Long-lasting severe immune dysfunction in Ebola virus disease survivors

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Abstract

Clinical follow-up of Ebola virus disease (EVD) survivors revealed a persistence of clinical symptoms and higher risk of mortality. Long-term analyses of the immune and inflammatory profiles of EVD survivors are currently lacking. Here, we evaluate immune profile status and gene expression profiles in 35 Guinean EVD survivors (EBOV_S) from the last West African outbreak, a median of 23 months (IQR [18-25]) after discharge from the Ebola treatment center. We show a persistent increase of several biomarkers of inflammation, immune activation and gut tissue damage in EBOV_S compared to healthy donors living in the same area. These results are confirmed by phenotypic characterization of immune cell subsets revealing increases in activation marker expression and the frequencies of $CD8^+$ T cells, exhausted B cells, non-classical NK cells and circulating dendritic cells. All survivors have EBOV-specific IgG antibodies and robust and polyfunctional EBOV-specific memory T-cell responses. Deep sequencing studies of the genes expressed in blood revealed a significant enrichment in genes associated with antiviral responses in EBOV_S.

This study assess the long-term persistence of immunological dysfunction in survivors and identify a set of biological and genetic markers that could be used to define a signature of “chronic Ebola virus disease (CEVD)”.

Introduction

In the 2013–2016 West African outbreak, Ebola virus infected more than 28,000 people, causing 11,310 deaths by May 11, 2016, in six countries (Sierra Leone, Liberia, Guinea, Mali, Nigeria, and Senegal) [1,2]. In the most affected countries, unprecedented follow-up of large numbers of Ebola Virus disease (EVD) survivors has revealed long-term clinical sequelae. These observations raise questions about the pathophysiology of EVD, the risk of virus re-emergence and patient clinical care and treatment of EVD [3,4].

Two large cohorts of survivors from the West African outbreak have been reported [3–5]. Their clinical follow-up revealed symptom persistence more than one year after acute EVD (Prevail III cohort) or discharge from the Ebola treatment center (ETC) (Postebogui cohort). The clinical symptoms recorded during follow-up were predominantly general symptoms, musculoskeletal pain, neurocognitive and ocular disorders.

The incidence of several new systemic symptoms was higher in survivors than in controls over six to 12 months of follow-up in the Prevail III cohort [4]. Symptom prevalence decreased overall, but the incidence of uveitis was significantly higher in survivors than in seronegative contacts.

In the Postebogui cohort, long-term follow-up of 802 survivors of the Guinea outbreak provided a temporal description of clinical incidence through the reporting of clinical events up to 600 days post-ETC discharge [3]. The frequencies of all clinical symptoms except ocular disorders decreased. Surprisingly, at inclusion, many patients presented symptoms similar to those experienced during acute-phase infection,

suggesting the persistence or recurrence of a pathogenic process. These studies, and smaller previous case series [6–9], have refined definitions of the clinical spectrum of post-EVD sequelae.

The symptoms and clinical findings reported for EVD survivors resemble those of chronic post-infectious and/or immune dysfunction diseases. Several hypotheses concerning EVD pathogenesis have been proposed based on the persistence or increase in incidence of clinical disorders long after acute Ebola infection [10].

The immune responses of EVD patients have been reported during acute-phase infection or soon after the resolution of infection [11–17]. These studies identified immune signatures associated with death from EVD rather than survival. Fatal EVD was characterized by high inflammatory marker levels and a high viral load [12,16,17]. Conversely, survivors had significantly lower levels of inflammation [15] and robust EBOV-specific T-cell responses [12]. Persistent immune activation and weak $CD8^+$ EBOV–specific T-cell responses were detectable for up to 46 days after viral clearance from plasma[13].

In this study, we took advantage of the long-term follow-up of EVD survivors (EBOV_S), for detailed analyses of inflammatory, immune functional and phenotypic characteristics and gene expression patterns, up to two years after acute EVD. Comparing these profiles with those of non-infected healthy donors (HD), we found that EVD survivors continued to display severe abnormalities of immune function and persistent EBOV-associated immune activation.

Materials And Methods

Participants

We enrolled a subgroup of post-EVD survivors from the Postebogui cohort in this ancillary immunological study. The design of the Postebogui cohort and patient characteristics have been described elsewhere[3,5,18]. Eligible patients with laboratory-confirmed EVD subsequently declared virus-free were recruited at the ETCs in Guinea between March 2015 and July 2016. All patients gave immunological study-specific written informed consent. Healthy volunteers enrolled in the PREVAC (Partnership for Research on Ebola Vaccination) vaccine trial Guinean center agreeing to participate in the immunological evaluation were included, at baseline, as controls. Whole blood, peripheral blood mononuclear cells (PBMC) and serum samples were collected and stored on site. The study protocols were approved by the Research Committee of the National Ebola Response Coordination and the National Ethics and Health Research Committee in Guinea and ethics committees in France (INSERM/CEEI, IRD/CCDE).

