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**An observational cohort study on the longitudinal antibody and T cell response in Ebola virus disease (EVD) survivors and contacts: characterisation of protective immunity & evidence of paucisymptomatic infection**

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**Summary**

**Background** - The 2013-2016 West African Ebola virus disease (EVD) epidemic caused international alarm due to its rapid and extensive spread resulting in a significant death toll and social unrest within the affected region.

**Methods** - Working with leaders of EVD survivor associations in two regions of Guinea, Guéckédou and Coyah, we recruited 117 survivors of EVD, 66 contacts from the households of individuals known to have suffered from EVD and 23 negative controls who had not knowingly associated with infected individuals or experienced EVD symptoms. We performed *ebolavirus zaire* (EBOV) glycoprotein (GP) specific T cell analysis on peripheral blood mononuclear cells (PBMCs) on location in Guinea. Plasma and PBMC were transported back to Europe for antibody quantification by ELISA, functional neutralising antibody analysis using live EBOV and T cell phenotype studies. We inform on the longitudinal cellular and humoral response amongst EVD survivors and highlight potentially paucisymptomatic infection.

**Findings** - Our results show that survivor neutralising antibody and GP specific T cell responses vary greatly between individuals but on average are ten-fold higher than that induced by single dose EBOV vaccines. Following reactivation with GP peptide, T cell responses were detectable and the dominant CD8+ T cell phenotype was shown to be IFNγ+, TNFα+, IL-2-. Both neutralising antibody and T cell responses were detected in 6/66 (9%) of EVD contacts and we note that 4/117 (3%) of EVD infected individuals lacked circulating EBOV antibodies post 3 months infection.

**Interpretation** - The continuous high-level neutralising antibody and T cell response may support the concept of long-term protective immunity in survivors. The existence of antibody and T cell responses in contacts of individuals with EVD adds further evidence to the existence of sub-clinical EBOV infection.

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**Introduction**

Dogma suggests that EVD survivors have long-term protection against re-infection with EBOV and studies in NHP 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 GP. (1-4) NHP vaccine studies also suggest CD8+ IFN+/TNF+/IL-2-/+ T cells are a potential correlate of protection. (5, 6) The recently licensed EBOV vaccine, ERVEBO® which is based on the viral GP, was evaluated in the 2013-2016 epidemic. Ring vaccination trials illustrated it provides a high level of protective immunity. (7) Prior studies on survivor EVD immunology have been performed but only antibody levels were monitored. (8) During historical EBOV outbreaks, plasma from survivors has been used to treat individuals suffering from acute EVD, however, a convalescent plasma therapy trial in West Africa failed to show significant clinical benefit or a correlation between neutralisation and efficacy. (9)These results suggest that both antibody and T cell responses may play a protective role in humans.

During the 1996 Gabon outbreak there were reports of a small number of individuals who were shown to be infected with EBOV but displayed a low virus load and presented only mild disease. (10) Similar observations were also reported in the recent West African epidemic. Unfortunately, such observations are rarely reported in detail and fail to include in depth immunology or molecular diagnostic data. However, a recent case report, with associated sequence data, strongly suggests that a paucisymptomatic mother was able to transmit EBOV to her baby. (11) A better understanding of the incidence of paucisymptomatic EVD is needed and may have implications in assessing future transmission risks. Additionally, systematic reviews of prior sero-epidemiology studies of populations in Africa report the existence of ~8% of individuals with antibodies to EBOV and other related members of the filovirus family. (12, 13) The absence of reported EVD outbreaks in such populations is puzzling but could be explained by antibody cross reactivity, sub-clinical EVD or increased rates of infectious diseases in general.

It is obvious that a complete understanding of naturally acquired immunity to EBOV will impact on our understanding of the design and evaluation of experimental vaccines, convalescent plasma therapy and therapeutic antibodies. Assessment of EVD survivor memory cell phenotype may also provide insight into chronic viral shedding from immune privileged sites. Furthermore, assessment of the immune response amongst individuals who were not shown to be EBOV positive, but were exposed to EVD patients, may reveal the extent of paucisymptomatic infections, this would help in assessment of the true incidence EBOV cases and associated case fatality rate.

