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Longitudinal antibody and T cell responses in Ebola virus disease survivors and contacts: an observational cohort study

Ruth Thom*, Thomas Tipton*, Thomas Strecker*, Yper Hall*, Joseph Akoi Bore*, Piet Maes*, Fara Raymond Koundouno, Sarah Katharina Fehling, Verena Krähling, Kimberley Steeds, Anitha Varghese, Graham Bailey, Mary Matheson, Saidou Kouyate, Moussa 3 Coné, Balla Moussa Keita, Sekou Kouyate, Amento Richard Ablam, Lies Laenen, Valentijn Vergote, Malcolm Guiver, Joseph Timothy, Barry Atkinson, Lisa Ottowell, Kevin S Richards, Andrew Bosworth, Stephanie Longet, Jack Mellors, Delphine Pannetier, Sophie Duraffour, César Muñoz-Fontela, Oumou Sow, Lamine Koivogui, Edmund Newman, Stephan Becker, Armand Sprecher, Herve Raoul, Julian Hiscox, Ana Maria Henao-Restrepo, Keita Sakoba, N'Faly Magassouba, Stephan Günther, Mandy Kader Konde, Miles W Carroll

Summary

Background The 2013–16 Ebola virus disease epidemic in west Africa caused international alarm due to its rapid and extensive spread resulting in a significant death toll and social unrest within the affected region. The large number of cases provided an opportunity to study the long-term kinetics of Zaire ebolavirus-specific immune response of survivors in addition to known contacts of those infected with the virus.

Methods In this observational cohort study, we worked with leaders of Ebola virus disease survivor associations in two regions of Guinea, Guéckédou and Coyah, to recruit survivors of Ebola virus disease, contacts from households of individuals known to have had Ebola virus disease, and individuals who were not knowingly associated with infected individuals or had not had Ebola virus disease symptoms to serve as negative controls. We did Zaire ebolavirus glycoprotein-specific T cell analysis on peripheral blood mononuclear cells (PBMCs) on location in Guinea and transported plasma and PBMCs back to Europe for antibody quantification by ELISA, functional neutralising antibody analysis using live Zaire ebolavirus, and T cell phenotype studies. We report on the longitudinal cellular and humoral response among Ebola virus disease survivors and highlight potentially paucisymptomatic infection.

Findings We recruited 117 survivors of Ebola virus disease, 66 contacts, and 23 negative controls. The mean neutralising antibody titre among the Ebola virus disease survivors 3–14 months after infection was 1/174 (95% CI 1/136–1/223). Individual results varied greatly from 1/10 to more than 1/1000 but were on average ten times greater than that induced after 1 month by single dose Ebola virus vaccines. Following reactivation with glycoprotein peptide, the mean T cell responses among 116 Ebola virus disease survivors as measured by ELISpot was 305 spot-forming units (95% CI 257–353). The dominant CD8+ polyfunctional T cell phenotype, as measured among 53 Ebola virus disease survivors, was interferon γ +, tumour necrosis factor+, interleukin-2-, and the mean response was 0·046% of total CD8+ T cells (95% CI 0·021–0·071). Additionally, both neutralising antibody and T cell responses were detected in six (9%) of 66 Ebola virus disease contacts. We also noted that four (3%) of 117 individuals with Ebola virus disease infections did not have circulating Ebola virus-specific antibodies 3 months after infection.

Interpretation The continuous high titre of neutralising antibodies and increased T cell response might support the concept of long-term protective immunity in survivors. The existence of antibody and T cell responses in contacts of individuals with Ebola virus disease adds further evidence to the existence of sub-clinical Ebola virus infection.

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Introduction

Dogma suggests that Ebola virus disease survivors have long-term protection against reinfection with Zaire ebolavirus and studies in non-human primates and rodents indicate that such protective effects are mediated to a great extent by both antibody and T cell responses to the viral envelope surface glycoprotein.^{1–4} Vaccine studies in non-human primates also suggest that CD8+, interferon (IFN)+, tumour necrosis factor+, interleukin 2

(IL-2)-/+ T cells are a potential correlate of protection.^{5,6} The Ebola virus vaccine, Ervebo, which is based on the viral glycoprotein, was evaluated during the 2013–16 epidemic. Ring vaccination trials estimated vaccine efficacy to be 100%.⁷ Previous studies on survivor Ebola virus disease immunology have been done but only antibody levels were monitored.⁸ During historical Ebola virus outbreaks, plasma from survivors had been used to treat individuals suffering from acute Ebola virus

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*These authors contributed equally

National Infection Service, Public Health England, Porton Down, UK (R Thom PhD, T Tipton PhD, Y Hall PhD, K Steeds PhD, A Varghese BSc, M Matheson PhD, B Atkinson PhD, L Ottowell PhD, K S Richards PhD, A Bosworth PhD, S Longet PhD, J Mellors BSc, E Newman PhD, Prof M W Carroll PhD); Institute of Virology, Philipps University of Marburg, Marburg, Germany (T Strecker PhD, S K Fehling PhD, V Krähling PhD, Prof S Becker PhD); Center for Training and Research on Priority Diseases including Malaria in Guinea, Conakry, Guinea (J Akoi Bore PhD, S Kouyate PhD, M 3 Coné, B Moussa Keita MD, S Kouyate MA, A Richard Ablam, Prof M Kader Konde MD); Rega Institute for Medical Research, KU Leuven, Leuven, Belgium (P Maes PhD, L Laenen PhD, V Vergote PhD); Bernhard Noct Institute for Tropical Medicine, Hamburg, Germany (F Raymond Koundouno BSc, S Duraffour, C Muñoz-Fontela PhD, Prof S Günther MD); Ministry of

Health Guinea, Conakry, Guinea (Joseph Akoi Bore, F Raymond Koundouno, L Koivogui BSc); German Center for Infection Research, Partner Site Gießen-Marburg-Langen, Marburg, Germany (V Krähling, Prof S Becker); Biodiscovery Institute, School of Medicine, University of Nottingham, UK (G Bailey PhD); Public Health Laboratory, National Infection Service, Public Health England, Manchester Royal Infirmary, Manchester, UK (M Guiver PhD); Department of Disease Control, London School of Hygiene and Tropical Medicine, London, UK (J Timothy PhD); Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool, Liverpool, UK (J Mellors, Prof J Hiscox PhD); P4 Jean Mérioux-Inserm Laboratory, Lyon, France (D Pannetier PhD, H Raoul PhD); German Center for Infection Research, Partner Site Hamburg-Lübeck-Borstel-Riems, Hamburg, Germany (S Duraffour PhD, C Muñoz-Fontela, Prof S Günther); National Ethics Committee for Health Research, Conakry, Guinea (O Sow BSc); Medecins San Frontieres Brussels, Brussels, Belgium (A Sprecher PhD); World Health Organization, Geneva, Switzerland (A M Henao-Restrepo MD); Projet Laboratoire Fièvres Hémorragiques, Conakry, Guinea (K Sakoba PhD, N Magassouba PhD); and Nuffield Department of Medicine, University of Oxford, Oxford, UK (Prof M W Carroll)

Correspondence to: Prof Miles W Carroll, Public Health England, Porton Down SP4 0JG, UK miles.carroll@phe.gov.uk

