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SUPPLEMENT ARTICLE

Ebola Virus Disease Is Characterized by Poor Activation and Reduced Levels of Circulating CD16$^+$ Monocytes

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A number of previous studies have identified antigen-presenting cells (APCs) as key targets of Ebola virus (EBOV), but the role of APCs in human Ebola virus disease (EVD) is not known. We have evaluated the phenotype and kinetics of monocytes, neutrophils, and dendritic cells (DCs) in peripheral blood of patients for whom EVD was diagnosed by the European Mobile Laboratory in Guinea. Acute EVD was characterized by reduced levels of circulating nonclassical CD16$^+$ monocytes with a poor activation profile. In survivors, CD16$^+$ monocytes were activated during recovery, coincident with viral clearance, suggesting an important role of this cell subset in EVD pathophysiology.

Keywords. Ebola virus; Ebola virus disease; monocytes; CD16; CD14.

Ebola virus (EBOV) causes severe disease in humans that is partially defined by alterations of the host immune response. In particular, professional antigen-presenting cells, including macrophages and dendritic cells (DCs), are readily infected with EBOV in vitro, and viral infection causes inhibition of DC activation [1,2] which is a necessary step for the initiation of adaptive antiviral immunity [3]. However, it is not known whether EBOV infection influences APC function in vivo, and the role of APCs on Ebola virus disease (EVD) pathophysiology remains to be elucidated.

Several subsets of APCs or their precursors can be found in human blood, including monocytes and DCs. Blood monocytes can be subdivided in 3 subsets based on phenotype and function: classical CD14$^+$ monocytes are defined by their capacity to produce proinflammatory cytokines and chemokines in response to lipopolysaccharide [4]. Conversely, nonclassical CD16$^+$ monocytes have been proposed to have an antiviral role [5, 6]. Indeed, CD16$^+$ monocytes can recognize viral RNA through their expression of Toll-like receptor 7 (TLR7) and TLR8 [5]. Intermediate CD14$^+$ CD16$^+$ monocytes have been also identified but are less well characterized functionally [4]. Precursors of conventional CD1c$^+$ and CD141$^+$ DCs, as well as plasmacytoid DCs and activated HLA-DR$^+$ neutrophils, can be also identified in human blood [7, 8]. The kinetics and phenotype of these APCs may be used to evaluate human immune responses to pathogens and vaccines [9].

To gain insight into the function of APCs during human EVD, we evaluated the alterations in this cell compartment in patients with EVD at the time of admission to the Ebola treatment center (ETC). The self-reported median time of admission after symptom onset among these patients was day 4 for both survivors and patients who died (Supplementary Figure 1). In addition, we monitored the activation status of APCs longitudinally in 8 patients, of whom 3 died and 5 survived. Our findings indicate that human EVD is characterized by a significant decrease in circulating CD16$^+$ monocytes and a poor activation of this cell subset. Survivors showed activation of CD16$^+$ monocytes during recovery, concurrent with viral clearance. Our work shows that a reduction of the CD16$^+$ monocyte population and poor activation of this cell subset are important features of human EVD.

METHODS

Patients

Patients with acute EVD (ie, those who tested positive for EBOV by reverse transcription–polymerase chain reaction [RT-PCR]) included in the study were attended at the ETC in Coyah (n = 28), under the care of medical teams deployed by the Cuban government. The median age of our patient cohort was 32 years (interquartile range, 20–43 years). Patient samples were obtained at the time of admission at the ETC, and patients with malaria parasite coinfection were excluded from the study. Additionally, 34 febrile EVD-negative controls were included in the study. The National Committee of Ethics in Medical Research of Guinea, as well as the Ethics Committee of the

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Medical Association of Hamburg, approved the use of diagnostic leftover samples and corresponding patient data for this study (permits 11/CNERS/14 and PV4910). Because 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.

