques (here called simply macaques) intramuscularly (i.m.) with a single inoculation of 1×10^{11} or 1×10^{10} viral particles (PU) of ChAd3 encoding EBOV GP and then challenged them i.m. with a lethal dose (1,000 PFU) of EBOV 5 weeks later. All vaccinated macaques survived the infection (Fig. 1a) with no detectable viremia (Fig. 1b), unlike unvaccinated control macaques, which succumbed to infection by day 6 and exhibited viral loads exceeding 1×10^7 PFU equivalents per milliliter in plasma (Fig. 1b). These data demonstrate that ChAd vectors generate acute protective EBOV immunity in macaques.

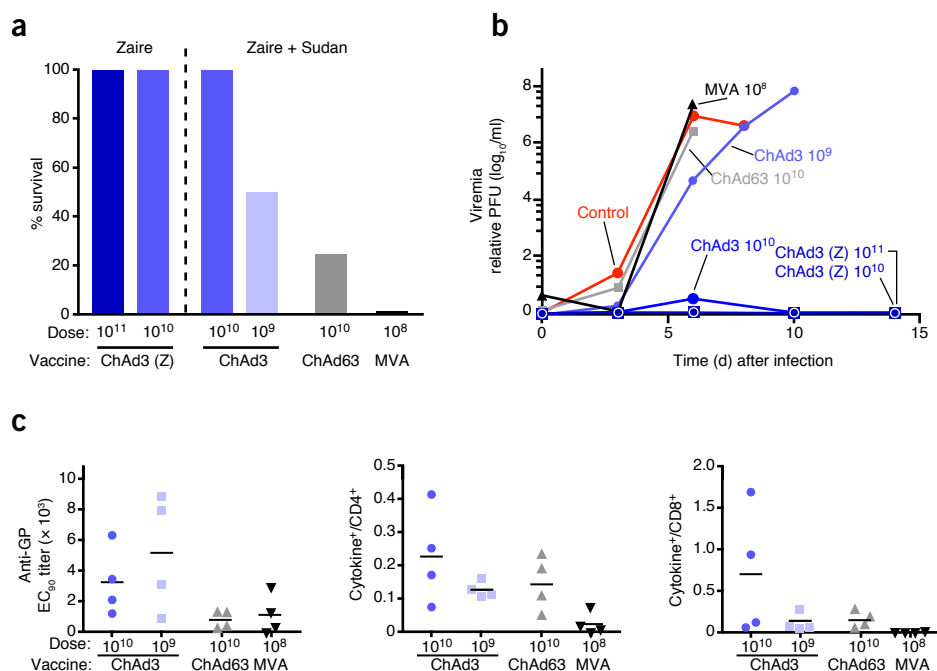
EBOV and SUDV are responsible for more deaths than any of the other three ebolavirus species, Bundibugyo (BDBV), Reston (RESTV) and Tai Forest (TAFV). The addition of SUDV GP to the vaccine would be advantageous in a natural outbreak setting, where the identity of the infecting species may not be established until days or weeks after the index case. To test for potential dilution effects from inclusion of an additional GP vector, we immunized four macaques with 1×10^{10} PU each of ChAd3 vectors encoding GPs from EBOV and SUDV, measured immune responses 3 weeks later and then challenged them i.m. with a lethal dose of EBOV 5 weeks after vaccination. As seen with the monovalent EBOV vaccine, the bivalent vaccine containing both GPs (Fig. 1a,b, ChAd3) protected the macaques from infection demonstrating that inclusion of an additional GP species did not interfere with the protection from EBOV observed with the monovalent vaccine.

To explore the relative potency of ChAd3 and ChAd63 compared to rAd5 vaccine vectors, we examined whether infection breakthrough occurs in the same vaccine dose range reported for the rAd5 vaccine¹⁶. In four macaques immunized with 1×10^9 PU of each ChAd3

¹Vaccine Research Center, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, Maryland, USA. ²United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, USA. ³Okairos, Rome, Italy. ⁴Centro Ingegneria Genetica (CEINGE), Naples, Italy. ⁵Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Naples, Italy. ⁶Keires AG, Basel, Switzerland. ⁷Present addresses: Integrated Research Facility, NIAID, NIH, Fort Detrick, Maryland, USA (A.N.H., J.C.J. and L.H.), and Sanofi, Cambridge, Massachusetts, USA (G.J.N.). ⁸Deceased. Correspondence should be addressed to N.J.S. (njsull@mail.nih.gov).

