Ubiquitination of TRAF6

We prepared whole-cell lysate from RAW 264.7 cells, 48 h after the transfection of HA-tagged ubiquitin cDNA. We added RANKL with or without 100 U ml^{-1}IFN-γ 5 h before protein extraction, and we added 50 μM MG132 1 h before protein extraction. Immunoprecipitates with an anti-TRAF6 polyclonal antibody (Santa Cruz) was first blotted with an anti-HA antibody (Boehringer). We blotted the same membrane with an anti-TRAF6 monoclonal antibody (Santa Cruz). For the detection of the endogenous ubiquitin in PA28$^{+/-}$ and PA28$^{-/-}$ mice, we performed immunoblotting with an anti-ubiquitin antibody (Novocastra).

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1. Roodman, G. D. Cell biology of the osteoclast. *Exp. Hematol.* **27,** 1229–1241 (1999).
2. Suda, T. *et al.* Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr. Rev.* **20,** 345–357 (1999).
3. Kong, Y. Y. *et al.* OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* **397,** 315–323 (1999).
4. Kong, Y. Y. *et al.* Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* **402,** 304–309 (1999).
5. Takayanagi, H. *et al.* Suppression of arthritic bone destruction by adenovirus-mediated csk gene transfer to synoviocytes and osteoclasts. *J. Clin. Invest.* **104,** 137–146 (1999).
6. Horwood, N. J. *et al.* Activated T lymphocytes support osteoclast formation *in vitro*. *Biochem. Biophys. Res. Commun.* **265,** 144–150 (1999).
7. Takahashi, N., Mundy, G. R. & Roodman, G. D. Recombinant human interferon-γ inhibits formation of human osteoclast-like cells. *J. Immunol.* **137,** 3544–3549 (1986).
8. Udagawa, N. *et al.* Interleukin-18 (interferon-γ-inducing factor) is produced by osteoblasts and acts via granulocyte/macrophage colony-stimulating factor and not via interferon-γ to inhibit osteoclast formation. *J. Exp. Med.* **185,** 1005–1012 (1997).
9. Ukai, T., Hara, Y. & Kato, I. Effects of T cell adoptive transfer into nude mice on alveolar bone resorption induced by endotoxin. *J. Periodontal. Res.* **31,** 414–422 (1996).
10. Chiang, C. Y., Kyritsis, G., Graves, D. T. & Amar, S. Interleukin-1 and tumor necrosis factor activities partially account for calvarial bone resorption induced by local injection of lipopolysaccharide. *Infect. Immun.* **67,** 4231–4236 (1999).
11. Huang, S. *et al.* Immune response in mice that lack the interferon-γ receptor. *Science* **259,** 1742–1745 (1993).
12. Manoury-Schwartz, B. *et al.* High susceptibility to collagen-induced arthritis in mice lacking IFN-γ receptors. *J. Immunol.* **158,** 5501–5506 (1997).
13. Vermeire, K. *et al.* Accelerated collagen-induced arthritis in IFN-γ receptor-deficient mice. *J. Immunol.* **158,** 5507–5513 (1997).
14. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. & Schreiber, R. D. How cells respond to interferons. *Annu. Rev. Biochem.* **67,** 227–264 (1998).
15. Taniguchi, T., Lamphier, M. S. & Tanaka, N. IRF-1: the transcription factor linking the interferon response and oncogenesis. *Biochim. Biophys. Acta* **1333,** M9–M17 (1997).
16. Wong, B. R. *et al.* The TRAF family of signal transducers mediates NF-κB activation by the TRANCE receptor. *J. Biol. Chem.* **273,** 28355–28359 (1998).
17. Lomaga, M. A. *et al.* TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev.* **13,** 1015–1024 (1999).
18. Naito, A. *et al.* Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells* **4,** 353–362 (1999).
19. Baumeister, W., Walz, J., Zuhl, F. & Seemuller, E. The proteasome: paradigm of a self-compartmentalizing protease. *Cell* **92,** 367–380 (1998).
20. Tanaka, K. & Kasahara, M. The MHC class I ligand-generating system: roles of immunoproteasomes and the interferon-γ-inducible proteasome activator PA28. *Immunol. Rev.* **163,** 161–176 (1998).
21. Preckel, T. *et al.* Impaired immunoproteasome assembly and immune responses in PA28$^{-/-}$ mice. *Science* **286,** 2162–2165 (1999).
22. Tanahashi, N. *et al.* Hybrid proteasomes. Induction by interferon-γ and contribution to ATP-dependent proteolysis. *J. Biol. Chem.* **275,** 14336–14345 (2000).
23. Firestein, G. S. & Zvaifler, N. J. How important are T cells in chronic rheumatoid synovitis? *Arthritis Rheum.* **33,** 768–773 (1990).
24. Kinne, R. W., Palombo-Kinne, E. & Emmrich, F. T-cells in the pathogenesis of rheumatoid arthritis: villains or accomplices? *Biochim. Biophys. Acta* **1360,** 109–141 (1997).
25. Takayanagi, H. *et al.* Involvement of receptor activator of nuclear factor κB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheum.* **43,** 259–269 (2000).
26. Gravallese, E. M. *et al.* Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. *Arthritis Rheum.* **43,** 250–258 (2000).
27. Billiau, A. Interferon-γ: biology and role in pathogenesis. *Adv. Immunol.* **62,** 61–130 (1996).
28. Matsuyama, T. *et al.* Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. *Cell* **75,** 83–97 (1993).
29. Meraz, M. A. *et al.* Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* **84,** 431–442 (1996).
30. Yasuda, H. *et al.* Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl Acad. Sci. USA* **95,** 3597–3602 (1998).