See Online for appendix 2

Research in context

Evidence before this study

We searched the PubMed database with MESH terms “Ebola”, “immunity”, “sero-prevalence”, “paucisymptomatic”, and for articles published between database inception and April 15, 2020, in any language. There was one report on long-term antibody and T cell responses in 11 survivors split across two small Sudan ebolavirus outbreaks. Neutralisation assays revealed titres of 0–1/80, and T cell proliferation and kinetics analyses clearly showed CD8+ T cell activation, but quantification by ELISpot was not done. A comprehensive analysis of ELISA responses in survivors from the 1995 Kikwit outbreak showed antibody titres plateaued at 3 weeks to 1 year after onset of symptoms. A report by Leroy and colleagues revealed the existence of paucisymptomatic individuals with Ebola virus infections. Additionally, work by Glynn and colleagues further demonstrates the presence of asymptomatic Ebola virus infection. Several reports showed persistence of Ebola virus in immune privileged sites, and an in-depth longitudinal analysis of the B cell response among four Ebola virus disease survivors has also been done. We found no other studies reporting the longitudinal analysis of cellular and humoral immunity among survivors over 3 consecutive years.

Added value of this study

This unique study reports an in-depth analysis of naturally acquired immunity to Ebola virus and enables a

disease; however, a convalescent plasma therapy trial in west Africa did not show significant clinical benefit or a correlation between neutralisation and efficacy.⁹ These results suggest that both antibody and T cell responses might have a role in protection in humans.

During the 1996 Ebola virus disease Gabon outbreak, a small number of individuals were shown to be infected with Ebola virus but had a low viral load and presented only mild disease.¹⁰ Similar observations were also reported in the 2013–16 west African epidemic.³¹ Such observations are rarely reported in detail and do not include in-depth immunology or molecular diagnostic data. However, a case report¹¹ with associated sequence data suggests that a paucisymptomatic mother was able to transmit Ebola virus to her baby. A better understanding of the incidence of paucisymptomatic Ebola virus disease is needed and might have implications in assessing future transmission risks. Additionally, systematic reviews^{12,13} of sero-epidemiology studies of populations in Africa reported that about 8% of individuals had antibodies specific to Ebola virus and other related members of the filovirus family. The absence of reported Ebola virus disease outbreaks in such populations is puzzling but could be explained by antibody cross reactivity, sub-clinical Ebola virus disease or increased rates of infectious diseases in general.

A complete understanding of naturally acquired immunity to Ebola virus will help improve the design and evaluation of experimental vaccines, convalescent

comprehensive comparison between naturally acquired and vaccine-induced immunity to Ebola virus both at the antibody and T cell level. The study also provides supporting evidence for the existence of paucisymptomatic Ebola virus disease and suggests that true incidence of Ebola virus infection in the west African outbreak was greater than recorded. Additionally, T cell phenotyping results support the preclinical findings of a potential correlate of protection to Ebola virus.

Implications of all the available evidence

We suggest that up to 9% of individuals with Ebola virus disease will present with mild symptoms, which will have implications with regard to monitoring and responding to future outbreaks. Additionally, the work here shows that T cell responses are relevant and long lasting among survivors of Ebola virus disease; therefore, future vaccine developers might wish to consider the T-cell response in more depth. The evaluation of convalescent plasma showed varying titres of anti-Ebola virus IgG, so pre-screening of IgG and neutralising titres before administration of convalescent plasma should be considered in future outbreaks.

plasma, and therapeutic antibodies. Assessment of the memory cell phenotype in survivors of Ebola virus disease might also provide insight into chronic viral shedding from immune privileged sites. Furthermore, assessment of the immune response of individuals who are not known to be Ebola virus-positive but exposed to Ebola virus disease might reveal the extent of paucisymptomatic infections and help in the assessment of the incidence of Ebola virus infection and the associated case fatality rate.

Methods

Study designs and participants

We did an observational study based on opportunistic sampling. Volunteers were recruited from two prefectures in Guinea, Guéckédou, the epicentre of the outbreak, and Coyah, which is around 300 miles away. Blood was collected from survivors of Ebola virus disease 3–14 months after infection. A second and third bleed of volunteers was done about 12 and 24 months later. Individuals presented their Ebola virus disease survivor certificate or were identified on Ebola treatment centre databases to verify that they were survivors. Contacts were defined as individuals who provided care for or were living in the same household of an Ebola virus disease confirmed case. Survivors and contacts were asked a number of questions (appendix 2, 2pp 2–4) regarding their contact with individuals with Ebola virus disease, as well as presentation of any Ebola virus disease-like

symptoms. Following immune analysis, where possible, a more in-depth interview took place to understand the degree of care they gave and discuss symptoms they might have had. Additionally, volunteers were recruited, who were not knowingly exposed to people with Ebola virus disease and did not attend high-risk events such as funerals. All volunteers were informed of the procedures and purpose of the study and only consenting participants were included. Ethical approval was obtained from the National Ethics Committee for Health Research, Guinea (33/CNERS/15) and from the National Research Ethics Service, UK. Written, informed consent was obtained from all participants.

Procedures

30–50 mL of blood was collected into edetic acid vacutainers. Plasma and peripheral blood mononuclear cells (PBMCs) were separated by layering the blood over a Ficoll gradient (GE Healthcare; Amersham, UK) and centrifugation. Plasma was aspirated and stored at -20°C for antibody studies. PBMCs were either used fresh, or frozen ($<-70^{\circ}\text{C}$) and shipped to the UK, where they were stored in liquid nitrogen. Participants were interviewed using the health surveillance questionnaire (appendix 2, pp 2–4) and with regards to health scores during the Ebola outbreak each category was given equal weighting with a maximum score of 13.

Freshly isolated PBMCs were prepared at 2×10^6 cells/mL in Leibovitz media supplemented with penicillin–streptomycin, foetal calf serum, L-glutamine, HEPES, and 2-mercaptoethanol was used for IFN γ ELISpot. Briefly, whole blood was layered onto LeucoSep-tubes (Sigma; Gillingham, UK) containing Ficoll-Paque Plus (GE Healthcare; Amersham, UK); the sample was centrifuged at 800 G for 20 min and PBMCs were collected from the buffy coat layer. The PBMCs were stimulated with Ebola virus glycoprotein peptide library (Mimotopes; Melbourne, Australia) at a final concentration of $2.5 \mu\text{g}$ per peptide per mL, as described previously.¹⁴ After 18–20 h incubation at 37°C , IFN γ release was determined by standard ELISpot protocol (Mabtech; Nacka Strand, Sweden) and spot forming cells enumerated using an S6 core analyser (Cellular Technology; Shaker Heights, OH). IFN γ release was calculated by subtracting the background from each well and taking the mean of three triplicate wells. The results were determined as spot forming units per one million cells and IFN γ response to the Ebola virus glycoprotein peptide were summed to determine the overall T-cell response.