BRIEF COMMUNICATIONS

Figure 1 Macaque survival, plasma viremia and immune responses for acute studies. Macaques ($n = 4$ per group) were immunized with indicated doses of ChAd3 encoding GP from EBOV (ChAd3 (Z)) or vectors encoding GP from both EBOV and SUDV (ChAd3, ChAd63 or MVA) or were unvaccinated (control). Five weeks after vaccination, macaques were infected with a lethal dose (1,000 PFU) of EBOV. **(a)** Survival. **(b)** Average viremia per vaccine group in **a**, assessed by real-time quantitative PCR, represented as relative PFU. Time points are represented as days after infection. For ChAd3 1×10^9 and ChAd63, viremia is shown only for the nonsurvivors. **(c)** Three weeks after vaccination with vectors (ChAd3, ChAd63 or MVA) encoding GP from EBOV and SUDV, GP-specific antibodies in plasma were measured by indirect antigen-capture ELISA (left; antibody titers are shown as individual animals in each group, with horizontal line indicating group mean); $n = 4$ per group; EC_{90} , 90% effective concentration as defined in the Online Methods. $CD4^+$ (middle) or $CD8^+$ (right) peripheral blood T cell responses are shown as a percentage of the memory population that produced TNF, IFN- γ or IL-2 by intracellular cytokine staining after stimulation with peptide pools overlapping the entire GP protein. Horizontal lines indicate group mean, and $n = 4$ per group. $CD4^+$ or $CD8^+$ T cell memory is defined using cell surface staining for CD95, CD28 and CD45RA markers as defined in the Online Methods. Graphs enumerating T cell responses represent T cells producing any of the three cytokines.



vector (encoding GPs from EBOV and SUDV), we measured immune responses 3 weeks after vaccination and then challenged the animals with EBOV 5 weeks after vaccination. Two of four vaccinated macaques survived with no detectable viremia, a protection rate matching that for rAd5, and two nonsurvivors developed viremia at levels comparable to the unvaccinated controls (**Fig. 1a,b**). ChAd3 at 1×10^{10} PU and 1×10^9 PU induced comparable titers of GP-specific antibodies (**Fig. 1c**), but 1×10^{10} PU resulted in a higher frequency of peripheral blood T cells secreting tumor necrosis factor (TNF), interferon- γ (IFN- γ) or interleukin-2 (IL-2) after GP stimulation (**Fig. 1c**), although these studies were not powered to detect statistically significant differences in immune responses. Unlike the acute protection observed with 1×10^{10} ChAd3, all ChAd63-vaccinated animals had detectable viremia by day 6 after infection (**Fig. 1b**), and only one animal survived infection (**Fig. 1a**). The survivor controlled viremia by day 8 after infection (data not shown). All ChAd63-vaccinated animals displayed antigen-specific immunologic responses (**Fig. 1c**), but these trended lower than those induced by the ChAd3 vaccine. Lastly, we examined the poxvirus vector, MVA, for protection against EBOV. Macaques received a single i.m. inoculation of 1×10^8 PFU MVA (encoding GPs from EBOV and SUDV). We measured immune responses 3 weeks after vaccination and gave the animals a lethal challenge of EBOV 2 weeks later. All vaccinated animals had detectable viremia by day 6 after infection (**Fig. 1b**), and none survived infection (**Fig. 1a**). Although the animals displayed antigen-specific antibody responses, like those induced by ChAd63, these responses were also lower than those induced by ChAd3 (**Fig. 1c**).

The lack of protection by ChAd63 was an unexpected result given the reported protection using this vector against malaria¹², so we vaccinated larger groups of macaques with ChAd3 ($n = 15$) or ChAd63 ($n = 14$) to increase statistical power to discern any differences in the antigen-specific antibody and T cell responses induced by these vaccines. The trends exhibited in the challenge studies toward higher humoral and

cellular immune responses with the ChAd3 vaccine were maintained; for example, the average anti-GP antibody titers in plasma were eightfold higher in ChAd3-vaccinated macaques ($P < 0.0001$ using the Mann-Whitney test) than in ChAd63-vaccinated animals (**Supplementary Fig. 1a**). This observation is consistent with the antibody correlate of immune protection for rAd-based EBOV vaccines¹⁷. The average frequency of antigen-specific T cells was also superior in the ChAd3 vaccine group, with the greatest difference observed in $CD8^+$ T cells (2.5-fold higher in ChAd3-vaccinated macaques as compared to ChAd63-vaccinated macaques, $P = 0.04$ using the Mann-Whitney test).

T cell quality influences vaccine performance¹⁸, so we interrogated the cytokine secretion patterns among antigen-specific T lymphocyte subsets in peripheral blood mononuclear cells. We defined populations of T lymphocytes producing any combination of IFN- γ , IL-2 or TNF at the single-cell level and calculated the relative proportions of these populations, each of which represents a distinct functional T cell subset. Whereas $CD8^+$ T lymphocytes simultaneously secreting TNF and IFN- γ have enhanced cytolytic activity¹⁹ and are induced by protective

Table 1 Durable vaccine protection against EBOV

Vector	Dose (PU)		Protection ^a
Single shot			
ChAd3	1 × 10 ¹¹	2/4	50%
ChAd3	1 × 10 ¹⁰	0/4	0%
Prime-boost			
ChAd3/ChAd3	1 × 10 ¹⁰ /1 × 10 ¹⁰	1/3	33%
ChAd3/ChAd63	1 × 10 ¹⁰ /1 × 10 ¹⁰	1/4	25%
ChAd3/MVA	1 × 10 ¹⁰ /1 × 10 ⁸	4/4	100%

Animals in single-shot groups were vaccinated with ChAd3 at the doses indicated and exposed to a lethal dose of EBOV 10 months after the prime vaccination. Animals in prime-boost groups were primed with ChAd3, boosted 8 weeks later and exposed to a lethal dose of EBOV as in the single-shot groups.