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Development of a preventive vaccine for Ebola virus infection in primates

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Outbreaks of haemorrhagic fever caused by the Ebola virus are associated with high mortality rates that are a distinguishing feature of this human pathogen. The highest lethality is associated with the Zaire subtype, one of four strains identified to date[1,2]. Its rapid progression allows little opportunity to develop natural immunity, and there is currently no effective anti-viral therapy. Therefore, vaccination offers a promising intervention to prevent infection and limit spread. Here we describe a highly effective vaccine strategy for Ebola virus infection in non-human primates. A combination of DNA immunization and boosting with adenoviral vectors that encode viral proteins generated cellular and humoral immunity in cynomolgus macaques. Challenge with a lethal dose of the highly pathogenic, wild-type, 1976 Mayinga strain of Ebola Zaire virus resulted in uniform infection in controls, who progressed to a moribund state and death in less than one week. In contrast, all vaccinated animals were asymptomatic for more than six months, with no detectable virus after the initial challenge. These findings demonstrate that it is possible to develop a preventive vaccine against Ebola virus infection in primates.

Genetic immunization has been shown to influence both humoral and cellular immune activation pathways and to protect against infection by human pathogens[3–6]. The effectiveness of plasmid vaccines is thought to result from host-cell protein synthesis and endogenous presentation of the immunogen, and possibly to immunostimulatory effects of plasmid DNA itself[7,8]. DNA vaccines have been shown to elicit specific immune responses to Ebola virus antigens and to protect guinea-pigs[9] and mice[10] against challenge with Ebola virus adapted to produce lethal infection in rodents[11,12]. Although both cell-mediated and humoral immune responses were elicited, antibody titre correlated with the degree of protection in animals immunized with plasmids encoding proteins

Table 1 Multivalent genetic immunization in guinea-pigs

ID	Immunization	ELISA IgG	Survival
1	Plasmid	0	No
2	Plasmid	0	No
3	Plasmid	0	No
4	Plasmid	0	No
5	GP(Z)	6,400	Yes
6	GP(Z)	6,400	Yes
7	GP(Z)	6,400	Yes
8	GP(Z)	3,200	Yes
9	GP(Z) + NP	6,400	Yes
10	GP(Z) + NP	6,400	Yes
11	GP(Z) + NP	6,400	Yes
12	GP(Z) + NP	6,400	Yes
13	GP(Z,IC,S) + NP	6,400	Yes
14	GP(Z,IC,S) + NP	1,600	Yes
15	GP(Z,IC,S) + NP	6,400	Yes
16	GP(Z,IC,S) + NP	6,400	Yes

Guinea-pigs were immunized intramuscularly three times at two-week intervals with 100 μg of DNA (Plasmid, 100 μg p1012; GP(Z), 100 μg pGP(Z); GP(Z) + NP, 75 μg pGP(Z) and 25 μg pNP; GP(Z, IC, S) + NP, 25 μg each of pGP(Z), pGP(IC), pGP(S) and pNP). Serum was collected six weeks after the first injection and pre-challenge titres for antibody to Ebola GP (ELISA IgG) were measured by ELISA[28] and are displayed as the reciprocal end-point dilution. Three months after the final immunization the animals were challenged as described previously[9].

from the Zaire subtype of Ebola virus.

A broadly effective vaccine would need to provide immunity to the multiple Ebola subtypes isolated in human infections (Zaire, Sudan and Ivory Coast), but a multivalent vaccine might dilute the specific immune response demonstrated for the single subtype vaccine. To address this concern, we analysed the efficacy of the original Ebola Zaire DNA vaccine in comparison to its use in combination with DNA from Ebola subtypes Sudan and Ivory Coast. As in a previous study[9], immunization with a single plasmid encoding Zaire-subtype virion glycoprotein, (GP(Z)), generated a substantial virus-specific antibody response and conferred protective immunity in guinea-pigs (Table 1). Inclusion of a plasmid expressing Ebola nucleoprotein, NP, did not affect the antibody titre to Ebola GP(Z) or diminish its protective efficacy. Further broadening of the vaccine components to include NP and three subtypes of Ebola glycoprotein, Zaire, Ivory Coast and Sudan (GP(Z,IC,S) + NP), yielded a pre-challenge immune response comparable to the single-plasmid vaccine. Moreover, complete protection from infection with Ebola Zaire was achieved in guinea-pigs that received the multivalent vaccine (Table 1, subjects 13–16). Anamnestic antibody was not induced by the virus challenge, indicating that the vaccine itself provided an immune response sufficient to efficiently clear the virus. These findings show that multivalent plasmid immunization did not substantially diminish glycoprotein (GP)-specific antibody production and its protective efficacy in a rodent model.