**Research In context**

***Evidence before this study***

We searched the PubMed database with MESH terms Ebola, Immunity, sero prevalence, paucisymptomatic and similar terms using an end date of April 2020. Importantly, there was a report on long-term antibody and T cell responses in 11 survivors split across two small *Sudan ebolavirus (SUDV)* outbreaks. Neutralisation assays revealed titres of 0 to 1:80, T cell proliferation and kinetics analyses clearly showed CD8+ T cell activation but quantification via ELISPOT was not performed. A comprehensive analysis of ELISA response in survivors from the 1995 EBOV Kikwit outbreak showed antibody levels plateaued at 3 weeks to 1 year post-onset of symptoms. A report by Leroy *et al* 2000 revealed the existence of paucisymptomatic EBOV-infected individuals. Additionally, work by Glynn *et al* further demonstrates the presence of asymptomatic EBOV infection. A number of reports demonstrate the persistence of Ebola virus in immune privileged sites and there is an in depth longitudinal analysis of the B cell response amongst four EVD survivors. We found no other studies reporting the longitudinal analysis of cellular and humoral immunity amongst survivors over three consecutive years.

***Added value of this study***

This unique study reports an in-depth analysis of naturally acquired immunity to EBOV and enables a comprehensive comparison between naturally acquired and vaccine-induced immunity to EBOV both at the antibody and T cell level.

***Implications of this study***

The long-term high-level immunity observed in survivors may result from re-stimulation of the immune response as a result of antigen seeping from immune privileged sites into the blood stream. The study also provides supporting evidence for the existence of paucisymptomatic EVD and suggests that true incidence of EBOV infection in the West African outbreak was greater than recorded. Additionally, T cell phenotyping results support the pre-clinical findings of a potential correlate of protection to EBOV.

**Methods**

**Study designs and participants**

We performed an observational study based on opportunistic sampling. A total of 206 volunteers were recruited from two prefectures of Guinea, Guéckédou (outbreak epicentre) and ~300 miles away in Coyah (Table 1 and Supplementary Figure 1). Blood was collected from 117 survivors, 3 to 14 months post-infection. A second and third bleed of volunteers was performed approximately 12 and 24 months later. Individuals presented their EVD survivor certificate or were identified on Ebola treatment centre (ETC) 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 EVD confirmed case. Survivors and contacts were asked a number of questions (Supplementary Figure 2) regarding their contact with individuals suffering from EVD, as well as presentation of any EVD-like symptoms. Following immune analysis, where possible, a further, more in-depth interview took place to understand the degree of care they gave and discuss symptoms they may have had. In addition, nine volunteers were recruited, who were not knowingly exposed to persons with EVD 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 (No. 33/CNERS/15) and from the National Research Ethics Service, UK.

**Sample collection**

Approximately, 30-50 ml of blood was collected into EDTA vacutainers. Plasma and PBMCs were separated by layering the blood over a Ficoll gradient (GE Healthcare; 17-1440-03) and centrifugation. Plasma was aspirated and stored at -20°C for antibody studies. PBMCs were either used fresh, or frozen (<-70°C) and shipped to the United Kingdom where they were stored in liquid nitrogen. Participants were interviewed using the health surveillance questionnaire (supplementary Figure 2) and with regards to health scores during the Ebola outbreak each category was given equal weighting with a maximum score of 13.

**IFNγ ELISpot**

Freshly isolated PBMC’s were prepared at 2 x 106 cells/ml in Leibovitz media supplemented with penicillin/streptomycin, foetal calf serum, L-glutamine, HEPES and 2-mercaptoethanol. PBMCs were stimulated with EBOV Mayinga GP peptide library (Mimotopes, Australia) at a final concentration of 2.5 µg/peptide, as described previously.(14) Following 18 to 20 hours incubation at 37°C, IFNγ release was determined by standard ELISpot protocol (Mabtech 3420-2A) and Spot Forming Cells (SFC) enumerated using an S6 core analyser (Cellular Technology Limited). IFNy 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 (SFU’s) per one million cells and IFNγ response to EBOV GP peptide were summed, to determine the overall T-cell response.

