Characterisation of the T-cell response to Ebola virus glycoprotein amongst survivors of the 2013–16 West Africa epidemic

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Zaire ebolavirus (EBOV) is a highly pathogenic filovirus which can result in Ebola virus disease (EVD); a serious medical condition that presents as flu like symptoms but then often leads to more serious or fatal outcomes. The 2013–16 West Africa epidemic saw an unparalleled number of cases. Here we show characterisation and identification of T cell epitopes in surviving patients from Guinea to the EBOV glycoprotein. We perform interferon gamma (IFNγ) ELISpot using a glycoprotein peptide library to identify T cell epitopes and determine the CD4+ or CD8+ T cell component response. Additionally, we generate data on the T cell phenotype and measure polyfunctional cytokine secretion by these antigen specific cells. We show candidate peptides able to elicit a T cell response in EBOV survivors and provide inferred human leukocyte antigen (HLA) allele restriction. This data informs on the long-term T cell response to Ebola virus disease and highlights potentially important immunodominant peptides.

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2013–16 saw the largest recorded epidemic of Ebola virus disease (EVD), resulting in over 30,000 cases and 11,000 case fatalities. During this period efforts were made to establish new and experimental therapeutics, this has culminated in two lead candidate vaccines which have undergone extensive safety and efficacy trials. The lead candidate vaccine uses Vesicular stomatitis Indiana virus (VSV) as a viral vector, and is known as rVSV-ZEBOV. This vaccine incorporates the glycoprotein (GP) of the Kikwit Ebola virus from the 1995 epidemic on the capsid surface, in place of its native GP. This substitution has resulted in a loss of tropism of VSV for its target, however, subsequent clinical trials investigating the safety of rVSV-ZEBOV reported mild to moderate adverse events in a small number of participants. There are a number of large animal studies demonstrating the efficacy of the rVSV-ZEBOV vaccine and a large clinical trial in Guinea has shown the vaccine to be effective and appropriate for ring vaccination strategy, with a reported 100% efficacy (95% CI 79·3–100·0; p = 0·0033). However, emerging evidence suggests that there have been cases of disease breakthrough associated with this vaccine.

Another vaccine candidate to be developed utilises a recombinant chimpanzee adenovirus subgroup 3 virus as a vector for EBOV, Mayinga strain, GP (ChAd3-EBO-Z). Research has investigated this vaccine on its own or in combination with a modified Vaccinia Ankara (MVA-BN-Filo) boost. The MVA-BN-Filo boost encodes for the same Mayinga strain GP as the ChAd3-EBO-Z, as well as the Sudan ebolavirus GP and Marburg virus GP, in addition, MVA-BN-Filo encodes for the Tai-forest Ebolavirus nucleoprotein (NP). A close relation to this vaccine combination has been developed and has recently received marketing authorisation from the European Union medicines agency. Both candidate vaccines are continuing to show success in the field, however, to what extent these vaccines need to mediate a cellular or humoral response to provide protection is still unclear.

Evidence from animal studies and survivor cohorts are helping us understand the naturally acquired immune response which in turn will help inform on vaccine design and may help elucidate the comparative need for a humoral or cellular response. Early work investigated the T cell response to mice vaccinated with Venezuelan equine encephalitis virus replicons, which expressed various EBOV proteins. This work found murine antigen-specific T cells to these EBOV proteins were generated, including the NP and GP. These T cells were expanded in vitro and adoptively transferred to EBOV naïve mice, when mice were challenged with an adapted EBOV strain it was found that they were protected from EVD. Seminal evidence for the importance of T cells to EVD survival following vaccination comes from the work of Sullivan et al. who vaccinated non-human primates (NHPs) with human recombinant adenovirus serotype 5 (rAdHu5) which encoded for EBOV GP. Cynomolgus macaques were vaccinated then exposed to EBOV. Interestingly, if post vaccinated animals underwent T cell depletion using an anti-CD3 monoclonal antibody (mAb) they lost their ability to control disease and succumbed to infection. Furthermore, if prior to challenge primates were CD8⁺ T cell depleted using a monoclonal antibody then, again, they were unable to control disease, this was not the case for CD4⁺ T cell depletion prior to challenge. However, work by Marzi et al. looking into the role of T cells following rVSV-ZEBOV vaccination in NHPs showed that CD8⁺ T cells were in fact dispensable and the humoral response, mediated by CD4⁺ T cells, was critical to vaccine-mediated protection.