Study Samples and Flow Cytometry

Real-time RT-PCR was performed on blood samples in ethylenediaminetetraacetic acid (EDTA)–lined tubes that were collected from patients with suspected acute EVD, using the RealStar Zaire Ebolavirus RT-PCR Kit 1.0 (Altona Diagnostics), at the European Mobile Laboratory (EMLab) unit in Coyah. Malaria was diagnosed using a rapid test. Leftover samples from diagnostic assays were shipped within 24 hours after collection to our immunology laboratory at Donka Hospital in Conakry and processed immediately. Thirty samples from convalescent patients, namely patients who recovered from acute EVD and were PCR negative for EBOV, were included in the study. Of these, 19 samples were from 12 surviving patients included in the acute EVD cohort.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole-blood specimens in EDTA-lined tubes. Red blood cells were lysed with Red Blood Cell Lysing buffer (BD Biosciences). Immunophenotyping was achieved via a multiparametric flow cytometry panel, using the following commercially available antibodies: CD3-PerCP/Cy5.5 (SK7), CD19-PerCP/Cy5.5 (HIB19), CD56-PerCP/Cy5.5 (HCD56), HLA-DR-PE/Cy7 (L243), CD11c-PB (Bu15), CD14-BV510 (M5E2), CD141-PE (M80), CD1c-APC (L161), and CD16-APC/Cy7 (3G8). All antibodies were from Biolegend. PBMCs were treated with fluorescence-activated cell-sorting block (Human TruStain Fc receptor blocking antibodies from Biolegend) for 20 minutes followed by staining with antibodies. After staining, samples were inactivated in Cytofix/Cytoperm (BD) buffer in the presence of 4% formaldehyde. Sample acquisition was done in a Guava easyCyte 8 flow cytometer from Millipore. Analysis of flow cytometry data was done with FlowJo software (Treestar).

Statistical Analysis

Nonparametric statistics was performed in GraphPad Prism software, using the Kruskal–Wallis test and the Dunn multiple comparison test. Sample distributions are illustrated via box plots. Boxes extend from the 25th to 75th percentiles, and the horizontal bar is plotted as the median. The bars (whiskers) represent the sample distribution down to the 10th percentile (lower bar) and up to the 90th percentile (upper bar).

RESULTS

To evaluate the phenotype of APCs in the peripheral blood of patients with EVD at the time of admission to the ETC, we used flow cytometry to identify classical $CD14^+$ and intermediate $CD14^+CD16^+$ monocytes, nonclassical $CD16^+$ monocytes, activated HLA-DR^+ neutrophils, and DCs (Figure 1*A*). We first compared the concentrations of these cell subsets in peripheral blood of patients with EVD and febrile patients who tested negative for EVD. We observed a reduction in the levels of all circulating monocyte subsets and $CD1c^+$ DCs in patients with EVD, compared with febrile controls, with a very significant decrease in $CD16^+$ monocytes (Figure 1*B* and Supplementary Figure 2). When patients with acute EVD were classified on the basis of outcome, the number of circulating $CD16^+$ monocytes was also significantly lower in patients who died, compared with survivors (Figure 1*B*). We did not find any significant differences between the groups with regard to the circulating levels of neutrophils, other DC subsets, or total peripheral blood mononuclear cells. To determine whether our findings indicated specific reduction of particular immune subsets, rather than a generalized loss of APCs, we also determined the percentages of monocytes and DCs within the APC gate. These analyses also indicated that monocytes and $CD1c^+$ DCs were reduced among the blood APCs, with a very significant reduction of $CD16^+$ monocytes in patients who died of EVD (Supplementary Figures 2 and 3).

To gain insight into the relevance of these findings for the pathophysiology of human EVD, we sought to determine the correlation between the levels of circulating monocytes and, as a readout of the viral load, the cycle threshold (Ct) value. Our results indicated that the concentration of $CD14^+$ and $CD16^+$ monocytes in blood samples from patients with EVD was positively correlated with the Ct value, which indicated that reduced numbers of these cells in peripheral blood was correlated with high viremia levels (Figure 2*A*). These results suggested that EVD is marked by a decrease in circulating monocytes and that this phenotype was exacerbated in patients with high viral loads, a known predictor of poor outcome [10].