**Enzyme linked-immunosorbent assay (ELISA)**

ELISA was carried out as described previously.(15) Briefly, high binding microtiter plates were coated with whole EBOV inactivated virions and incubated for 16 to 20 hours. Following washing in PBS/0.1% Tween20 (PBST) and blocking (PBS/5% milk powder); 1:200 dilutions of plasma sample were added to the plates and incubated for one hour. Polyclonal-horse radish peroxidase (HRP) antibody (Dako, P0214; dilution 1:1,000) in conjunction with TMB substrate was used to develop the reaction. Optical density (OD) was determined at 450 nm minus 630 nm (reference wavelength). Each sample was analysed in duplicate on mock and viral antigen. The mean OD value of each sample on the mock antigen was subtracted from the mean OD value of the respective sample on the EBOV antigen. Arbitrary ELISA units (AEU) were extrapolated by linear regression analysis using standard curves generated from patient antiserum. Further specificity was assessed using a EBOV Makona GP (sourced from Oxford University, Nuffield Department of Medicine UK) specific ELISA, for which Nunc Maxisorb 96-well plates were coated overnight (16 to 18 hours) with purified EBOV-GP antigen (0.5 µg/ml). Plasma was serially diluted, starting at 1:200 and the bound IgG was detected using goat anti-human IgG specific antibody conjugated to alkaline phosphatase (AP) (1:15000). AP-Yellow substrate was added and the OD measured at 405 nm using a VERSAmax plate reader controlled by SoftMax Pro (V4.7.1) Enterprise software. The plates were read using a predefined Softmax template which fits a 4-parameter logistic (4PL) curve to the dose response data. The cut-off is defined as the mean negative value plus five standard deviation (SD).

**Virus neutralisation assay**

The activity of the EBOV-specific antibodies present in plasma was determined by neutralisation of EBOV variant Mayinga (1976) as previously described.(5) Briefly, following heat treatment for complement inactivation, plasma was serially diluted in supplemented Dulbecco's modified Eagle's medium (DMEM) in 96-well culture plates, 100 TCID50 units of EBOV, variant Mayinga were added to the plasma dilutions. Following incubation at 37°C for one hour, Vero cell suspension in supplemented DMEM was added. Plates were then incubated at 37°C with 5% CO2 and cytopathic effects were evaluated at seven days post-infection. Neutralisation titres were calculated as Geometric Mean Titre (GMT) of four replicates. A titre of 1:8 or above is classified as positive.

**Western blot**

Recombinant GP, nucleoprotein (NP) and viral protein 35 (VP35) were generated based on the EBOV strain Makona in HEK293T cells and whole cell lysates were used. VP40 was based on EBOV strain Kikwit and was obtained from Stratech scientific. Proteins were heat denatured and loaded onto 4-12% BisTris gels and separated by size by SDS-PAGE. The proteins were then transferred to PVDF membrane and blocked overnight in block buffer (PBST buffer with 5% milk (w/v)). Plasma was diluted 1:1000 in block buffer and incubated with the EBOV-protein containing blots for four hours at room temperature. The blots were washed for five minutes in PBST. Secondary antibody; Anti-Human IgG (y-Chain Specific) peroxidase conjugate developed in goat; F(ab')2 fragment (Sigma Aldrich; A2290), was prepared at 1:1000 dilution in block buffer. The blots were incubated with secondary antibody for one hour at room temperature. Membranes were washed and blots developed with ECL prime, incubating for five minutes. Images were captured at five and ten-minute exposure and presence of immunoreactivity determined against a molecular marker standard.

**T cell phenotyping studies**

Intracellular cytokine staining (ICS) was performed as has been described previously.(5) Briefly, PBMCs were resuspended in warmed complete media (RPMI supplemented with penicillin/streptomycin, foetal calf serum, L-glutamine, HEPES and 2-mercaptoethanol), and rested overnight at 37°C. The following day cells were adjusted to 1x106 cells/ml in media containing anti-CD28 BUV737, CD49d and CD107a-PerCP cy5.5 (1 µg/ml). Samples were then left either untreated (NT) or were stimulated with EBOV GP peptide pool, containing 187 15mer overlapping peptides at 2.5 µg/peptide or 1 µg/ml SEB for 16-18 hours, as previously described.(5, 6) Two hours 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) 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, CD45RO-BV605. Cells were then washed, fixed and permeabilised using BD cytofix/cytoperm before staining for intracellular cytokines using IFNγ-AF488, TNFα- BV421 and IL-2-PE. Samples were then washed, resuspended and acquired using a BD Fortessa machine and FACS Diva software. Sample analysis utilised FlowJo™, Pestle and SPICE software as described in previous publications.(5) All antibodies were obtained from Biolegend with the exception of CD95-BUV395 and CD28 BUV737 which were obtained from Becton Dickinson (BD).