ELISA was done as described previously.¹⁵ Briefly, high binding microtitre plates were coated with whole Ebola virus inactivated virions and incubated for 16–20 h. After washing in PBS and 0.1% Tween20 (PBST) and blocking (PBS and 5% milk powder), 1/200 dilutions of plasma sample were added to the plates and incubated for 1 h. Horseradish peroxidase-conjugated polyclonal antibody (P0214, Dako; Santa Clara, CA; dilution 1/1000) in

conjunction with TMB substrate was used to develop the reaction. Optical density was determined at 450 nm minus 630 nm (reference wavelength). Each sample was analysed in duplicate on mock and viral antigen. The mean optical density of each sample on the mock antigen was subtracted from the mean optical density of the respective sample on the Ebola virus antigen. Arbitrary ELISA units were extrapolated by linear regression analysis using standard curves generated from patient antiserum. Further specificity was assessed using a Zaire, Makona Ebola virus glycoprotein (sourced from Nuffield Department of Medicine, Oxford University, Oxford, UK) specific ELISA, for which Nunc Maxisorb 96-well plates (Merck; Darmstadt, Germany) were coated overnight (16–18 h) with purified Ebola virus glycoprotein antigen ($0.5 \mu\text{g}/\text{mL}$). Plasma was serially diluted, starting at 1/200 and the bound IgG was detected using goat anti-human IgG Fc γ specific antibody conjugated to alkaline phosphatase (1/15000). Alkaline phosphatase-yellow substrate (Sigma) was added and the optical density measured at 405 nm using a VERSAmax plate reader (Molecular Devices; Wokingham, UK) controlled by SoftMax Pro Enterprise software (version 4.7.1). The plates were read using a predefined Softmax template, which fits a four-parameter logistic curve to the dose response data. The cutoff was defined as the mean negative value plus five SDs.

The activity of the Ebola virus-specific antibodies present in plasma was determined by neutralisation of Ebola virus variant Mayinga (1976) as previously described.⁵ Briefly, after heat treatment for complement inactivation, plasma was serially diluted in supplemented Dulbecco's modified Eagle's medium (DMEM) in 96-well plates, 100 median tissue culture infectious dose (TCID₅₀) units of Ebola virus variant Mayinga were added to the plasma dilutions. Following incubation at 37°C for 1 h, Vero cell suspension in supplemented DMEM was added. Plates were then incubated at 37°C with 5% CO₂ and cytopathic effects were evaluated at 7 days after infection. Neutralisation titres were calculated as geometric mean titre of four replicates. A titre of 1/8 or above is classified as positive.

Recombinant glycoprotein, nucleoprotein, and viral protein 35 were generated based on the Ebola virus strain Makona in HEK293T cells and whole cell lysates were used. Viral protein 40 was based on Ebola virus strain Kikwit and was obtained from Stratech Scientific (Ely, UK). Proteins were heat denatured and loaded onto 4–12% BisTris gels and separated by size by SDS-PAGE. The proteins were then transferred to polyvinylidene difluoride membrane and blocked overnight in block buffer (PBST buffer with 5% milk). Plasma was diluted 1/1000 in block buffer and incubated with the Ebola virus-protein containing blots for 4 h at room temperature. The blots were washed for 5 min in PBST. Secondary antibody (goat anti-human IgG γ -chain

specific] peroxidase conjugate) F(ab')₂ fragments (Sigma; A2290), were diluted 1/1000 in block buffer. The blots were incubated with secondary antibody for 1 h at room temperature. Membranes were washed and blots developed with ECL prime, incubating for 5 min. Images were captured at 5 min and 10 min exposure and presence of immunoreactivity determined against a molecular marker standard.

Intracellular cytokine staining was done as has been described previously.⁵ Briefly, PBMCs were resuspended in warmed complete media (Roswell Park Memorial Institute medium supplemented with penicillin-streptomycin, fetal calf serum, L-glutamine, HEPES, and 2-mercaptoethanol) and rested overnight at 37°C. The following day cells were adjusted to 1×10⁶ cells/mL in media containing anti-CD28 BUV737, CD49d, and CD107a-PerCP cy5.5 (1 µg/mL). Samples were then left either untreated or were stimulated with an Ebola virus glycoprotein peptide pool, containing 187 15mer overlapping peptides at 2.5 µg/peptide or 1 µg/mL staphylococcal enterotoxin B for 16–18 h, as previously described.^{5,6} 2 h into the incubation, brefeldin A and monensin (1 µg/mL) were added to block cytokine secretion from the cell. The following day samples were washed in cold FACS wash and LIVE/DEAD fixable aqua dye (Life Technologies; Carlsbad, CA) was added. Samples were washed, then incubated with a cell surface cocktail of antibodies including CD3-APC 750, CD4-BV786, CD8-AF700, CD19-BV510, CD14-BV510, CCR7-APC, CD95-BUV395, and CD45RO-BV605. Cells were then washed, fixed and permeabilised using Becton Dickinson (London, UK) Cytofix/Cytoperm, and stained for intracellular cytokines using IFN γ -AF488, TNF-BV421, and IL-2-PE. Samples were then washed, resuspended, and acquired using a Becton Dickinson Fortessa machine and FACS Diva software. Sample analysis used FlowJo, Pestle, and SPICE software as described previously.⁵ All antibodies were obtained from Biolegend (London, UK), with the exception of CD95-BUV395 and CD28 BUV737, which were obtained from Becton Dickinson.

Statistical analysis

The data collected from all the volunteers were categorised into three groups, survivor, contact, and negative, which were sub-divided by region and sex. Measurements for ELISpot, ELISA and neutralisation were tested independently. Statistical analysis on the fixed effect coefficient for year was done using R version 4.0.1 with the lme4 package version 1.1–23 and fitted models were assessed for violation of assumptions. Cytokine responses were determined by subtracting the untreated response from that of the stimulation; negative values were set to 0.001 and statistical differences were determined using Mann-Whitney test. Correlations were determined using Spearman correlation analysis. Statistical tests were done using GraphPad Prism version 8.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

206 volunteers were recruited from the Guéckédou and Coyah prefectures (table; appendix 2, p 1): 117 survivors of Ebola virus disease, 66 contacts, and 23 negative controls. Glycoprotein-specific IgG titres were significantly correlated with whole Ebola virus-specific antibody titres (r 0.85; p <0.0001; appendix 2, p 6). 113 (96%) of the 117 survivors had detectable concentrations of IgG to Ebola virus 3–14 months after infection, with responses measured at one dilution and defined as arbitrary units. 11 (17%) of 66 contacts had concentrations of Ebola virus-specific IgG that were above background (figure 1A). The activity of the Ebola virus-specific IgG was measured by the ability to neutralise the infection of Ebola virus-Mayinga in vitro. Testing for functional activity using our neutralisation assay showed that 113 (96%) of 117 survivors showed detectable titres of Ebola virus-specific neutralising antibody (figure 1B). Notably, there was a greater than 100-fold range in neutralisation titres (1/10–>1/1000) among survivors, with a mean of 1/174. Plasma from six (9%) of the 66 contacts also showed Ebola virus-specific neutralising antibodies, and the mean of these six samples was 1/133, which paralleled their detectable concentrations of Ebola virus glycoprotein-specific IgG. Further characterisation of five of these samples by Western blot revealed the presence of antibodies not only to glycoprotein but also to nucleoprotein, viral protein 40, and viral protein 35 (appendix 2, p 7). All negative controls had undetectable neutralising activity. PBMCs from 15 (13%) of 116 survivors and isolated 4 months after infection did not produce an IFN γ response following glycoprotein peptide stimulation. During 2015 sampling, ELISpots were only done on contacts in the Coyah cohort, with the exception of G012 and G034 from the Guéckédou cohort; however, of the 42 contacts tested, eight had a detectable IFN γ response to Ebola virus glycoprotein. Six of these also had detectable Ebola virus-specific ELISA and neutralisation titres. Ebola virus disease survivors typically had a score of 7.5 out of 13 in health surveillance questionnaires, whereas close contacts would report a score of 1.3 out of 13. Of the contacts that showed a humoral and cellular response, only two of the five we were able to question reported a score greater than 2 out of 13, suggesting they were seropositive contacts that were displaying more overt symptoms during the outbreak.

