

# Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans

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**Protective immunity against *Mycobacterium tuberculosis* depends on the generation of a T<sub>H</sub>1-type cellular immune response, characterized by the secretion of interferon- $\gamma$  (IFN- $\gamma$ ) from antigen-specific T cells. The induction of potent cellular immune responses by vaccination in humans has proven difficult. Recombinant viral vectors, especially poxviruses and adenoviruses, are particularly effective at boosting previously primed CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses against a number of intracellular pathogens in animal studies<sup>1–7</sup>. In the first phase 1 study of any candidate subunit vaccine against tuberculosis, recombinant modified vaccinia virus Ankara (MVA) expressing antigen 85A (MVA85A) was found to induce high levels of antigen-specific IFN- $\gamma$ -secreting T cells when used alone in bacille Calmette-Guérin (BCG)-naïve healthy volunteers. In volunteers who had been vaccinated 0.5–38 years previously with BCG, substantially higher levels of antigen-specific IFN- $\gamma$ -secreting T cells were induced, and at 24 weeks after vaccination these levels were 5–30 times greater than in vaccinees administered a single BCG vaccination. Boosting vaccinations with MVA85A could offer a practical and efficient strategy for enhancing and prolonging antimycobacterial immunity in tuberculosis-endemic areas.**

*Mycobacterium bovis* BCG does not protect against adult pulmonary tuberculosis, but does protect against disseminated disease in childhood when administered at birth in developing countries<sup>8,9</sup>. Although repeated vaccination with BCG appears not to enhance protection against tuberculosis further<sup>10</sup>, the protective effect of BCG in childhood might be retained by a vaccination strategy that improved BCG rather than replaced it. The secretion of IFN- $\gamma$  is central to the activation of *M. tuberculosis*-infected macrophages, and the measurement of IFN- $\gamma$  release from antigen-specific T cells provides the best available immunological correlate of protection against tuberculosis<sup>11–13</sup>. HLA class II-restricted CD4<sup>+</sup> T cells are essential for protective immunity against *M. tuberculosis* and class I-restricted CD8<sup>+</sup> T cells also have a role<sup>14–16</sup>.

Heterologous prime-boost immunization strategies induce higher levels of effector T-cell responses in animals and humans than homologous boosting with the same vaccine<sup>1,2,17</sup>. In tuberculosis, incorporating BCG into such a heterologous prime-boost regime retains the protective effects of BCG. Antigen 85A is highly conserved amongst all mycobacterial species and is present in all strains of BCG<sup>18</sup>. It is immunodominant in animal and human studies, and is protective in small animals<sup>5,19,20</sup>. The immunogenicity and protective efficacy of boosting BCG with viral vectors expressing antigen 85A in several animal models has previously been documented (ref. 21 and unpublished data).

Here we report the results of a series of phase 1 studies in human volunteers evaluating the safety and immunogenicity of MVA85A, first in BCG-naïve healthy volunteers and subsequently in volunteers who have been vaccinated with BCG 0.5–38 years previously.

Immunization with MVA85A was safe and well-tolerated (Table 1). The kinetics and magnitude of the antigen-specific T-cell response induced by vaccination with BCG alone, MVA85A alone and BCG prime-MVA85A boost were compared. All three vaccination regimes induced substantial immune responses using purified protein derivative (PPD) from *M. tuberculosis*, antigen 85 protein or overlapping peptides from antigen 85A as antigen in the assays (Table 2 and Fig. 1). There was a significant main effect of vaccine in the PPD ( $F = 3.624$ ;  $P = 0.037$ ), antigen 85 ( $F = 16.605$ ;  $P < 0.001$ ) and summed pooled antigen 85A peptide groups ( $F = 39.982$ ;  $P < 0.001$ ). Immunization with BCG induced moderate levels of antigen-specific IFN- $\gamma$ -secreting T cells, which peaked 4 weeks after immunization (Table 2 and Fig. 1b–d). Responses to the pooled antigen 85A peptides were notably weak following BCG vaccination (Fig. 1d). Only 4 of the 11 volunteers responded to any of the 7 peptide pools in the *ex vivo* ELISPOT assay. These peptide pool responses were all attributable to peptides 12, 13, 27 and 28, and were completely abrogated by CD4<sup>+</sup> T-cell depletion.