**Statistical analysis**

The data collected from all the volunteers were categorised into three groups; survivor, contact and negative; and these 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 for Figure 2A-2C was performed using R statistical software (version 4.0.1, lme4 package version 1.1-23) and fitted models were assessed for violation of assumptions. Cytokine responses were determined by subtracting the NT 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 performed using GraphPad Prism v8.

**Role of the funding source**

The funding agencies had no role in the study design, data collection, data analysis, interpretation or preparation of the manuscript. 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**

The presence of EBOV-specific IgG was measured in plasma samples collected in 2015 from volunteers using an ELISA based on whole inactivated EBOV Makona (Figure 1A) and recombinant GP (Supplementary figure 3). The GP-specific antibody titres correlated with high significance to the whole EBOV-specific antibody titres (r= 0.85; p < 0.0001) (Supplementary figure 4). From a total of 117 survivors recruited (3-14 months post-infection), 96% had detectable levels of IgG to EBOV, with responses measured at one dilution and defined as arbitrary units. A small number of survivor samples 4/117 (3%) did not have detectable EBOV-specific neutralising IgG antibodies in their plasma, and 11/66 (16%) of contacts had levels of EBOV-specific IgG that were above background and higher than the average IgG titre (GMT) detected following 28 days post-vaccination.(5, 16) (Figure 1A). The activity of the EBOV-specific IgG was measured by the ability to neutralise the infection of EBOV Mayinga *in vitro*. Testing for functional activity using our neutralisation assay revealed that 113/117 96% of survivors showed detectable levels of EBOV-specific neutralising antibody (Figure 1B). Notably there was >100-fold range in neutralisation titres (1:10 to >1:1000) amongst survivors with a mean of 1:180 which is approximately ten fold higher than that seen after vaccination with single dose candidate EBOV vaccines.(5, 16) Plasma from six of the 66 contacts also showed EBOV-specific neutralising antibodies and the mean of these six samples was 1:133, this paralleled their detectable levels of EBOV GP specific IgG. Further characterisation of five of these samples by Western blot revealed the presence of antibodies not only to GP but also to NP, VP40 and VP35 (Supplementary figure 5). These six individuals are illustrated in Figure 1 as coloured squares. From our sampling cohort, this would indicate that 6/66 (9%) of the contacts had detectable neutralising antibody with levels comparable to that of some EVD survivors. Importantly, all negative controls demonstrated undetectable levels of neutralising activity. T cell responses to EBOV-GP were assessed by measuring IFNy release using ELISpot following re-stimulation of PBMCs with EBOV-GP peptides as previously reported.(5) Cells were stimulated with two GP peptide pools separately and the sum of the IFNy responses are reported here (Figure 1C). Testing of EVD survivors revealed that 15/116 (13%) did not produce an IFNγ response following GP peptide stimulation. Unfortunately, during 2015 sampling ELISpots were only carried out 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 IFNγ response to EBOV-GP. Six of these also had detectable EBOV-specific ELISA and neutralisation titres. To further support the existence of paucisymptomatic individuals, volunteers answered a number of health surveillance questions aimed at elucidating the relative magnitude of EVD symptoms they experienced during the 2013-16 epidemic. Figure 1D reveals that EVD survivors would typically report a score of 7·5/13 whereas close contacts would report a score of 1·3/13. Interestingly, 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 2/13 suggesting they were seropositive contacts that were displaying more overt symptoms during the outbreak.