We were able to longitudinally map the responses of 96 survivors and found that ELISA (figure 2A), neutralisation (figure 2B), and ELISpot responses (figure 2C) appeared stable over time. Mixed-effects linear regression for each outcome showed that there was

	Guéckédou			Coyah			Total		
	Survivor	Contact	Negative	Survivor	Contact	Negative	Survivor	Contact	Negative
Total	46	26	9	71	40	14	117	66	23
Male	21	7	8	32	23	9	53	30	17
Female	25	19	1	39	17	5	64	36	6
Returning in 2016-17	33	19	0	63	23	0	96	42	0

Table: Numbers of participants from Coyah and Guéckédou sample sites

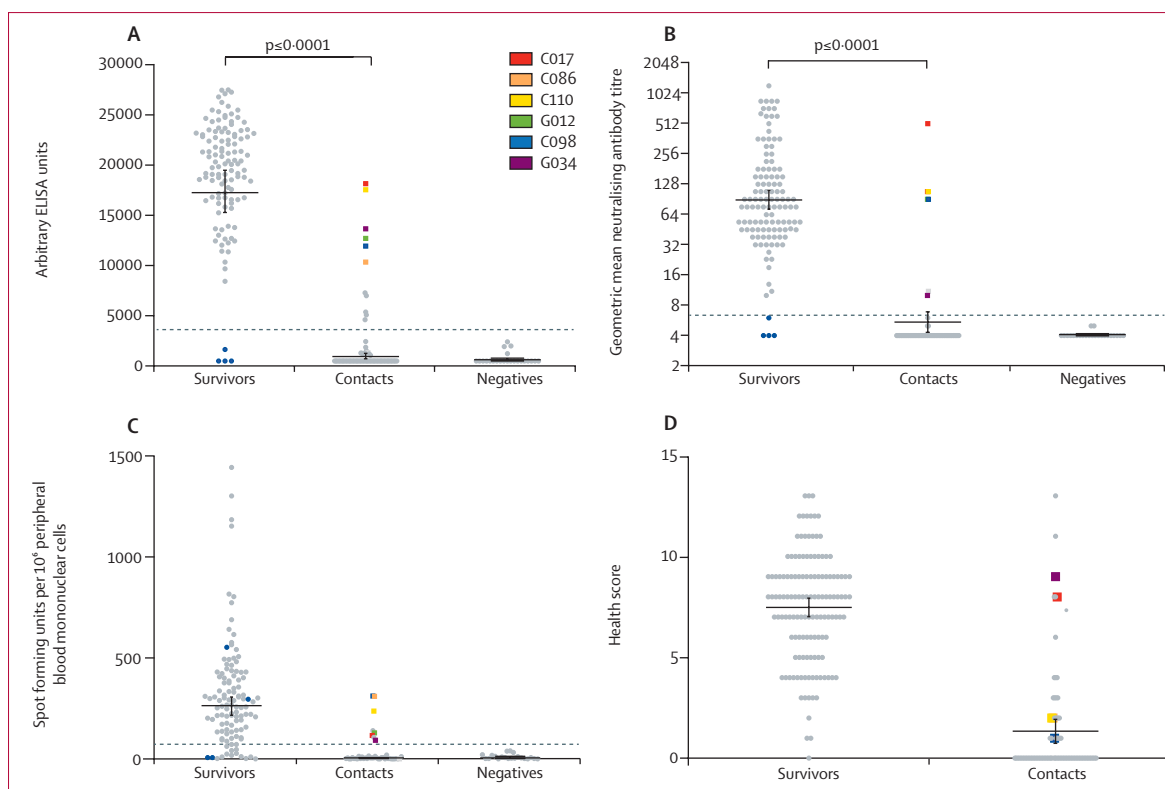


Figure 1: Characterisation of immune responses of Ebola virus disease survivors and close contacts

(A) Ebola virus-specific antibody titre against whole virus preparation as measured by ELISA in plasma samples collected from survivors ($n=117$), contacts ($n=66$), and negative controls ($n=23$). (B) Geometric mean neutralising antibody titre against live Ebola virus (strain Mayinga) using plasma samples collected from survivors ($n=117$), contacts ($n=66$), and negative controls ($n=23$). Results displayed on a log₂ scale. (C) ELISpot to detect IFN γ secretion using an Ebola virus glycoprotein peptide library and peripheral blood mononuclear cell samples from survivors ($n=116$), contacts ($n=42$), and negative controls ($n=21$). (D) Quantified results from health questionnaire given to all survivors and contacts. A score of 13 represents severe symptoms and 0 represents no symptoms during the 2013-16 outbreak. Bars represent the geometric mean plus or minus the standard error of the mean. Dashed lines represent the mean plus five SDs. Mann-Whitney tests were done for all statistical analyses. Blue dots represent seronegative survivors. Coloured squares represent six (9%) of the 66 contacts who had Ebola virus-specific neutralising antibodies. IFN γ =interferon γ .

statistical evidence for the effect of year for all three measured outcomes (assessed by ANOVA against nested intercept-only models; ELISA $p<0.0001$, neutralisation $p<0.0001$, ELISpot $p=0.0019$). Comparison of each model to a reduced model with a single linear parameter fitted for year showed that the linear predictor sufficiently explained the annual variance in the outcome for ELISA data only ($p=0.14$). For the ELISA outcome, the fixed effect coefficient for year showed an inverse relationship (-1022.08 arbitrary ELISA units, 95% CI -1453.54 to -590.62), suggesting an annual decrease in antibody titre. However,

for all outcomes, the magnitude of serological responses across years and individuals remained higher than responses measured among control group samples (figure 1). These prolonged, elevated, responses could theoretically be due to re-exposure to Ebola virus antigen or simply reflect the basal immunological state to such a pathogenic infection. To address this issue, we plotted 2015 survivor results in relation to their time since resolution of infection. Results were consistent over time (figure 2D).