^aProtection is shown as the number surviving/total challenged and as a survival percentage.

rAd-based EBOV vaccines²⁰, T cells simultaneously producing TNF, IL-2 and IFN- γ are associated with immunologic memory²¹. ChAd3 and ChAd63 bivalent vaccines resulted in a similar proportional representation of antigen-specific T cells producing one, two or three cytokines (**Supplementary Fig. 1b**). However, the magnitude of each subset was higher for ChAd3 than ChAd63 (**Supplementary Fig. 1b**), suggesting that T cell magnitude rather than quality drives differences in acute EBOV protection for these vaccine vectors.

Next, we investigated whether ChAd3 could provide durable immunologic protection against EBOV challenge when the vaccine was administered as a single shot. Although durable protection for Marburg virus has been shown with a replicating vector (VSV) vaccine¹⁵, the

requirements for vaccine protection against this virus are less stringent than for EBOV²², and no vaccine has been shown to elicit durable protection from EBOV in NHPs. We inoculated the macaques i.m. with 1×10^{11} or 1×10^{10} PU ChAd3 vaccine and exposed them to a lethal dose of EBOV 10 months later. Even with this extended interval between vaccination and challenge, 1×10^{11} PU ChAd3 provided protection in half of the vaccinated macaques (**Table 1**). The lowest vaccine dose that was uniformly protective in the acute vaccine-challenge regimen, 1×10^{10} PU, failed to elicit durable protection. In all vaccinated animals, acute humoral and cellular responses declined over time (**Fig. 2a**). But the macaques receiving 1×10^{11} PU ChAd3 exhibited a trend toward higher antigen-specific antibody titers and memory CD8⁺ T cell frequencies

