Ebola and Marburg virus infection in bats induces a systemic response

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Abstract. The filoviruses Ebola (EBOV) and Marburg (MARV) cause fatal disease in humans and nonhuman primates but are associated with subclinical infections in bats, with Egyptian rousette bat (ERB, Rousettus aegyptiacus) being a natural MARV reservoir. To understand the nature of this resistance, we have analyzed how EBOV and MARV affect the transcriptomes of multiple ERB tissues. We have found that while the primary locus of infection was the liver, gene expression was affected in multiple tissues, suggesting a systemic response. We have identified transcriptional changes that are indicative of inhibition of the complement system, induction of vasodilation, changes in coagulation, modulation of iron regulation, activation of a T cell response, and converting macrophages from the M1 to M2 state. We propose that these events are facets of a systemic anti-inflammatory state that enables effective control of the infection in bats and suggest that dissecting this state can inform how to control a filovirus infection in humans.

Introduction

Ebola (EBOV) and Marburg (MARV) filoviruses cause a severe, frequently fatal disease in humans[1]. For example, the 2004-2005 outbreak of MARV killed 227 out of 252 (90%) infected individuals[2], while an ongoing EBOV outbreak has killed 2,264 out of 3,444 (66% case fatality)[3]. EBOV and MARV kill by causing a multisystem disease state involving hypotension, multisystem organ failure, sepsis-like symptoms, and disseminated intravascular coagulation (DIC) due to profound immune dysregulation, including cytokine storm[4]. Despite the aggressive use of a recently approved Ebola vaccine, control of the ongoing outbreak has been difficult, indicating the need to look for new prevention and therapeutic approaches by understanding better the pathobiology of these viruses. An intriguing clue is a remarkable fact that EBOV and MARV are well tolerated by their natural reservoir hosts – bats.

MARV has been isolated from the Egyptian rousette bat (ERB, Rousettus aegyptiacus)[5–7], and ecological and experimental studies have demonstrated that ERB are a reservoir for the virus[6,8]. Experimental infections of ERBs with MARV have consistently demonstrated that despite viral replication in multiple tissues, animals develop a mostly subclinical disease, which is characterized by mild pathology involving transient elevation of alanine aminotransferase, elevated lymphocyte and monocyte counts, and some evidence of minimal inflammatory infiltration in the liver[9,10]. Clinical signs of disease are absent[9–13]. Transmission has been demonstrated between co-housed ERBs, and virus is known to be shed in saliva, urine, and feces[8]. However, ERBs do not appear to develop a chronic infection when exposed to MARV, and instead clear the virus and develop at least temporary immunity, including MARV-specific IgG[14].

Whether ERB are a reservoir for EBOV is unclear. While detecting of EBOV RNA and anti-EBOV antibodies in ERB [15,16], [17–19] suggest that these bats could be a reservoir for this virus, the failure of isolating infectious EBOV from a wild bat[20] and finding that these animals are refractory to the virus[21] argue against this possibility.

The ability of bats to tolerate viral infections has been a topic of considerable interest, and several models have been proposed to explain this phenomenon. Most of these are centered on the innate immune system, which includes the inflammatory response (induced by cytokines), phagocytosis, natural killer cells, and the complement system. One model posits that bats constitutively express interferons to maintain a basal level of innate immune activity, ready for pathogens to appear[22], although the universality of this model in bats has been questioned[23,24]. An alternative model claims that the resistance is due to a weakened innate immune response, which is attenuated by changes in some proteins such as the stimulator of interferon genes (STING or TMEM173)[25]. Along these lines, a genomic analysis [24] suggested that tolerance of viral infection, rather than enhanced antiviral defenses, explained the bat's ability to asymptomatically host viruses that cause human diseases. The similarity of innate immune responses to MARV and EBOV in bat and human cell lines[26] seems to contradict these theories and suggests that the control of viral infections in bats is more complex. Moreover, while these models can explain how viruses can survive in the animal, they cannot explain how the infection is eliminated.