Flow cytometry and intracellular cytokine staining showed that the majority of IFN γ , as a proportion of their

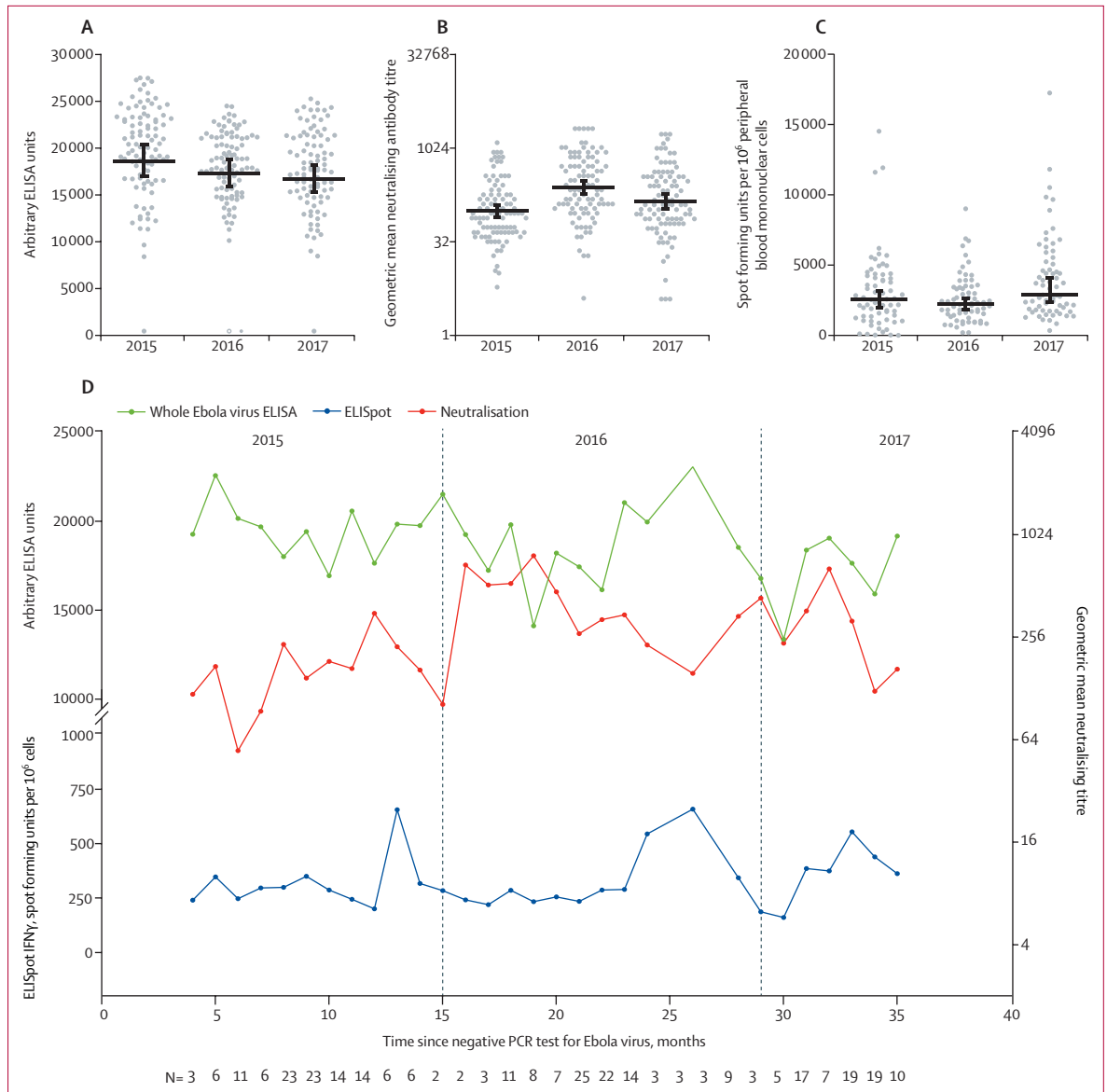


Figure 2: Longitudinal response following Ebola virus infection

(A) Ebola virus-specific antibody titre against whole virus preparation as measured by ELISA in plasma samples collected from matched survivors (n=96) in 2015–17. (B) Neutralising antibody titre against live virus preparation (Mayinga) using plasma samples collected from matched survivors (n=96) in 2015–17. (C) ELISpot to detect IFN γ secretion using an Ebola virus glycoprotein peptide library and peripheral blood mononuclear cell samples from matched survivors (n=63) in 2015–17. (D) Time in months from when each volunteer was confirmed Ebola virus-negative following second negative PCR and so were discharged from the Ebola treatment centre. Data include antibody titre to whole Ebola virus as determined by ELISA, antibody activity as determined by neutralisation assay, and T-cell responses as determined by ELISpot. Parts A–C show the geometric mean with 95% CI. IFN γ =interferon γ .

parent subset, was coming from CD8+ T cells (figure 3). CD8+ T cells originating from Ebola virus disease survivors (n=51) produced significantly more IFN γ than peripheral blood mononuclear cells originating from ELISpot negative controls (n=26; p<0.0001). Furthermore, when these IFN γ responses were backgated to their parent phenotype markers, the majority of antigen-specific CD8+ T cells showed intermediate CCR7 expression and low CD45RO expression, corresponding with a naive like phenotype. By contrast, CD4+ T cells

showed primarily CD45RO positive and CCR7 low or intermediate expression consistent with a central memory or effector memory phenotype.¹⁷

A previous report¹⁸ suggested a role for so called polyfunctional CD8+ T cells, which produce multiple cytokines, in the control of Ebola virus disease; we therefore investigated the incidence of IFN γ +, TNF+ or IFN γ +, TNF+, IL-2+ CD8+ T cells among 53 survivor PBMC samples collected in 2016 (15–28 months after infection). When these PBMCs were stimulated overnight

