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BRIEF REPORT

Transient Liver Damage and Hemolysis Are Associated With an Inhibition of Ebola Virus Glycoprotein-Specific Antibody Response and Lymphopenia

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Numerous studies have demonstrated the importance of the adaptive immunity for survival following Ebola virus (EBOV) infection. To evaluate the contribution of tissue damage to EBOV-induced immune suppression, acute liver damage or hemolysis, 2 symptoms associated with lethal EBOV infection, were chemically induced in vaccinated mice. Results show that either liver damage or hemolysis was sufficient to inhibit the host humoral response against EBOV glycoprotein and to drastically reduce the level of circulating T cells. This study thus provides a possible mechanism for the limited specific antibody production and lymphopenia in individuals with lethal hemorrhagic fever infections.

Keywords. Ebola virus; humoral response; lymphopenia; tissue damage.

Understanding the difference between survivors and nonsurvivors of Ebola virus (EBOV) infection can provide critical clues to develop novel therapeutics. Compared with fatal infections, survivors typically have lower viral loads, they do not experience a loss of circulating T and NK cells, and are able to mount a robust humoral response against EBOV [1, 2]. In addition, severe liver damage is observed in individuals who succumbed to EBOV infection. Hemolysis and muscle damage are readily detectable in EBOV-infected individuals and in the lethal animal model of the disease [3, 4].

Although the positive role of the specific adaptive response to help control EBOV replication is well documented, the underlying mechanisms responsible for the low humoral response and the lymphopenia in EBOV-infected fatal cases remain poorly understood [5]. Previous studies have documented the impact of viral particles or proteins on the host cellular and humoral response. In vitro, the ability of EBOV glycoprotein (GP) and abortive infection to induce T-cell death has been described [6, 7]. Contradictory evidence exists regarding the role of EBOV soluble GP (sGP). Despite the multiple mechanisms developed by sGP to impede the host humoral response, infection using guinea pig-adapted viruses with lower or ablated sGP expression resulted in a modest increase in survival, arguing against a substantial role of sGP in EBOV pathogenicity [2, 8].

To date, the impact of liver damage, hemolysis, or myolysis resulting from EBOV infection on the host's immune response has not been investigated. It is worth pointing out that aseptic injuries, such as major surgeries and trauma, are associated with immune suppression in affected individuals. During the resulting posttraumatic immunosuppression, these individuals are highly susceptible to opportunistic infections and organ failure [9]. A transient reduction in T-cell frequency, proliferation, and cytokine production was previously reported in patients undergoing major surgery [10, 11].

We investigated the contribution of EBOV-induced liver damage, hemolysis, and myolysis to the global inhibition of the host adaptive immune response. To do so, GP-specific antibody titers as well as immune cell frequencies were investigated in mice infected with a replication-competent vesicular stomatitis virus (VSV) vector expressing EBOV GP (rVSV-GP) in the presence or absence of different drugs inducing liver damage, hemolysis, or myolysis, 3 hallmarks of lethal EBOV infection. The use of rVSV-GP, a replication-competent vaccine, instead of wild-type EBOV, allows the analyses of the immune response to EBOV-GP alone with damages induced by chemicals independently of the inhibitory mechanisms triggered by EBOV viral proteins.

METHODS

Ethic Statement

Mice experiments were approved by Université Laval Animal Care Committee. Animals were all acclimatized for at least 7 days.

Mice Immunization and Treatments

Female 5 to 6-week-old BALB/c mice were obtained from Charles River (Quebec, Canada). Mice were injected intraperitoneally (IP) with 10^6 50% tissue culture infectious dose ($TCID_{50}$) of rVSV-GP. After 24 hours, mice were mock treated (phosphate-buffered saline, PBS) or treated IP with 1.3 mg

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phenylhydrazine (PHZ), or 0.6 mg concanavalin A (ConA), or 50 µg of CD4-depleting antibody (GK1.5). Alternatively, mice received intramuscularly 50 µL of 1.2% barium chloride ($BaCl_2$) in demineralized water. Naive mice were used as control.

Tissue Damage Validation

To monitor liver damage, serum alanine transaminase level levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. For hemolysis, 5% Drabkin's reagent in Brij L23 solution was used as previously described [12].

Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from 20 mg of spleen using Qiazol (Qiagen) according to the manufacturer's instructions. For quantitative reverse transcription polymerase chain reaction (qRT-PCR), 750 ng of purified RNA was used per sample.

