Pathogenicity of Ebola and Marburg Viruses Is Associated With Differential Activation of the Myeloid Compartment in Humanized Triple Knockout-Bone Marrow, Liver, and Thymus Mice

Kerry J. Lavender,1 Brandi N. Williamson,2 Greg Saturday,3 Cynthia Martellaro,2 Amanda Griffin,3 Kim J. Hasenkrug,1 Heinz Feldmann,3 and Joseph Prescott1

1Laboratory of Persistent Viral Diseases, 2Laboratory of Virology, and 3Rocky Mountain Veterinary Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, Montana

Ebola virus (EBOV) and Marburg virus (MARV) outbreaks are highly lethal, and infection results in a hemorrhagic fever with complex etiology. These zoonotic viruses dysregulate the immune system to cause disease, in part by replicating within myeloid cells that would normally innately control viral infection and shape the adaptive immune response. We used triple knockout (TKO)-bone marrow, liver, thymus (BLT) humanized mice to recapitulate the early in vivo human immune response to filovirus infection. Disease severity in TKO-BLT mice was dissimilar between EBOV and MARV with greater severity observed during EBOV infection. Disease severity was related to increased Kupffer cell infection in the liver, higher levels of myeloid dysfunction, and skewing of macrophage subtypes in EBOV compared with MARV-infected mice. Overall, the TKO-BLT model provided a practical in vivo platform to study the human immune response to filovirus infection and generated a better understanding of how these viruses modulate specific components of the immune system.

Keywords. Ebola virus; humanized mice; immune response; Marburg virus; myeloid cell.
METHODS

Biosafety
All infectious virus work was conducted in the biosafety level 4 (BSL-4) laboratory at Rocky Mountain Laboratories (Hamilton, MT) following established BSL-4 standard operating procedures that were approved by the Institutional Biosafety Committee.

Ethics Statement
Animal experiments were approved by the Institutional Animal Care and Use Committee of Rocky Mountain Laboratories, and they were performed following the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) by certified staff in an AAALAC-approved facility.

Humanized Triple Knockout-Bone Marrow, Liver, and Thymus Mice
C57BL/6 Rag2−/−γc−/−CD47−/− (TKO) mice were engrafted using the BLT method, as previously described [18, 19]. In brief, 6- to 10-week-old mice received 5.0 Gy whole body irradiation before transplantation of 17- to 22-week-gestation human thymus and liver under the kidney capsule, followed by intravenous injection of autologous liver-derived CD34+ hematopoietic progenitor cells. Mice were produced using 2 separate human donors and distributed equally between groups. Animals were housed under specific pathogen-free conditions. Mice were humanely euthanized at the designated time points, or when indicated by clinical illness scores.

Virus Inoculation
The TKO-BLT mice (23 and 24 weeks post-engraftment) received sham injections with sterile Dulbecco’s modified Eagle's medium (DMEM) or inoculated with 10^6 focus-forming units (FFUs) of either EBOV-Makona (Ebola virus/H.sapiens-tc/GIN/2014/Makona; first passage after isolation) [20, 21] or MARV-Angola (Marburg virus/H.sapiens-tc/AGO/2005/Angola-368; second passage after isolation) [22] diluted in DMEM. Inoculation was intramuscular (IM) in the hind limbs.

Quantitative Reverse-Transcription Polymerase Chain Reaction
Ribonucleic acid (RNA) was extracted from homogenized tissue samples using the RNeasy Mini Kit (QIAGEN, Carlsbad, CA) following manufacturer’s instructions and according to our established protocols [23]. Viral RNA was quantitated using a one-step real-time reverse-transcription polymerase chain reaction (RT-PCR) targeting nucleoprotein (NP) genes with a Rotor-Gene probe kit (QIAGEN) (primer and probe sequences available on request). Standard dilutions of RNA extracted from a previously titrated virus stock were assayed in parallel to calculate FFU equivalents of the viral RNA.