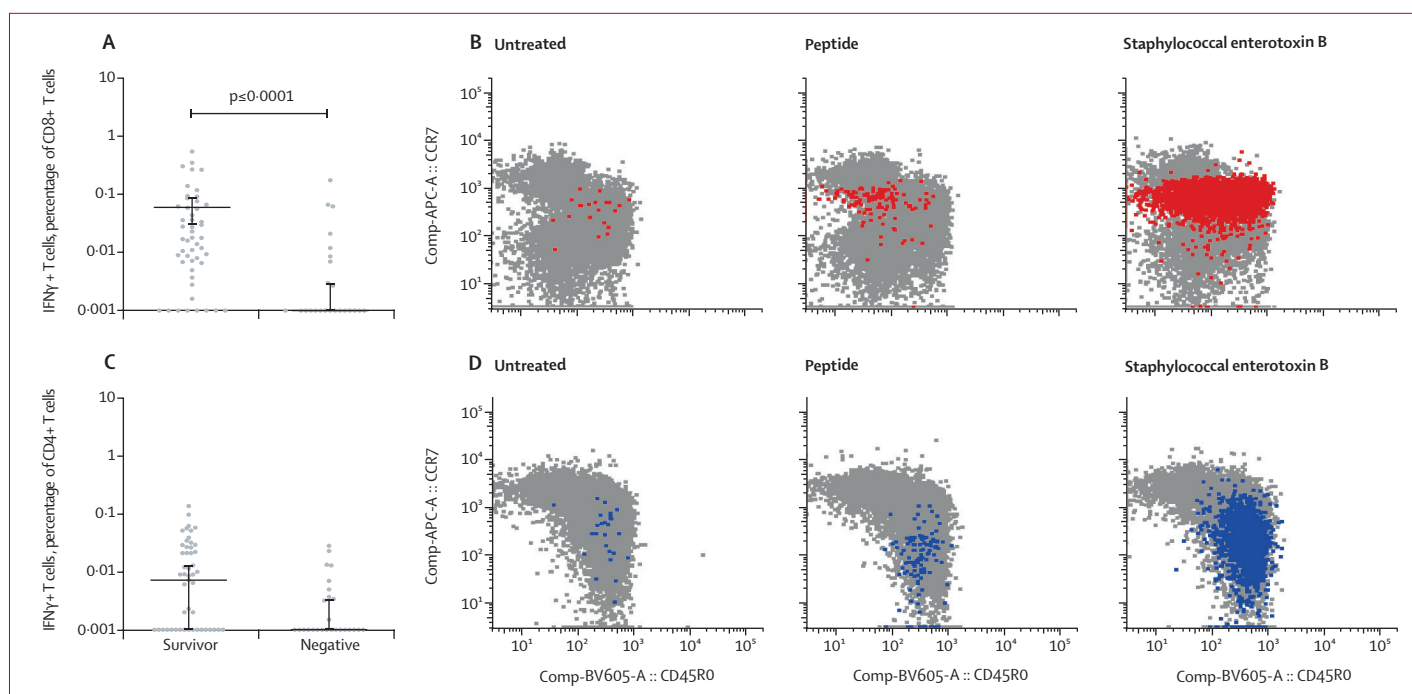


Figure 3: T cell activation in response to Ebola virus glycoprotein peptide

Samples collected from 2016 were used to analyse the T cell response to Ebola virus disease. (A) The sum of CD8+ T cells producing IFN γ in response to overnight stimulation with glycoprotein peptides (2.5 μg per peptide per mL). Bars represent the geometric mean plus or minus the standard error of the mean. (B) Representative dot plot of CD8+ T cell phenotype. IFN γ producing cells are highlighted in red. (C) The sum of CD4+ T cells producing IFN γ in response to overnight stimulation with glycoprotein peptides (2.5 μg per peptide per mL). Bars represent the geometric mean \pm the standard error of the mean. (D) Representative dot plot of the CD4+ T cell phenotype. IFN γ producing cells are highlighted in red. IFN γ =interferon γ .

with Ebola virus glycoprotein peptide pools, a significant proportion of T cells were IFN γ +, TNF+, and CD8+ compared with 26 contacts who were seronegative by ELISA and neutralisation ($p=0.0004$; figure 4). Significant populations of IFN γ + ($p=0.018$) only and IFN γ +, TNF+ ($p=0.0075$) CD8+ T cells were identified among the survivors' PBMCs compared with ELISpot negative controls. Survivor samples had a significantly larger proportion of CD107a expressing cells than their comparative contacts ($p=0.032$), and when backgated, the majority of these CD107a+ cells were IFN γ +, TNF+, CD8+ T cells (figure 4B). CD8+ T cells originating from survivors of Ebola virus disease showed significantly greater amounts of CD107a than negative controls ($p=0.032$). There was a strong correlation between total Ebola virus ELISA and neutralisation assay results (appendix 2, p 8). We also observed a significant correlation between ELISpot and ELISA as well as ELISpot and neutralisation results (appendix 2, p 8). We did not find a significant correlation or difference between either age, sex, or virus load at time of diagnosis (cycle threshold values) and any of the immunological parameters measured (appendix 2, pp 8–9).

Discussion

We report the most comprehensive study of Ebola virus disease survivor immunity to date and uniquely assess both neutralising antibody and T cell responses.

Neutralising antibody titres from survivors 3–35 months after infection were ten-fold larger than those seen 1 month after vaccination with an efficacious single dose vaccine^{5,7}. This finding is compelling evidence for long-term protection against reinfection with Ebola virus and bodes well for survivors if the disease returns to west Africa, suggesting that they could continue to have a role in front-line activities to control future outbreaks. However, the absence of humoral immunity in a small percentage of survivors might explain observations that a minority of Ebola virus disease survivors can be reinfected, suggesting it would be essential to assess their immunity before the risk of potential re-exposure. Of the four survivors who displayed no antibody response, two showed a detectable ELISpot response; however, this response was not present in subsequent samples and misuse of survivor certificates cannot be ruled out in this instance. Absence of antibody responses has been documented for various emerging diseases, including severe acute respiratory syndrome coronavirus 2 antibody negative samples from COVID-19 convalescent individuals.¹⁹ From these data we would suggest that sero-epidemiology studies for Ebola virus might underestimate the prevalence of the disease. The disparity between vaccinees and survivors also highlights the need to periodically boost vaccine immunity or use alternative heterologous vaccine vector approaches that might induce a more durable response. However, it is yet to be

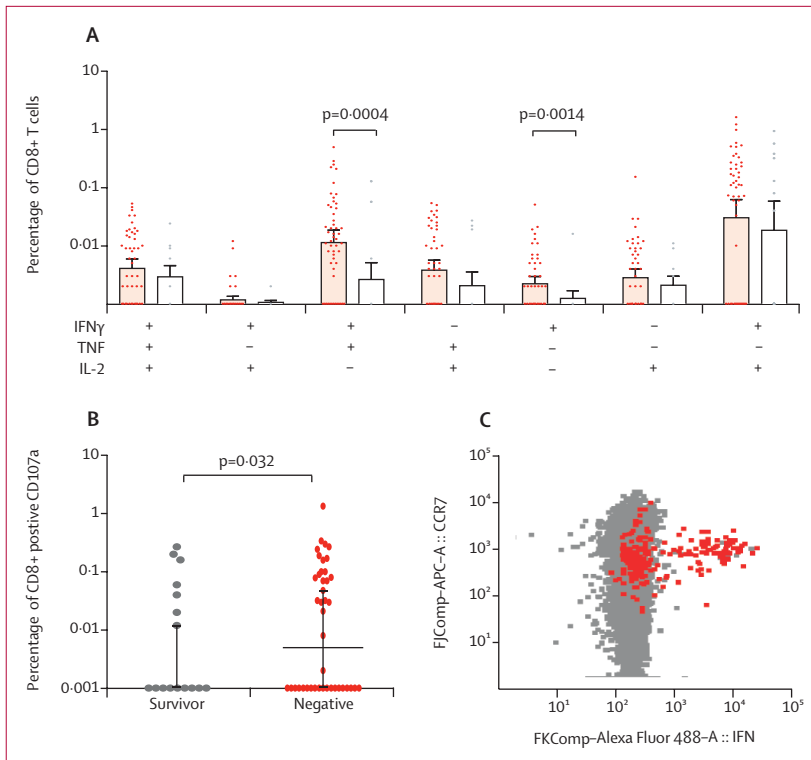


Figure 4: Polyfunctional CD8+ T cell analysis

(A) Cytokines secreted by CD8+ T cells. Survivors' PBMCs are represented by red dots and ELISpot negative controls by grey dots. Samples that returned a negative result during analysis were assigned a value of 0.001. (B) CD8+ T cell activation as measured by intracellular staining for the degranulation marker CD107a. (C) Representative dot plot of survivor PBMCs stimulated with Ebola virus glycoprotein peptide. CD107a+ events are highlighted in red. PBMC=peripheral blood mononuclear cell. IL-2=interleukin 2. IFN γ =interferon γ . TNF=tumour necrosis factor.

determined what constitutes a protective antibody titre. The continuously high titres of neutralising antibody and T cell responses are perhaps unsurprising, because Ebola virus has been reported to persist for more than 1 year in immune privileged sites.^{11,20,21} Furthermore, where we have found responses to be stable up to 3 years after infection, studies of survivors from Ebola virus disease outbreaks suggest that ELISA antibody responses continue to be relatively stable for more than 1 year and that low titres of neutralising antibodies have been found in survivors 40 years after infection.^{22,23} However, further work is needed to show antigen persistence in these survivor samples.