In BCG-naïve volunteers, a single immunization with MVA85A induced high levels of antigen-specific IFN- $\gamma$ -secreting T cells, which peaked 7 d after vaccination in 13 of 14 volunteers (Table 2 and Fig. 1b,c). By 4 weeks this response had fallen to a level just above

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**Table 1** Adverse events after immunization with MVA85A

	Adverse event	Number of subjects ( <i>n</i> = 31)
Local	Redness	31
	Pruritus	31
	Pain	30
	Induration	31
Systemic <sup>a</sup>	Fever <sup>b</sup>	5
	Flulike	11
	Arthralgia	10
	Headache	11
	Myalgia	12
	Nausea	3
	Vasovagal syncope	1 (previous history of vasovagal attacks)
	Alterations in hematology/biochemistry	0

There were no differences in either the frequency or severity of adverse events reported between the BCG-naïve and BCG-primed groups. <sup>a</sup>All systemic symptoms resolved within 7 d. <sup>b</sup>Range 37.7–38.1 °C; all resolved spontaneously within 24 h.

baseline. We did not see a boosting effect of the second vaccination with MVA85A, administered at week 3. One volunteer did not develop insert-specific T cells following immunization with MVA85A; however, this volunteer did develop specific T-cell responses to the MVA vector, despite having no previous history of vaccinia immunization (data not shown).

In contrast to BCG vaccination, vaccination with MVA85A induced strong responses to several peptide pools in 13 of 14 responding volunteers (Table 2 and Fig. 1d). We saw responses to a broad range of peptides across the whole length of antigen 85A. These individual peptide responses were all completely abrogated by CD4<sup>+</sup> T-cell depletion (data not shown).

In 16 of 17 volunteers in the BCG prime-MVA85A boost group, a substantial and significant ( $P < 0.001$ ) rise in antigen-specific T cells was seen 1 week after vaccination (Table 2 and Fig. 1). The peak response 1 week after immunization was significantly higher ( $P < 0.05$ ) in the BCG prime-MVA85A boost group than in either the BCG or MVA85A alone groups (Fig. 1b–e). These responses were

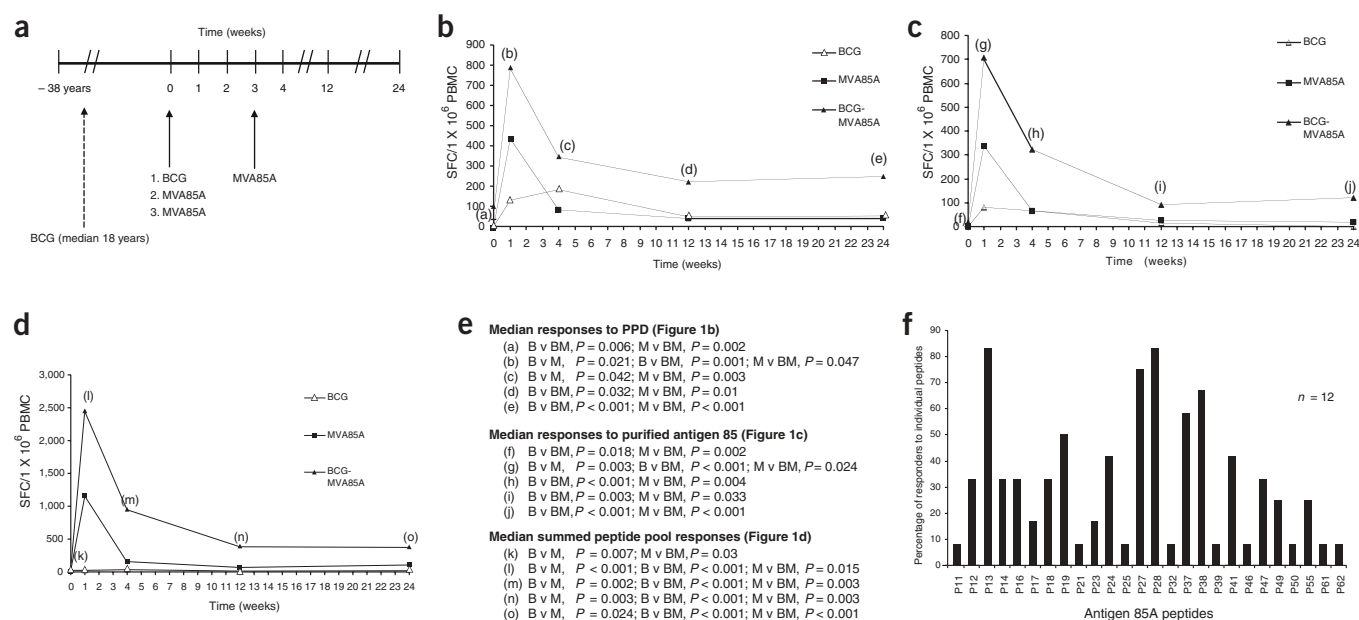
sustained at a significantly higher level than after vaccination with either BCG or MVA85A alone, for at least 24 weeks (Fig. 1e). The baseline responses at screening were higher in the volunteers previously vaccinated with BCG than in the BCG-naïve group, as would be expected. Nonetheless, the responses 24 weeks after vaccination with MVA85A in the BCG prime-MVA85A boost group were significantly higher than baseline counts in this group for PPD (Wilcoxon  $z = -3.010$ ,  $P = 0.003$ ), antigen 85 (Wilcoxon  $z = -3.516$ ,  $P < 0.001$ ) and the summed pooled peptides (Wilcoxon  $z = -3.408$ ,  $P = 0.001$ ).