Flow Cytometry
Spleens were weighed and macerated through a 70-μM filter before ACK lysis (Gibco, Grand Island, NY) and enumeration. Approximately 1–2 × 10^6 cells were stained for flow cytometry. Antibody stains are available upon request. Samples were run on an LSR II (BD Biosciences) and analyzed using FlowJo 9.9 (TreeStar, Ashland, OR).

Histology and Immunohistochemistry
Tissues were fixed and processed as previously described [24]. Specific immunoreactivity was detected using polyclonal rabbit serum against EBOV VP40 or MARV NP [25] followed by a BioGenex (Fremont, CA) biotinylated anti-rabbit antibody.

Statistics
Statistical calculations were performed with GraphPad Prism (GraphPad Software, La Jolla, CA).

RESULTS

Infection of Triple Knockout-Bone Marrow, Liver, and Thymus Mice With Wild-Type Ebola Virus and Marburg Virus Induced Differential Disease Severity
To assess how the immune response during the first few weeks of filovirus infection relates to disease progression, we inoculated TKO-BLT mice with either EBOV or MARV and monitored weight loss. In addition, necropsies were performed on subsets of mice at 3, 8, and 22 days post-inoculation (DPI) to determine tissue viral loads, antigen localization, and to comprehensively phenotype immune cells by flow cytometry. Unlike nonhumanized mice, HIS mice can be infected with wild-type (WT) EBOV via the IM route [24, 26]. Therefore, we IM inoculated TKO-BLT mice with 10^3 FFU of either WT EBOV-Makona or MARV-Angola.

Compared with control animals that showed no weight loss, TKO-BLTs inoculated with EBOV first exhibited significant weight loss at 13 DPI and MARV-inoculated mice slightly later, at 16 DPI (Figure 1A). By the study’s end point at 22 DPI, MARV-infected animals had a significantly lower average weight loss of 5.89% (±2.93%) compared with EBOV-infected animals that had an average weight loss of 13.84% (±5.79%) (Figure 1A). Although 2 of 8 animals (25%) in the MARV group met the criteria for euthanasia at 16 and 18 DPI, 5 of 9 animals (56%) inoculated with EBOV met the criteria for euthanasia between 16 and 22 DPI (Figure 1B), but this difference was not statistically different by χ^2 analysis at this time point. More importantly, the 2 euthanized MARV mice exhibited marked malaise and pale extremities indicating anemia in combination with weight loss. Thus, morbidity may have been related to severe anemia that occasionally develops in TKO-BLT mice rather than viral infection. These data suggested that although both viruses produced morbidity in TKO-BLT mice, EBOV disease was significantly more severe than MARV during the first 3 weeks of infection.

Ebola Virus and Marburg Virus Replicated to Similarly High Levels in Spleen and Liver of Triple Knockout-Bone Marrow, Liver, and Thymus Mice but Exhibited Differential Liver Tropism
Ebola virus and MARV viral RNA levels were then quantitated to assess whether the difference in disease severity could be
attributed to differences in viral loads. Samples were collected from mice euthanized at 3, 8, and 22 DPI. Liver (Figure 1C) and spleen (Figure 1D) samples were used for virus-specific quantitative RT-PCR analysis of viral RNA loads. There were no significant differences between the mean EBOV and MARV RNA loads in the livers at any time point (Figure 1C) or in the spleen at 3 and 22 DPI (Figure 1D). The mean EBOV RNA load in the spleen at 8 DPI was approximately 2 logs higher than MARV (Figure 1D); however, this difference between the groups was not statistically significant. Therefore, differences in tissue viral loads could not account for the differential disease severity observed between the EBOV- and MARV-infected animals.