Antibody and T cell responses have been shown to be long-lived amongst EVD survivors. Therefore, the investigation into the natural immune response to EBOV may help better inform on vaccine design and the relative importance of cellular or humoral immunity. Recent work found that during the 2013–16 West Africa epidemic patients with elevated levels of the T cell inhibitory molecules PD-1 and CTLA-4 were more likely to succumb to disease and longitudinally characterised T cell response in two western repatriated patients, found a decrease in CD4⁺ T cells leading to a flip in the CD4⁺:CD8⁺ T cell ratio. It was also found that T cells showed elevated PD-1 expression and that there was impaired IFNγ production which was associated with virus reactivation. Similarly, work by McElroy et al. investigated the cellular response to four acute EBOV infected patients at Emory University hospital, where they found striking activation of both CD4⁺ and CD8⁺ T cells to several EBOV proteins. Work by Sakabe et al. 2018 identified a number of antigen-specific T cells amongst survivors of the 2013–16 West African epidemic and concluded that responses to the NP were immunodominant—suggesting NP should be included in any vaccine design. Here, we assess the T cell response from a number of EVD survivors to the EBOV glycoprotein, we inform on a number of antigen-specific peptides, the resulting T cell phenotype and the associated HLA dynamics of the cohort studied.

**Results**

IFNγ is a potent antiviral cytokine which is critical to the control and elimination of many intracellular pathogens. It is primarily produced by natural killer cells and antigen specific CD4⁺ and/or CD8⁺ T cells. To determine to what extent survivors (two years post recovery) of EVD can mount a long-term immune response to the EBOV GP we used ELISpot to measure IFNγ release following overnight peripheral blood mononuclear cell (PBMC) stimulation with a GP peptide library. This peptide library consists of 187 peptides, each 15 amino acids long, overlapping by 11 thereby offset by 4 amino acids (Supplementary Fig. 1). Comparison of the summed frequency of IFNγ spot forming units (SFU) measured in response to stimulation with the peptide library indicated that EVD survivors have significantly elevated GP-specific IFNγ SFU frequencies compared with negative controls (p < 0·0001) with a median value of 331 SFU amongst the survivors and 6 amongst the negative controls. It can also be seen in Fig. 1c that the majority of EVD survivors are mounting a T cell response to the soluble region of the GP and that these T cell responses correlate with whole virus antibody levels (Supplementary Fig. 2). Furthermore, individual EVD survivor response to peptide pools (Fig. 1c and Supplementary Fig. 3) showed considerable heterogeneity, although the majority of survivors responded to peptides within the GP1-2 and GP1-4 peptide pools.

We next performed more in-depth peptide screening, via IFNγ ELISpot, to each individual peptide within our glycoprotein peptide pool. This analysis was performed on 15 EVD survivors, fresh in the field (Fig. 2a, Supplementary Fig. 4) and highlights a number of immunogenic regions, particularly within GP1-2 and GP1-4. Additionally, frozen PBMC that were transported back to the UK were used to perform peptide mapping on the EVD survivors who showed a response to peptide pools SP, GP1-1, GP1-2 or GP1-4 (Fig. 2b–e). Frozen samples were chosen for more in-depth mapping if they had an ELISpot reading that was five standard deviations above the mean negative value for the corresponding peptide pool shown in Fig. 1. From these additional IFNγELISpot studies we found several candidate peptides generated an IFNγ response, in particular, peptides 79 and 82 from peptide pool GP1-4 were studied in greater depth due to this region previously being highlighted as potentially immunogenic after vaccination.

