Unique human immune signature of Ebola virus disease in Guinea

Paula Ruibal1,2,3,4, Lisa Oestereich2,3,4, Anja Lüdtke1,2,3,4, Beate Becker-Ziaja2,3,4, David M. Wozniak2,3,4, Romy Kerber2,3,4, Miša Korva5,6, Mar Cabeza-Cabrero2,3,4, Joseph A. Bore4, Fara Raymond Koundouno4, Sophie Duraffourg2,3,4, Romy Weller2,3,4, Anja Thorenz2,4, Eleonora Cinmini4,5, Domenico Volia4,6, Chiara Agrati4,5, Johanna Repits4, Babak Afsrugh2,4,5, Lauren A. Cowley2,3,4, Didier Ngabo4,5, Julia Hinzmann4,5,11, Marc Mertens4,5,11, Inès Vitoriano4,5,16, Christopher H. Logue4,9, Jan Peter Boettcher3,4,11, Elisa Pallasch2,3,4, Andreas Sachse4,11, Amadou Bah4,13, Katja Nitzsche3,4,11, Eeva Kuismaa3,4,11, Janine Michiel4,5,11, Tobias Holm4,5,3, Elsa-Giulie Zekeng9, Isabel García-Dorival14,15, Roman Wölfle13,4,15, Kilian Stoecker3,4,15, Erna Fleischmann2,4,15,16, Thomas Strecker3,4,16, Antonio Di Caro4,5,8, Tatjana Avsić-Zupanc4,5, Andreas Kurth4,11, Silvia Meschi4,8, Stephane Mély1,12,17, Edmund Newman4,9, Anne Bocquim4,17, Zoltan Kis4,18,19, Anne Kelterbaum3,4,16, Peter Molkenthin4,13,15, Fabrizio Carletti4,8, Jasmine Portmann1,20, Svenja Wolff3,4,16, Concetta Castilletti18,14, Gordian Schmied1,16, Alexandra Fizet1,21, Lisa J. Ottowell4,9, Eva Herker4,16, Thomas Jacobs2, Birte Kretschmer22, Ettore Severi19, Nobila Ouédraogo4,16,19, Mar Lago23, Anabel Negrado24, Leticia Franco24, Pedro Anda20, Stefan Schmiedel25, Benno Kreuel2,3,22, Dominic Wichmann2,25, Marylyn M. Addo3,25, Ansgar W. Lohse2,22, Hilde De Clerck26, Carolina Ncaclare2,27, Sylvie Jonckheere2,26, Michel Van Herp2,26, Armand Sprecher2,26, Gao Xiaojiang27,28, Mary Carrington27,28, Osvaldo Miranda29, Carlos M. Castro29, Martin Gabriel2,3,4, Patrick Drury30, Pierre Formenty30, Boubacar Diallo30, Lamone Kivogu11, N’Faly Magassouba32, Miles W. Carroll4,9, Stephan Günther3,4,8 & César Muñoz-Fontela2,3,4

Despite the magnitude of the Ebola virus disease (EVD) outbreak in West Africa, there is still a fundamental lack of knowledge about the pathophysiology of EVD1. In particular, very little is known about human immune responses to Ebola virus2,3. Here we evaluate the physiology of the human T cell immune response in EVD patients at the time of admission to the Ebola Treatment Center in Guinea, and longitudinally until discharge or death. Through the use of multiparametric flow cytometry established by the European Mobile Laboratory in the field, we identified an immune signature that is unique in EVD fatalities. Fatal EVD was characterized by a high percentage of CD4+ and CD8+ T cells expressing the inhibitory molecules CTLA-4 and PD-1, which correlated with elevated inflammatory markers and high virus load. Conversely, surviving individuals showed significantly lower expression of CTLA-4 and PD-1 as well as lower inflammation, despite comparable overall T cell activation. Concomitant with virus clearance, survivors mounted a robust Ebola-virus-specific T cell response. Our findings suggest that dysregulation of the T cell response is a key component of EVD pathophysiology.