Because the liver is a major target for filovirus replication, and liver dysfunction likely contributes significantly to disease [10], we compared viral burden, cell tropism, and pathology in the livers between MARV- and EBOV-infected mice. No discernable difference in pathology was detected between the infections (Supplementary Figure 1), but immunohistological staining of viral antigen in the liver showed differing characteristics between EBOV and MARV. Marburg virus antigen was first detected at 8 DPI (Supplementary Figure 1C) and was highest at 22 DPI (Supplementary Figure 1D). Antigen was seen in a number of cell types of murine origin. Viral antigen was observed primarily in hepatocytes, although some endothelial cells stained positive, and even fewer Kupffer cells contained antigen. Ebola virus was also first detectable at 8 DPI (Supplementary Figure 1G) and increased over time to 22 DPI (Supplementary Figure 1H). Ebola virus was detected in a similar number of cells as MARV at the corresponding time points, suggesting no differences in overall viral burden (Supplementary Figure 1). However, in contrast to MARV-infected animals, EBOV-infected animals had antigen primarily in Kupffer cells and endothelial cells at 22 DPI, with fewer hepatocytes staining positive (Supplementary Figure 1H). Thus, differences in disease severity between MARV- and EBOV-infected mice could have been related, at least in part, to differential cell tropism in the liver.

Marburg Virus-Infected Mice Had More Phenotypically Functional Dendritic Cells Than Ebola Virus-Infected Mice, but Markers of T-Cell Activation Were Similar in Both Infections

Plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) are major components of the early response to viral infection and play key roles in limiting viral spread and shaping downstream innate and adaptive immune responses [27]. Therefore, we analyzed DCs isolated from spleens of EBOV- and MARV-infected mice for phenotypic markers of functionality at three time
points over the 22 days of infection. Both EBOV and MARV induced early activation of mDC at 8 DPI as indicated by significantly increased CD80 expression, but this increase was only maintained until 22 DPI during MARV infection (Figure 2A). Marburg virus also induced significant expression of the chemokine receptor CCR7 on mDCs at 8 DPI, which was sustained to 22 DPI (Figure 2B, left). In addition, although neither EBOV nor MARV induced increased expression of the chemokine receptor CXCR3, mDCs from EBOV infection had significant downregulation of the receptor by 22 DPI (Figure 2B, right). Similarly to what was seen in mDCs, pDCs from MARV-infected mice also appeared more phenotypically functional than those from EBOV-infected mice. In contrast to pDCs from EBOV-infected mice, pDCs from MARV-infected mice demonstrated increased expression of CCR7 that started at 8 DPI and was sustained until termination at 22 DPI. In addition, CXCR3 expression was upregulated at 8 DPI on pDCs and sustained to termination, solely during MARV infection. Overall, greater expression of activation markers on mDCs and pDCs from MARV-infected mice suggested that greater functionality of these cell types may have contributed to reduced disease severity compared with the more dysfunctional phenotype observed in EBOV infection.

Myeloid DC can play a key role in the activation of T cells. Therefore, we also compared the activation status of CD4+ and CD8+ T cells harvested from EBOV- and MARV-infected TKO-BLT mice at 3, 8, and 22 DPI. There was no difference in the frequency of activated CD38+ T cells (Supplementary Figure 2A and B) nor any indication of a difference in the kinetics of T cell activation, as indicated by the timing of expression of the early

**Figure 2.** Dendritic cells from Ebola virus (EBOV)-infected mice exhibited a dysfunctional phenotype. Groups of infected triple knockout-bone marrow, liver, thymus (TKO-BLT) mice were euthanized at days 3, 8, and 22, and splenocytes were analyzed by flow cytometry for differences in expression levels of the costimulatory receptor CD80 on myeloid dendritic cells (mDC) (A) and chemokine receptors CCR7 (left) and CXCR3 (right) on mDC (B) and plasmacytoid DCs (pDC) (C) compared with control animals; n = 6 mice per group at each time point. One-way analysis of variance with Dunnett’s post-test; *, P < .05; **, P < .01; ***, P < .001. Abbreviation: MFI, mean fluorescence intensity.
activation marker CD69 (Supplementary Figure 2C and D). These results suggested that despite the presence of more phenotypically functional mDC in MARV infection, they were not associated with an increase in the frequency of CD4+ and CD8+ T cells that became activated, nor were they associated with an increase in the kinetics of T cell activation during MARV infection.