To better understand the longitudinal nature of the immune response to EBOV, we sampled from the same set of volunteers in 2016 and 2017, and again looked at humoral and cellular responses in plasma and peripheral blood (Figure 2). 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 surprisingly stable over time. However, to assess the effect of year on serological outcome measures after accounting for individual-level variation, we performed mixed-effects linear regression for each outcome. There was 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 = 0.002). To assess the directional effect of year on each outcome, each model was compared to a reduced model with a single linear parameter fitted for year. There was evidence 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 demonstrated an inverse relationship (-1022.08 AEU, 95% CI -1453.54 – -590.62) suggesting an annual decrease in antibody titre. Importantly, however, for all outcomes, the magnitude of serological responses across years and individuals remained clearly higher than responses measured among control group samples, as can be seen in Figure 1. These prolonged, elevated, responses could theoretically be due to re-exposure to EBOV antigen or simply be a reflection of the basal immunological state to such a pathogenic infection. To address this issue, we plotted survivor results in relation to their time since resolution of infection, this was done in the hope of seeing a tailing off of the primary immune response. However, results again showed a remarkable level of consistency over time (Figure 2D).

We next performed in depth flow cytometry studies to better characterise the response of survivors and to help address the question of potential antigenic re-stimulation, to this extent we performed intracellular cytokine staining experiments on PBMCs collected from volunteers and shipped back to the UK (Figure 3). As in ELISpot we looked for the presence of IFNγ following overnight stimulation with GP peptides and found that the majority of IFNγ, as a proportion of their parent subset, was coming from CD8+ T cells. Furthermore, when these IFNγ responses were backgated to their parent phenotype markers it can be seen that the majority of CD8+ T cells show CCR7 intermediate and CD45RO low expression, corresponding with a naïve like phenotype. In contrast, CD4+ T cells showed primarily as CD45RO positive and CCR7 low/intermediate expression consistent with a central memory or effector memory phenotype.(17)

Previous reports have suggested the importance of multiple cytokine producing “polyfunctional” CD8+ T cells in the control of EVD(18), we therefore investigated the incidence of IFNγ/TNFα+ or IFNγ/TNFα/IL-2+ CD8+ T cells amongst survivor PBMC samples collected in 2016 (15-28 months post-infection). We found that when these PBMCs were stimulated overnight with EBOV GP peptide pools there were a significant proportion of IFNγ/TNFα+ CD8+ T cells compared to contacts who were seronegative by ELISA and neutralisation (Figure 4). Finally, we measured the expression of CD107a (marker for degranulation) following GP peptide stimulation. Results in Figure 4B show that survivor samples illustrate a significantly higher proportion of CD107a expressing cells than their comparative contacts and when backgated the majority of these CD107a+ cells are also IFNγ/TNFα+ CD8+ T cells. Finally, we looked to see if there was a correlation between immunological and clinical parameters. As expected, we found there was a strong correlation between total EBOV ELISA and neutralisation assay results. Interestingly, we also observed a significant correlation between ELISpot and ELISA as well as ELISpot and neutralisation results (Supplementary figure 6). We did not find a significant correlation or difference between either age, sex or virus load at time of diagnosis (Ct values) and any of the immunological parameters measured (Supplementary figure 6 and Supplementary figure 7).

**Discussion**

We report the most comprehensive study of EVD survivor immunity to date and uniquely assess both neutralising antibody and T cell responses. The fact that neutralising antibody titres of survivors 3 – 35 months after infection are greater than tenfold compared to one month after vaccination with an efficacious single dose vaccine(5, 7) provides compelling evidence for long-term protection against re-infection with EBOV. This bodes well for survivors if the disease returns to West Africa and suggests they could continue to play a role in front line activities to control future outbreaks. However, the absence of humoral immunity in a small percentage of survivors may explain observations that a minority of EVD survivors can be re-infected suggesting it would be essential to assess their immunity before the risk of potential re-exposure. Interestingly, 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. Furthermore, there is precedence for the lack of antibody response to various emerging diseases, this has recently been documented with regards to COVID-19 convalescent individuals that are SARS-CoV-2 antibody negative.(19) From this data we would suggest that sero epidemiology studies for EBOV may underestimate the true prevalence of the disease. The disparity between vaccinees and survivors also highlights the potential need to periodically boost vaccine immunity or use alternative heterologous vaccine vector approaches that may induce a more durable response, however, it is yet to be determined what constitutes a protective antibody titre. The continuously high levels of both neutralising antibody and T cell responses are perhaps not unsurprising since it has been reported that EBOV can persist for over one year in immune privileged sites.(11, 20, 21) Furthermore, where we have found responses to be stable up to three years post-infection, studies of survivors from EVD outbreaks suggests that ELISA antibody responses continue to be relatively stable for over one year and that low titres of neutralising antibodies have been found in survivors 40 years post-infection.(22, 23) However, further work would be needed to show antigen persistence amongst these survivor samples.