Blood EBOV-RNA and serum EBOV antibody determinations

The Real-Star Filovirus Screen RT-PCR kit 1.0 (Altona Diagnostics GmbH, Hamburg, Germany) was used to test for EBOV RNA at the European West African Mobile Laboratory (EuWamlab) or the *Institut National de Sante Publique* (INSP) in Conakry. EBOV-specific IgG was quantified in Luminex assays, as previously

described [19]. Samples were considered positive for IgG against EBOV if they reacted simultaneously and repeatedly with nucleoprotein (NP) and glycoprotein (GP) or NP ± viral protein (VP40) + GP (weakly positive: 200 to 399 MFI) [20].

Quantification of serum analytes

We quantified 29 analytes (see supplementary appendix) in serum samples with the Human XL Cyt Disc Premixed Mag Luminex Perf Assay Kit, Human Magnetic Luminex Assay kits and Human Quantikine ELISA kits (R&D Systems).

Cell phenotyping

Immune phenotyping was performed with an LSRII Fortessa 4-laser (488, 640, 561 and 405 nm) flow cytometer (BD Biosciences), and FlowJo software version 9.9.6 (Tree Star Inc.) The antibodies used are described in the supplementary appendix. $CD4^+$ and $CD8^+$ T cells were analyzed for CD45RA and CCR7 expression, to identify the naive, memory and effector cell subsets, and for co-expression of the activation markers HLA-DR and CD38. $CD19^+$ B-cell subsets were analyzed for the CD21 and CD27 markers. Antibody-secreting cells (ASC, plasmablasts) were identified as $CD19^+$ cells expressing CD38 and CD27. We used CD16 and CD56 to identify NK cell subsets. HLA-DR, CD33, CD45RA, CD123, CD141 and CD1c were used to identify dendritic cell (DC) subsets, as previously described. [21]

Characterization of EBOV-specific immune responses

Cellular responses to EBOV peptides were assessed with EpiMax technology [22]. Briefly, PBMC were stimulated *in vitro* with 158 overlapping 15-mer peptides (11-amino acid overlaps), covering the Ebola virus Mayinga variant GP, in two pools of 77 (EBOV1) and 81 peptides (EBOV2) (JPT Technologies). Cell functionality was assessed by intracellular cytokine staining (ICS), with Boolean gating (Fig. 1A, supplementary appendix). The flow cytometry panel included a viability marker, CD3, CD4 and CD8 to determine T-cell lineage, and CD107a, IFN-γ, TNF-α, MIP–1β and IL–2 antibodies. Distributions were plotted with SPICE version 5.22, downloaded from http://exon.niaid.nih.gov/spice [23].

RNA isolation and mRNA sequencing

Total RNA was purified from whole blood with the Tempus™ Spin RNA Isolation Kit (Invitrogen). Gene expression profiles were analyzed by mRNA sequencing (see supplementary appendix). Only genes with adjusted *P*-values (FDR) ≤ 0.05 and a fold-change in expression ≥1.5 were considered to be differentially expressed. The differentially expressed genes were subjected to functional enrichment analysis with Ingenuity Pathway software.

Statistical analysis

Graphpad Prism software version 6 was used for nonparametric statistics and plots, as described in the figure legends.

Results

Participants

We enrolled 35 EBOV_S, with a median age of 30 years (interquartile range (IQR): 25–36) in this study. Median [IQR] time between ETC discharge and enrollment was 23 months [19–25]. No EBOV RNA was detectable in the blood at time of sampling. The enrolled subjects received only supportive care (no experimental drugs, no convalescent plasma) during the acute phase of EBOV infection and were seronegative for HIV, HCV and HBV. On inclusion in Postebogui cohort, 23 of the 35 patients (66%) had post-EVD symptoms similar to those for the whole cohort [3] (Table 1). The median mean fluorescence intensity (MFI) [IQR] of EBOV-specific antibodies was 3341 [1365–7154], 930 [548–1621], 640 [413–919], and 1288 [649–2328] against NP, GP-Kissidougou, GP-Mayinga, and VP40, respectively. By contrast, none of the serum samples from HD tested positive for these antibodies (Fig. 2, supplementary appendix). Thus, 23 months after EVD infection, EBOV_S had EBOV-specific IgG.

Serum proteins

Eleven of the 29 soluble mediators quantified in serum were present at significantly higher levels in EBOV_S than in controls. Median [IQR] pro-inflammatory cytokine concentrations were 33 pg/ml [15.9–118.9] vs. 14 pg/ml [9.8–21.6] (P = 0.001) for IL–8 and 16.5 pg/ml [11–28] vs. 12.6 pg/ml [9.3–16.4] for TNFα (P = 0.015). For the anti-inflammatory cytokine IL–1RA, median [IQR] concentrations were 433.8 pg/ml [315–657] vs. 327.9 pg/ml [221–419] (P = 0.0004) (Fig.1A). The T-cell function markers sCD40L and CCL5 were also present at significantly higher levels in survivors than in HD (P = 0.0002 and P = 0.005, respectively) (Fig. 1B).