**Superior Activation of Myeloid Cells, Including CD14+ Macrophages, Occurs in Marburg Virus Compared With Ebola Virus Infection**

Because myeloid cells other than mDCs are thought to support infection and become dysfunctional during filovirus infection, we focused on the overall human myeloid compartment present in spleen to see whether we could detect additional phenotypic differences between MARV and EBOV infection. First, we looked at the entire CD33+ myeloid compartment to determine whether there had been any disease-induced influx of myeloid cells. We found that no significant infiltration of human CD33+ cells had occurred during the 22 days of infection (Figure 3A). We also looked at the frequency of activated myeloid cells in the spleen as indicated by CD69 expression and found, similar to what we had observed for mDCs (Figure 2A), that the frequency of activated CD33+ myeloid cells increased significantly in both MARV and EBOV infection at 8 DPI but only in MARV infection was the increase sustained until 22 DPI (Figure 3B). When we focused specifically on CD14+ MΦ, we again saw no significant influx of these cells into the spleens of MARV- or EBOV-infected mice over the 22 days of infection (Figure 3C). Upon assessment of the frequency of CD14+ cells expressing the CD69 activation marker, we observed a significant increase in activated MΦ at 22 DPI in MARV-infected mice but not in EBOV-infected mice (Figure 3D). Overall, these data showed that there was no significant increase in the proportion of myeloid cells into the spleens of filovirus-infected mice. However, the cells from MARV-infected TKO-BLT mice appeared more functional and activated than those from EBOV-infected mice.

**Macrophage Subsets Differentially Expand, Mature, and Become Activated in Marburg Virus and Ebola Virus Infection**

We next investigated whether we could detect phenotypic differences in the CD14+ cells present in spleens of TKO-BLT mice, which might provide clues to the difference in disease severity between MARV- and EBOV-infected mice. Initially, we examined whether there were any differences in the frequencies of CD14+CD163− M1-like MΦ (Figure 4A, left) compared with CD14+CD163+ M2-like MΦ (Figure 4A, right) over the course of infection. We found slightly more CD14+CD163+ cells in EBOV-infected spleens at 22 DPI than in MARV-infected spleens, but the difference was not significant (Figure 4A,

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**Figure 3.** Frequency and activation of total CD33+ myeloid and CD14+ monocyte-derived macrophages (MΦ) in spleen of Marburg virus (MARV)- and Ebola virus (EBOV)-infected mice. Leukocytes were purified from triple knockout-bone marrow, liver, thymus (TKO-BLT) spleens and analyzed at 3, 8, and 22 days post-inoculation by flow cytometry. Frequencies of CD33+ myeloid cells (A) and CD69+ activated myeloid cells (B) in spleen were compared among mock, MARV-, and EBOV-infected mice. In addition, frequencies of CD14+ MΦ (C) and CD69+ activated MΦ (D) in spleens were compared at each time point; n = 6 mice per group at each time point. One-way analysis of variance with Dunnett’s post-test; *, P < .05.
Closer inspection of the CD14⁺CD163⁺ subset showed a significant increase in the frequency of mature CD14⁺CD163⁺ cells in EBOV-infected mice as evidenced by a greater frequency of these cells that were CD33⁻ at 22 DPI (Figure 4B, left). It is interesting to note that there was also a significantly reduced frequency of these mature CD33⁻CD14⁺CD163⁺ cells in EBOV-infected mice expressing CD69 at 22 DPI (Figure 4B, right). In contrast to EBOV, both CD14⁺CD163⁻ and CD14⁺CD163⁺ cells from MARV-infected mice demonstrated a significant increase in the frequency of cells expressing the activation marker CD69 at 22 DPI (Figure 4C). Overall, these data suggested that EBOV infection drove a terminally differentiated and exhausted M2-like MΦ response, whereas MARV infection drove a more functional and balanced M1- and M2-like MΦ response.