We found steady state titres of Ebola virus glycoprotein antibody measured by ELISA and neutralisation from groups of survivors 3–14 months after the onset of symptoms. This finding contrasts with a study of four Ebola virus disease survivors in which longitudinal analysis suggest a continuous increase in neutralising titre from up to 900 days after recovery in some individuals.²⁴ Obvious study differences, including an absence of longitudinal sampling and of experimental treatment, might explain the differing results. Of note, the longitudinal analysis in our study uses only data from participants who provided a full 3 years' worth of data;

therefore, data from participants with missing or incomplete data was discarded, which is a limitation of this study with regard to the stability over time. Long-term, potent Ebola virus responses could be a result of continuous exposure to viral antigen as it seeps back into the systemic environment from immune privileged locations. There is evidence that relapse and transmission can occur long after the primary infection has been resolved,²⁵ and it would be of great interest to sample such immune privileged sites from our cohorts to see if there is live virus present. However, such an approach was not possible owing to biosafety constraints and the limitations of our ethical approval. We suggest that our studies do not support the presence of recirculating antigen 15 months or more after recovery, because we believe that CD8+ T cell phenotyping revealed a possible subset of T memory stem cells. T memory stem cells, are a self-renewing population of lymphocytes that are CD45RO⁻, CCR7⁺, CD27⁺, and CD95⁺.²⁶ In support of this suggestion, the phenotype of IFN γ -producing CD4+ T cells was CD45RO⁺ (figure 3D), suggesting that these cells are memory T cells and not terminally differentiated effectors that have recently been exposed to antigen. LaVergne et al²⁷ found that post Ebola syndrome was associated with greater activation of CD4+ and CD8+ T cells, so it would be of interest to correlate the magnitude of our T cell responses with post Ebola syndrome in any follow up studies.

Plasma from survivors of Ebola virus disease, containing various levels of neutralising antibodies, has been used to treat acute cases of Ebola virus infection during several prior outbreaks.²⁸ However, the 2013–16 outbreak enabled a more comprehensive study to be done, which reported an absence of statistically significant improvement in survival associated with plasma treatment.⁹ In the same trial, neutralising antibody titres of plasma harvested from Ebola virus disease survivors were on average ten times smaller than seen in our study. However, differences between assay conditions could account for this outcome, which might have affected the subsequent analysis of functional antibody activity and clinical outcome.⁹ Furthermore, Sahr et al²⁹ showed a positive effect with plasma therapy in a small clinical trial in Sierra Leone.

Immune analysis of contacts of individuals with Ebola virus disease, who did not have a confirmed positive PCR test or report with typical disease to a treatment centre, revealed that up to 9% have both detectable humoral and cellular immunity. This finding could be explained by the existence of cross-reactive antibodies to Ebola virus proteins, a hypothesis that has been suggested by a previous study that analysed serum from German citizens and found a number of filovirus specific seropositive cases.³⁰ However, our seropositive contacts showed both neutralising antibody and T cell responses, and we were able to show that five individuals also had antibodies specific to Ebola virus nucleoprotein, viral protein 35, and viral protein 40, thus confirming that they must have

come into contact with the virus. The extent to which these contacts were symptomatic remains unknown and is difficult to address after the event and largely relies on anecdotal evidence. Matters are further complicated owing to the stigma surrounding Ebola virus disease in west Africa, meaning that individuals who were infected at the time were likely to have downplayed their symptoms or not sought treatment. However, owing to the health questioning and interviews that took place we are confident that the paucisymptomatic contacts within our cohort did have mild disease that did not prevent them from conducting their normal duties. Therefore, these observations strongly suggest that four potential outcomes from Ebola virus exposure exist: symptomatic infection resulting in death, symptomatic infection with survival and immunity, paucisymptomatic infection with survival and immunity, and no infection and no immunity. Additional support for the existence and potential transmission from paucisymptomatic people comes from a report whereby a mother was able to transmit Ebola virus to her child through breastmilk. Both parents reported no symptoms of Ebola virus disease and blood samples were PCR negative; however, semen and breast milk samples were shown to be PCR positive, and breastfeeding was concluded to be the most likely route of transmission.¹¹ Furthermore, a number of serological studies have suggested the existence of paucisymptomatic infection in west Africa.^{31,32} It might not be possible to categorically show that these positive contacts were paucisymptomatic or showed mild disease; however, this study and others^{10,31} suggest that this aspect of Ebola virus transmission and the role it might have in spreading Ebola virus disease needs to be considered during any future outbreaks. Additionally, the number of individuals infected with Ebola virus could be significantly larger than the officially reported numbers, which will also affect the extent of the reported mortality rate.

We show here that survivors of Ebola virus disease have a long-lasting T cell response. The T cell responses in our survivor cohort, measured some years after recovery from disease, are similar to that seen 6 months after vaccination with chimpanzee adenovirus and modified vaccinia Ankara boost.⁵ The importance of this T cell activity to Ebola virus vaccine design has been the subject of debate. The ChAd3, MVA boost regime has been shown to induce a strong T cell response and primate studies have revealed that CD8⁺ polyfunctional T cells might have a key role in protection from Ebola virus challenge, following vaccination with an adeno vaccine vector.¹⁸ Our results show that the IFN γ ⁺ and TNF⁺ double-positive CD8⁺ T cell population, which is known to be involved in long-term protection in primates, is also the dominant CD8⁺ T cell population in responses to restimulation with glycoprotein peptide in Ebola virus disease survivors. Therefore, further work should be done to determine the activity and proportion of CD8⁺ polyfunctional T cells in response to Ebola virus vaccination.

Contributors

PM, M3C, SG, and MKK conceived the study and experimental design with input from GB, MM, LL, MG, DP, OS, LK, EN, SB, HR, JH, AMH-R, and NM. Sample collection within Guinea was led by JAB and MKK with assistance from M3C, BMK, SK, and ARA. Sample processing in Guinea was led by RT, TT, YH, JAB, and PM with help from FRK, KiS, AV, VV, BA, LO, AB, SD, and EN. Analysis of samples in the UK was done by RT, TT, YH, and KSR. Processing of whole virus ELISA and live virus neutralisation was led by TS with help from SKF and VK. The manuscript was primarily written by RT, TT, YH, VK, M3C, SL, JM, CM-F, SB, and SG with input from all authors.

Declaration of interests

SG received grants from European Commission and German Research Foundation for the conduct of this study. All other authors declare no competing interests.