The persistence of markers of chronic activation in the blood of survivors suggested possible microbial translocation from a leaky gut, as described in other chronic infectious diseases [24,25]. Survivors had significantly higher levels of soluble CD14 (P<0.0001), lipopolysaccharide binding protein (LBP) (P = 0.007), fatty acid binding protein (iFABP), a marker of gut epithelial damage (P = 0.006) and soluble CD163, a specific marker of monocyte/macrophage activation, (P = 0.014) (Fig. 1C). Consistently, epidermal growth factor (EGF), a secreted gastric protection protein [26] was also present at higher levels in EBOV_S than in HD: 483.6 pg/ml [356–665] vs. 282.6 pg/ml [230–388] (P <0.0001). The EGF-like molecule, amphiregulin (AREG), was also present at higher levels in EBOV_S: 8.4 pg/ml [6.2–13.8] vs. 5.45 pg/ml [4–7.7]; (P = 0.001) (Fig. 1C).

Cellular phenotype

Cell phenotyping on blood from survivors (representative data, Fig. 3 in supplementary appendix) showed a higher frequency of total $CD8^+$ T cells among $CD3^+$ cells (P = 0.0004) and higher levels of activated $CD8^+$ T cells ($CD8^+HLADR^+CD38^+$) (*P* = 0.01) than in controls (Fig. 2A). Total $CD19^+$ B-cell frequency was similar in the two groups, as was the frequency of plasmablasts (data not shown) but the frequencies of activated memory and exhausted B cells were higher in EBOV_S (*P* = 0.006 and *P*<0.0001, respectively) (Fig. 2B). There was a trend towards lower total NK cell frequencies in EBOV_S, but the activation marker NKG2D was significantly downregulated relative to controls (*P* = 0.004). Moreover, the frequency of non-classical CD56-$CD16^+$ NK cells remained high in EBOV_S (*P*<0.0001) (Fig. 2C). Blood analysis revealed a severe deficit of total DCs (HLA-DR^+, Lin^-) (*P* = 0.01), mostly due to a deficit of plasmacytoid DC (pDC) ($HLADR^+/Lin^-/CD45RA^+/CD123^+$) (*P* = 0.006), whereas conventional DC (cDC) levels remained in the normal range. Higher levels of expression were observed for the activation marker CD40 in both cDC (*P* = 0.02) and pDC (*P* = 0.02), with higher levels of HLA–ABC expression in pDC (*P* = 0.02) (Fig. 2D).

EBOV-specific cellular responses

A high frequency of functional $CD4^+$ and $CD8^+$ T cells producing cytokines in response to various EBOV peptides was detected in survivors. Median frequencies [IQR] of $CD4^+$ and $CD8^+$ $cytokine^+$ specific T cells were 4.89% [1.93–11.2] and 12.4% [8.9–25.9], respectively, for the EBOV1 peptide pool and 5.18% [3.4–12.6] and 11.8% [5.1–19], respectively, for the EBOV2 peptide pool (*P*<0.0001 for all comparisons to non-stimulated conditions) (Fig. 3A). EBOV stimulation elicited IFN-γ^+, TNF-α^+, MIP–$1\beta^+$ and IL–2^+ $CD4^+$ T cells (*P*<0.0001, for each cytokine) and IFN-γ^+, TNF-α^+, MIP–$1\beta^+$ $CD8^+$ T cells (*P*<0.0001 for each cytokine) (Supplementary Fig. 1B). EBOV-specific $CD8^+$ T cell expressing the cytotoxicity markers CD107a and IFN-γ after peptide stimulation were also detected at high frequency (*P*<0.0001, for all stimulation conditions) (Fig. 3B). A large proportion of the EBOV-specific $CD4^+$ and $CD8^+$ T cells were polyfunctional, producing up to four cytokines simultaneously (Fig. 3C).

Gene expression profiles

An analysis of whole-blood gene expression profiles (26 EBOV_S and 33 HD) revealed differential expression for 559 annotated genes (112 upregulated and 447 downregulated in survivors) defining the cluster of survivors (Figure 4A). The differentially expressed genes characteristic of survivors included genes involved in immune responses: acute-phase response signaling, interferon signaling, the complement system, phagosome formation and pattern recognition receptors (PRR) (Figure 4B). EBOV_S displayed a clear upregulation of genes relating to the antiviral response involving IFN signaling (*IFIT1, IFI6, IFIT3, ISG15, OAS1, IFITM3* and *MX1),*, the complement system *(C4BPA, SERPING1, C1QC, C1QB, C1QA)* and pattern recognition receptor (PRR) *(C1QA, C1QB, C1QC, IL10, OAS1, OAS3, PRKD1)* signaling pathways (Figure 4C).