DISCUSSION

In this study, we used the TKO-BLT mouse to compare the immunity and disease progression of two highly pathogenic filoviruses. The TKO-BLT mice have features that differ from other filovirus animal models, including the other HIS models recently used for filovirus research [24, 28, 29]. The TKO-BLT mice have one of the most complete and functional HISs available in a HIS mouse. Therefore, we chose this system to study the earliest interactions between WT filoviruses and bona fide human immune cells during in vivo infection [19].

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Figure 4. Differential expansion, maturation, and activation of monocyte-derived macrophages (MΦ) subsets in Ebola virus (EBOV)- and Marburg virus (MARV)-infected triple knockout-bone marrow, liver, thymus (TKO-BLT) mice. The MΦ subsets from the spleens of control, MARV-, and EBOV-infected TKO-BLT mice were analyzed by flow cytometry at 3, 8, and 22 days post-inoculation. The overall frequency of CD14⁺CD163⁻ M1-like (A, left), CD14⁺CD163⁺ M2-like (A, right), and mature CD33⁻CD14⁺CD163⁺ M2-like (B, left) MΦ in spleen were compared between the infection groups at each time point. In addition, the frequency of CD33⁻CD14⁺CD163⁺ mature M2-like (B, right), CD14⁺CD163⁻ M1-like (C, left), and CD14⁺CD163⁺ M2-like (C, right) MΦ expressing the activation marker CD69 was compared between groups over the time of infection; n = 6 mice per group at each time point. One-way analysis of variance with Dunnett's post-test (B, left and C) and unpaired t test (B, right); *P < .05, **P < .01.
Although EBOV-Makona and MARV-Angola are equivalently pathogenic in humans and NHPs, in TKO-BLT mice we observed a clear difference in disease progression during the first few weeks of infection. Ebola virus progressed more rapidly in TKO-BLT mice, with over half of the animals requiring euthanasia by 22 DPI and the surviving animals continuing to lose weight at that time point. In contrast, only 25% of MARV-inoculated animals developed disease severe enough to require euthanasia, and the remaining animals had significantly less weight loss than the EBOV-inoculated animals. Although the more rapid weight loss indicated a more rapid disease progression during in Ebola infection, we could not definitively determine a difference in survival because both groups of infected animals were continuing to lose weight at the final time point. More rapid weight loss occurred in Ebola infection despite both viruses replicating to similar levels in the spleens and livers, which are target organs for filoviruses [30]. The unusual disparity in disease severity between EBOV and MARV infection seen here, compared with what is often observed at the population level during outbreaks where both viruses are highly lethal, may be due to several factors. The data herein are based off of only two human donors. It is possible that these two donors would have responded differently to EBOV and MARV. One strong advantage of the TKO-BLT system is the ability to assess the immune responses to several viruses in a single donor. The HIS mouse models provide a means to correlate cellular responses with differential disease outcomes.

Myeloid cells, including DCs, are early targets of filovirus infection and replication and likely play key roles in disease outcome [31–35]. Furthermore, functional adaptive immune responses correlate with disease outcome [36] and are at least partially shaped by innate cells, including DCs. In vitro studies have demonstrated that both EBOV and MARV severely impair normal function of DCs, potentially contributing to disease [6, 37]. The TKO-BLT mice contain human myeloid cells [19], and we observed several differential myeloid cell responses between EBOV and MARV that correlated with differing pathogenicity. Although both viruses replicated similarly, MARV infection appeared to initiate a more functional innate immune response, with sustained activation of both pDCs and mDCs, in contrast to transient and dysfunctional activation in EBOV infection. Although differences in the activation and maturation status of DCs was associated with disease severity, it is currently unclear what mechanism may be involved in mediating this difference. Given that there were no differences in viral loads or levels of T-cell activation between MARV- and EBOV-infected mice, it remains to be determined what role DCs had in mediating disease outcome.