Because protection in the rodent model of Ebola virus infection correlated with antibody titres, and efficient humoral responses may influence clinical outcome in human disease[13,14], we considered it important to elicit a strong humoral response for vaccines tested in primates, although cell-mediated immunity is coordinately induced and probably contributes to protection[9]. Recently, regimens of DNA priming followed by administration of viral vectors have demonstrated enhanced immune responses compared to vaccines using DNA alone[15–18]. Recombinant, replication-deficient adenoviruses can be grown to high titre, infect antigen-presenting cells, and induce potent immune responses[19–21]. Adenoviruses have shown a boosting effect in mice[22], but the combination of DNA and adenovirus has not been tested for efficacy in an infectious challenge model, and the success of this approach in primates is yet unknown.

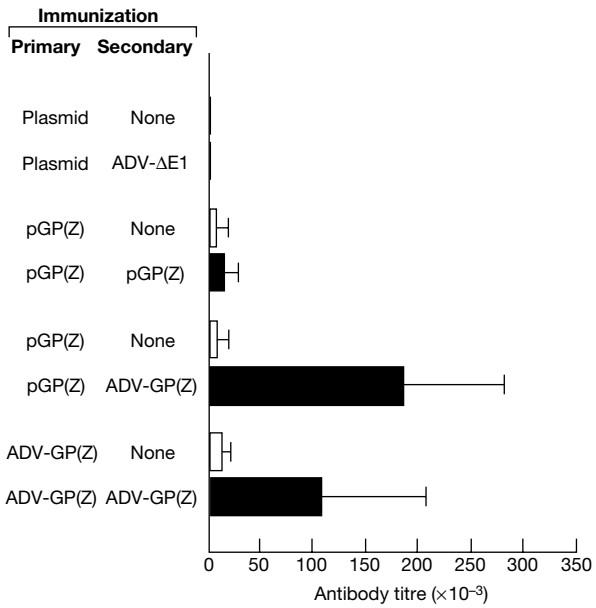

Figure 1 Ebola-specific antibody responses generated by different DNA/adenovirus prime-boost combinations. See Methods. Data are the means of the reciprocal end-point dilution for each group of mice and error bars represent the standard deviation.

We therefore developed a recombinant adenoviral vector that directs high-level GP expression (ADV-GP(Z)) and used this vector to test whether a modified prime-boost strategy would augment the antibody response to Ebola virus obtained with naked DNA alone. Mice were injected with DNA and adenovirus vectors either singly or in combinations, and cell-mediated and humoral immune responses were assessed. A 10-fold to 100-fold increase in antibody titre was found in mice injected with DNA followed by an adenovirus boost, compared with DNA immunization alone (Fig. 1). An increase in cytotoxic T-cell responses was also observed with this combination (data not shown). Immunization with ADV-GP(Z) alone yielded antibody titres that were not