Discussion

The 2013-2016 Ebola outbreak in West Africa revealed how little was known about the pathogenicity of this virus and markers predictive of its clinical outcome. Several studies have investigated the acute phase of infection in EVD patients, or the period shortly after viral clearance. By contrast, we studied long-term survivors of the recent outbreak in Guinea. By comparing these survivors with a cohort of volunteers who had not had EVD and lived in the same areas, we were able to identify a profile specific to survivors. Our results, obtained with a large array of assays, highlight the existence of a consistent, intense chronic immune activation and inflammatory profile in survivors. Up to two years after healing and discharge from the ETC, survivors have persistently high serum levels of pro-inflammatory cytokines (IL-8 and TNF-α) and chronic immune activation markers (CCL5 and sCD40L). Consistent with these observations, an abnormal expression of activation markers was observed on circulating dendritic cells, and the balance of immune cells was shifted towards circulating activated $CD8^+$ T cells, exhausted B cells and non-classical NK cells, a population reported to be abundant in other chronic viral infections [27,28] and in patients with acute EVD who subsequently survived [29]. Finally, deep sequencing analyses of gene expression in the blood showed a significant enrichment in the expression of genes associated with IFN signaling, complement, pattern recognition receptor and acute-phase response signaling. Previous studies have reported similar profiles for patients with acute EVD [14,30].

It has been suggested that survivors have a profile of weaker inflammation, and less intense cytokine and chemokine “storms” than fatal cases, in whom these abnormalities are correlated with viral replication and tend to increase until death. Our data extend these findings, by showing that the long-term persistence of these abnormalities, in the absence of detectable viral replication, may be a signature of a “chronic Ebola virus disease (CEVD)”, and shedding light on its pathophysiology.

Clinically, the production of a damage-associated molecular pattern (DAMP) during acute EVD was thought to be associated with tissue damage leading to a pathogenic activation cascade and, ultimately, to a clinical syndrome resembling septic shock [17]. We did not perform gut biopsies on these survivors, for practical reasons, but our results show that the chronic activation profile in CEVD is associated with high blood levels of markers of intestinal permeability and microbial translocation from a leaky gut (sCD14, iFABP, LBP, sCD163). Acute EVD is characterized by severe gastrointestinal symptoms [31,32]. Our data suggest there may be substantial long-term gut damage, resulting in structural impairment of the epithelial barrier, as reported in several other chronic infectious diseases [24,25]. Consistently, levels of EGF, which is present at higher levels during the acute phase of EVD in individuals who survive than in those who die [15] and AREG, which restores tissue integrity following inflammation-associated damage [33], remained higher in EVD survivors than HD.

Identifying the immune responses associated with protection against Ebola infection or survival is crucial. The development of strong T-cell responses during acute infection has been shown to be important for viral clearance and survival, whereas B-cell responses seem to be less efficient with very

few potent B-cell clones [12,34]. However, data remain scarce for humans. We show here that survivors display strong, robust polyfunctional memory T-cell responses to various Ebola epitopes. The antiviral functional capacity of these cells was not studied, but the phenotype of $CD8^+$ T cells after *in vitro* stimulation (strong cytotoxicity marker expression) suggested that they remained cytotoxic. We were unable to study the progression of these responses longitudinally, but the high frequency of these cells two years after viral clearance is intriguing. Survivors also maintained strong specific IgG responses against various Ebola proteins, confirming previous studies from precedent outbreaks [35,36] . These data and the recent demonstration of a change in IgG isotype during maturation of the anti-Ebola IgG repertoire in four survivors [37] suggest that specific anti-Ebola responses change over time, consistent with possible persistent exposure to viral proteins captured at immunological sites or localized smoldering viral replication at sites of immune privilege (testes, eyes, central nervous system) [38-40]. The ability of these responses to protect against secondary infection or control residual replication, thereby containing viral reservoirs, is unknown, but would have major implications for vaccine development.

Ebola disease remains a public threat. The mobilization of national and international organizations, community health workers and patients, civil society and policy makers is crucial to contain epidemic spread and decrease mortality. Survivors of the 2013-2016 epidemic in West Africa and the current outbreak in the Democratic Republic of Congo ("*les vainqueurs d'Ebola*") are at risk of developing a profile of severe immune dysfunction and increased morbidity that we propose to call "CEVD". Our data provide a set of biological and genetic markers for assessing clinical outcomes and highlight the importance of developing such leading-edge studies despite limited infrastructures in an epidemic context and the need for resources for the long-term follow-up of survivors.