To further explore the relationship between reduced levels of monocytes and EVD severity, we determined the concentration of these cells in all diagnostic EVD samples for which Ct values were available. Samples were classified into 3 groups: those with Ct values of < 25, 25–33, or > 33. A fourth group was formed by samples from convalescent patients with EBOV-negative PCR results, and, finally, another group was formed by samples from EVD-negative febrile patients. The samples from patients with a high viremia level (Ct value, < 25) showed significantly reduced levels of $CD16^+$ monocytes, compared with samples from patients with a low viremia level (Ct value, > 33), samples from PCR-negative convalescent patients, and samples from other acute febrile patients (Figure 2*B*). We also observed significantly reduced levels of $CD14^+$ monocytes and intermediate monocytes in samples with a Ct value of < 25 as compared to those from convalescent patients, further suggesting that, during EVD, a generalized decrease in peripheral blood monocytes occurs. In addition to circulating monocytes, the concentration

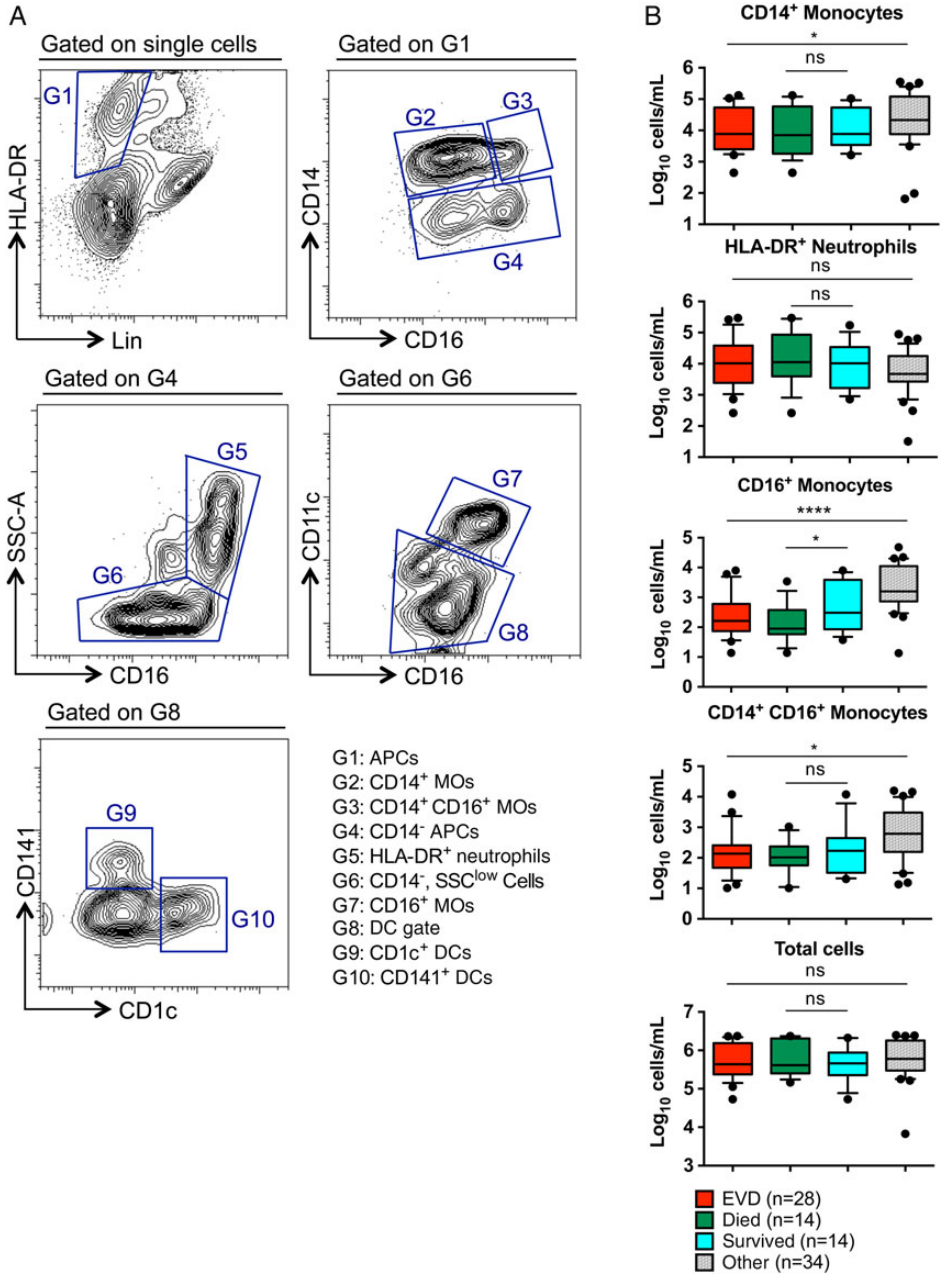

Figure 1. Analysis of antigen-presenting cells (APCs) in peripheral blood of patients with Ebola virus disease (EVD). *A*, Gating strategy for flow cytometric analysis of APCs in peripheral blood. APCs were gated as follows: G1, APCs; G2, $CD14^+$ monocytes (MOs); G3, $CD14^+CD16^+$ MOs; G4, $CD14^-$ APCs; G5, $HLA\text{-}DR^+$ neutrophils; G6, $CD14^-$, SSC^{low} cells; G7, $CD16^+$ MOs; G8, DC gate; G9, $CD1c^+$ DCs; and G10, $CD141^+$ DCs. *B*, Box plots depicting the concentrations of $CD14^+$ monocytes, $HLA\text{-}DR^+$ neutrophils, $CD16^+$ monocytes, $CD14^+CD16^+$ monocytes, and total peripheral blood mononuclear cells (total cells). Graphs indicate comparison between acute EVD cases (red) with febrile controls (gray), as well as between individuals who died (green) and those who survived (turquoise). "Other" denotes febrile EVD-negative controls. All patient samples were obtained at admission to the Ebola treatment center. $*P \leq .05$ and $****P \leq .0001$, by the Kruskal–Wallis test and the Dunn multiple comparison test. Abbreviation: ns, not significant.

of conventional $CD1c^+$ DCs was reduced in samples from patients with high-level viremia, compared with those from febrile controls (Supplementary Figure 2).

EBOV infection of monocyte-derived DCs results in inhibition of DC activation, mainly because of the ability of viral protein 24 (VP24) and VP35 to antagonize the type I interferon pathway [11, 12]. However, to the best of our knowledge, EBOV-derived effects on APC activation have never been demonstrated in vivo. Previous studies have indicated that low levels of expression of major histocompatibility complex class II (HLA-DR) molecules in monocytes and other APCs is associated with reduced activation and loss of antigen presentation capacity [13, 14]. Thus, to determine the activation status of APCs in patients with EVD, we measured the expression levels