A potential source of the difficulty to understand how bats tolerate or eliminate the viruses that are deadly to humans is the lack of studies that analyze the response to infection in bats rather than in cultured bat cells. The results obtained using cell lines have been contradictory. Some studies claim both EBOV and MARV replicate to similar levels in ERB and human derived cell lines[26], with a robust innate immune response mounted by ERB and to a lesser degree, human cells, while others claim MARV inhibited the antiviral program in ERB cells, like in primate cells, and did not induce almost any IFN gene [27], or little anti-viral gene induction[28]. An experiment with the pig (PK15A) and bat (EhKiT) cells suggested they responded to EBOV through the upregulation of immune, inflammatory, and coagulation pathway, in contrast to a limited response in the human (HEK293T) cells[29]. To comprehensively understand the pathways involved in the bat filoviral response, we infected bats, rather than their isolated cells, and analyzed tissue-specific RNA expression through mRNA-seq in the organs of the infected animals.

To probe the complexity of the response, we have attempted to test two hypotheses: that the response of bats to filoviruses is systemic, involving multiple interrelated processes, and that the differences in the responses to infection between bats and humans are due to evolutionarily divergent genes. To test these hypotheses, we have analyzed how EBOV and MARV affect global gene expression patterns in various tissues, with a particular focus on evolutionarily divergent genes. Our analysis of these transcriptomes begins to reveal a systemic organismal response that facilitates the ability of bats to survive filovirus infections and suggest potential therapeutic strategies for controlling human infection. This is the first in vivo study that focuses on the coordinated transcriptional response to filoviruses at the level of individual organs in bats.

RESULTS

Inoculation of bats with MARV and EBOV results in detectable viral replication only in some organs

Eleven ERBs were inoculated subcutaneously with 10^4 PFU of MARV or EBOV. Following inoculation, animals were observed at least daily, and bled every other day. Viremia was monitored via ddPCR, and animals were euthanized shortly after becoming viremic. Bats inoculated with MARV or EBOV showed no apparent clinical signs of disease or changes in behavior, with no significant effect on body weight and temperature (Fig. 1-A, B). MARV and EBOV were detected by ddRT-PCR in the blood of infected bats, with MARV detected earlier and at a higher copy number than EBOV (Fig. 1-C). MARV was detected by plaque assay in livers and spleens of all inoculated animals, and in the salivary glands (2 animals) and kidneys (1 animal) of some animals (Fig 1-D). By contrast, EBOV was present above the limit of detection in the livers of two inoculated animals, which is contrast to prior reports that ERB are refractory to EBOV infection [13,30]. We did not detect EBOV by plaque assay in other organs (Fig 1-E).

Two of the three EBOV-inoculated animals presented with histopathological lesions in the liver, consisting of pigmented and unpigmented infiltrates of aggregated mononuclear cells compressing adjacent tissue structures, and eosinophilic nuclear and cytoplasmic inclusions, changes consistent with previous reports[13,30]. In EBOV-infected animals, focal immunostaining with both pan-filovirus

and EBOV-VP40 antibodies was observed in the liver of one animal, but very few foci were found, suggesting limited viral replication.

MARV-inoculated animals showed histopathology like that observed in prior experimental infection studies[10]. Immunohistochemistry with a pan-filovirus antibody suggested that MARV was present in mammary glands and testes, despite the lack of histopathological lesions in these organs. (Fig 1-F).

MARV and EBOV infection affects the transcriptome of multiple organs

To examine the response to filovirus infection, we sequenced mRNA from liver, spleen, kidney, lungs, salivary glands, large and small intestine, and testes collected from filovirus inoculated and uninfected bats (Methods, Table S1). Consistent with prior reports that liver is the primary target of MARV[31], and our findings (Fig. 1, Table S2), MARV transcripts were most abundant in this tissue (79 transcripts-per-million, tpm), but were also present in spleen (56 tpm), intestine (10 tpm) and lungs(2 tpm) (Table S2). EBOV transcripts were detected at very low levels (< 1 tpm) in the livers of inoculated bats and were not detectable in other tissues.