During the initial months of the EVD outbreak in Guinea, we transferred leftover blood samples from EVD patients diagnosed by the EMLab in Guéckédou (n = 47) to Europe (Extended Data Fig. 1a and 1b). Immunophenotyping analysis of these samples, indicated a significantly higher expression of CTLA-4 in CD8+ T cells from EVD patients compared to non-EVD patients (n = 61) (Extended Data Fig. 1c). The levels of CTLA-4 were significantly higher in CD8+ T cells from fatal EVD cases compared to survivors (Extended Data Fig. 1d). CTLA-4 plays an important role in inhibiting T cell function, an immune homeostasis mechanism to control excessive or persistent T cell activation4,5. Owing to its regulatory properties, there are licensed therapeutics to either antagonize or enforce CTLA-4 function6,7. Thus, we hypothesized that our findings could reflect a pathophysiological mechanism of EVD that might be amenable to therapeutics. However, we had concerns regarding the quality of the material that arrived at our laboratory after days of transport and we therefore established flow cytometry directly in Guinea to further evaluate T cell immunity in EVD. Leftover diagnostic blood samples from 157 EVD patients tested by the EMLab at the Coyah Ebola Treatment Center (ETC) were transferred to our laboratory in Conakry within 24 h after collection (Extended Data Fig. 2a, b). The median day of admission at the ETC for both fatalities and survivors was day 4 post symptom onset and, thus, outcome was not associated with the time elapsed between onset of disease and admission (Fig. 1a).

First, we evaluated the immune status of EVD patients upon their arrival to the ETC. Owing to the limitation to six parameters in our field analyses (Extended Data Fig. 3), we focused on the evaluation of individual markers of T cell function. We determined the expression levels of HLA-DR and Ki-67 as markers of activated T cells8, as well as PD-1 and CTLA-4, which together are key regulators of T cell homeostasis5,9. The percentage of CD4+ and CD8+ T cells expressing

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CTLA-4 and PD-1 was significantly higher in fatal cases compared with survivors (Fig. 1b). Sometimes more than 50% of total CD8+ T cells expressed CTLA-4 (Fig. 1c). Fatal cases also showed higher frequency of Ki-67+ CD8+ T cells and HLA-DR+ CD4+ T cells than survivors (Fig. 1b).

High expression of both PD-1 and CTLA-4 has been correlated with functional exhaustion (loss of function) of T cells in chronic inflammatory conditions9–11. However, the role of these molecules during acute infection is less well understood. In previous studies, expression of CTLA-4 and PD-1 in T cells correlated with the extent of pro-inflammatory responses against hantavirus and influenza virus among others9–11. Thus, we next evaluated the levels of pro- and anti-inflammatory cytokines in fatal and non-fatal EVD. In agreement with previous reports12–14, fatal EVD cases had significantly higher levels of serum pro-inflammatory cytokines (TNF also known as TNFα) and IL-8 (Fig. 1d). Moreover, the levels of pro-inflammatory chemokines (MIP-1α, MIP-1β and MCP1) were also significantly upregulated in fatalities (Fig. 1d). Levels of anti-inflammatory cytokines such as IL-10 were also higher in fatalities suggesting the onset of compensatory homeostatic mechanisms in response to excessive inflammation, consistent with expression of T cell inhibitory molecules such as CTLA-4 and PD-1.

To gain insight into the correlation between CTLA-4 expression and the functional status of T cells in patients we performed analyses of T cells from cryopreserved peripheral blood leukocytes, which allowed evaluation of T cell marker co-expression (Extended Data Fig. 4). We used co-expression of CD38 and HLA-DR as well as CD38 and Ki-67 to define activated T cells3,8,13, and co-expression of CTLA-4 and PD-1 to identify effector T cells that had activated inhibitory mechanisms13. Our results indicated robust CD8+ T cell activation in both fatal and non-fatal EVD cases with no statistically significant difference between the two groups. The frequency of activated CD8+ T cells (around 30% of CD38+ HLA-DR+ and 18% of CD38+ Ki-67+) was similar to that previously reported in acute survivors medevac EVD patients3, and to that described in other acute infections13 and after vaccination8. However, the frequency of CD8+ T cells co-expressing PD-1 and CTLA-4 was significantly higher in fatalities (Fig. 2a, b). The results with CD4+ T cells corresponded to those of CD8+ T cells, with no differences in the activation status between fatal and non-fatal EVD, but a higher frequency of cells co-expressed CTLA-4 and PD-1 in fatal
cases (Fig. 2c, d). CTLA-4+ T cells in both CD8+ and CD4+ subsets expressed high levels of CD38 and HLA-DR, suggesting a discrete subset of effector T cells that have initiated a compensatory homeostatic mechanism (Fig. 2b, d).