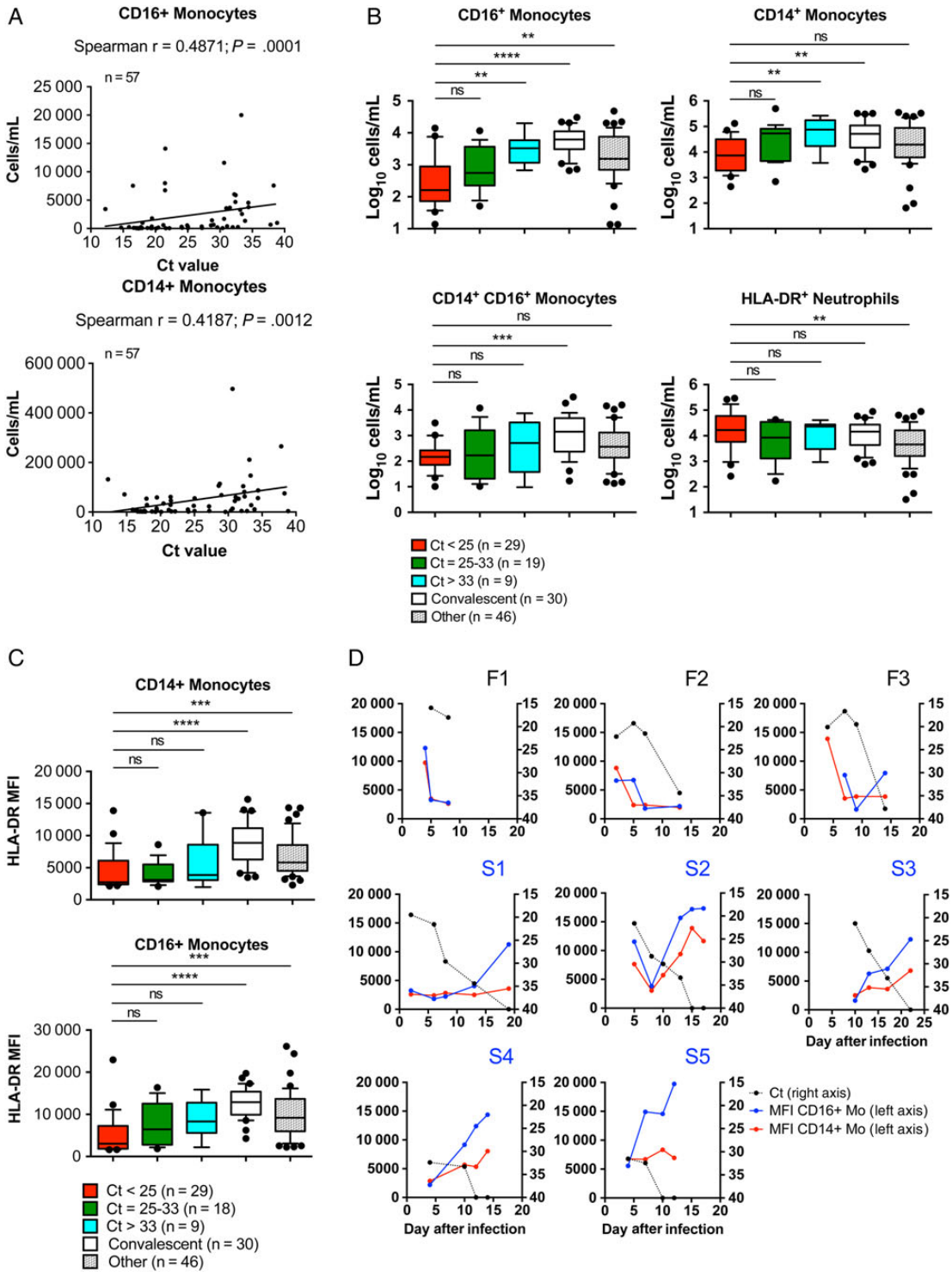

Figure 2. Relationship between antigen-presenting cell (APC) concentrations, activation status, and Ebola virus disease (EVD) severity. *A*, Graphs showing the correlation between the concentrations of $CD16^+$ and $CD14^+$ monocytes and the cycle threshold (Ct) values in 57 samples that tested positive for EVD at the European Mobile Laboratory (EMLab) unit in Coyah, Guinea. Lines represent linear regression. *B*, Box plots showing the concentrations of $CD16^+$ monocytes, $CD14^+$ monocytes, $CD14^+$ $CD16^+$ monocytes, and $HLA\text{-}DR^+$ neutrophils in patient samples. The samples were grouped according to their Ct values. Three groups were distinguished for patients with EVD: Ct < 25 (red), Ct = 25–33 (green), and Ct > 33 (turquoise). The fourth group comprised convalescent patients with Ebola virus (EBOV)–negative polymerase chain reaction results (white), and the fifth group comprised patients with other undiagnosed febrile illnesses (gray). *C*, Box plots representing HLA-DR expression on $CD14^+$ and $CD16^+$ monocytes grouped as in panel *B*. HLA-DR expression was assessed by calculation of the median fluorescence intensity (MFI) of this molecule on the cell surface. *D*, Longitudinal analysis of HLA-DR expression of $CD14^+$ and $CD16^+$ monocytes (left axis) and Ct values (right axis; reversed) for patients who died of EVD (F1–3) and those who survived EVD (S1–5). Correlation analysis was done using the nonparametric Spearman correlation test. $*P \leq .05$, $**P \leq .01$, $***P \leq .001$, and $****P \leq .0001$, by the Kruskal–Wallis test and the Dunn multiple comparison test. Abbreviation: ns, not significant.