Monocytes and MΦ are also targets for filovirus infection, which may affect their function [4, 5, 34]. These cells are key innate controllers of infection. Resident MΦ and incoming monocytes in infected tissues can activate and differentiate into effectors that act directly to kill virus and clear dead and dying cells. In addition, MΦ secrete mediators that shape the immune response and therefore affect disease severity. Marburg virus infection resulted in sustained myeloid cell activation. However, CD33+ cells were only transiently activated by EBOV, and MΦ were not significantly activated at any time point. We saw that the frequency of mature M2-like MΦ was significantly higher in EBOV-infected mice, although there was significantly less activation of these mature M2-like cells compared with MARV and control animals. This suggested that EBOV induces an accumulation of terminally differentiated M2-like MΦ in spleen that are exhausted, suppressed, or otherwise dysfunctional. In contrast, both M1-like and M2-like MΦ were activated by MARV infection and indicated a more balanced activation of MΦ subsets in response to MARV infection. Myeloid dysfunction in EBOV infection may be due to lack of recognition or direct antagonism by the virus as has been demonstrated in vitro [38]. Overall, mice infected with MARV consistently contained innate cells with a more phenotypically functional profile that may have contributed to reduced disease severity by mediating a more functional and less pathogenic immune response to infection.

The pathogenicity of filoviruses is multifactorial, and liver damage is a contributing factor. Marburg virus has been shown to be more hepatotropic in NHPs and replicates in the liver to a greater extent than EBOV [41]. We measured similar MARV and EBOV RNA levels in the liver, but the staining pattern was markedly different between the two viruses. Both viruses infected endothelial cells and hepatocytes, but MARV was largely localized to hepatocytes, whereas EBOV was found primarily in Kupffer cells. In HIS mice, Kupffer cells are of murine origin [42] and are a form of specialized resident liver macrophage that secretes soluble mediators in response to liver injury. Such mediators can shape the myeloid immune response and contribute to disease outcome [43]. Because EBOV infection can disturb macrophage responses, the greater burden of EBOV infection in Kupffer cells suggests their responses may also have been dysregulated, thereby affecting disease outcome.

Humanized mouse models of viral infection require careful interpretation because antigen recognition and effector functions of the human compartment will not always be able to model a fully immune competent animal. Regardless, HIS mice have been successfully used to study human immune responses to many pathogens [39], and the TKO-BLT model in particular has been regularly used for human immunodeficiency virus (HIV)-1 studies [40–42]. In contrast to HIV-1 where only human CD4+ cells serve as targets of viral infection, in the case of filoviruses human leukocyte subsets and several murine cell types all support filovirus replication in these animals. During the early stages of infection, filoviruses initially infect myeloid cells [32]. Therefore, assessment of human myeloid cell responses and their ability to prime the solely human lymphoid system, as shown here, may represent what occurs during early stages of infection in humans. As infection progresses and murine parenchymal cells become infected, BLT mice may be less...
likely to model all downstream features of the immune response and the resulting pathogenic processes, particularly due to the inability of the human T cells, which are educated on human thymic tissue, to specifically recognize antigen presented in the context of murine MHC on infected mouse cells. Although there may ultimately be a disconnect between the human immune response and the murine background, many of the early events we investigated here involving the innate immune compartment may model early features of bona fide human infection. In addition, the ability to infect HIS mice with WT filoviruses via a peripheral route, whereas non-HIS mice require rodent-adapted viruses injected intraperitoneally, indicates a requirement for cellular components of human origin in the periphery for transmission and supports their involvement in the earliest stages of the disease process.

CONCLUSIONS

Elucidation of the features of the immune system that contribute to filovirus disease is difficult. Although the TKO-BLT model has important caveats to take in consideration, it represents a valuable model to assess the complex in vivo interactions of human immune cells upon filovirus infection. Our data support previous findings that suggest filovirus infection results in ineffective innate cell responses. In addition, this study provides new information regarding specific differences between MARV and EBOV infection and demonstrated associations among disease severity, liver cell tropism, and activation of myeloid cell subsets.

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 notes of myeloid cell subsets.

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