To determine the contribution of either CD4⁺ or CD8⁺ T cells in the response seen to peptide pool GP1-4 we used survivor PBMC depleted for either CD4⁺ or CD8⁺ T cells and then
assayed for their IFNγ response to peptides that make up the GP1-4 peptide pool (Fig. 3). It was found that the peptides within sub-pool 2 were responsible for CD4+ T cell activation (p = 0.0264) whereas the peptides within sub-pool 3 were responsible for CD8+ T cell activation (p = 0.0255). GP1-4 sub-pool 2 contained peptides 74–80 and included peptide 79 which from Fig. 2a, e appeared to be immunogenic. GP1-4 sub-pool 3 contained peptides 81–88, which included peptide 82, again this looked to be immunogenic in Fig. 2a, e.

We next used flow cytometry studies to better characterise the immune response seen in Fig. 3. EVD survivor PBMC samples were stimulated overnight with GP peptide pool (all 187 peptides), peptide 79, peptide 82 or Staphylococcal enterotoxin B (SEB), which was used as a positive control. The following day, cells were stained and acquired on the flow cytometer (Supplementary Fig. 5). The phenotype of cells that produced IFNγ and TNFα (double positive) or IFNγ and TNFα and IL-2 (Triple positive) in response to GP peptide pool stimulation can be seen in Fig. 4a, b. With regards to CD4+ T cells this phenotype primarily expressed CD45RO+ and CCR7+/- consistent with a central memory phenotype, in contrast, antigen-specific CD8+ T cells, were CCR7+/- and CD45RO- which is indicative of a naïve or effector cell phenotype20. Antigen specific CD8+ cells were also CD28+, CD95+ (Supplementary Fig. 6) and CD107a+.

**Fig. 1 Cellular immune response, using fresh PBMC, to EBOV GP (Mayinga) peptide library as measured by IFNγ ELISPOT.**

a. Schematic representation of the EBOV glycoprotein highlighting notable regions and peptide pools used in ELISPOT analysis. SP = Signal peptide.

b. The summed ELISPOT response to all GP peptides in the library amongst 57 EVD survivor and 18 non-exposed, negative, PBMC samples. c. ELISPOT response amongst 57 EVD survivors to either sGP or exclusive GP portions of the glycoprotein. d. The ELISPOT response amongst 57 EVD survivors to each peptide pool. For graphs b–d bars represent the median values with the upper 95% confidence interval. Two-tailed Mann–Whitney U test used to look for significance in (b) (p = <0.0001) and two-tailed Wilcoxon test in (c) (p = <0.0001). Dashed black line is the lower limit of detection (LLD) represents the in house cut off value (23 SFU), this is the mean of all negative results in (b) plus 3 standard deviations (SD) and discriminates between a positive and negative responder.
Fig. 2 Cellular immune response to EBOV GP peptides as measured by IFNγ ELISpot. a Response to 187 individual glycoprotein peptides amongst 15 fresh PBMC samples. Bars are stacked and indicate the sum of all results to each peptide amongst 15 EVD survivors. b Response amongst 6 EVD survivors to individual 15 mer peptides which make up the SP peptide pool. c The response amongst 12 EVD survivors to individual 15 mer peptides that make up the GP1-1 peptide pool. d Response amongst 18 EVD survivors to individual 15 mer peptides which make up the GP1-2 peptide pool. e The response amongst 14 EVD survivors to individual 15 mer peptides that make up the GP1-4 peptide pool. For graphs (b–e) red dots indicate individual data points and bars with error represent the median with the upper 95% confidence interval. Black dashed line represents the lower limit of detection (LLD) for the GP1-2 (19 SFU) or GP1-4 (16 SFU) peptide pool respectively, this was calculated using the mean of the negative samples plus three SD.
glycoprotein and its expression has been associated with CD8+ T cell degranulation^{20,21}. Antigen-specific CD4+ T cells showed a tendency to produce IFNγ, TNFa, and IL-2 whereas CD8+ antigen-specific cells primarily produced IFNγ and TNFa only, which is a functional profile consistent with the phenotypes described above (Fig. 4b). With regards to CD107a expression, there was a trend for CD8+ EVD survivor T cells to express more CD107a in response to GP peptide stimulation however this did not reach significance (Supplementary Fig. 7).