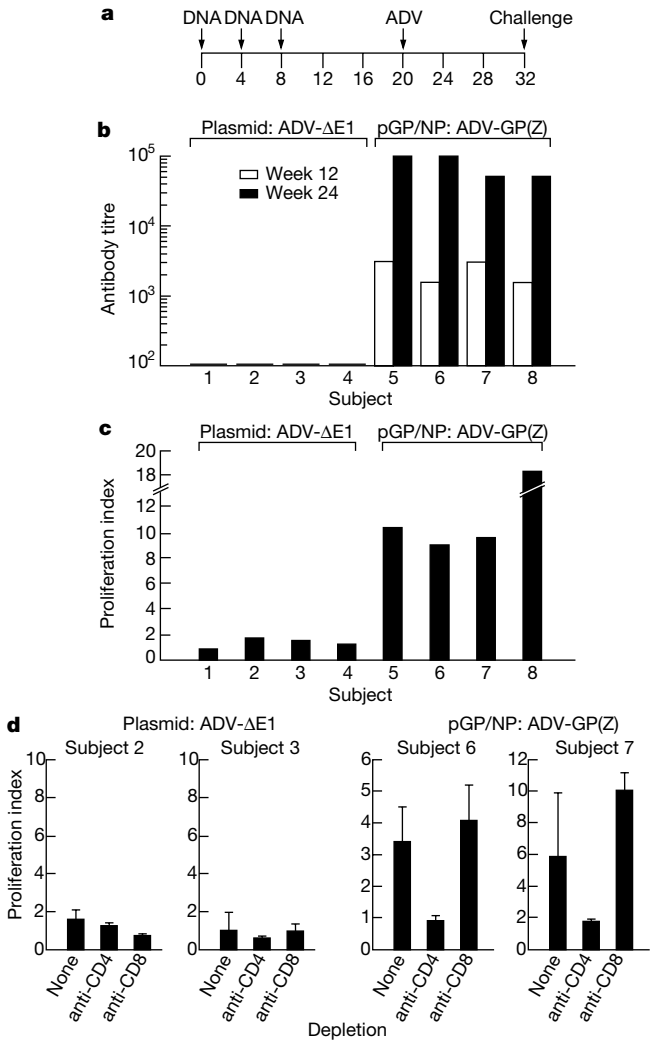

Figure 2 DNA-adenovirus immunization of cynomolgus macaques. **a**, Immunization schedule for DNA and/or adenovirus injections, and challenge with the wild-type Mayinga strain of the Zaire subtype of Ebola virus. **b**, Elisa titres of Ebola-specific antibodies in serum. Serum was collected at week 12 (open bar) and 2 days before immunization at week 24 (closed bar). **c**, Lymphoproliferative responses to Ebola-secreted glycoprotein (sGP) after immunization. Bars represent the average fold-proliferation of all four blood samples for each subject. The standard deviation is not shown because the baseline level of induction varied between experiments. However, PBMC from all eight animals were assayed within the same experiment for each time point, and the averages displayed are representative of the results obtained for any single time point. **d**, Lymphoproliferative responses to Ebola sGP in bulk PBMC following depletion of lymphocyte subsets. PBMC from week 24 were treated with Dynal magnetic beads coated with the indicated antibody to deplete $CD4^+$ or $CD8^+$ cell subsets. Cells remaining after depletion were normalized for input cell number and stimulated as described in the Methods. Results are shown for two control (subjects 2 and 3) and two vaccinated (subjects 6 and 7) monkeys.

significantly different from those obtained with the DNA prime, adenovirus boost, immunization. These data suggest that immunogenicity of the Ebola GP DNA vaccine in mice is improved by boosting with recombinant adenovirus and that this strategy might represent a useful approach to enhance immune responses in non-human primates.

Whereas the rodent model has been useful in the development of a vaccine strategy, Ebola virus isolated directly from humans must first be adapted by multiple, sequential passages in rodents in order to produce a lethal infection in mice or guinea-pigs[11,12]. Primate models of Ebola infection are thought to have a stronger predictive value for human disease and immune protection. We therefore conducted studies in non-human primates using a bimodal DNA/ADV vaccine and the multiple plasmid strategy that correlated with protection in guinea-pigs. Cynomolgus macaques (*Macaca fascicularis*) received three injections of naked DNA vectors at four-week intervals (Fig. 2a) and, after several months of rest, which has been shown to boost immune responses[23], were boosted with recombinant adenovirus expressing only the Zaire glycoprotein (Fig. 2a). Control animals received empty vectors (plasmid DNA and ADV-ΔE1 recombinant adenovirus), and vaccinated animals received the multicomponent DNA vaccine containing NP and three subtypes of Ebola GP (pGP/NP), followed by ADV-GP(Z). As expected, anti-Ebola serum antibodies could not be detected in control animals, but in animals receiving the Ebola vaccine, an antigen-specific antibody response was detected at week 12, one month after the third DNA injection (Fig. 2b). After boosting with recombinant adenovirus, antibody titres increased 10-fold to 20-fold over the levels obtained with DNA alone. Three months after the final immunization, antibody levels remained high (data not shown), except for one animal (subject 8) whose titre dropped slightly from 5×10^4 to 1.3×10^4.

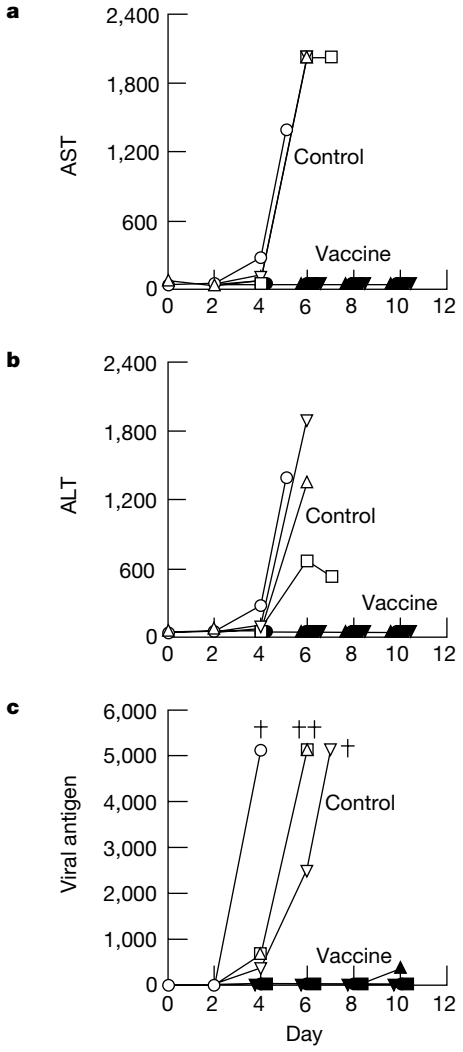

Figure 3 Protection of cynomolgus macaques against lethal challenge with Ebola virus after DNA-adenovirus immunization. **a**, **b**, Hepatic enzyme levels in monkeys after challenge with Ebola virus. Liver enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) levels in the non-human primate sera were measured by standard recommended procedures using a General chemistry 12 reagent disk for the Piccolo Analyzer (Abaxis). Results are shown for four immunized (closed symbols) and four control (open symbols) monkeys. **c**, Plasma viraemia in monkeys after infection with Ebola virus. Crosses represent time of death in control animals (days 5 (subject 1) and 6 (subjects 2 and 4)). One control animal, subject 3, was euthanized on day 7 when it was moribund (see Methods). One vaccinated animal that was resistant to infection, subject 5, was euthanized on day 10 for histological examination of tissues. By day 17, none of the animals had detectable viraemia, and they remained aviremic for the duration of the observation period (six months). Data are the reciprocal end-point dilution of serum for each monkey. Results are shown for four immunized (closed symbols) and four control (open symbols) monkeys.