Although viral transcripts were detected primarily in the liver, gene expression patterns were altered in all analyzed tissues and involved thousands of genes, suggesting a systemic response (Fig. 2). The changes were highest in the livers of MARV-infected animals relative to other organs, consistent with the possibility that these changes were induced by the virus, and differed between MARV and EBOV (Fig. 3, S1), indicating that the observed changes in gene expression patterns are related to the infectious agent.

Evolutionarily divergent bat genes as tools for understanding the response to filovirus infection

To identify genes that may be relevant to the difference in resistance to filoviruses between humans and bats, we reasoned that homologous genes with greater evolutionary divergence between bats and humans are also likely to diverge in function or regulation. This hypothesis made the divergent genes our primary suspects. We also reasoned that focusing first on divergent genes would also simplify the computational analysis of the transcriptomes, further increasing the chance of identifying relevant pathways.

To identify divergent genes, we relied on BLASTn[32]. Genes detected as homologues (16004, 87% out of 18443 bat genes in our database) using BLASTn default settings were labelled “similar”. The remaining 2439 genes (13%) were considered “divergent”. Of these genes, 1,548 transcripts (8% of the total), could be identified as homologous by reducing the word-size in BLASTn from 11, the default, to 9. This approach is equivalent to matching at the protein level, but we find that using nucleotide level matches provides a cleaner separation of the two classes than using translated proteins (Fig. 4, Methods). From this divergent set, using expression levels, we narrowed down to a subset of 264 genes that expressed at more than 20 tpm in at least one set of liver samples (MARV, EBOV, or uninfected). Of these 264 genes, 151 were differentially expressed in the livers of either MARV or EBOV infected bats relative to uninfected animals (Fig. 4, Methods). These 151 genes were then used in the first step of pathway analysis.

The most abundant group in this set comprised genes related to mitochondria (20 genes), followed by genes involved in the vascular system (19), innate immunity (16), tissue regeneration and apoptosis (15), macrophages (13), inflammation (10), metabolism and fatty-acid oxidation (8), T cells (4), complement system (2), digestion (5), and toxin processing (3).

Finding these sets led us focus on the entire transcriptomes (both homologous and divergent genes) of the corresponding systems : i) innate immune system which includes the inflammatory response, phagocytosis by macrophages, natural killer cells, and the complement system; ii) inflammatory response, including acute phase proteins, macrophages activities involving metabolism, fatty-acid oxidation, mitochondrial abundance and function, and tissue regeneration and apoptosis; and iii) the vascular system, involving the regulation of blood pressure, coagulation, and iron homeostasis.

MARV and EBOV infection induce inflammation, indicated primarily by an acute phase response

Acute phase proteins (APP) are produced by hepatocytes in the liver in response to inflammatory cytokines, such as Interleukin-1(IL-1), IL-6, and TNFα, and are an important part of the innate immune response[33–35]. Serum concentration of positive APPs[36], including SAA1 and SAA2 can increase more than 10-fold as a part of the response, while the concentration of negative APPs, including transferrin and albumin, decreases[37].

We found that MARV, and to a lesser extent EBOV, infection induced APP response in liver, spleen and kidney, with the largest changes in APP expression (>10-fold) observed in the liver (Table I, Fig. 5). However, SAA1 and SAA2 expression also increased to a similar degree in most tissues, not only in the tissues in which the viruses were detected. At the same time, we detected no expression of C-reactive protein (CRP), an APPs used as a marker for measuring inflammation acute-phase-response in humans (Table 1, Fig. 5). Bats may lack a CRP response as we could not detect the CRP transcript in public mRNA-seq data from lab-infected samples from various species of bats (data not shown). Consistent with the induction of SAA1 and SAA2, we also detected induction of other markers of inflammation including, ORM2, CP, HAMP and the microsomal glutathione S-transferases, MGST1 and MGST2[38] (Table 1, Fig. 5).

MARV and EBOV infection is associated with an early transition from M1- to M2-dominated populations of macrophages

Macrophages recognize and phagocytize foreign organisms and damaged host cells, as a part of the innate immune response, and are an important early target for filoviruses[31]. Macrophages can either be in the M1 state, an inflammatory state enabling apoptosis, or in the M2 state, anti-inflammatory state assisting tissue regeneration. A key difference between the M1 and M2 states lies in their metabolism, with the M1 state characterized by hypoxia and glycolysis metabolism[39] and the M2 state is characterized by fatty acid metabolism and abundant mitochondria[40].