Declarations

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Author Contributions

YL, CL and AW conceived and designed the study. AKK, SM, J-CF, AT, HR, ED, CL-M, LK participated in sample collection. EF, CLe and AA performed experiments and analyzed data. AW, HH, CLe, YL and AA analyzed and interpreted data. AW and YL drafted the first version and wrote the final version of the manuscript. All authors approved the final version.

Conflict of interest statement

None of the authors has any conflict of interest to declare.

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Table 1

	HD	EBOV_S
No. of subjects	39	35
Median Age (yr)	25 [21-36]	30 [25-36]
Sex (male)	31 (80%)	19 (54%)
Median time from ETC discharge to inclusion (months)	N/A	23 [19-25]
EBOV RT PCR *	N/A	Negative (100%)
Clinical events¥		
Joint pain	N/A	14 (40%)
Fatigue	N/A	12 (34%)
Ocular disorders	N/A	6 (17%)
Headache	N/A	6 (17%)
Muscle pain	N/A	5 (14.3%)
Fever	N/A	4 (11.4%)
Abdominal pain	N/A	2 (5.7%)
Anorexia	N/A	1 (2.8%)

Table 1: Characteristics of survivors (EBOV_S) and healthy donors (HD)

*Blood samples were taken from patients enrolled in the Postebogui cohort. Before PBMC and serum freezing, EBOV RT-PCR was performed on each sample to exclude the presence of EBOV in the blood. ¥ Symptoms and findings on physical examination for the EBOV_S on inclusion in the Postebogui cohort.