Enzyme-Linked Immunosorbent Assay

Plates (96 well) were coated overnight with 50 ng of EBOV VLP or VSV M protein per well. Plates were blocked with PBS 5% milk prior to incubation with 2-fold serial dilution of sera, followed by 15 ng of either anti-mouse immunoglobulin G (IgG) or immunoglobulin M (IgM) antibodies. The absorbance was read at 405 nm after incubation with ABTS substrate. Sample were run in duplicate.

Flow Cytometry

Spleens were collected 5 days after rVSV-GP infection and homogenized. The resulting cell suspension was stained with a cocktail of antibodies against CD3, CD4, CD45, CD8, CD11b, B220, and Ly6-G, and a viability dye, then run onto a FACSAria Fusion (BD Biosciences) and analyzed using Flow Jo version 10 (Treestar).

Statistics

One-way analysis of variance followed by the Dunn test was used to compare immune cells frequencies and humoral responses. All statistical analyses were performed using GraphPad Prism software version 5.0.

RESULTS

Mouse Model of Tissue Damage

We hypothesized that the tissue damage build-up, in addition to viral antigens, was responsible for the lack of robust adaptative response observed in lethal EBOV infection. To test this hypothesis, mice were injected with a replication-competent rVSV-GP to mimic EBOV infection. Nonlethal doses of PHZ or ConA were injected in mice IP 24 hours postinfection to induce hemolysis and liver damage. Intramuscular injection of $BaCl_2$, which induces temporary skeletal muscle damage (rhabdomyolysis), was also studied. Elevated creatinine and creatine phosphokinase was reported during EBOV infection, suggesting muscle damage in infected individuals [4]. Finally, administration of CD4-depleting mAb (GK1.5), which was previously shown to inhibit antigen-specific antibody production, was used as a positive control when studying the EBOV GP-specific immune response [13].

The ability of PHZ, ConA, and GK1.5 to induce hemolysis, liver damage, and CD4 T-cell depletion was confirmed using the Drabkin reagent, serology, and fluorescence-activated cell sorting (FACS) (Figure 1A–1C). Muscle damage by $BaCl_2$ was confirmed by visual inspection. To ensure that changes in immune response did not result from lower viremia, the impact of the above treatments on splenic viral load in rVSV-GP–infected mice was evaluated by qRT-PCR. No significant impact on viremia was observed 5 days postinfection (dpi) with rVSV-GP; viral load averaged around 3.5, 1.6, 4.1, and 2.1×10^4 viral copies per µg/RNA in rVSV-GP–infected mice treated with PBS, ConA, PHZ, and $BaCl_2$, respectively (Figure 1D).

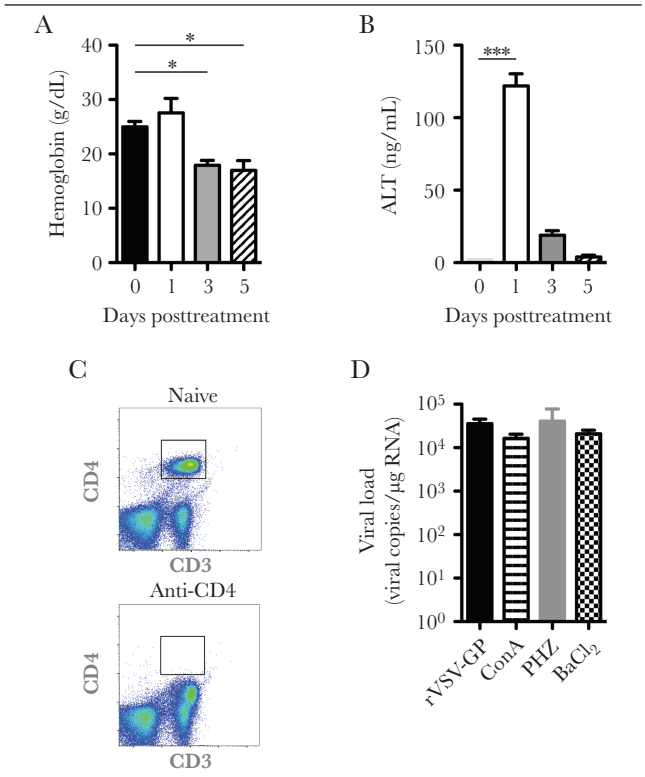

Figure 1. Validation of a mouse model of tissue damage: (*A*) hemoglobin (n = 4/group); (*B*) ALT (n = 4/group); and (*C*) CD4 frequency (n = 3/group) were monitored using Drabkin's reagent, ELISA, and fluorescence-activated cell sorting in mice treated with PHZ, ConA, and CD4-depleting antibodies (anti-CD4). *D*, Splenic viral load in recombinant vesicular stomatis encoding Ebola virus glycoprotein (rVSV-GP)–infected mice treated with phosphate-buffered saline, 0.6 mg ConA, 1.3 mg PHZ, or 50 µL of 1.2% $BaCl_2$ was measured by qRT-PCR (n = 7/group). Mean ± standard error of the mean are depicted. *** $P < .001$, * $P < .05$. Abbreviations: ALT, serum alanine transaminase; ConA, concanavalin A; ELISA, enzyme-linked immunosorbent assay; PHZ, phenylhydrazine; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