We have found that key markers of the M1 state were upregulated in livers of infected bats (more so in MARV infected animals). These included IRF5, NF-κB, AP1G1 (a subunit of the AP-1 complex), STAT1, and SOCS3 (Fig. 5, S4, S5). Likewise, HIF1A[41–43], which promotes mitophagy and glycolysis metabolism to induce M1 polarization was also upregulated in infected livers, again

more so in MARV infection. PKM, which activates HIF1A, and the pyruvate dehydrogenase, PDK1, involved in the response to hypoxia were also upregulated, to a greater degree in MARV than EBOV (Fig. 5, S4, S5)[44].

The M2 state markers, MRC1, arginase-1(ARG1), IL-10 and TGF-β[45], were highly expressed in livers of bats infected with both viruses (Fig. 5,S4,S5), suggesting the presence of M2 macrophages. Several genes related to fatty acid oxidation in M2 macrophages were upregulated by filovirus infection (Tables S3-8). CPT2, a gene associated with fatty acid transport was upregulated under filoviral infection (greater in MARV infection). Infected bats also exhibited upregulation of multiple markers of mitochondria abundance another characteristic of M2 macrophages. These included TFAM, OPA1, MFN1/2, and DNM1L. Two genes involved in mitochondrial biogenesis[46], HGF-MET and PPARGC1A, are also upregulated upon MARV infection.

Prolonged M1 activity can be harmful to tissues as these cells can induce inflammation and apoptosis. Thus a negative feedback system that shifts macrophages from the M1 state to the M2 state[47,48], controlling inflammation during infection and facilitating the transition to tissue repair and regeneration[49,50]. In our data, the transcriptomes of the MARV-infected liver samples suggest a more equal representation of M1 and M2 macrophages, while in the EBOV-infected liver samples, gene expression suggests an M2-dominated macrophage population, suggesting a conversion from M1 to M2 state is underway over the course of the infection, as the virus is cleared.

The M1 to M2 transition is associated with a change in cellular energy metabolism. GPD2, the mitochondrial glycerol-3-phosphate dehydrogenase, identified as a contributor to the shift in core macrophage metabolism associated with the M1 to M2 transition during infection[51], was found to be upregulated by filovirus infection (Fig. 5, S6A). Inactivating HIF1A also promotes M2 polarization. HIF1AN, the inhibitor of HIF1A, is upregulated in filovirus infected bats. Increased availability of iron also promotes the M1 to M2 polarization shift [52]. Gene expression patterns in EBOV-infected bats, notably ferritin and HBB expression, suggest that iron levels may be elevated in these animals. This supports our other findings of an M2 polarization bias.

Expression of key components of the classical complement pathway is inhibited by filovirus infection

The complement pathway, a part of the immune system, has three branches: the classical pathway, the mannose-binding lectin pathway and the alternative pathway[53]. The classical pathway recognizes antigens bound to antibodies; the lectin pathway binds to sugar molecules on the virus particles, and the alternative pathway is a component of the innate defense against infections.

Several key gene associated with the complement pathway were upregulated by filovirus infection, including C3P1, C4B, C5, C9, C6, and MASP1, while others (C1R, C3, C8G, and MASP2) were downregulated or not expressed (Fig. 5, S6B). This indicates that the complement pathway is impacted by filovirus infection in the liver and suggests that aspects of the immune response dependent upon complement such as some forms of antibody-mediated viral neutralization, are compromised.

Infected bats exhibit transcriptional signatures of T cell activity

Previous studies on the adaptive immune response to Ebola and Marburg viruses in humans, non-human primates, and non-primate mammals, shows that long-term immunity is conferred by both T cell and antibody responses. Mostly CD8+ T cells were elicited and helpful against Ebola in mice[54,55], while SUDV infection in humans[56]) and MARV infection in cynomolgus monkeys[57] and humans[58]) elicited mostly CD4+ T cells . In most human EBOV infections, CD8+ T cells against the EBOV NP protein dominated the responses, while a minority of individuals harbored memory CD8+ T cells against the EBOV-GP [59].