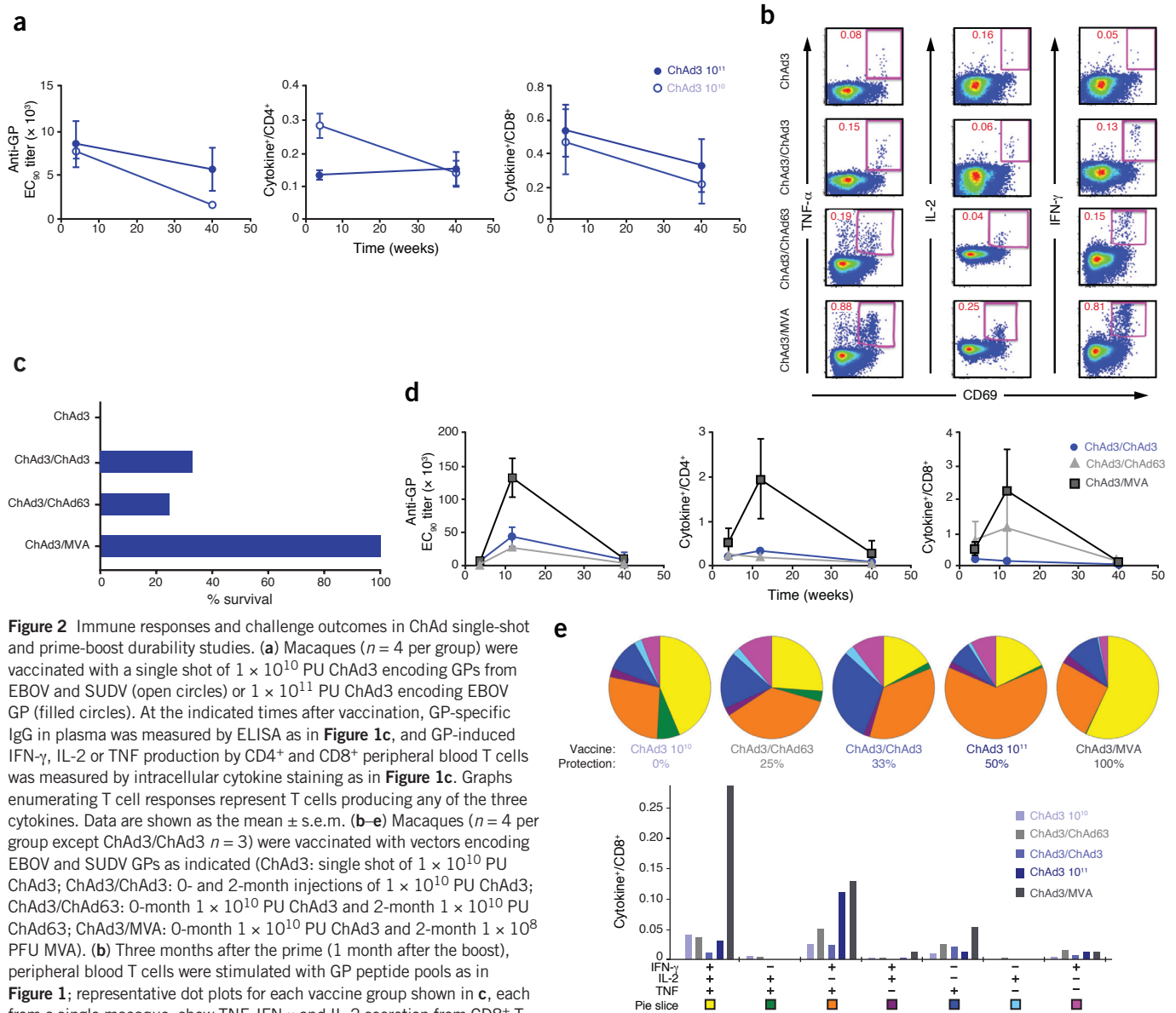


Figure 2 Immune responses and challenge outcomes in ChAd single-shot and prime-boost durability studies. **(a)** Macaques ($n = 4$ per group) were vaccinated with a single shot of 1×10^{10} PU ChAd3 encoding GPs from EBOV and SUDV (open circles) or 1×10^{11} PU ChAd3 encoding EBOV GP (filled circles). At the indicated times after vaccination, GP-specific IgG in plasma was measured by ELISA as in **Figure 1c**, and GP-induced IFN- γ , IL-2 or TNF production by CD4⁺ and CD8⁺ peripheral blood T cells was measured by intracellular cytokine staining as in **Figure 1c**. Graphs enumerating T cell responses represent T cells producing any of the three cytokines. Data are shown as the mean $\pm$ s.e.m. **(b–e)** Macaques ($n = 4$ per group except ChAd3/ChAd3 $n = 3$) were vaccinated with vectors encoding EBOV and SUDV GPs as indicated (ChAd3: single shot of 1×10^{10} PU ChAd3; ChAd3/ChAd3: 0- and 2-month injections of 1×10^{10} PU ChAd3; ChAd3/ChAd63: 0-month 1×10^{10} PU ChAd3 and 2-month 1×10^{10} PU ChAd63; ChAd3/MVA: 0-month 1×10^{10} PU ChAd3 and 2-month 1×10^8 PFU MVA). **(b)** Three months after the prime (1 month after the boost), peripheral blood T cells were stimulated with GP peptide pools as in **Figure 1**; representative dot plots for each vaccine group shown in **c**, each from a single macaque, show TNF, IFN- γ and IL-2 secretion from CD8⁺ T cells. **(c)** Ten months after the prime, macaques were administered a lethal dose (1,000 PFU) of EBOV. Graph indicates percentage of surviving animals. **(d)** At the indicated times after vaccination, GP-specific IgG in plasma was measured by ELISA, and GP-induced IFN- γ , IL-2 and TNF production by CD4⁺ and CD8⁺ peripheral blood T cells was measured by intracellular cytokine staining. Graphs enumerating T cell responses include T cells producing one or any combination of those three cytokines. Data are shown as the mean $\pm$ s.e.m. **(e)** Qualitative profiles of antigen-specific T cell responses in peripheral blood 1 week before EBOV challenge. Program with Integrated Circuit Emphasis (SPICE), a data-mining software application used to analyze flow cytometry data sets, was used to define T cell populations producing any combination of TNF, IFN- γ or IL-2 at the single-cell level. The pie slices depict as averages for each vaccine group the relative proportions of each population (color defined below the bar graph) as a fraction of CD4⁺ or CD8⁺ T memory cells, and the bar graph shows the magnitude each T cell subset.

than those receiving the 1×10^{10} PU dose; the frequency of antigen-specific CD4⁺ T cells was similar in both groups.

For EBOV⁸ and other pathogens¹⁸, prime-boost vaccination elicits more potent immune responses than single-shot immunization. For example, prime-boost vaccinations elicit a higher frequency of the CD8⁺ T cells¹⁸ needed for rAd5 vector vaccine-mediated acute protection against EBOV⁷. To determine whether we could achieve complete long-term protective immunity with a prime-boost vaccine, we evaluated MVA and ChAd vectors for their capacity to boost immunological memory in ChAd3-primed macaques. We first tested whether ChAd3 could boost responses in a homologous prime-boost regimen (ChAd3/ChAd3). We primed macaques with 1×10^{10} PU ChAd3 and boosted with the same vaccine 8 weeks later. When exposed to infectious EBOV 10 months later, only one of three animals was protected (**Table 1** and **Fig. 2c**). Therefore, we compared boosting of ChAd3-primed responses with ChAd63 expressing EBOV and SUDV GPs to boosting with MVA expressing both GPs, as heterologous combinations boost more efficiently than homologous combinations^{1,3}. We performed the ChAd3/ChAd63 and ChAd3/MVA vaccination and challenge using the same schedule and doses as the homologous regimen, except for MVA administered at a dose of 1×10^8 PFU. ChAd3/ChAd63 protected only one animal, but MVA boosting of ChAd3 provided uniform protection in this group (**Table 1** and **Fig. 2c**).

To explore the basis for the superior protection achieved with ChAd3/MVA, we evaluated peripheral blood CD8⁺ T cell responses 3 months after the priming vaccination. ChAd3/MVA (1×10^{10} PU/ 1×10^8 PFU) vaccine recipients contained the highest frequency of CD8⁺ T cells producing TNF, IL-2 or IFN- γ compared to all other ChAd3-primed groups (**Fig. 2b**). GP-specific antibody and CD4⁺ T cell responses were also highest in ChAd3/MVA-vaccinated macaques after 3 months (**Fig. 2d**). However, although we detected differences in humoral and cellular immune responses between ChAd3/MVA and nonprotective vaccines shortly after the booster vaccine, we observed only small differences at the time of challenge 10 months after vaccination (**Fig. 2d**, week 40).