Figures

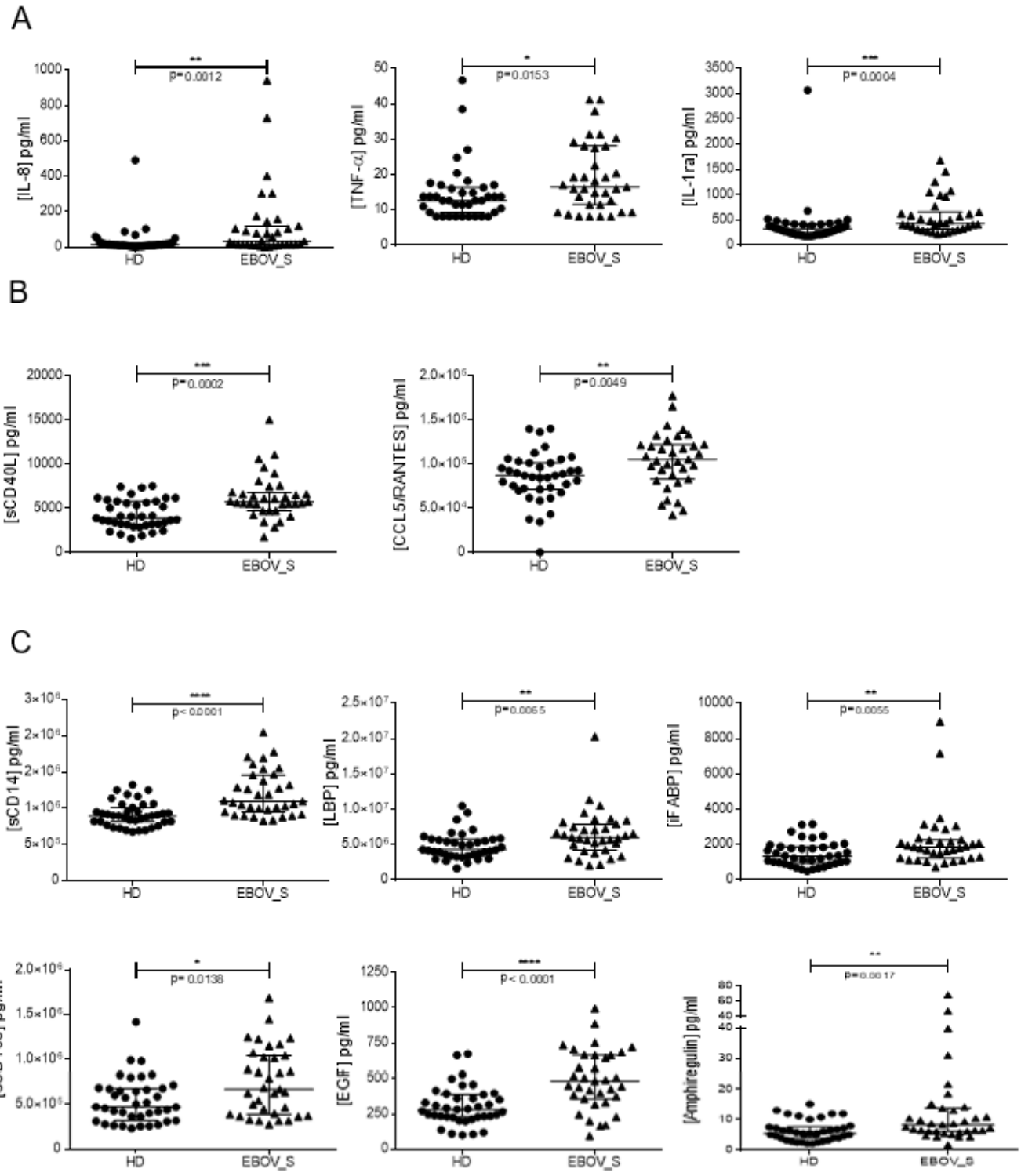

Figure 1

: Quantification of serum soluble mediators differentially expressed in HD and EBOV_S. Measurement of serum soluble mediators (pg/ml) from n=39 HD and n=35 EBOV_S with the Bio-Plex 200 SystemTM (Bio-rad). Pro-inflammatory and anti-inflammatory cytokines (A). Markers of T-cell function (B). Markers of gastric tissue integrity (C). The differences between HD and EBOV_S were evaluated in nonparametric Mann-Whitney U tests. Median values ± IQR are shown.

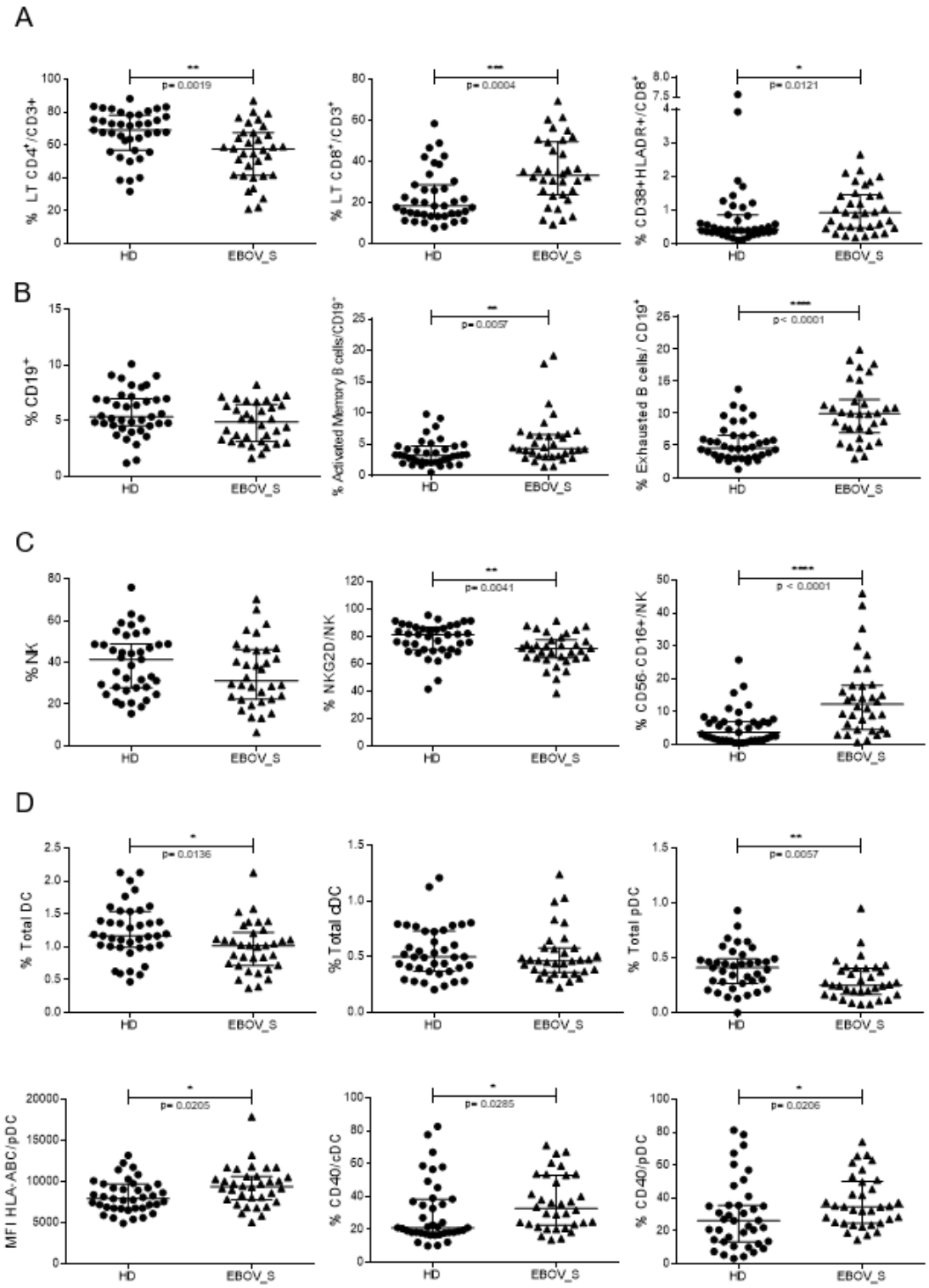

Figure 2

PBMC phenotypic characterization in EBOV_S. Cumulative T-cell frequency and CD8 T-cell activation analyses (A), B-cell subsets (B), NK cell subsets (C), dendritic cell subsets and activation (D). DC gating strategy is shown in supplementary Figure 3. The differences between HD and EBOV_S were evaluated with nonparametric Mann-Whitney U tests. Median values ± IQR are shown.