Consistent with this, in MARV-infected bats, CD4 expression (specific to CD4+ T cells) was higher, while in EBOV-infected bats, CD8 expression (specific to CD8+ T cells) was higher, the overall levels are low, because the tissue samples are heterogenous and expression of these markers is not high in the T cells to begin with. T cell markers (such as CCL3, ANAX1, TIMD4 and MAGT1) are also upregulated in liver, suggesting a T cell response is mounted.

EBOV and MARV infection affects the vascular system

The vascular system carries nutrients, oxygen and the cells and molecules involved in the immune response and inflammation. The proper functioning of the system requires control of iron metabolism, blood pressure, and blood coagulation. We found that MARV and EBOV affected the expression of genes involved in all these processes.

Genes involved in iron homeostasis. The absorption and availability of iron, an essential component of heme needed for oxygen transport, is tightly regulated[60]. Most iron is in hemoglobin (66%), the remainder is stored mostly in macrophages in the liver, which take up iron through the CD163 receptor. Iron is exported from macrophages and absorbed from food[61] through Ferroportin (SLC40A1/FPN1).

MARV and EBOV changed the expression of multiple genes involved in iron homeostasis. Hepcidin (HAMP)[62] , which controls iron homeostasis by binding ferroportin, leading to its degradation as well as blocking the export of iron, was induced in infected livers (Fig. 5,S8 Table 1). Infection induced ceruloplasmin (an APP, Table 1), which enables the formation of the transferrin-iron complex and is also involved in processing copper[63]. In the cytosol, iron is bound to ferritin (comprised of a heavy chain, FTH1 and a light chain FTL), synthesized by cells in response to increased iron[64]. In mitochondria, iron is bound to FTMT, the mitochondrial ferritin[65]. Both FTH1 and FTMT were downregulated in MARV-infected bats but upregulated in EBOV-infected animals (Fig. 5, S8). MARV infection was associated with lowered hemoglobin expression, suggesting impairment of red blood cell production, potentially resulting in anemia. Consistent with this conclusion, CD164, which suppresses hematopoietic cell proliferation, was also upregulated by MARV infection (Fig. 5, S8), while HBB was suppressed EBOV-infected samples.

These observations suggest that hematopoiesis was impaired in MARV-infected bats, but not in EBOV-infected bats, and that regulation of iron by HAMP in bats might diverge from the homologous process in humans.

Genes regulating vasodilation. The primary means of blood pressure regulation is renal expression of renin, which converts angiotensinogen (AGT) to angiotensin I. Angiotensin converting enzyme

(ACE) converts angiotensin I to angiotensin II, which constricts blood vessels to increase blood pressure. AGT is down regulated by MARV and EBOV infection, which would be expected to deplete the substrate for ACE, limiting the potential for blood pressure to increase even with upregulation of ACE (Fig. 5, S8). Low blood pressure would be consistent with our finding that filovirus infection induced expression of Prostaglandin I2 synthase (PTGIS), a potent vasodilator and inhibitor of platelet aggregation. However, blood pressure was not directly measured in the bats before euthanasia.

Genes involved in blood coagulation. Mechanisms that control blood pressure also impact coagulation. MARV and EBOV induced PTGIS, which reduces blood pressure and also inhibits platelet aggregation (Fig. 5, S9) and repressed AGT, the precursor of angiotensin II which enhances production of active plasmin to increase coagulation[66]. MARV and EBOV also induced CYP11B1, which increases cortisol that acts to reduce inflammation, and CYP11B2, which increases aldosterone levels that increases blood volume[67]. Together, these would be expected to reduce the effects of inflammation on the vascular system.