We found steady state levels of EBOV GP antibody measured by ELISA and neutralisation from groups of survivors 3 to 14 months post onset of symptoms. This is in contrast to a study of four EVD survivors in which longitudinal analysis suggest a continuous increase in neutralising titre from up to 900 days post-recovery.(24) Obvious study differences, including a lack of longitudinal sampling and an absence of experimental treatment, in our report may explain our different results. It is also important to note that the longitudinal analysis seen in figure 2 uses only data from participants who provided a full three years’ worth of data, therefore, participants with missing or incomplete data was discarded and this is a limitation to this study with regards to the stability over time. It is possible that the long-term potent EBOV responses could be a result of continuous exposure to viral antigen as it seeps back into the systemic environment from immune privileged locations. Indeed, 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. However, due to bio-safety constraints and limitations of our ethical approval this was not possible. We suggest that our studies do not support the presence of re-circulating antigen post >15 months recovery, since we believe that CD8+ T cell phenotyping reveal a naïve, possibly, stem cell memory antigen specific subset. Stem cell memory, are a recently characterised self-renewing population of lymphocytes that are CD45RO-, CCR7+, CD27+ & CD95+.(26) To support this the phenotype of IFNγ producing CD4+ T cells is CD45RO+ (Figure 3D), suggesting that these are memory T cells and not terminally differentiated effectors that have recently been exposed to antigen. Interestingly, LaVergne *et al* found that post Ebola syndrome (PES) was associated with greater activation amongst CD4 and CD8+ T cells, it would therefore be of interest to correlate the magnitude of our T cell responses with PES in any follow up studies.(27)

Plasma from EVD survivors, containing various levels of neutralising antibodies, has been used to treat acute cases of EBOV infection during several prior outbreaks.(28) However, the 2013-2016 outbreak enabled a more comprehensive study to be performed, which reported an absence of statistically significant improvement in survival associated with plasma treatment.(9) Though van Griensven *et al* (2016) show a trend between the level of EBOV specific IgG and survival this was not seen for neutralising antibody titre. Surprisingly, in this trial neutralising antibody titres of plasma harvested from EVD survivors were on average 10-fold lower than seen in our study. However, it is possible that differences between assay conditions could account for this outcome, which may have impacted on the subsequent analysis of functional antibody activity and clinical outcome.(9) Furthermore, work by Sahr *et al* (2017) show a positive effect with plasma therapy in a small clinical trial in Sierra Leone.(29)

Immune analysis on contacts of individuals with EVD, who did not have a confirmed positive PCR test or report with classical disease to a treatment centre, reveals up to 9% have both detectable humoral and cellular immunity. This could possibly be explained by the existence of cross-reactive antibodies to EBOV proteins, this hypothesis 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 in addition to this we were able to show that five individuals also had antibodies specific to EBOV NP, VP35 and VP40 thus confirming that they must have come into contact with EBOV. The question remains, to what extent were these contacts symptomatic. This is difficult to address after the event and largely relies on anecdotal evidence. Matters are further complicated due to the significant stigma surrounding EVD in West Africa, meaning that individuals who were infected at the time were likely to have played down their symptoms or not sought treatment. However, due to the health questioning and interviews that took place we are confident that the paucisymptomatic contacts within our cohort truly did have mild disease which did not prevent them from conducting their normal duties. Therefore, these observations strongly suggest that there are four potential outcomes from EBOV exposure: i) symptomatic infection resulting in death, ii) symptomatic infection with survival and immunity iii) paucisymptomatic infection with survival and immunity iv) no infection and no immunity. Additional support for the existence and potential transmission from paucisymptomatic persons comes from a report whereby a mother was able to transmit EBOV to her child via breastmilk. Both parents reported no symptoms of EVD 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 may never be possible to categorically show that these positive contacts were paucisymptomatic or showed mild disease, however, this study and others taken as a whole would suggest that this aspect of EBOV transmission and the role it may play in spreading EVD would need to be considered during any future outbreaks. Additionally, it is quite possible that the true number of individuals infected with EBOV may be significantly higher than that officially reported which will also impact on the extent of the reported mortality rate of the infection.