Whether or not CTLA-4 overexpression affects T cell-mediated viral clearance is controversial. While some reports have correlated the frequency of CD8+ T cells expressing CTLA-4 with poor viral clearance14, others do not support this hypothesis17. To explore the relationship between CTLA-4 and PD-1 expression and virus loads during EVD, we analysed whether the percentage of T cells expressing CTLA-4, PD-1, or both, correlated with the threshold cycle (Ct) values of the Ebola virus (EBOV) real-time PCR. Fatal EVD cases had significantly lower Ct values at the time of admission in agreement with the Ct value negatively correlated with the percentage of CD8+ T cells expressing CTLA-4 alone and in combination with PD-1 correlated with high viraemia.

Acute lymphopenia triggers proliferation of naive T cells with very low expression of CTLA-4 and PD-1 (ref. 21). Thus, we reasoned that differences in Ebola virus (EBOV)-induced lymphopenia15, could be a confounding variable in our study. However, there were no differences in circulating peripheral blood T cells between fatal and non-fatal EVD cases (Extended Data Fig. 5b). A recent study comparing infection with the Makona versus Mayinga variants of EBOV in non-human primates found evidence of initial leukocytosis followed by moderate lymphopenia with no overall differences between both EBOV variants22. Initial leukocytosis in patients infected with the Makona variant of EBOV has been also observed23,24, which indicates the need of longitudinal evaluation of haematological parameters in patients infected with EBOV Makona, an approach that was not possible in our study due to the lack of statistically relevant numbers of fatal cases with longitudinal sampling.
To link the observed activation of CD8+ T cells with EBOV-specific responses, we sought to track EBOV-specific CD8+ T cells during infection through the use of HLA class I dextramers. We first performed in silico analysis of EBOV nucleoprotein-derived peptides predicted to bind with high affinity to selected HLA alleles common in West Africa (HLA-A*02:01, HLA-A*23:01 and HLA-B*35:01) and designed dextramers (Extended Data Fig. 6). We chose the EBOV nucleoprotein based on previous reports indicating that nucleoprotein drives most of the CD8+ T cell response. Dextramer-matching HLA alleles were identified in 26 patients via sequencing of the HLA locus. Six patients had more than one sample for longitudinal evaluation of EBOV-specific T cell responses. These included two fatal cases (F1 and F2) and four survivors (S2–S5). S3 and S4 were treated with favipiravir, and all the other patients received only supportive therapy.

At the time of admission, the differences in the percentage of EBOV-specific T cells between fatalities and survivors were not statistically significant (Fig. 3a). However, differences became evident during later stages of infection. The fatal cases with longitudinal sampling (F1 and F2) showed a high frequency of PD-1+ CTLA-4+ CD8+ T cells until death, but barely detectable EBOV-specific CD8+ T cells (Fig. 3b, e). In contrast, an increase in the frequency of EBOV-specific CD8+ T cells was observed in survivors in coincidence with virus clearance and decrease of the PD-1+ CTLA-4+ CD8+ T cell population (Fig. 3c–e).

These findings were substantiated by the kinetics of CTLA-4 expression in two EVD patients, one fatality and one survivor, evacuated to Europe, as well as five survivors treated in Coyah, Guinea. Longitudinal analysis of these patients revealed persistent upregulation of CTLA-4 in the fatal case and transient upregulation in survivors (Extended Data Fig. 7). Of note, the increase of EBOV-specific T cells in the surviving patients coincided with contraction rather than expansion of the CD38+ HLA-DR+ subset (Fig. 3e). Despite the fact that the phenotype of activated T cells (CD38+ HLA-DR+) suggests engagement of the T cell receptor (TCR) and not bystander T cell activation, our findings may point to the presence of non-EBOV-specific T cells within the CD38+ HLA-DR+ compartment.