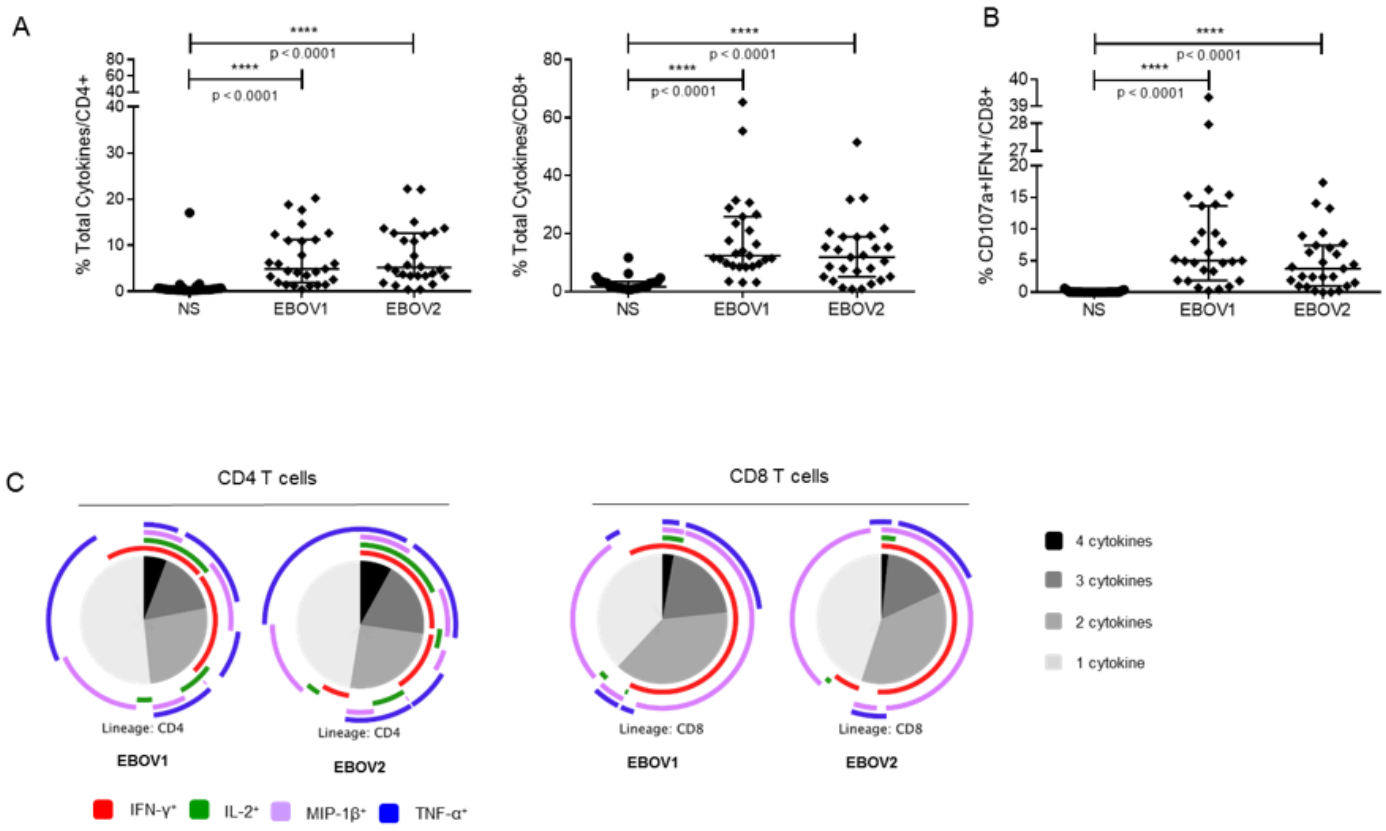

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Figure 3

Characterization of functional responses in EBOV_S. EBOV GP-specific CD4+ T-cell (left panel) and CD8+ T-cell (right panel) responses of EBOV_S (n=27) after nine days of EBOV GP-specific (EBOV1 and EBOV2 peptide pools) T-cell expansion in vitro (all cytokines) (A). Analysis of the co-expression of CD107a and IFN-γ by EBOV GP-specific CD8 T cells from EBOV_S (n=27) after nine days of antigen-specific T-cell expansion in vitro (B). Median values ± IQR are shown, and Friedman's test was used for comparisons. Functional composition of EBOV GP-specific CD4+ and CD8+ T-cell responses (C). Responses are color-coded according to the combinations of cytokines produced. The arcs identify cytokine-producing subsets (IFN-γ, IL-2, MIP-1β, TNF-α) within the CD4+ and CD8+ T-cell populations.