DISCUSSION

Recently, multiple filoviruses associated with bats have emerged or re-emerged as threatening human pathogens, such as EBOV, MERS-CoV and SARS-CoV-2. As a result, the role of bats as reservoirs for a diverse array of viruses and their ability to tolerate viral infections that cause severe disease in humans have become a topic of considerable interest. A number of hypotheses have been proposed to explain this unique aspect of bat biology, most of which are centered on the innate immune system. In these hypotheses, various aspects of bat innate immunity are either more or less potent than their human counterparts. One hypothesis in posits that some bat species[22–24] constitutively express interferons, leading to a basal level of innate immune activation. However, prior work with filoviruses demonstrating that the innate response in bat cells is robust, and similar to that observed in human cell lines[26] is inconsistent with this hypothesis. Another hypothesis suggests that components of the innate immune response (e.g., STING/TMEM173) are less effective in bats[25], allowing viruses to survive in the host. Although this mechanism helps to explain the ability of bats to serve as reservoirs for a diverse range of viruses, it is less useful in explaining the ability of bats to survive and clear infection and may indicate at the involvement of the adaptive immune system in virus clearance.

All MARV-inoculated bats were productively infected, and our virology and histopathology data in MARV-infected bats are consistent with previous reports, including viral replication in the mammary glands and testes[13] Evidence of successful, if limited, infection was identified in two of three EBOV inoculated animals. In particular, virus was detected by plaque assay in the livers of two of three animals, and immunohistochemistry identified a small number of foci in the liver of one animal. This contrasts with prior reports[30] and suggests that ERBs may not be truly refractory to EBOV infection. However, given the very low titers detected, and the limited nature of the observed immunostaining, it is unlikely that the virus could be maintained in animals in nature

There are hundreds of genes involved in the interferon response, some key components can mutate to change specificity of their interactions, but most, especially those in the core ISG category[68], evolve slowly and have conserved function and sequence[69]. Our analysis of gene divergence shows that the majority of interferon response genes are not divergent from their human homologs, consistent with prior observations that the innate responses are quite similar between human and bat cell lines[26]. This implies that other systems are involved in generating the difference in response between bats and humans.

We developed a framework to understand the observations with the interconnections between various systems as pertains to our study shown in Fig. 6. A key feature of filovirus infection is an inflammatory response leading to the expression of APPs and stimulation of M1 macrophages. C-reactive protein (CRP), which binds to micro-organisms, assists in complement binding to foreign and damaged cells, and enhances phagocytosis by macrophages (opsonin-mediated phagocytosis)[70] appears to be absent in bats, based on the lack of CRP sequences in our mRNA-seq data. In mice, CRP is not an acute phase protein[71], and as such, it is unclear if this apparent lack of CRP is consequential in regards to the innate immunity of bats. Aside from CRP, however, the other APPs are conserved. We found evidence that the effector component of the antibody response may be weakened by incomplete complement activation. This is consistent with the previous reports that antibody-mediated virus neutralization is not the dominant mechanism of filovirus clearance in R. aegyptiacus bats[72]. The robust CD8+ T cell activity implied by our mRNA-seq data suggests that control and clearance of filovirus infection in bats may instead depend upon a robust T cell response. This is consistent with what is known in humans, where individuals who recover from filovirus infections tend to mount robust T cell responses[73–75], and have higher levels of CD40L expression, a marker for T cell activity[75].

The macrophage response was one of the more notable points of divergence between the human response to filovirus infection and what we observed in infected bats. We identified markers of both M1 and M2 macrophages in ERBs infected with MARV, suggesting that macrophage populations in the animals were in the process of switching from the classically pro-inflammatory M1 polarization to the M2 state, which is conventionally associated with anti-inflammatory processes, tissue repair, and regeneration. In particular, the modulation of the innate response facilitated by M2 macrophages is important for T cell mediated clearing of the virus. In EBOV-infected animals, where viral replication was far more limited, our sequencing data indicate that the macrophage population was further along in the transition to M2 polarization by the time of euthanasia. The generalized anti-inflammatory state observed in bats during filovirus infection, especially the early switch to M2 macrophage polarization, may be key to preventing the immunopathology associated with filovirus infection in humans, including cytokine storm and DIC. Supporting this, an mRNA-seq study conducted with PBMCs isolated from EBOV-infected humans found that individuals who succumbed to disease showed stronger upregulation of interferon signaling and acute phase response-related genes than survivors during the acute phase of infection[76], suggesting that a tempered response may be more beneficial.