The main hypothesis we formulate on the basis of these data is that differences between the T cell response of fatal and surviving EVD patients are centred in the mechanisms that regulate T cell homeostasis. While all patients showed T cell activation irrespective of outcome, it was the expression of the regulatory molecules CTLA-4 and PD-1 on peripheral blood T cells that marked fatal EVD and correlated with high viraemia, a known predictor of poor outcome. We hypothesize that this upregulation reflects a compensatory mechanism to excess inflammatory stimuli, which is consistent with the concomitant expression of pro-inflammatory cytokines in fatal cases. However, our findings do not indicate causality between expression...
of CTLA-4/PD-1 and poor outcome: on one hand it is possible that high viraemia triggers overwhelming inflammation thereby causing CTLA-4 and PD-1 upregulation on T cells, and on the other hand, it is plausible to assume that expression of these molecules inhibits T cell function leading to poor viral clearance. Indeed, both possibilities have been reported in the literature, and their elucidation requires evaluation of CTLA-4 function during EVD in adequate infection models. Interestingly, during severe EVD, both PD-1 and CTLA-4 are upregulated while in other acute human infections such as hantavirus disease and paediatric influenza, only CTLA-4, not PD-1, was upregulated. These results suggest that different infections trigger specific regulatory mechanisms, the balance of which is vital for optimal T cell function. Robust T cell activation was observed in both fatal and non-fatal EVD cases as reflected by similar levels of discrete CD8+ and CD4+ T cells co-expressing CD38 and HLA-DR. These results are in agreement with the strong T cell activation observed in surviving US patients and strengthen the notion that EVD is characterized by immune activation. Although limited, our dextramer data suggest that both fatal and non-fatal cases are able to mount EBOV-specific T cell responses. However, while in survivors the PD-1+ CTLA-4+ CD8+ T cell compartment contracted in conjunction with viral clearance, formation of EBOV-specific T cells and recovery, this did not seem to be the case in fatal EVD. One plausible explanation for this finding is that the high expression of CTLA-4 observed in activated T cells of fatal cases promotes cell extrinsic inhibition of EBOV-specific T cells. T-cell-extrinsic CTLA-4 functions have been demonstrated in vivo and involve different mechanisms such as stripping of T cell co-stimulatory molecules from antigen-presenting cells, stimulation of regulatory T cells and local tryptophan starvation among others. Further functional experiments with adequate in vivo models are needed to explore the role of regulatory T cell molecules during EVD and to test whether their modulation may serve as a putative post-exposure therapy against EVD.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the open content of the paper. Correspondence and requests for materials should be addressed to C.M.-F. (cesar.munoz-fontela@hpi.uni-hamburg.de) or S.G. (guenther@bni.uni-hamburg.de).
METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Patients. EVD patients included in the study were managed at the Ebola Treatment Centers in Guéckédou (n = 47) and Coyah (n = 157) under the medical care of Médecins sans Frontières and doctors deployed by the Cuban government, respectively. Patients with malaria co-infection were excluded from the study. Two patients evacuated into Europe for medical treatment were included in the study for longitudinal analysis. One patient was treated at the University Medical Center Hamburg-Eppendorf, Hamburg, Germany, and the other one at the Hospital La Paz, Madrid, Spain. Demographic and outcome data for EVD patients were obtained from databases of the World Health Organization. The National Committee of Ethics in Medical Research of Guinea as well as the Ethics Committee of the Medical Association of Hamburg approved the use of diagnostic leftover samples and corresponding patient data for this study (permits Nº11/CNERS/14 and PV-4910). This study was also approved by the protocol review office of the US National Cancer Institute institutional review board. As the samples had been collected as part of the public health response to contain the outbreak in Guinea, informed consent was not obtained from patients. Informed consent was obtained from the two EVD patients treated in Hamburg and Madrid.

Study samples and flow cytometry analysis. Real-time RT-PCR was performed on EDTA-blood of patients with suspected EVD using the RealStar Ebolavirus RT-PCR Kit 1.0 (Altona Diagnostics) at the European Mobile Laboratory (EMLab) units in Guéckédou and Coyah. Malaria was diagnosed using a rapid test. Whole blood samples from Guéckédou were shipped to the biosafety level 4 (BSL-4) laboratory in Hamburg within 1–3 weeks after collection and processed immediately upon arrival via multiparametric flow cytometry.