With regards to characterising the T cell response to individual peptides that were found to elicit an IFNγ ELISpot response we found that peptide 82 elicited a CD8+ specific T cell response (Fig. 4d) which was primarily associated with IFNγ and TNFa production, cytokine-producing cells again showed a CCR7+/− and CD45RO− phenotype. In contrast, peptide 79 was primarily associated with CD4+ T cells producing IFNγ, TNFa and IL-2 (Fig. 4c). Additionally, we found that peptide 3 was associated with a CD8+ T cell response, unfortunately, we could not determine whether the ELISpot response seen for peptides within GP1-2 were CD4+ or CD8+ mediated (Supplementary Fig. 8).

Finally, genomic DNA was used to HLA genotype EVD survivor samples and the most common MHCI and MHCII frequencies can be seen in Supplementary Fig. 9. We have shown that peptide 82 elicits a CD8+ T cell response, therefore, to determine the most likely candidate HLA alleles responsible for binding peptide 82 we performed in silico analysis using the immune epitope database and analysis resource (IEDB) research tool. Results in Table 1 indicate that as expected a number of different HLA alleles are capable of presenting common fragments of peptide 82 to CD8+ T cells.

**Discussion**

We were readily able to detect T cell responses to EBOV GP amongst survivors of EVD years after infection, however, to what extent these cytotoxic responses are important to acute infection is still being debated. Previous work by Dahlke et al. studied the T cell response amongst a repatriated EVD survivor in Germany. They found that CD8+ T cells dominated during the recovery phase of EVD and GP-specific CD8+ T cells were detectable but of low magnitude at 46 days post-recovery. However, this patient received multiple experimental treatments which may have altered lymphocyte dynamics during the recovery phase^{22}. We again see GP-specific responses of low magnitude within our cohort which is on average greater than 2 years post EVD recovery. Comparison with vaccine data will be important and it has been shown that T cell responses to the same GP peptide pool are detectable 12 months post-rVSV-ZEBOV vaccination^{23}. Recent work by Powlsen et al. has characterised the T cell response to EBOV GP following vaccination with ChAd3-MVA and showed a number of immunogenic peptides, one of which (TTIGEWAFW) falls within the motif we found to elicit a CD8+ T cell response amongst EVD survivors (peptide 82)^{19}. Based on our IEDB predictions we believe that this peptide is able to bind to a number of different HLA and therefore could provide a broad response amongst various ethnic backgrounds which...
would favour its incorporation into future therapeutic vaccine platforms. Work by Ahmad et al. used immune-informatics tools to make in silico predictions on potential B and T cell epitopes of interest\(^\text{24}\). One of their top hits (IRGFPRCRY) was contained within our peptide 36, which we show here in Fig. 2 to give a response by IFN\(\gamma\) ELISpot, although we were unable to categorically determine whether this peptide resulted in CD4\(^+\) or CD8\(^+\) T cell activation. Nevertheless, our results would support the validity of these reverse vaccinology approaches in vaccine design.

Important work by Sakabe et al. used an expression system to probe the immunogenicity of various EBOV proteins amongst EVD survivors from Sierra Leone and they found a broad response to various EBOV products. Interestingly, only half of the
survivor cohort they investigated responded to EBOV GP protein whereas the majority of people responded to VP24, VP40 or NP protein\textsuperscript{17}. Therefore, future work should look to broaden investigation into the immune response to a range of EBOV proteins with a view to incorporating greater antigenic diversity into the various vaccine platforms. The frequency of cytokine-producing T cell responses we detected when performing our ICS assays was of low magnitude, but are similar to those seen by Sakabe et al. whose cohort would have been at a similar time post EVD recovery\textsuperscript{17}.