To determine the memory cellular responses required for durable protection, we examined the functional quality of CD8⁺ T cells just before EBOV exposure. At the time of infectious challenge (10 months after vaccination), macaques vaccinated with 1×10^{10} PU ChAd3 exhibited an increase in the proportional representation of polyfunctional triple-cytokine-producing cells (**Fig. 2e**) compared to responses observed 4 weeks after vaccination (**Supplementary Fig. 1b**), and this resulted in a reduction in the relative proportion of effector CD8⁺ T cells (coproducing TNF and IFN- γ) and a loss of protection (**Fig. 2c**). Increasing the vaccine dose to 1×10^{11} PU preserved the frequency and proportion of effector TNF and IFN- γ coproducers and yielded higher vaccine protection (50%) (**Fig. 2c,e**). Vaccination with ChAd3/ChAd3 and ChAd3/ChAd63 (1×10^{10} PU of each vector) generated a CD8⁺ T cell quality between that achieved with 1×10^{10} and 1×10^{11} PU ChAd3 and resulted in intermediate protection (**Figs. 1a** and **2e**). Only vaccination with ChAd3/MVA (1×10^{10} PU/ 1×10^8 PFU) provided uniform protection, and this was associated with TNF- and IFN- γ -coproducing effector cells whose proportional representation of the antigen-specific CD8⁺ T cell response was lower but whose absolute frequency in the total CD8⁺ T cell memory pool was higher and matched the absolute frequency for this population achieved with 1×10^{11} PU ChAd3. The response to ChAd3/MVA was clearly differentiated from that achieved with 1×10^{11} PU ChAd3 and other less protective vaccines by the combination of high-frequency TNF and IFN- γ coproducers combined with a fivefold higher level of triple-cytokine-producing CD8⁺ T cells (**Fig. 2e**).

We have demonstrated for the first time, to our knowledge, that both acute and durable immunity against EBOV can be achieved using a single inoculation (partial protection) or a prime-boost vaccine regimen (uniform

protection). This vaccine will be beneficial for populations at acute risk during natural outbreaks or others with a potential risk of occupational exposure. Although additional work is needed to fully unravel the protective mechanisms associated with these vaccine regimens, ChAd3-mediated acute protection associated strongly with antibody responses, consistent with the established immune correlate for an rAd5 EBOV vaccine¹⁷. Long-term protection required generation of both effector and memory CD8⁺ T cell response cytokine qualities, suggesting that when the initial CD8⁺ T cell effector response has fully contracted, central memory cells are needed to reestablish the population needed for virus clearance. Rapid mobilization of effector cells is probably required owing to the aggressive replication of this virus, which targets all tissues, and whose pathogenic mechanism involves destruction of host lymphocytes.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank M. Cichanowski for graphics, A. Tislerics and B. Hartman for assistance with the manuscript and R. Seder for review and helpful suggestions. S. Peretto and S. Norris and D. Follmann for technical discussions, and the Vaccine Research Center's Nonhuman Primate Immunogenicity Core for NHP sample processing. We thank the Vaccine Research Center Laboratory Animal Medicine, S. Rao, A. Taylor, J.P. Todd and H. Bao for protocol support and the NIH Division of Veterinary Resources for animal care. We also thank H. Esham for technical assistance and data management and D. Alves for pathology assistance. TPG shuttle vector was provided by A. Siccardi (Istituto San Raffaele). This work was supported by the Intramural Research Program of the US NIH NIAID Vaccine Research Center. Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the US Army or the US Department of Defense.

AUTHOR CONTRIBUTIONS

D.A.S., C.A., G.J.N., J.M., R.A.K. and N.J.S. designed these studies. D.A.S., A.N.H., C.A., J.C.T. and N.J.S. wrote animal study protocols and executed *in vivo* studies. D.A.S., C.A., A.W.L.-K., K.E.F., M.M.D. and M.R. conducted immune assessments. A.N.H., J.C.T., J.C.J. and L.H. executed challenge studies and performed post-challenge assays. V.A., A.A., F.G., C.C. and L.W. constructed, produced and characterized vectors. S.C., A.F., A.N. and R.C. identified chimp adenoviruses.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the [online version of the paper](#).

Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

- Sullivan, N.J., Sanchez, A., Rollin, P.E., Yang, Z.Y. & Nabel, G.J. *Nature* **408**, 605–609 (2000).
- Shiver, J.W. *et al.* *Nature* **415**, 331–335 (2002).
- Brown, S.A. *et al.* *Viruses* **2**, 435–467 (2010).
- Ledgerwood, J.E. *et al.* *Vaccine* **29**, 304–313 (2010).
- Barnes, E. *et al.* *Sci. Transl. Med.* **4**, 115ra1 (2012).
- Quinn, K.M. *et al.* *J. Immunol.* **190**, 2720–2735 (2013).
- Sullivan, N.J. *et al.* *Nat. Med.* **17**, 1128–1131 (2011).
- Geisbert, T.W. *et al.* *J. Virol.* **85**, 4222–4233 (2011).
- Barouch, D.H. *et al.* *J. Immunol.* **172**, 6290–6297 (2004).
- Colloca, S. *et al.* *Sci. Transl. Med.* **4**, 115ra2 (2012).
- Kobinger, G.P. *et al.* *Virology* **346**, 394–401 (2006).
- Reyes-Sandoval, A. *et al.* *Infect. Immun.* **78**, 145–153 (2010).
- O'Hara, G.A. *et al.* *J. Infect. Dis.* **205**, 772–781 (2012).
- Sheehy, S.H. *et al.* *Mol. Ther.* **20**, 2355–2368 (2012).
- Mire, C.E. *et al.* *PLoS ONE* **9**, e94355 (2014).
- Sullivan, N.J. *et al.* *PLoS Med.* **3**, e177 (2006).
- Sullivan, N.J., Martin, J.E., Graham, B.S. & Nabel, G.J. *Nat. Rev. Microbiol.* **7**, 393–400 (2009).
- Seder, R.A., Darrah, P.A. & Roederer, M. *Nat. Rev. Immunol.* **8**, 247–258 (2008).
- Lichterfeld, M. *et al.* *Blood* **104**, 487–494 (2004).
- Hensley, L.E. *et al.* *PLoS Pathog.* **6**, e1000904 (2010).
- Darrah, P.A. *et al.* *Nat. Med.* **13**, 843–850 (2007).
- Geisbert, T.W. *et al.* *J. Virol.* **84**, 10386–10394 (2010).

ONLINE METHODS

Construction of ChAd3 and ChAd63 expressing EBOV or SUDV GP. Ebola GP—spanning open reading frames of Zaire and Sudan were codon modified for increased expression in primate cells as described previously²² and were modified by introducing two tetracycline operator binding sites within the HCMV promoter. To this end, ChAd3 vector expressing EBOV GP was constructed by two partially overlapping primers containing TetO sequences; forward primer (PBI for 5') -GATAGAGATCTCCCTATCAGTGATAGAGATCGTCGACGAGCTCGTT-TAGTGAACCGTCAGATC-3' and reverse primer (PBI rev 5') -GATAGGGAGATCTCTATCACTGATAGGGAGAGCTCTGCTTATATAGACCTCCCA-3' were designed around the HCMV promoter TATA box. PBI for was then combined with primers intA rev2 5'-ACGGTGACTGCAGAAAAGACCCATGG-3' annealing on the 3' end of intron A region, whereas the PBI reverse primer was combined with the primer 5'-CATTGCATACGTTGTATCCATATC-3' annealing at the 5' end in two separate PCR reactions. The two PCR products, encompassing the entire HCMV plus intronA, were digested with BglII, gel purified and fused by a standard ligation reaction. The DNA ligation product was then digested with SnaBI and SacII and cloned into EBOV GP open reading frame digested with SnaBI-SacII, thus generating EBOV-TetO. The EBOV GP expression cassette was excised by SfiI-BsrGI digestion and inserted into ChAd3 vector by homologous recombination as described²³. ChAd3 vector expressing SUDV GP was constructed by introducing two tetracycline operator binding sites (TetO) within the HCMV promoter. To this end, two partially overlapping primers containing TetO sequences (PBI for 5'-GATAGAGATCTCCCTATCAGTGATAGAGATCGTCGACGAGCTCGTTTAGTGAACCGTCAGATC-3' and PBI rev 5'-GATAGGGAGATCTCTATCACTGATAGGGAGAGCTCTGCTTATATAGACCTCCCA-3') were designed around the HCMV promoter TATA box. PBI for was then combined with intA rev2 5'-ACGGTGACTGCAGAAAAGACCCATGG-3' annealing on the 3' end of intron A region, whereas the PBI rev was combined with the primer 5'-CATTGCATACGTTGTATCCATATC-3' annealing at the 5' end of HCMV promoter in two separate PCR reactions. The two PCR products, encompassing the entire HCMV plus intronA, were digested with BglII, gel purified and fused by a standard ligation reaction. The DNA ligation product was then digested with SnaBI and SacI and cloned into the SUDV GP open reading frame digested with SnaBI-SacI, thus generating SUDV-TetO.

SUDV-TetO was cleaved with BsrGI and SfiI to excise a 3.75-kb fragment containing hCMV with TetO, SUDV GP coding region and BGH polyA sequences. The 3.75-kb, BsrGI and SfiI fragment was cloned into ChAd3 acceptor vector linearized with the restriction endonuclease SnaBI by homologous recombination as described^{10,24}.