Primate cellular responses to Ebola antigens were next examined with an *in vitro* lymphocyte-proliferation assay. In control monkeys, antigen-specific lymphocyte proliferation, measured by ^{3}H-thymidine uptake, was equivalent to that in matched, unstimulated cells, resulting in a proliferation index near 1.0 for each animal (Fig. 2c). In contrast, peripheral blood mononuclear cells (PBMC) from animals immunized with the multivalent vaccine showed 9-fold to 20-fold increased stimulation, demonstrating a robust immune response to Ebola antigen at the cellular level. Depletion of CD4-positive lymphocytes reduced the antigen-stimulated proliferative response of PBMC from vaccinated monkeys to the level observed in control animals (Fig. 2d). Depletion of CD8-positive lymphocytes, however, did not affect Ebola-antigen-specific lymphocyte proliferation. Therefore, the CD4-positive subset of lymphocytes, which provide the T-cell help required for high antibody titres, contributes to the vaccine-induced cellular immune response.

To determine the protective efficacy of this vaccination regimen, monkeys were challenged with a lethal dose of the wild-type Mayinga strain from the Zaire subtype of Ebola virus. In the control monkeys, blood chemistry revealed an increase in hepatic enzymes (Fig. 3a and b) that is characteristic for Ebola virus infection[24]. No such increase was observed in vaccinated subjects. The elevation of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was parallel to a dramatic increase in viraemia in all of the control animals (Fig. 3c). In contrast, no substantial increase in viral load was observed in vaccinated monkeys. The kinetics of disease progression was similar among the control animals, and the disease incidence was 100% in this group. Death occurred between days 5 and 6 for 3 animals, and the last monkey, moribund, was euthanized on day 7 (Fig. 3c). In contrast, 4 out of 4 monkeys immunized with the combination DNA-adenovirus vaccine survived this lethal challenge of Ebola virus, and sterilizing immunity was achieved in 3 out of 4 subjects. The remaining animal showed a small transient rise in viral antigen; however, when followed long-term, all vaccinated animals showed no signs or symptoms of infection, and there was no detectable viraemia for more than six months after infection, as measured by ELISA detection of viral antigen (Fig. 3a) and end-point titration analysis of cultured virus (data not shown). The vaccine recipient (subject 8) that exhibited a transient low level of viraemia on day 10 had undetectable levels by day 17.

As the natural reservoir for Ebola virus is unknown, the potential for traditional public health measures to prevent future outbreaks is limited, thus increasing the urgency for the development of a vaccine and therapeutics in humans. The present findings demonstrate that primates can be immunized against the lethal effects of Ebola virus infection, and that sterilizing immunity can be achieved

using a heterologous prime-boost immunization strategy. A multicomponent genetic vaccine expressing Ebola virus structural proteins from diverse geographic isolates generated a strong antigen-specific immune response and resulted in the survival of all immunized primates after challenge with a lethal dose of Ebola Zaire, the subtype of this virus associated with the highest number of deaths in human infections. The results of this study suggest that T-cell mediated and humoral immunity contribute to virus clearance in non-human primates, consistent with previous studies in rodents[9,25]. Two immune parameters, antibody titre (1:75,000 versus <1:100, $P = 0.001$) and the cellular proliferative response (~12-fold versus 1.4-fold, $P = 0.0014$), provided highly significant immune correlates of protection. Studies investigating the correlates of immune protection from Ebola virus infection in humans are hampered by the aggressive nature of the virus and necessarily high level of biosafety containment. With the model of primate immunity presented here, it should now be possible to elucidate the mechanisms of immune protection from Ebola virus infection, to advance immune-based anti-viral therapies, and to develop a human vaccine for this pathogen and possibly other infectious causes of haemorrhagic fever. □

Methods

Vector construction

The construction of DNA vectors expressing Ebola Zaire GP, secreted GP (sGP), and NP has been described previously[9]. The GP Sudan (Genbank accession number U28134) and Ivory Coast (Genbank accession number U28066) expression vectors were constructed similarly. Briefly, GP open reading frames were generated from polymerase chain reaction after reverse transcription of RNA (RT-PCR) products of infected cell RNA using the following primers: 5′-ATC TTC AGG ATC TCG CCA TGG A-3′ (Sudan GP gene; NcoI > ATG), 5′-GAT ATT CAA CAA AGC AGC TTG CAG-3′ (Sudan GP gene; C-terminus GP stop), 5′-CTA ATC ACA GTC ACC ATG GGA-3′ (IC GP gene; NcoI > ATG), 5′-AAA GTA TGA TGC TAT ATT AGT TCA-3′ (Ivory Coast GP gene; carboxy-terminus GP stop) yielding the TA clones PCR2.1 Sudan and PCR2.1 Ivory Coast. The Sudan glycoprotein was digested from plasmid PCR2.1 with XbaI/HindIII, Klenow treated, and cloned into the XbaI site of p1012 (ref. 9). Ivory Coast GP was digested from plasmid PCR2.1 with EcoRI, Klenow treated, and cloned into the XbaI site of p1012 (ref. 9).