Although highly heterogeneous, the EVD survivors we studied, in general, responded to peptide pools GP1-2 and GP1-4 which correspond to a portion of the receptor-binding domain and glycan cap of the GP. Once EBOV enters its target cell via the cytosolic or vacuolar pathways and that this compartment can be compromised when a naive or effector phenotype; however, these cells were also CD28\textsuperscript{+} and CD95\textsuperscript{+} (Supplementary Fig. 5) so could potentially be stem cell memory T cells\textsuperscript{31,32}. Future work may wish to consider the use of tetramers or scRNAseq to further characterise the T cell responses that are important for an effective immune response to EBOV infection.

Methods

Study designs and participants. During 2017 a total of 62 volunteers were recruited from Guinea, Coyah. Blood was collected from 57 survivors, approximately two years post EVD. Additionally, blood was collected from negative controls, these were five EBOV naive West African participants who had not knowingly associated or exposed themselves to EVD patients. Patients presented their EVD survivor certificate or were identified on Ebola treatment centre (ETC) databases, to verify that they were survivors. 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.

Table 1 In silico predictions for peptide 82/HLA binding to CD8\textsuperscript{+} TCR.

| ID    | Sequence | HLA-A\textsubscript{1} | HLA-A\textsubscript{2} | HLA-B\textsubscript{1} | HLA-B\textsubscript{2} | HLA-C\textsubscript{1} | HLA-C\textsubscript{2} | ELISpot (SFU/10\textsuperscript{6} cells) | ICS (% IFN\textgamma\gamma + /TNF\textalpha) |
|-------|----------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------------------------|------------------------------------------|
| C012  | IDTTIGEAWFVETKK | 02:01                  | 68:02                  | 35:01                  | 53:01                  | 04:01                  | 16:01                  | 130 (0.066)                             | LLD                                      |
| C021  | IDTTIGEAWFVETKK | 30:01                  | 68:02                  | 18:01                  | 42:01                  | 05:01                  | 17:01                  | 99 (0.000)                              | LLD                                      |
| C105  | IDTTIGEAWFVETKK | 26:01                  | 45:01                  | 56:01                  | 01:02                  | 16:01                  | 85 (0.080)                            | LLD                                      |
| C112  | IDTTIGEAWFVETKK | 02:01                  | 68:02                  | 18:01                  | 42:01                  | 05:01                  | 17:01                  | 99 (0.000)                              | LLD                                      |
| C003  | IDTTIGEAWFVETKK | 02:01                  | 23:01                  | 51:01                  | 36:01                  | 06:02                  | 16:01                  | 63 (0.009)                              | LLD                                      |
| C126  | IDTTIGEAWFVETKK | 02:01                  | 35:01                  | 52:01                  | 57:03                  | 04:01                  | 18:01                  | 55 (0.006)                              | LLD                                      |
| C092  | IDTTIGEAWFVETKK | 24:02                  | 33:01                  | 27:05                  | 40:02                  | 02:02                  | 02:02                  | 48 (0.016)                              | LLD                                      |
| C078  | IDTTIGEAWFVETKK | 03:01                  | 30:01                  | 35:01                  | 35:01                  | 04:01                  | 16:01                  | 44 (0.000)                              | LLD                                      |
| C081  | IDTTIGEAWFVETKK | 23:01                  | 30:02                  | 07:02                  | 58:01                  | 04:01                  | 07:01                  | 24 (0.014)                              | LLD                                      |
| C093  | IDTTIGEAWFVETKK | 02:01                  | 33:03                  | 07:02                  | 58:01                  | 03:02                  | 07:02                  | 4 (0.004)                                | LLD                                      |

Bold HLA cells indicate predicted good binders (+2%) as determined by the IEDB database (IEDB.org). Bold sequence text indicates the predicted sequence which will bind to the highlighted HLA.
Intracellular cytokine staining (ICS) was performed on flow cytometry to confirm the depletion of the desired target cell population.