The breadth of peptide responses seen in the MVA85A alone (not shown) and BCG prime-MVA85A boost groups (Fig. 1f) were very similar. But the magnitude of responses was significantly higher ( $P < 0.05$ ) in the BCG prime-MVA85A boost group (Fig. 1e). Peripheral blood mononuclear cells (PBMC) from 12 volunteers in the BCG prime-MVA85A boost group were assayed with all 66 peptides from antigen 85A. Several of these peptides were recognized by more than 50% of subjects (Fig. 1f), showing the promiscuous recognition of these peptides by different HLA class II molecules, as previously reported<sup>19</sup>.

The magnitude of T-cell responses seen in the MVA85A alone group are stronger by a factor of about 10 than those seen with other recombinant MVAs used to date<sup>17,22</sup>. A recombinant MVA expressing an antigen from *P. falciparum* induced a mean summed peptide response of 90 spot-forming cells (SFC)/10<sup>6</sup> PBMC 7 d after vaccination<sup>17</sup>. In contrast, we saw a mean response to the summed peptides of 1,365 SFC/10<sup>6</sup> PBMC 7 d after vaccination with MVA85A (Table 2). One explanation for this is that these volunteers have some pre-existing antimycobacterial immunity that is being boosted by immunization with MVA85A. We used a cultured, rather than an *ex vivo* ELISPOT assay to investigate this further. The cultured ELISPOT assay has previously been shown to measure central memory T cells, rather than the activated effector T cells that are measured by *ex vivo* ELISPOT<sup>23,24</sup>. We performed a cultured IFN- $\gamma$  ELISPOT assay on the prevaccination screening PBMC from four of the volunteers in the MVA85A alone group. Cells were cultured with *M. tuberculosis* PPD, *M. avium* PPD and recombinant antigen 85A. All four volunteers responded to *M. avium* PPD, two of four responded to *M. tuberculosis* PPD and two of four responded to recombinant antigen 85A (Fig. 2). None of these

**Table 2** Arithmetic mean (SE) and median (IQR) ELISPOT responses to PPD, antigen 85 and summed pooled peptides in each vaccination group at each timepoint