To make ADV-GP, the BamHI/EcoRI fragment of GP(Z) was digested from PGEM-3Zf(−)-GP, treated with Klenow, and inserted into HindIII/XbaI/Kle/CIP-treated pRc/CMV plasmid. The resulting plasmid (PRC/CMV-GP(Z)) was digested by NruI/DraIII and treated with Klenow. The NruI/DraIII/Kle fragment containing the CMV enhancer, GP(Z) DNA and bovine growth hormone polyadenylation signal was inserted into the BglII site of the adenoviral shuttle plasmid pAdBgIII[26]. The adenovirus, a first generation dl 309-based Ad5 vector, contained a deletion in E1 to render the vector replication defective and a partial deletion/substitution in E3, which disrupts the coding sequences for the E3 proteins with a relative molecular mass of 14,700, 14,500 and 10,400 (M_r 14.7, 14.5 and 10.4 K), respectively. The recombinant adenovirus expressing Zaire GP, ADV-GP(Z), was made according to previously published methods[27]. The dose of adenovirus administered, 10^{10} plaque-forming units (PFU) per animal (approximately 3×10 PFU kg^{-1}), is within the range used safely in human gene-therapy trials.

Animal study and safety

Eight cynomolgus macaques (*M. fascicularis*), 3 years old and weighing 2–3 kg, obtained from Covance, were used for the immunization and challenge experiment. To obtain blood specimens and administer vaccines, the monkeys were anaesthetized with Ketamine. The animals were housed singly and received regular enrichment according to the Guide for the Care and Use of Laboratory Animals (DHEW No. NIH 86–23). Just before the Ebola virus challenge and up to the end of the experiment, the animals were maintained in the Maximum Containment Laboratory (BSL-4) and fed and checked daily. One animal was euthanized that appeared moribund and was subsequently necropsied for pathologic examination. In addition, a single asymptomatic vaccinated animal was euthanized for pathologic and virologic analysis.

Mouse immunization

DNA and adenovirus vectors expressing Ebola Zaire glycoprotein or nucleoprotein (GP or NP) were constructed as described previously[9,26] with gene expression under the control of the cytomegalovirus enhancer and promoter. Mice were immunized intramuscularly with 100 μg of DNA (pGP or a p1012 plasmid control) or 10^8 PFU of adenovirus (ADV-GP or ADV-ΔE1 control virus) on days 0, 14 and 28, and blood was collected on day 28 (open bars). On day 42, mice received an intramuscular boost with DNA or adenovirus and titres were re-measured on day 56 (solid bars). ELISA IgG titres were determined using 96-well plates coated with a preparation of Ebola virus antigen derived from purified virions and enriched for membrane-associated proteins (GP, VP40 and VP24)[28]. Specific antigen binding was detected using a goat anti-human IgG(H+L)-horseradish peroxidase conjugate and ABTS/peroxide (substrate/indicator).

Macaque immunization

For the DNA immunizations, animals received 1 mg each of DNA expressing GP(Zaire), [GP(Z)], GP(Ivory Coast) [pGP(IC)], GP(Sudan) [pGP(S)] and NP(Zaire) administered as a mixture [pGP/NP], or 4 mg empty [pGP(Z)] control plasmid bilaterally (2 mg per side) in the deltoid muscle. Immunization at weeks 0 and 4 were by IM injection, and at week 8 by Biojector. For the adenovirus boost, animals received 10^{10} PFU of ADV-GP (Zaire subtype) or ADV-ΔE1 (empty vector) divided into two doses administered bilaterally in the deltoid muscle. At week 32, all animals received an intraperitoneal injection of approximately six PFUs of Ebola virus (Zaire 1976 isolate; Mayinga strain)[29] in 1 ml Hanks' buffered salt solution. The virus was isolated directly from patient blood and used after a single passage in Vero cells.

ELISA IgG titres were determined as above for control (Plasmid: ADV-ΔE1) and immunized (pGP/NP: ADV-GP(Z)) monkeys. The reciprocal end-point of dilution for each subject was at week 12 and week 24. Serum antibody levels were measured by ELISA as described[28]. Blood was collected from control (plasmid: ADV-ΔE1) or immunized (pGP/NP: ADV-GP(Z)) animals 1–3 days before immunizations at weeks 4, 8 and 20, and at week 24. Blood was separated over a Percoll gradient to obtain the lymphocyte enriched population. Lymphocytes were stimulated as described previously[9] for five days *in vitro* using supernatant from cells transfected with either sGP or empty plasmid, and proliferation was measured by ^{3}H-thymidine uptake. The proliferation index was calculated as the proliferation in wells receiving sGP divided by proliferation in wells receiving control supernatant.

Viral detection in macaques

The presence of circulating Ebola virus antigen was detected as described[28] by capturing VP40 protein from serial dilutions of monkey plasma. 96-well plates coated with anti-VP40 mAb were used to capture antigen, and detection was with a rabbit anti-Ebola virus serum.