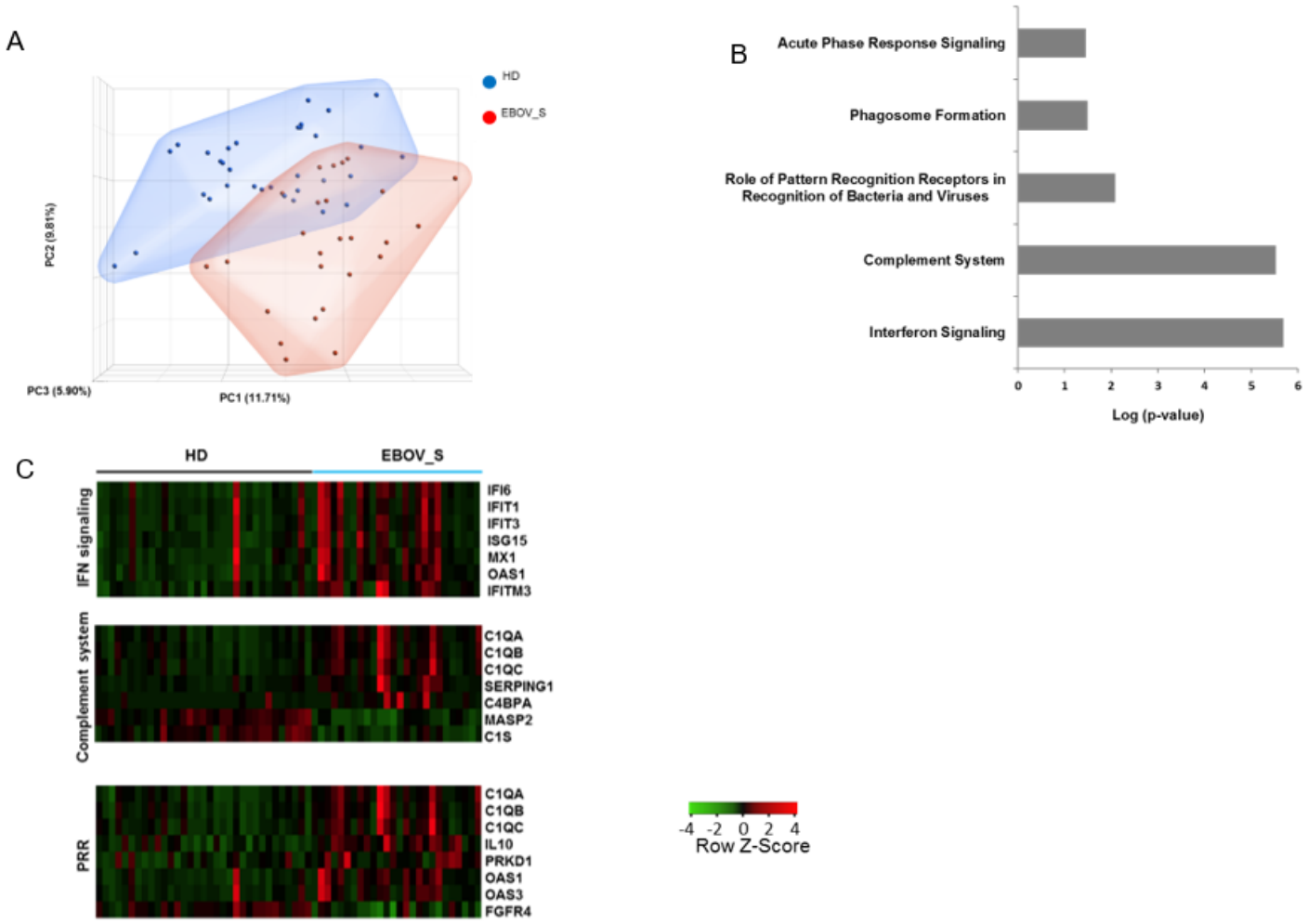

Figure 4

Whole-blood gene expression profiles of EBOV_S and HD. Unsupervised principal component analysis (PCA) of EBOV_S (n=26) and HD (n=33). EBOV_S are indicated in red and HD in blue (A). Ingenuity Pathway software analysis of the genes involved in immune responses differentially expressed in EBOV_S and HD (B). Heatmap of genes from the main pathways associated with differentially expressed genes in EBOV_S and HD, including IFN signaling, the complement system and PRR signaling pathways (C).

Supplementary Files

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