HLA analysis. HLA typing was performed using the Fluidigm/MiSeq NGS (next-generation sequencing) method. Briefly, ~120 ng of genomic DNA was used for PCR amplification with Fluidigm Access Array (Fluidigm Singapore PTE Ltd, Singapore). Locus-specific primers were designed to amplify a total of 23 polymorphic exons of HLA-A (exons 1 to 4), HLA-B (exons 1 to 4), HLA-C (exons 1 to 4), DQA1 (exons 1, 2, 3), DQB1 (exons 2, 3), DRB1 (exons 2, 3), and DRB3, 4, 5 (exon 2) genes. The 23 PCR amplicons were pooled, concentrated, adjusted, and subjected to sequencing on an illumina MiSeq sequencer (illumina, San Diego, CA).

Intracellular cytokine staining (ICS). BMCMCs were resuspended in warmed complete media and rested overnight at 37 °C and 5% CO2. The following day, 2×10^5 cells/ml in media containing anti-CD28, CD49d and rm depletion of the desired target were added to 2×10^6 cells/ml in media containing anti-CD28, CD49d and CD4 and CD8 and acquired on the flow cytometer to confirm depletion of the desired target cell population.

T cell depletion. Frozen PBMC were thawed in warm media and rested overnight. The following day, >2×10^6 cells were washed and resuspended in staining buffer (PBS, 0.5% FCS, 0.5 mM EDTA). Cells were then incubated with either anti-CD4 (Miltenyi; 130-045-101) or CD8 (Miltenyi; 130-045-201) microbeads following manufacturer’s instructions. Samples were then passed through a magnetised LD column and collected into 5 ml FACs tubes. Sample was stained for CD3, CD4 and CD8 and acquired on the flow cytometer to confirm depletion of the desired target cell population.

HLA analysis. HLA typing was performed using the Fluidigm/MiSeq NGS (next-generation sequencing) method. Briefly, ~120 ng of genomic DNA was used for PCR amplification with Fluidigm Access Array (Fluidigm Singapore PTE Ltd, Singapore). Locus-specific primers were designed to amplify a total of 23 polymorphic exons of HLA-A (exons 1 to 4), HLA-B (exons 1 to 4), HLA-C (exons 1 to 4), DQA1 (exons 1, 2, 3), DQB1 (exons 2, 3), DRB1 (exons 2, 3), and DRB3, 4, 5 (exon 2) genes. The 23 PCR amplicons were pooled, concentrated, adjusted, and subjected to sequencing on an illumina MiSeq sequencer (illumine, San Diego, CA). HLA alleles and genotypes were assigned using the Oximun HLA Explore (beta version) software (Oximun, Budapest, Hungary).

Intracellular cytokine staining (ICS). BMCMCs were resuspended in warmed complete media and rested overnight at 37 °C and 5% CO2. The following day, cells were adjusted to 2×10^6 cells/ml in media containing anti-CD28, CD49d and CD107a-PerCP cy5.5 (1 µg/ml). Two hours into the incubation, brefeldin A and monensin (1 µg/ml) were added to block cytokine production. Samples were then left either untreated (NT) or with MVA.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files.

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Author contributions
T.T wrote the manuscript with input from K.E., M. Carroll, M. Carrington, Y.H., S.L., J.M. and A.W. T.T, Y.H. and J.A.B were responsible for sample collection and data analysis. M. Carroll, M.K.K., S.G. and Y.H. were responsible for project conception and experimental design. A. White, L. S. Sibley, C. Sarfas aided in the design of flow cytometry studies. Y.Y., M.M. and M. Carrington performed HLA genotyping studies.

Competing interests
The authors declare no competing interests.

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