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1. Feldmann, H., Nichol, S. T., Klenk, H. D., Peters, C. J. & Sanchez, A. Characterization of filoviruses based on differences in structure and antigenicity of the virion glycoprotein. *Virology* **199,** 469–473 (1994).
2. Sanchez, A., Trappier, S. G., Mahy, B. W. J., Peters, C. J. & Nichol, S. T. The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. *Proc. Natl Acad. Sci. USA* **93,** 3602–3607 (1996).
3. Tang, D. C., DeVit, M. & Johnston, S. A. Genetic immunization is a simple method for eliciting an immune response. *Nature* **356,** 152–154 (1992).
4. Ulmer, J. B. *et al.* Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259,** 1745–1749 (1993).
5. Wang, B. *et al.* Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc. Natl Acad. Sci. USA* **90,** 4156–4160 (1993).
6. Sedegah, M., Hedstrom, R., Hobart, P. & Hoffman, S. L. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc. Natl Acad. Sci. USA* **91,** 9866–9870 (1994).
7. Krieg, A. M. *et al.* CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374,** 546–549 (1995).
8. Sato, Y. *et al.* Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273,** 352–354 (1996).
9. Xu, L. *et al.* Immunization for Ebola virus infection. *Nature Med.* **4,** 37–42 (1998).
10. Vanderzanden, L. *et al.* DNA vaccines expressing either the GP or NP genes of Ebola virus protect mice from lethal challenge. *Virology* **246,** 134–144 (1998).
11. Connolly, B. M. *et al.* Pathogenesis of experimental Ebola virus infection in guinea pigs. *J. Infect. Dis.* **179,** S203–S217 (1999).
12. Bray, M., Davis, K., Geisbert, T., Schmaljohn, C. & Huggins, J. A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. *J. Infect. Dis.* **178,** 651–661 (1998).
13. Baize, S. *et al.* Defective humoral responses and extensive intravascular apoptosis are associated with fatal outcome in Ebola virus-infected patients. *Nature Med.* **5,** 423–426 (1999).
14. Maruyama, T. *et al.* Ebola virus can be effectively neutralized by antibody produced in natural human infection. *J. Virol.* **73,** 6024–6030 (1999).
15. Sedegah, M. *et al.* Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine. *Proc. Natl Acad. Sci. USA* **95,** 7648–7653 (1998).
16. Hanke, T. *et al.* Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. *Vaccine* **16,** 439–445 (1998).
17. Robinson, H. L. *et al.* Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nature Med.* **5,** 526–534 (1999).
18. Schneider, J. *et al.* Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nature Med.* **4,** 397–402 (1998).
19. Davis, A. R. *et al.* Expression of hepatitis B surface antigen with a recombinant adenovirus. *Proc. Natl Acad. Sci. USA* **82,** 7560–7564 (1985).
20. Natuk, R. J. *et al.* Adenovirus-human immunodeficiency virus (HIV) envelope recombinant vaccines elicit high-titered HIV-neutralizing antibodies in the dog model. *Proc. Natl Acad. Sci. USA* **89,** 7777–7781 (1992).
21. Xiang, Z. Q., Yang, Y., Wilson, J. M. & Ertl, H. C. A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. *Virology* **219,** 220–227 (1996).
22. Xiang, Z. Q., Pasquini, S. & Ertl, H. C. Induction of genital immunity by DNA priming and intranasal booster immunization with a replication-defective adenoviral recombinant. *J. Immunol.* **162,** 6716–6723 (1999).
23. Letvin, N. L. *et al.* Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. *Proc. Natl Acad. Sci. USA* **94,** 9378–9383 (1997).
24. Fisher-Hoch, S. P. *et al.* Pathophysiology of shock and hemorrhage in a fulminating viral infection (Ebola). *J. Infect. Dis.* **152,** 887–894 (1985).

25. Wilson, J. *et al.* Epitopes involved in antibody-mediated protection from Ebola virus. *Science* **287,** 1664–1666 (2000).
26. Ohno, T. *et al.* Gene therapy for vascular smooth muscle cell proliferation after arterial injury. *Science* **265,** 781–784 (1994).
27. Aoki, K., Barker, C., Danthinne, X., Imperiale, M. J. & Nabel, G. J. Efficient generation of recombinant adenoviral vectors by Cre-lox recombination *in vitro*. *Mol. Med.* **5,** 224–231 (1999).
28. Ksiazek, T. G. *et al.* Enzyme immunosorbent assay for Ebola virus antigens in tissues of infected primates. *J. Clin. Microbiol.* **30,** 947–950 (1992).
29. Kiley, M. P., Regnery, R. L. & Johnson, K. M. Ebola virus: identification of virion structural proteins. *J. Gen. Virol.* **49,** 333–341 (1980).

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Coenzyme Q is an obligatory cofactor for uncoupling protein function

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Uncoupling proteins (UCPs) are thought to be intricately controlled uncouplers[1–3] that are responsible for the futile dissipation of mitochondrial chemiosmotic gradients, producing heat rather than ATP. They occur in many animal and plant cells[4–9] and form a subfamily of the mitochondrial carrier family[10]. Physiological uncoupling of oxidative phosphorylation must be strongly regulated to avoid deterioration of the energy supply and cell death, which is caused by toxic uncouplers. However, an H^+ transporting uncoupling function is well established only for UCP1 from brown adipose tissue[2,8,9,11], and the regulation of UCP1 by fatty acids, nucleotides and pH remains controversial[2,12–14]. The failure of UCP1 expressed in *Escherichia coli* inclusion bodies to carry out fatty-acid-dependent H^+ transport activity inclusion bodies[15] made us seek a native UCP cofactor. Here we report the identification of coenzyme Q (ubiquinone) as such a cofactor. On addition of CoQ_{10} to reconstituted UCP1 from inclusion bodies, fatty-acid-dependent H^+ transport reached the same rate as with native UCP1. The H^+ transport was highly sensitive to purine nucleotides, and activated only by oxidized but not reduced CoQ. H^+ transport of native UCP1 correlated with the endogenous CoQ content.