Time after vaccination (weeks)		PPD			Antigen 85		Summed pooled peptides	
	Vaccine group	Number	Arithmetic mean (SE)	Median (25–75%)	Arithmetic mean (SE)	Median (25–75%)	Arithmetic mean (SE)	Median (25–75%)
0	BCG	11	15 (6)	0 (0–28)	6 (5)	0 (0–0)	15 (4)	20 (0–22)
	MVA85A	14	18 (7)	0 (0–22)	4 (4)	0 (0–0)	3 (3)	0 (0–0)
	BCG-MVA85A	17	129 (33)	60 (28–218)	24 (6)	18 (0–37)	26 (11)	0 (0–50)
1	BCG	11	173 (52)	132 (42–249)	79 (22)	82 (23–122)	38 (9)	28 (20–52)
	MVA85A	14	460 (93)	434 (175–553)	419 (98)	338 (140–540)	1365 (378)	1,153 (531–1,432)
	BCG-MVA85A	17	917 (148)	783 (403–1,653)	895 (150)	707 (438–1,653)	3,248 (592)	2,455 (1,315–5,187)
4	BCG	11	233 (50)	182 (104–314)	64 (12)	67 (37–94)	31 (7)	35 (13–40)
	MVA85A	14	107 (21)	81 (53–167)	117 (42)	65 (33–138)	306 (95)	156 (60–533)
	BCG-MVA85A	17	362 (86)	343 (165–421)	341 (67)	322 (180–405)	1123 (266)	953 (609–1,219)
12	BCG	11	76 (25)	47 (27–85)	21 (8)	15 (0–33)	15 (9)	0 (0–17)
	MVA85A	14	70 (25)	41 (22–92)	59 (26)	27 (18–52)	167 (58)	68 (21–205)
	BCG-MVA85A	17	299 (83)	223 (68–387)	227 (72)	92 (42–287)	739 (199)	390 (212–910)
24	BCG	11	58 (17)	53 (10–95)	12 (6)	0 (0–22)	23 (7)	20 (0–35)
	MVA85A	14	62 (19)	38 (26–63)	32 (13)	18 (0–45)	113 (27)	105 (32–152)
	BCG-MVA85A	16	328 (89)	249 (150–385)	240 (77)	119 (82–293)	669 (177)	385 (223–1,043)



**Figure 1** Median IFN- $\gamma$  ELISPOT responses after vaccination in each vaccination group: BCG alone; MVA85A alone; BCG prime-MVA85A boost. (a) Timeline for vaccinations (weeks) in each group. (b) Tuberculin PPD responses. (c) Purified antigen 85 protein responses. (d) Summed pooled peptide responses. (e) For each of the three antigens measured, the responses between each vaccine group at each timepoint were compared using the Mann-Whitney statistical test. Statistically significant comparisons are indicated. (f) T-cell epitope display after MVA boost in BCG vaccinated individuals. CD4<sup>+</sup> T-cell depletion completely abrogated all individual peptide responses.

volunteers had any baseline responses to either *M. tuberculosis* PPD or purified antigen 85 on the screening *ex vivo* ELISPOT.

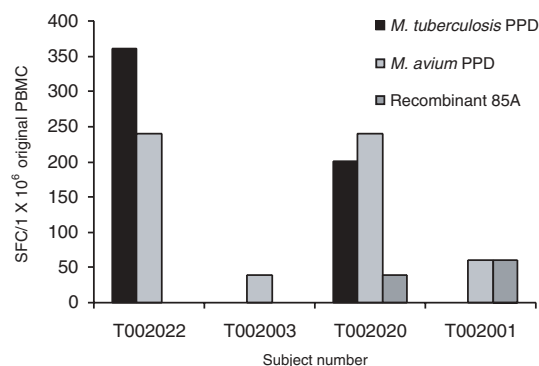
These are the first results of the clinical evaluation of any subunit candidate tuberculosis vaccine since BCG was first used over 80 years ago. We observed induction of high levels of antigen-specific T cells when BCG-naïve volunteers are immunized with a single low dose of MVA85A. The cultured ELISPOT data presented here show that four of four volunteers tested have pre-existing central memory responses to *M. avium* PPD, supporting the hypothesis that vaccination with MVA85A is boosting these pre-existing antimycobacterial immune responses. Other groups have also found evidence of pre-existing immunity to environmental mycobacteria in healthy UK adults who are otherwise mycobacterially naïve<sup>25</sup>. The leading explanation for the variable efficacy of BCG in different continents is that prior exposure to environmental mycobacteria either inhibits the replication and 'take' of BCG when this is used in subsequent vaccination, or masks the effect of BCG vaccination by providing a similar degree of antimycobacterial immunity<sup>13,26</sup>. In contrast, we find here that the immunogenicity of MVA85A appears to be enhanced by pre-existing antimycobacterial immunity induced by environmental mycobacteria.