Leftover samples from Coyah were shipped within 24 h after collection to the EMLab immunology laboratory at Donka Hospital in Conakry. PBMCs were isolated after sedimentation of cells in EDTA whole blood tubes. Red blood cells were lysed with Red Blood Cell Lysing buffer (BD Biosciences). PBMCs were processed immediately upon reception for immunophenotypic analysis or were cryopreserved and transported to the BSL-4 laboratory in Hamburg. Immune phenotyping was achieved via multiparametric flow cytometry panel using a battery of commercially available antibodies as follows: anti-human (h) CD3-APCCy7 (clone UCHT1), anti-hCD4-FITC (clone OKT4), anti-hCD8-PerCP Cy5.7 (clone RPA-T8), anti-hCD152 (CTLA-4)-PE (clone L3D10), anti-hCD279 (PD-1)-APC (clone EH12-2HT), anti-hHLA-DR-PerCP Cy5.5 (clone L243), anti-hKi67-PE (clone 16A8), anti-hCD38-APC (clone HB-7). All antibodies were from Biolegend. Single-cell PBMC suspensions were incubated with live/dead discrimination dye (Zombie-NIR from Biolegend) followed by FACS block (Human TruStain Fc receptor blocking antibodies from Biolegend) for 20 min followed by staining with antibodies against extracellular antigens. After extracellular staining samples were inactivated in Cytofix/Cytoperm (BD) buffer in the presence of 4% formaldehyde followed by staining with intracellular antibodies (anti-CD3, Ki-67 and CTLA-4). Sample acquisition was done in a Guava easyCyte 8 Flow Cytometer from Milliporein Guinea. In Hamburg, samples were thawed at 37°C and the sample volume transferred to ice-cold 15-ml Falcon tubes where 5 ml ice-cold R8 medium (RPMI + 8% FBS) were added. Sample tubes were then centrifuged to remove the cryopreservant (10% DMSO) at 1,000 r.p.m. for 10 min and resuspended in R8 medium. Samples were centrifuged once more and the pellets were washed with 12 ml of ice-cold R8 medium. Samples were processed as indicated above. In experiments involving dextramer staining, samples were incubated in a volume of 50 μl of PBS with 5 μl of the indicate dextramers (1:10 dilution) before extracellular antibody staining. All dextramers were purchased from Immudex. Samples were acquired in a LSR Fortessa instrument (BD). Flow cytometry analysis was done with FlowJo software (Treestar).

In silico peptide analysis. Prediction of EBOV nucleoprotein-derived peptide binding to selected human HLA alleles was achieved using an artificial neural network method at the Immune Epitope Database and Analysis Resource (IEDB) (http://www.iedb.org). Selected peptides (IC50 < 50 nM) were then cross-checked with two additional matrix-prediction algorithms, BIMAS (http://www-bimas. cit.nih.gov) and SYFPEITHI (http://www.syfpeithi.de). Peptides predicted by all three bioinformatic tools were selected and screened for similarity to the human genome using the NIH Blast server. Peptides showing homology to the human proteome were discarded.

Multiplex ELISA. Concentrations of selected cytokines and chemokines were measured in 1:2 diluted plasma samples using Milliplex Human Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Missouri, USA) on the Luminex platform LX200 (Luminex, Austin, USA). The procedure was performed according to the manufacturer’s instructions.

HLA genotyping. High-resolution genotyping for HLA class I loci was performed by PCR-sequence-based typing, as recommended by the 13th International Histocompatibility Workshop (available at: http://www.ihwg.org). HLA sequences were analysed using the ASSIGN software (Conexio Genomics).