We show here that survivors of EVD have a long-lasting T cell response. In fact, the T cell responses in our survivor cohort, measured a number of years after recovery from disease, are similar to that seen 6 months post vaccination with ChAd and MVA boost.(5)The importance of this T cell activity to EBOV 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 may play a key role in protection from EBOV challenge, following vaccination with an Adeno vaccine vector.(18) Our results show that the same CD8+ IFNγ/TNFα+ double positive T cell population determined to be important in primates for long-term protection is also the dominant CD8+ T cell population, in response to re-stimulation with GP peptide, in EVD survivors. Therefore, further work should be undertaken to determine the activity and proportion of CD8+ polyfunctional T cells in response to EBOV vaccination.

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**Contributors**

MC, MKK & SG conceived the study and experimental design with input from NFM, AMH, HR, JH, SB, EN, GB, MM, LL, MG, DP, OS, LK & AT. Sample collection within Guinea was led by MKK & JAB with assistance from M3C, BMK, SK, ARA. Sample processing in guinea was led by YH, TT, RT & JAB with help from PM, FRK, EN, AB, KS, AV, VV, BA, LO & SD. Analysis of sample in the UK was performed by TT, RT, YH and KSR. Processing of Whole virus ELISA and Live virus neutralisation was led by TS with help from SKF & VK. Writing of the manuscript was primarily undertaken by TT, RT, MC, YH, SL, JM, CMF, SB, SG, VK with input from all authors.

**Conflict of interest**

Stephan Günther received grants from European Commission and German Research Foundation for the conduct of this study. The other authors have no conflict of interest to report.

Table 1: Participant information from Coyah and Guéckédou sample sites

|  |  |  |  |
| --- | --- | --- | --- |
|   | **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 | 5 |
| **Female** | 25 | 19 | 1 | 39 | 17 | 5 | 64 | 36 | 0 |
| **Returning 2016/17** | 33 | 19 | 0 | 63 | 23 | 0 | 96 | 42 | 0 |

**References**

1. Mohamadzadeh M, Chen L, Schmaljohn AL. How Ebola and Marburg viruses battle the immune system. Nat Rev Immunol. 2007;7(7):556-67.

2. Olinger GG, Bailey MA, Dye JM, Bakken R, Kuehne A, Kondig J, et al. Protective cytotoxic T-cell responses induced by venezuelan equine encephalitis virus replicons expressing Ebola virus proteins. J Virol. 2005;79(22):14189-96.

3. Warfield KL, Olinger G, Deal EM, Swenson DL, Bailey M, Negley DL, et al. Induction of humoral and CD8+ T cell responses are required for protection against lethal Ebola virus infection. J Immunol. 2005;175(2):1184-91.

4. Bradfute SB, Bavari S. Correlates of immunity to filovirus infection. Viruses. 2011;3(7):982-1000.

5. Ewer K, Rampling T, Venkatraman N, Bowyer G, Wright D, Lambe T, et al. A Monovalent Chimpanzee Adenovirus Ebola Vaccine Boosted with MVA. N Engl J Med. 2016;374(17):1635-46.

6. Stanley DA, Honko AN, Asiedu C, Trefry JC, Lau-Kilby AW, Johnson JC, et al. Chimpanzee adenovirus vaccine generates acute and durable protective immunity against ebolavirus challenge. Nat Med. 2014;20(10):1126-9.

7. Henao-Restrepo AM, Longini IM, Egger M, Dean NE, Edmunds WJ, Camacho A, 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(9996):857-66.

8. Peters CJ. Ebola and hantaviruses. FEMS Immunol Med Microbiol. 1997;18(4):281-9.

9. van Griensven J, Edwards T, de Lamballerie X, Semple MG, Gallian P, Baize S, et al. Evaluation of Convalescent Plasma for Ebola Virus Disease in Guinea. N Engl J Med. 2016;374(1):33-42.

10. Leroy EM, Baize S, Volchkov VE, Fisher-Hoch SP, Georges-Courbot MC, Lansoud-Soukate J, et al. Human asymptomatic Ebola infection and strong inflammatory response. Lancet. 2000;355(9222):2210-5.