The levels in the BCG-MVA vaccinees appear to be the highest reported T-cell responses induced by vaccination. Of particular relevance to the induction of long-lived memory immune responses, these antigen-specific T-cell responses remained significantly higher ( $P < 0.05$ ) in the BCG-MVA85A group than in either the BCG or MVA85A alone group for at least 24 weeks after vaccination (Fig. 1). These data suggest that immunization with MVA85A boosts the pre-existing antimycobacterial immunity induced by BCG in these volunteers. Taken together, these results show that vaccination with MVA85A boosts pre-existing antimycobacterial immune responses induced either by environmental mycobacteria or BCG vaccination. The immunogenicity of repeated BCG immunization using these sensitive T-cell assays has not been studied and it is possible that

repeated BCG vaccination could result in similar increases in T-cell responses seen after MVA85A boosting.

All of the individual peptide responses seen following vaccination with BCG and MVA85A are completely abrogated by CD4<sup>+</sup> T-cell depletion. We have not detected any responses resulting from CD8<sup>+</sup> T cells. This may be a technical issue that relates to the use of whole protein and peptides in the assay, rather than live BCG in a restimulation assay.

The data presented here show that MVA85A is safe and highly immunogenic, and encourage the further evaluation of this promising vaccine in tuberculosis-endemic countries. A booster MVA85A vaccination might be used either after BCG in infants or to boost immunity in teenagers before the peak of tuberculosis incidence in adolescents in developing countries. Studies are underway to evaluate the safety and immunogenicity of MVA85A in individuals latently infected with *M. tuberculosis*, aimed at assessing its potential utility as



**Figure 2** Screening blood cultured ELISPOT responses to *M. tuberculosis* PPD; *M. avium* PPD and recombinant antigen 85A; from four volunteers in the MVA85A-alone study, before vaccination.

a postexposure vaccine. Another target group for a new tuberculosis vaccine will be HIV-infected individuals, who are at high risk of tuberculosis. Recent data on the use of MVA as an investigational therapeutic HIV vaccine suggest that MVA85A should be safe in this population<sup>27</sup>. More generally, the capacity of MVA to boost strongly pre-existing naturally evoked T-cell responses suggests potential therapeutic uses in other chronic infections.

## METHODS

**The MVA85A vaccine.** The construction of MVA85A has previously been described<sup>5</sup>. Clinical-grade MVA85A was produced to good manufacturing practice standard by Impfstoffwerke Dessau-Tornau. A Doctors and Dentists Exemption Certificate was issued from the Medicines and Healthcare products Regulatory Agency, London, for the use of MVA85A in clinical trials.

**Clinical trials.** We recruited volunteers for immunization studies under protocols approved by the Oxfordshire Research Ethics Committee and enrolled them only after obtaining written informed consent. The age range for inclusion was 18–55 and all volunteers tested seronegative for HIV, HBV and HCV at screening. We performed routine laboratory hematology and biochemistry before vaccination and all values were within normal limits. We followed all volunteers for 6 months, and took blood samples at regular timepoints. Those who received MVA85A immunizations completed a diary card recording local and systemic side effects and body temperature for 7 d following vaccination. The demographic details of the volunteers are summarized in **Supplementary Table 1** online.

**Vaccinations.** We conducted the first two studies in BCG-naïve healthy volunteers. Those with a negative (Grade 0) Heaf test (equivalent to a tuberculin skin test of 0 mm) were vaccinated with either BCG (a single immunization with BCG Glaxo strain, 100 l administered intradermally,  $n = 11$ ) or MVA85A (5  $\times 10^7$  plaque-forming units (p.f.u.) administered intradermally, 2 immunizations given 3 weeks apart,  $n = 14$ ). In the third study, volunteers who had previously been vaccinated with BCG were recruited ( $n = 17$ ). The median time between BCG vaccination and immunization with MVA85A was 18 years (range 0.5–38 years). We enrolled volunteers with a Heaf test not greater than grade II (equivalent to a tuberculin skin test of <15 mm) in strength in the study and immunized them with a single dose of 5  $\times 10^7$  p.f.u. of MVA85A intradermally into the skin overlying the deltoid on the contralateral arm to BCG vaccination. In total, we vaccinated 31 healthy volunteers with MVA85A. Of the 14 BCG-naïve volunteers, 11 received 2 immunizations, given 3 weeks apart. The remaining three received a single immunization. All of the 17 BCG-primed volunteers received a single MVA85A immunization. All volunteers completed the 6-month follow up period and there were no serious or severe adverse events in any of these studies.