ChAd63 vector expressing EBOV GP construction. pV1J TetO hCMV-bgh polyA, which contains hCMV promoter with TetO sequences and multiple cloning sites, was constructed by modifying pV1JNS (Invitrogen) with the insertion of two copies of TetO downstream of the HCMV TATA box. To this end, human CMV tetracycline-regulated promoter (TetO2) was amplified from pcDNA5/FRT/TO-TOPO plasmid (Invitrogen) with the following oligos: SnaBI for 5'-GTACATCTACGTATTAGTCATCGCTATTAC-3' and PmeI rev: 5'-CATAGAGGTTTAAACGGAAGATCTCACTCTTGGCACGGGGAATCCGCGTTCCAATGCA CCGTTCCTCCGCGCGGAGGCTGGATCGGTCCCGG-3'; the PCR product was then cut with SnaBI and PmeI, filled-in by Klenow and cloned into pV1JNS linearized by SnaBI/BglII digestion, finally obtaining pVJTetOhCMVbghpolyA. The human codon-optimized gene encoding the full length of Ebola GP Zaire was excised as a 2.3-kb EcoRV SphI fragment. The fragment was inserted into the EcoRV SphI site of pVJTetOhCMVbghpolyA to form shuttle vector pvj tetO hCMV hEB Z-bghpolyA. The shuttle vector pvj tetO hCMV hEB Z-bghpolyA was then digested with restriction endonucleases SpeI and SfiI to obtain a 3-kb fragment that contains a partial CMV promoter, the TetO sites, the EBOV GP gene and partial BGH poly A sequences. This 3-kb fragment was cloned into ChAd63 acceptor vector linearized with the restriction endonuclease SnaBI by homologous recombination as described^{13,24}.

ChAd63 vector expressing SUDV GP construction. The Sudan-TetO similarly constructed as above was digested with SpeI and SfiI to obtain a 3-kb fragment that contains partial CMV promoter, the TetO sites, the SUDV GP gene and the partial BGH polyA sequences. This 3-kb fragment was crossed over into ChAd63 acceptor vector linearized with the restriction endonuclease SnaBI by homologous recombination as described^{10,24}.

ChAd3 and ChAd63 EBOV GP rescue and amplification. To amplify both ChAd3 and ChAd63 EBOV GP vectors, 293 cells expressing tetracycline repressor were seeded at 5×10^6 cells in 6-cm cell culture dishes and transfected with 10 µg of either ChAd3 or ChAd63 EBOV GP vector DNA released from plasmid sequences by PmeI endonuclease digestion as previously described¹⁰. Transfected cells were collected 10 days post-transfection and lysed by freeze-thaw. Rescued EBOV vectors were then amplified by serial passage on Procell-92 cells. The vector was purified by standard CsCl gradient methods.

MVA vector generation. EBOV and SUDV genes were cloned under the control of P7.5 vaccinia virus early promoter in the TPG shuttle vector²³, kindly provided by A. Siccardi, Istituto San Raffaele, Milan, Italy. To this end, the EBOV GP coding region was amplified from the plasmid VRC 6001 by PCR using the following primers: EBOLA FOR1 (5'-GTCTGGGGATCCGCGACTTCGCGCCCGT CGTCGACACGTGTGAT-3') and EBOLA REV1 (5'-GGAGATCTGGCGCGCCTCAATTCGAGCTCGGTACCT-3'). The PCR product was then digested with AscI and BamHI and cloned into TPG plasmid, thus generating TPG- Ebola Zaire shuttle vector. The recombinant MVA expressing EBOV GP was obtained by *in vivo* recombination as previously described²³.

Animal immunization. Macaques were immunized intramuscularly (i.m.) in both deltoids. In **Figure 1**, 10^{11} ChAd3 contained the native-sequence EBOV GP insert (ChAd3(Z)), 10^{10} ChAd3 contained either the native (ChAd3(Z)) or codon-optimized GP sequence. ChAd63 contained codon-optimized GP and MVA reported in all figures contained native GP. MVA was always administered at a dose of 10^8 PFU. Doses of other vaccines were given as indicated in the figures and legends. In **Figure 2** prime-boost vaccines, and all ChAd groups in **Supplementary Figure 1**, ChAd3 and ChAd63 contained codon-optimized GP. MVA was constructed using the native GP sequence.

Infectious challenge with EBOV. Animal studies conducted at USAMRIID were approved by the USAMRIID Institutional Animal Care and Use Committee (IACUC). Animals were transferred 1 week before challenge to the Bio-Safety Level-4 (BSL-4) facility for exposure to a lethal (1,000 PFU) i.m. EBOV challenge. All acute challenges occurred 5 weeks post vaccination. In the ChAd3 10^{11} PU durability study, animals were challenged 43 weeks' post vaccination. ChAd3 10^{10} PU, ChAd3/ChAd3 and ChAd3/MVA studies were challenged at 39 weeks' post prime in three independent groups. All challenge studies include a single unvaccinated animal (control); the use of historical control ($n > 50$) allows for one unvaccinated control to be used in each challenge experiment. Experimental groups consist of four animals per group with the exception of the ChAd3/ChAd3 durability study ($n = 3$). One animal in this experimental group had a rectal prolapse during the vaccination period and was humanely euthanized at that time. While at USAMRIID, the monkeys were fed and checked daily. During the EBOV challenge study, blood was collected from the NHPs for hematological, biochemical and virological analyses. Following the development of clinical signs, animals were checked multiple times daily. Institute scoring criteria were used to determine timing of humane euthanasia under anesthesia to minimize pain and distress.