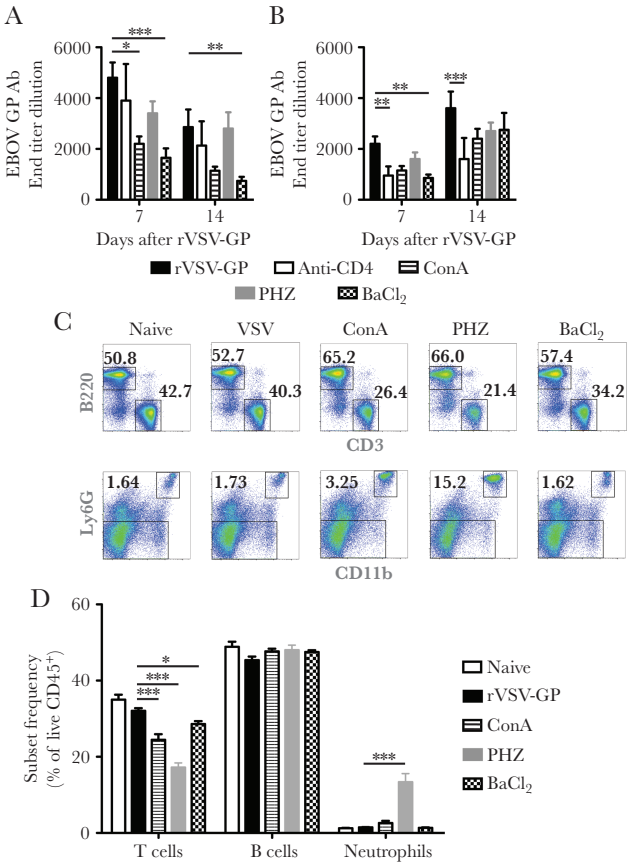

Figure 2. Tissue damage is sufficient to impact the humoral response and induce lymphopenia. Humoral response (*A* and *B*) and immune cells frequency (*C* and *D*) were analyzed in recombinant vesicular stomatis encoding Ebola virus glycoprotein (rVSV-GP)–infected mice treat with phosphate-buffered saline or 50 µg CD4-depleting antibodies (anti-CD4), 0.6 mg ConA, 1.3 mg PHZ, or 50 µL 1.2% $BaCl_2$. EBOV GP-specific IgM (*A*) and IgG (*B*) were evaluated by ELISA. T-cell ($CD3^+B220^-$), B-cell ($B220^+CD3^-$), and neutrophil ($Ly6\text{-}G^+CD11b^+$) frequencies were monitored by fluorescence-activated cell sorting at 5 dpi. Naive mice were used as controls. Representative plots (*C*) and cumulative data (*D*) are presented. Mean ± standard error of the mean (n = 7/group) and significant *P* values are indicated. *** $P < .001$, ** $P < .01$, * $P < .05$. Abbreviations: Ab, antibody; ConA, concanavalin A; dpi, days postinfection; EBOV, Ebola virus; ELISA, enzyme-linked immunosorbent assay; GP, glycoprotein; IgG, immunoglobulin G; IgM, immunoglobulin M; PHZ, phenylhydrazine.

Acute Tissue Damage Is Sufficient to Inhibit GP-Specific Antibody Response in rVSV-GP–Infected Mice

The levels of GP-specific IgM or IgG at 7 or 14 dpi were measured by ELISA in all the above groups. Seven and 14 dpi, the IgM responses against GP was lower in ConA- and $BaCl_2$-treated mice compared with mice receiving rVSV-GP alone. In ConA- and $BaCl_2$-treated mice, the mean end titer dilution significantly decreased from 4800 (standard error of the mean [SEM] 605) in mock-treated rVSV-GP mice to 2200 (SEM 293; $P < .05$) and 1650 (SEM 374; $P < .001$) at 7 dpi, and from 2857 (SEM 695) to 1143 (SEM 162; $P > .05$) and 742 (SEM 162; $P < .01$) 14 dpi, respectively (Figure 2A). IgG-specific responses against GP were significantly inhibited by CD4 depletion both 7 and 14 dpi (*P* values <.01 and .001, respectively). Although IgG response in ConA- or $BaCl_2$-treated mice appeared lower at 7 and 14 dpi, this decrease only reached statistical significance in $BaCl_2$-treated mice 7 dpi (Figure 2B). IgM response against VSV matrix protein was also decreased by $BaCl_2$ treatment, while IgG response against VSV was mainly undetectable, preventing any comparison (Supplementary Figure 1). Together, the above results suggest that acute tissue damage, including liver damage and rhabdomyolysis, is capable of limiting the IgM humoral response against EBOV.