11. Sissoko D, Keita M, Diallo B, Aliabadi N, Fitter DL, Dahl BA, 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(4):513-6.

12. Nyakarahuka L, Kankya C, Krontveit R, Mayer B, Mwiine FN, Lutwama J, 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(1):708.

13. Mulangu S, Alfonso VH, Hoff NA, Doshi RH, Mulembakani P, Kisalu NK, et al. Serologic Evidence of Ebolavirus Infection in a Population With No History of Outbreaks in the Democratic Republic of the Congo. J Infect Dis. 2018.

14. Ledgerwood JE, Costner P, Desai N, Holman L, Enama ME, Yamshchikov G, et al. A replication defective recombinant Ad5 vaccine expressing Ebola virus GP is safe and immunogenic in healthy adults. Vaccine. 2010;29(2):304-13.

15. Krahling V, Becker D, Rohde C, Eickmann M, Eroglu Y, Herwig A, et al. Development of an antibody capture ELISA using inactivated Ebola Zaire Makona virus. Med Microbiol Immunol. 2016;205(2):173-83.

16. Agnandji ST, Huttner A, Zinser ME, Njuguna P, Dahlke C, Fernandes JF, et al. Phase 1 Trials of rVSV Ebola Vaccine in Africa and Europe. N Engl J Med. 2016;374(17):1647-60.

17. Akondy RS, Fitch M, Edupuganti S, Yang S, Kissick HT, Li KW, et al. Origin and differentiation of human memory CD8 T cells after vaccination. Nature. 2017;552(7685):362-7.

18. Zhou Y, Sullivan NJ. Immunology and evolvement of the adenovirus prime, MVA boost Ebola virus vaccine. Curr Opin Immunol. 2015;35:131-6.

19. Wang B, Wang L, Kong X, Geng J, Xiao D, Ma C, et al. Long-term coexistence of SARS-CoV-2 with antibody response in COVID-19 patients. J Med Virol. 2020.

20. Varkey JB, Shantha JG, Crozier I, Kraft CS, Lyon GM, Mehta AK, et al. Persistence of Ebola Virus in Ocular Fluid during Convalescence. N Engl J Med. 2015;372(25):2423-7.

21. Sissoko D, Duraffour S, Kerber R, Kolie JS, Beavogui AH, Camara AM, 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(1):e80-e8.

22. Ksiazek TG, Rollin PE, Williams AJ, Bressler DS, Martin ML, Swanepoel R, 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, Steffen I, Doshi RH, Hoff NA, et al. Ebola Virus Neutralizing Antibodies Detectable in Survivors of theYambuku, Zaire Outbreak 40 Years after Infection. J Infect Dis. 2018;217(2):223-31.

24. Davis CW, Jackson KJL, McElroy AK, Halfmann P, Huang J, Chennareddy C, et al. Longitudinal Analysis of the Human B Cell Response to Ebola Virus Infection. Cell. 2019;177(6):1566-82 e17.

25. Mate SE, Kugelman JR, Nyenswah TG, Ladner JT, Wiley MR, Cordier-Lassalle T, et al. Molecular Evidence of Sexual Transmission of Ebola Virus. N Engl J Med. 2015;373(25):2448-54.

26. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, et al. A human memory T cell subset with stem cell-like properties. Nat Med. 2011;17(10):1290-7.

27. LaVergne SM, Sakabe S, Kanneh L, Momoh M, Al-Hassan F, Yilah M, 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.

28. Mendoza EJ, Racine T, Kobinger GP. The ongoing evolution of antibody-based treatments for Ebola virus infection. Immunotherapy. 2017;9(5):435-50.

29. Sahr F, Ansumana R, Massaquoi TA, Idriss BR, Sesay FR, Lamin JM, et al. Evaluation of convalescent whole blood for treating Ebola Virus Disease in Freetown, Sierra Leone. J Infect. 2017;74(3):302-9.

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. 1992;181(1):43-55.

31. Glynn JR, Bower H, Johnson S, Houlihan CF, Montesano C, Scott JT, 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(6):645-53.

32. Diallo MSK, Rabilloud M, Ayouba A, Toure A, Thaurignac G, Keita AK, 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(3):308-16.