Statistical analysis. Non-parametric statistics and plots were performed in Graphpad Prism software as described in the figure legends. Sample distribution is illustrated throughout the manuscript using box-and-whisker plots. In all figures the boxes extend from the 25th to 75th percentiles and the horizontal bar is plotted as the median. The bars (whiskers) represent sample distribution down to the 10th percentile (lower bar) and up to the 90th percentile (upper bar).
Extended Data Figure 1 | Initial immunophenotyping data from Guéckédou. a, Graph depicting the number of samples tested by the EMLab unit in Guéckédou by function of time since the beginning of the outbreak. The blue square indicates the period in which leftover whole blood samples from the diagnostic activities were shipped to the BSL-4 laboratory in Hamburg for initial immunophenotyping. b, Demographic data of the Guéckédou EVD patient cohort. Adults were ≥18 years of age and paediatric patients were <18 years of age. c, Comparison of the expression of CTLA-4 assessed by median fluorescence intensity ratio (MFIR) in CD4+ and CD8+ T cells of EVD patients (POS, black boxes) and non-EVD controls (NEG, green boxes). MFIR represents the ratio between the CTLA-4-specific signal divided by the fluorescence minus one (FMO) signal of the same cell population. d, Comparison between CTLA-4 MFIR values in CD8+ T cells from fatal (black) versus surviving (blue) EVD cases. In all panels, the ends of the whiskers in the box-and-whisker plots represent the 10th and 90th percentile, respectively. Statistical analysis was performed by non-parametric Mann–Whitney test; *P ≤ 0.05; ***P ≤ 0.0001.
Extended Data Figure 2 | Epidemiological data of patients tested by EMLab unit in Coyah. a, b, Demographic data of the Coyah EVD patient cohort. Adults were ≤18 years of age and paediatric patients were <18 years of age. The median age of the 157 patients in the study was 26 years (interquartile range (IQR) 20–38 years). Percentages of males and females were comparable within all groups, with adults accounting for 79% of patients. c, Box-and-whisker plots depicting statistical association between Cₜ values and outcome. The case-fatality ratio (CFR) was 51.6%. Fatalities and survivors were compared via non-parametric Mann–Whitney test; ***P < 0.001. d, Correlation between Cₜ value and age of the patients. The Cₜ value did not correlate with age. However, survivors clustered in a group characterized by Cₜ value higher than 18 and by age less than 40 years (cluster encircled in blue). Statistical significance was tested by non-parametric Spearman correlation analysis. e, Cₜ values correlated negatively with symptom scoring, so that low Cₜ values were associated with severe disease symptoms. Symptom score was calculated as the summation of individual symptoms (bleeding, liver dysfunction, respiratory distress, kidney failure, neurological symptoms and anorexia) from '0' (no symptoms) to '6' (all symptoms present). In the box-and-whisker plot the ends of the whiskers represent the 10th and 90th percentile, respectively. Statistical analysis was performed by non-parametric Mann–Whitney test; ***P ≤ 0.0001.
Extended Data Figure 3 | Gating strategy for flow cytometry studies in Guinea. All samples evaluated in the field were aliquoted for four panels. All panels had the following common gating: G1, lymphocyte gate; G2, live cells; G3, singlets; G4, T cells; G5, CD4+ T cells; G6, CD8+ T cells. Panels 1, 2, 3 and 4 evaluated expression of HLA-DR, Ki-67, CTLA-4 and PD-1, respectively, in either CD4+ or CD8+ T cells.
Extended Data Figure 4 | Gating strategy for flow cytometry in Hamburg. Cryopreserved PBMC samples from Coyah were thawed as indicated in the Methods. The following gates were used for sample analysis: G1, lymphocyte gate; G2, live cells; G3, singlets; G4, T cells; G5, CD4⁺ T cells; G6, CD8⁺ T cells. In G5 or G6, samples were evaluated for co-expression of the indicated cell markers. Dextramer staining was evaluated in G6 following protocols described in the Methods.
Extended Data Figure 5 | Correlation of double positive PD-1+/CTLA-4+ CD8+ T cells with Ct values and lymphopenia. a, Graph showing correlation between the frequency of CD8+ T cells co-expressing PD-1 and CTLA-4 and the Ct value. Correlation analysis was done via non-parametric Spearman correlation test. b, Box-and-whisker plots depicting the concentration of CD4+ and CD8+ T cells in blood of fatal and surviving EVD patients. The ends of the whiskers in the box-and-whisker plots represent the 10th and 90th percentile, respectively.
Extended Data Figure 6 | In silico peptide analysis and dextramer design. a, Selection of peptides consisting of nine amino acid residues corresponding to the EBOV nucleoprotein sequence predicted to bind the indicated HLA alleles. IC₅₀ values for peptide binding to HLA were predicted by the artificial neural network (ANN) at the Immune Epitope Database and Analysis Resource (IEDB) (http://www.iedb.org). Blue squares = Peptides chosen for dextramer design.

b, Dextramer background was determined by staining of HLA-matched healthy donor peripheral blood leukocyte samples. T cells were gated as indicated in Extended Data Fig. 4. Plots in the upper row represent staining of a FMO (fluorescent minus one) sample in which the APC channel was left empty. Lower rows show background dextramer staining as indicated. The mean background staining plus minus standard deviation is indicated for each dextramer and the FMO.
Extended Data Figure 7 | Longitudinal analysis of CTLA-4 expression in the CD8⁺ T cell compartment during the course of EVD in two patients. a, Graph depicts the levels of expression of CTLA-4 in CD8⁺ T cells of a fatal versus a surviving EVD case over the course of the disease. Both patients were treated in Europe. Samples were taken at consecutive days starting immediately after patient admission as indicated. MFIR represents the ratio between the CTLA-4-specific signal divided by the fluorescence minus one (FMO) signal of the same cell population. b, Longitudinal analysis of CTLA-4 expression in CD8⁺ T cells and Ct values in survivors from Coyah.