Animal study and safety. Research at the VRC was conducted under a protocol approved by the Vaccine Research Center IACUC—approved in compliance with the Animal Welfare Act, PHS Policy and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. Animal study protocols were approved by both the Vaccine Research Center and United States Army Medical Research Institute of Infectious Diseases IACUCs. All animals were Vietnamese-origin cynomolgous macaques (*Macaca fascicularis*), female, were approximately 3–5 years of age and were obtained from Covance. Animals were randomly assigned to vaccine groups based on sequential selection from a population inventory. Sample sizes of four animals per BSL4 infectious challenge group provide 85% power for a one-sided Fisher's exact test using $\alpha = 0.05$ with true survival rates of 0.98 versus 0.02. Prior to blood sampling or vaccination, animals were anesthetized with ketamine or telazol. The investigators were blinded to allocation during experiments and outcome assessment. Un-blinding was performed at the end of the challenge study.

Anti-EBOV GP IgG ELISA. Anti-EBOV GP IgG ELISA titers were measured as described previously⁸. ELISA titers are expressed as EC₉₀, reciprocal serum dilution values, which represent the dilution at which there is a 90% decrease in antigen binding.

Intracellular cytokine staining. Whole-blood samples were collected and peripheral blood mononuclear cells (PBMCs) were isolated as described previously⁸. PBMCs were stimulated for 6 h at 37 °C with or without co-stimulation (CD28 and CD49d) plus Brefeldin A (Sigma-Aldrich) and either dimethyl sulfoxide (DMSO) or a pool of peptides spanning the entire EBOV GP open reading frame. The peptides were 15-mers overlapping by 11 amino acids reconstituted in fresh sterile DMSO. Following peptide stimulation, PBMCs were stained with a mixture of antibodies against lineage markers using one of the two following antibody panels (given as protein the antibody is directed to followed by the fluorescent label). Panel 1 consisted of primary staining with CD4 Alexa 700 (clone RPA-T4), CD8 PerCP-Cy5.5 (clone SK1), CD28 PE-Cy7 (clone 28.2), CD95 Cy5PE (clone DX2), CD14 PB (M5E2), CD20 PB (clone L27), and the viability dye ViVid to allow discrimination between live and dead cells, followed by 2 washes. The cells were then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained with CD3 APC (clone SP34-2), TNF- α FITC (MAb11), IFN- γ APC (clone B27), IL-2 PE (clone MQ14H12). Panel 2 consisted of primary staining with CD4 QD605 (clone M-T477) or CD4 BV421 (clone OKT4), CD45RA CY7PE (clone L48), CD28 Cy5PE (clone CD28.2), and AquaViD a viability dye to allow for discrimination between live and dead cells, followed by 2 washes. The cells were then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained with CD8 PB (clone RPA-T8) or CD8 BV570 (clone RPA-T8), CD69 ECD (clone TP1.55.3), CD3 Cy7APC (clone SP34-2), IFN- γ APC (clone B27), IL-2 PE (clone MQ1-17H12), TNF- α FITC

(clone Mab11). Cytokine-positive cells were defined as a percentage within CD4⁺ and CD8⁺ T cell memory subsets. Memory subsets were defined as either CD45RA⁺CD95⁺, CD28⁺CD95⁺ or CD45RA⁺CD28⁺. Samples were acquired on an LSR II cytometer (BD Biosciences), collecting up to 1,000,000 events and analyzed using FlowJo (Tree Star, Inc.) and SPICE software²⁵ (http://www.niaid.nih.gov/LABSANDRESOURCES/RESOURCES/BIOINFORMATICS/SOFTWAREAPPLICATIONS/Pages/spice.aspx#niaid_inlineNav_Anchor). Boolean gating was used to define subsets of T cells expressing all combinations of IFN- γ , IL-2, and/or TNF- α .

Detection of EBOV. RNA was isolated from plasma of EBOV-exposed NHP by real-time qPCR as described previously²⁶. Primers and probe were specific for the EBOV GP gene (GenBank accession no. [AF086833](#)) (Forward, 1,000 nM: TTTTCA ATC CTC AAC CgT AAg gC; Reverse, 1,000 nM: CAg TCC ggT CCC AgA ATg Tg (Oligos Etc.); Probe, 100 nM: 6FAM - CAT gTg CCg CCC CAT CgC TgC - TAMRA (Applied Biosystems)). The absolute quantification was compared to a viral RNA standard curve using LC480 software (version 1.5.0.39) and a standard calibrator on each plate. The viral standard curve was created through serial dilution and extraction of virus stock with known PFU/mL to yield PFU equivalents (relative PFU).

Statistical analyses. Comparison of anti-GP ELISA IgG titers and intracellular cytokine production by T cell memory subsets were calculated using the Mann-Whitney test (GraphPad Prism software).

23. Di Lullo, G. *et al. J. Virol. Methods* **163**, 195–204 (2010).

24. Chartier, C. *et al. J. Virol.* **70**, 4805–4810 (1996).

25. Roederer, M., Nozzi, J.L. & Nason, M.C. *Cytometry A* **79**, 167–174 (2011).

26. Malhotra, S. *et al. PLoS Negl. Trop. Dis.* **7**, e2171 (2013).