of HLA-DR in $CD14^+$ and $CD16^+$ monocytes in diagnostic samples stratified according to the Ct values. All acute PCR-positive samples showed significant reduced expression of HLA-DR in both monocyte subsets, compared not only with samples from convalescent patients, but also with samples from febrile controls (Figure 2*C*). These data strongly suggested that inhibition of monocyte activation is a prominent feature of EVD.

To explore the kinetics of monocyte subsets during EVD recovery, we studied the activation status of monocytes over time in 8 patients with EVD for whom longitudinal samples were available. Three of these patients died (F1–F3), and 5 survived (S1–S5). While all 3 fatal cases displayed low levels of expression of HLA-DR in both monocyte compartments, all surviving patients had increased expression of HLA-DR over the course of disease, in particular in the $CD16^+$ subset, which coincided with viral clearance (Figure 2*D*). Taken together, our results suggested that a significant reduction in the levels of circulating monocytes is correlated with EVD severity and that robust activation of $CD16^+$ monocytes is associated with survival.

DISCUSSION

The main conclusion to be drawn from our data is that human EVD is characterized by a decrease in circulating monocytes and a poor activation profile of these blood cells. Both the reduced levels of blood monocytes and their low activation was correlated with high viremia levels. The reduced levels of circulating cells was especially significant in nonclassical $CD16^+$ monocytes, even compared with findings for other acutely febrile patients. In addition, the levels of blood $CD16^+$ monocytes were significantly reduced in patients who died of EVD, compared with those in survivors, suggesting a putative association between $CD16^+$ monocyte function and EVD outcome. Conversely, survivors of EVD showed robust activation of this cell subset during recovery, which coincided with viral clearance. These findings suggested that loss of $CD16^+$ monocyte function is an important component of EVD pathophysiology.

It is conceivable that monocytes may be directly infected by EBOV, which could explain why these cells are lost in samples from patients with high viremia levels. We failed to demonstrate direct infection or apoptosis of APC subsets in field conditions, and, thus, further research is necessary to address this hypothesis.

An important function of $CD16^+$ monocytes is to patrol the endothelium and to extravasate to peripheral inflammation sites, where they can differentiate into macrophages and DCs [5]. Thus, another explanation for the loss of these cells in peripheral blood of patients with high-level viremia could be massive extravasation to infection sites. In this scenario, it would be highly relevant to determine whether $CD16^+$ monocytes could be acting as so-called viral vessels favoring systemic virus dissemination. A previous study has indicated that EBOV may attach to the surface of monocytes preferentially infecting these cells during their differentiation into DCs or macrophages [15].

It is also plausible that $CD16^+$ monocytes play a more active role in the EBOV-specific antiviral response. In fact, these cells have been shown to respond poorly to bacterial cues but to be able to sense viruses through TLR7/8, leading to production of specific antiviral cytokines [5]. Our finding that surviving patients with EVD were able to upregulate expression of HLA-DR in $CD16^+$ monocytes over time suggests activation and possible involvement in viral clearance.

In summary, our study indicates that the levels and functional status of monocytes and in particular nonclassical $CD16^+$ monocytes in peripheral blood of patients with EVD is an important component of EVD pathophysiology and suggests that this cell subset may play a key role in host immunity against EBOV.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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