Acute Tissue Damage After rVSV-GP Infection Is Associated With T-Cell Depletion

The impact of liver damage, hemolysis, or rhabdomyolysis on T-cell levels was investigated. The frequencies of splenic neutrophils, B cells, and T cells in mice infected with rVSV-GP followed by mock, ConA, PHZ, or $BaCl_2$ treatment were analyzed by flow cytometry. Naive mice were used as controls.

There was no significant change in B-cell frequency between the different groups 5 dpi. At the same time point, splenic neutrophil frequency rose from 1.5% to 13.4% of live hematopoietic cells ($CD45^+$) between mock and PHZ-treated mice following rVSV-GP injection ($P < .001$). No difference in splenic neutrophils frequency was noted between the remaining groups and mice that received rVSV-GP alone (Figure 2C and 2D, and Supplementary Figure 2).

In contrast, splenic T-cell frequency was significantly reduced from 32% of $CD45^+$ cells in mice injected with rVSV-GP alone to 24.5%, 17.2%, or 28.6% of $CD45^+$ cells in ConA-, PHZ-, or $BaCl_2$-treated mice, respectively ($P < .001$, $P < .001$, and $P < .04$, respectively; Figure 2C and 2D, and Supplementary Figure 2). Of note, PHZ, ConA, or $BaCl_2$ treatment equally affected both CD4 and CD8 T cells (Supplementary Figure 3). The above data indicate that transient hemolysis, liver damage, or, to a lower, extent rhabdomyolysis, is associated with a significant reduction in T-cell frequency in treated mice.

DISCUSSION

This study investigated the contribution of liver damage or hemolysis, 2 hallmarks of lethal EBOV infections, as well as rhabdomyolysis, on the host immune response after viral infection. Chemical induction of transient liver damage or muscle damage using ConA or $BaCl_2$, respectively, was sufficient to significantly reduce the early magnitude of the GP-specific IgM response. ConA-induced liver damage and PHZ-induced hemolysis significantly reduced the T-cell frequency in mice infected with rVSV-GP compared with their mock-treated counterpart. Following PHZ treatment, neutrophils infiltrated the spleen, as observed during EBOV infection. PHZ damaged red blood cells (RBCs) are removed from the circulation by the spleen. Neutrophils then infiltrate the spleen to phagocyte the damaged RBCs.

These data are from the imperfect mice model of EBOV infection and could be solidified in nonhuman primates.

However, ethical considerations regarding the use of these chemicals in primates are important. Overall, the data obtained in mice support the concept that tissue damages accumulating during a worsening EBOV infection can contribute to the poor humoral response and lymphopenia observed in lethal human infection. Hemolysis, liver damage, and myolysis concomitantly occur during EBOV infection [3]. Their effect on the immune response were evaluated independently in our study. As a result, the present study may underevaluate the impact of tissue damage on the host immune response. Finally, other mechanisms, including the inhibition of type I interferon and antigen presentation by dendritic cells, contribute to the lack of robust adaptive responses during EBOV infections [14].

In summary, the current study supports the concept that increasing liver damage and hemolysis triggered by EBOV infection cause a meaningful reduction in the production of EBOV-specific antibodies and decrease the level of circulating T lymphocytes. This newly proposed mechanism offers a rational basis for the lack of robust adaptive response in lethal infections caused not only by EBOV and other viral hemorrhagic fever viruses but also in severe malaria and sepsis, in which viral, fungal, parasitic, or bacterial infections lead to dysregulated systemic inflammation (cytokines storms) and organ damage [15]. Additional work will be needed to identify the pathways triggered by EBOV-induced tissue damage that initiate and cause impairment of the adaptive immune response.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. H. F.-B. and G. K. designed the experiments and wrote the manuscript. H. F.-B., Q. X., G. G. B., H. A, J. P., and G. W. conducted the experiments.

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Potential conflicts of interests. All authors: No reported conflicts of interest. 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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