Whereas UCP1 from brown adipose tissue (BAT) can be isolated and is thus functionally well characterized[11], the isolation of more recently discovered UCPs has not been possible because of their low tissue content[8,9]. For this reason and for mutagenesis studies, we expressed recombinant UCPs in yeast and in *E. coli*[15–20]. Our inability to reconstitute H^+ transport activity from UCP1 expressed as inclusion bodies (IB-UCP1) in *E. coli* prompted us to consider several causes. (1) The reconstituted IB-UCP1 may not attain the full native configuration. (2) The reconstituted IB-UCP1 may still contain traces of the anion detergent sarcosyl used to solubilize UCP1, which, as a fatty-acid analogue, may prevent fatty-acid binding. (3) A covalent or (4) non-covalent cofactor present in the native UCP1 may be missing.

To first bring soluble IB-UCP1 into a native state, we thoroughly removed the sarcosyl used for solubilization of inclusion bodies by substitution with digitonin and anion-exchange treatment. We succeeded in restoring the nucleotide-binding capacity of soluble IB-UCP1 (Fig. 1a) with the fluorescent derivative dansyl-GTP, which had been used to test the nucleotide binding of native UCP1 (ref. 21). The decrease of fluorescence caused by competition with excess ATP corresponded to the specific binding of UCP1. We further tested for native protein configuration with the carboxyl reagent, Woodward reagent K, which blocks with high specificity

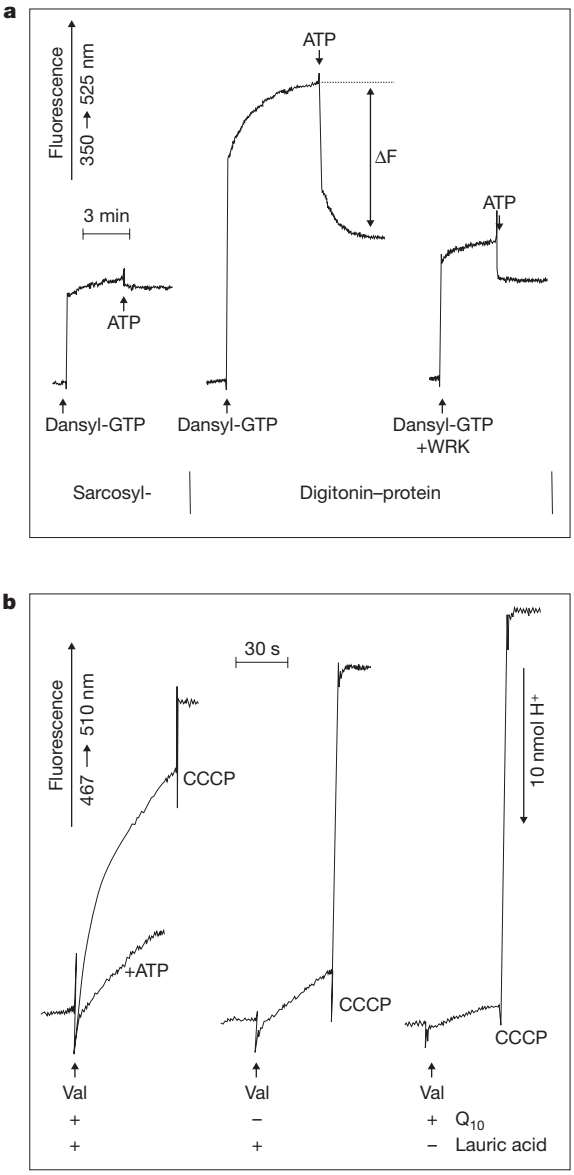

Figure 1 Functional integrity of refolded recombinant IB-UCP1 from *E. coli*. **a**, Fluorescence response of 2′-O-(dimethylamino-naphthaline-1-sulfonyl)-GTP (dansyl-GTP) binding to sarcosyl- and digitonin-treated IB-UCP1. Dansyl-GTP (5 μM) was added to a solution of 45 μg ml^{-1} UCP1 in 10 mM MES/HEPES buffer containing 0.3% digitonin, pH 6.5 at 10 °C. To differentiate the UCP1-linked binding, 0.5 mM ATP was added to displace the fluorescent ligand. To confirm binding to native UCP1, 10 μM Woodward reagent K was added 30 min before dansyl-GTP. **b**, Recording of H^+ influx into vesicles reconstituted with digitonin-treated IB-UCP1. H^+ influx was monitored by the fluorescence of pyranine. Vesicles (50 μl), containing 1.25 μg protein and 0.42 mg phospholipid, were added to a medium containing 0.5 mM HEPES pH 7.3, 1 μM pyranine, 0.5 mM EDTA, 280 mM sucrose in a final volume of 330 μl at 10° C; 125 μM lauric acid and 2 nmol CoQ_{10} were added where indicated. Valinomycin (Val; 2.5 μM) was added to induce K^+ efflux and thus a membrane potential. The uncoupler carbonylcyanide m-chlorophenylhydrazone (CCCP) (1 μM) was added to a determine the vesicle capacity for H^+ uptake.