Data sharing

De-identified individual participant data that underlie the results reported in this article and the study protocol will be made available to researchers who provide a methodologically sound proposal beginning 3 months and ending 5 years following publication. Proposals should be directed to miles.carroll@phe.gov.uk, and to gain access, data requestors will need to sign a data access agreement.

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References

- Mohamadzadeh M, Chen L, Schmaljohn AL. How Ebola and Marburg viruses battle the immune system. *Nat Rev Immunol* 2007; 7: 556–67.
- Olinger GG, Bailey MA, Dye JM, et al. Protective cytotoxic T-cell responses induced by Venezuelan equine encephalitis virus replicons expressing Ebola virus proteins. *J Virol* 2005; 79: 14189–96.
- Warfield KL, Olinger G, Deal EM, et al. Induction of humoral and CD8⁺ T cell responses are required for protection against lethal Ebola virus infection. *J Immunol* 2005; 175: 1184–91.
- Bradfute SB, Bavari S. Correlates of immunity to filovirus infection. *Viruses* 2011; 3: 982–1000.
- Ewer K, Rampling T, Venkatraman N, et al. A monovalent chimpanzee adenovirus Ebola vaccine boosted with MVA. *N Engl J Med* 2016; 374: 1635–46.
- Stanley DA, Honko AN, Asiedu C, et al. Chimpanzee adenovirus vaccine generates acute and durable protective immunity against ebolavirus challenge. *Nat Med* 2014; 20: 1126–29.
- Henao-Restrepo AM, Longini IM, Egger M, et al. Efficacy and effectiveness of an rVSV-vectored vaccine expressing Ebola surface glycoprotein: interim results from the Guinea ring vaccination cluster-randomised trial. *Lancet* 2015; 386: 857–66.
- Peters CJ. Ebola and hantaviruses. *FEMS Immunol Med Microbiol* 1997; 18: 281–89.
- van Griensven J, Edwards T, de Lamballerie X, et al. Evaluation of convalescent plasma for Ebola virus disease in Guinea. *N Engl J Med* 2016; 374: 33–42.

- 10 Leroy EM, Baize S, Volchkov VE, et al. Human asymptomatic Ebola infection and strong inflammatory response. *Lancet* 2000; **355**: 2210–15.
- 11 Sissoko D, Keita M, Diallo B, et al. Ebola virus persistence in breast milk after no reported illness: a likely source of virus transmission from mother to child. *Clin Infect Dis* 2017; **64**: 513–16.
- 12 Nyakarahuka L, Kankya C, Krontveit R, et al. How severe and prevalent are Ebola and Marburg viruses? A systematic review and meta-analysis of the case fatality rates and seroprevalence. *BMC Infect Dis* 2016; **16**: 708.
- 13 Mulangu S, Alfonso VH, Hoff NA, et al. Serologic evidence of Ebola virus infection in a population with no history of outbreaks in the Democratic Republic of the Congo. *J Infect Dis* 2018; **217**: 529–37.
- 14 Ledgerwood JE, Costner P, Desai N, et al. A replication defective recombinant Ad5 vaccine expressing Ebola virus GP is safe and immunogenic in healthy adults. *Vaccine* 2010; **29**: 304–13.
- 15 Krähling V, Becker D, Rohde C, et al. Development of an antibody capture ELISA using inactivated Ebola Zaire Makona virus. *Med Microbiol Immunol (Berl)* 2016; **205**: 173–83.
- 16 Agnandji ST, Huttner A, Zinser ME, et al. Phase 1 trials of rVSV Ebola vaccine in Africa and Europe. *N Engl J Med* 2016; **374**: 1647–60.
- 17 Akondy RS, Fitch M, Edupuganti S, et al. Origin and differentiation of human memory CD8 T cells after vaccination. *Nature* 2017; **552**: 362–67.
- 18 Zhou Y, Sullivan NJ. Immunology and evolution of the adenovirus prime, MVA boost Ebola virus vaccine. *Curr Opin Immunol* 2015; **35**: 131–36.
- 19 Wang B, Wang L, Kong X, et al. Long-term coexistence of SARS-CoV-2 with antibody response in COVID-19 patients. *J Med Virol* 2020; **92**: 1684–89.
- 20 Varkey JB, Shantha JG, Crozier I, et al. Persistence of Ebola virus in ocular fluid during convalescence. *N Engl J Med* 2015; **372**: 2423–27.
- 21 Sissoko D, Duraffour S, Kerber R, et al. Persistence and clearance of Ebola virus RNA from seminal fluid of Ebola virus disease survivors: a longitudinal analysis and modelling study. *Lancet Glob Health* 2017; **5**: e80–88.
- 22 Ksiazek TG, Rollin PE, Williams AJ, et al. Clinical virology of Ebola hemorrhagic fever (EHF): virus, virus antigen, and IgG and IgM antibody findings among EHF patients in Kikwit, Democratic Republic of the Congo, 1995. *J Infect Dis* 1999; **179** (suppl 1): S177–87.
- 23 Rimoin AW, Lu K, Bramble MS, et al. Ebola virus neutralizing antibodies detectable in survivors of the Yambuku, Zaire outbreak 40 years after infection. *J Infect Dis* 2018; **217**: 223–31.
- 24 Davis CW, Jackson KJL, McElroy AK, et al. Longitudinal analysis of the human B cell response to Ebola virus infection. *Cell* 2019; **177**: 1566–82.
- 25 Mate SE, Kugelman JR, Nyenswah TG, et al. Molecular evidence of sexual transmission of Ebola virus. *N Engl J Med* 2015; **373**: 2448–54.
- 26 Gattinoni L, Lugli E, Ji Y, et al. A human memory T cell subset with stem cell-like properties. *Nat Med* 2011; **17**: 1290–97.
- 27 LaVergne SM, Sakabe S, Kanneh L, et al. Ebola-specific CD8⁺ and CD4⁺ T cell responses in Sierra Leonean Ebola virus survivors with and without post viral sequelae. *J Infect Dis* 2020; **jiaa268**.
- 28 Mendoza EJ, Racine T, Kobinger GP. The ongoing evolution of antibody-based treatments for Ebola virus infection. *Immunotherapy* 2017; **9**: 435–50.
- 29 Sahr F, Ansumana R, Massaquoi TA, et al. Evaluation of convalescent whole blood for treating Ebola virus disease in Freetown, Sierra Leone. *J Infect* 2017; **74**: 302–09.
- 30 Becker S, Feldmann H, Will C, Slenczka W. Evidence for occurrence of filovirus antibodies in humans and imported monkeys: do subclinical filovirus infections occur worldwide? *Med Microbiol Immunol (Berl)* 1992; **181**: 43–55.
- 31 Glynn JR, Bower H, Johnson S, et al. Asymptomatic infection and unrecognised Ebola virus disease in Ebola-affected households in Sierra Leone: a cross-sectional study using a new non-invasive assay for antibodies to Ebola virus. *Lancet Infect Dis* 2017; **17**: 645–53.
- 32 Diallo MSK, Rabilloud M, Ayouba A, et al. Prevalence of infection among asymptomatic and paucisymptomatic contact persons exposed to Ebola virus in Guinea: a retrospective, cross-sectional observational study. *Lancet Infect Dis* 2019; **19**: 308–16.