**Immunogenicity measures.** We used the *ex vivo* IFN- $\gamma$  ELISPOT assay as the main immunological measure used to determine vaccine immunogenicity. We performed the assay on blood taken at the following time points: at screening before the tuberculin skin test, and then at 1, 4, 12 and 24 weeks after vaccination. These measurements were carried out on fresh PBMCs using tuberculin PPD (20 g/ml, SSI), purified antigen 85 complex (10 g/ml)<sup>28</sup>, and 7 pools of 9–10 15-mer peptides, overlapping by 10 amino acids (10 g/ml final concentration of each peptide in ELISPOT well.) Briefly, we plated 300,000 PBMCs per well in 100 l R10 (RPMI plus 10% fetal calf serum) directly onto the ELISPOT plate (MAIP S4510, Millipore) in the presence of antigen, and incubated the plate for 18 h. We used streptokinase (250 U/ml), streptodornase (12.5 U/ml) and phytohaemagglutinin (10 g/ml) in all assays as positive controls. Assays were performed in duplicate and the results were averaged.

**Epitope mapping.** Responses to individual peptides were either tested for on the first or subsequent sample after vaccination. Magnetic bead depletions (Dynal) were performed on individual peptide responses. CD4<sup>+</sup> and CD8<sup>+</sup> T-cell depletions were performed by 30-min incubation with monoclonal antibodies to CD4 and CD8 conjugated to ferrous beads at a ratio of five beads: one cell using M-450 (Dynal) in 200 l R10 on ice. Antibody-coated cells were removed using a magnet (Dynal). Samples were analyzed according

to undepleted and CD4<sup>+</sup> and CD8<sup>+</sup> T cell-depleted groups. We confirmed cell depletions by FACS scanning. Depletions were always >90% for CD8<sup>+</sup> T cells and >97% for CD4<sup>+</sup> T cells (data not shown).

**Analysis of immunogenicity.** We analyzed the ELISPOT data by subtracting the mean number of spots in the medium and cells-alone control wells from the mean counts of spots in wells with antigens or peptide pools, and cells. We disregarded counts less than 5 spots/well. A well was considered positive if the count was at least twice that in the negative control wells and at least 5 spots more than the negative control wells. For the peptide pool wells, we summed the results across all the peptide pools for each volunteer at each timepoint. This will potentially count twice a T cell that responds to any of the 10-mer overlap regions that occur in two pools with adjacent peptides.

**Statistical analysis.** We performed analysis of variance for repeated measurements using the baseline result at screening as a covariate on log-transformed data to compare between groups. We then used a Mann-Whitney test for all comparisons between groups and a Wilcoxon test for the paired comparison of screening and 24-week samples in the BCG prime-MVA85A boost group.

**Cultured ELISPOT method.** For cultured ELISPOT, we stimulated 1  $\times 10^6$  cryopreserved PBMC with 20 g/ml of *M. tuberculosis* PPD, *M. avium* PPD or 10 g/ml recombinant antigen 85A in a 24-well plate. After a 3-d incubation period at 37 °C and 5% CO<sub>2</sub> atmosphere, we removed 500 l of the cell culture supernatant and replaced it with 5 IU/ml Lymphocult-T (Biotest) in R10. This was repeated on day 7. On day 9 we washed the cells three times and left them to rest overnight in 37 °C and 5% CO<sub>2</sub> atmosphere in R10. On day 10, cells were washed and resuspended in 2 ml of R10, 50 l of cultured cells (2.5  $\times 10^4$  of the initially plated cells) were transferred to duplicate wells of an ELISPOT plate and stimulated for 18 h with PPD-T 20 g/ml, PPD-A 20 g/ml and Ag85A 10 g/ml. We then developed the ELISPOT plate as previously described.

*Note: Supplementary information is available on the Nature Medicine website.*

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## COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see *Nature Medicine* web site for details).

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