Comparing our observations to human responses to filoviruses is limited by the scarcity of studies in humans. Nevertheless, this comparison suggests potential directions to explore. In one study, individuals who succumbed to the disease showed stronger upregulation of interferon signaling and acute phase responses compared to survivors during the acute phase of infection[76], consistent with the anti-inflammatory response gene expression signature identified in this study in bats. However, most of the genes used in the study by Liu et al. to classify survivors are either barely expressed in bats or do not respond to filoviral infection (Table 2), the differences that provide potential clues to find why bats can tolerate the infection.

A study of patients infected with Sudan Ebola virus (SUDV) analyzed protein levels for a panel of genes using a Luminex multiplex assay (using antibodies)[77]. The panel was based on results from other studies and pathways involved in the response to infections. The patients were classified into 3 possible dichotomies (fatal/non-fatal, hemorrhaging/non-hemorrhaging, or high/low viremia) correlated with genes that characterized these states. Most of these genes either are barely expressed, if at all, or are unaffected by infection in bats, except for ferritin (FTL, FTH1) whose expression is lowered by MARV infection, consistent with the observation that ferritin is higher is fatal human cases (Table 3).

Our data suggest that the vascular response in bats differs from that in humans. Humans infected with EBOV or MARV frequently present with hemorrhagic manifestations and dysregulated coagulation in the form of disseminated intravascular coagulation[78]. We identified transcriptional patterns consistent with vasodilation and reduced potential for coagulation. This could result in a state in which blood pressure is lower than normal, and coagulation is reduced. This state m be protective, as it might be expected to prevent DIC. Our findings are consistent with results from a study in humans infected with EBOV[77] which analyzed 55 biomarkers in blood. This report found that viremia was associated with elevated levels of tissue factor and tissue plasminogen activator, consistent with coagulopathy.

Our results also suggest that reducing the hyperinflammatory response[79] or controlling the coagulopathies[80] in humans during filovirus infection may have a therapeutic benefit by preventing damage to the host and allowing other processes to clear the infection. This could be achieved by the inhibition of IL-6 by agents such as siltuximab (Sylvant)[81], or by targeting the IL-6 receptor via an antibody such as tocilizumab (Actemra)[82]

In bats, filovirus infection upregulates MGST1 and MGST2 which both induce leukotrienes (LTC4) and prostaglandin E, both of which are mediators of inflammation[38]. This is a potential druggable target, as these are targeted by several therapeutic agents. Thus, this inflammation could also be targeted by another class of anti-inflammatory agents such as LTC4 inhibitors, used to treat asthma.

Our observations let us to surmise that upon filovirus infection bats may naturally vasodilate and reduce their blood pressure (mimicking the action of ACE inhibitors) while the endothelial system becomes anti-thrombotic. In fact, field trials of ACE inhibitors and statins in human Ebola virus disease have already seen some success[83]. Along these lines, another potentially useful drug is prostaglandin I_2 (PGI_2, known as epoprostenol as a drug), a powerful vasodilator and anti-coagulant

that acts by binding to the prostacyclin receptor. This has potential for use in human filovirus infections as a means of emulating the physiological conditions (low blood pressure and coagulation) in bats that our data suggest may have protective effects[84].

In humans, high levels of HAMP causes iron to be sequestered in the liver, reducing levels of iron in blood (lower ferritin). Our observations indicate that in EBOV infected bats high HAMP expression is decoupled from the levels of iron, as both ferritin and HAMP are induced. Thus, HAMP inhibitors, which are used to treat anemia, might recreate in humans the state seen in bats under filoviral infection. Two HAMP inhibitors, Heparin[85] and erythropoietin (EPO)[86,87], have additional beneficial effects, anti-coagulation and RBC synthesis respectively, which might make them particularly efficacious. Vitamin D is also a HAMP inhibitor[88].

Overall, the changes in gene expression patterns that we have observed in infected bats suggest that the filovirus pathogens induce a systemic response that involves pathways regulating coagulation, vasodilation, iron homeostasis, inflammation, the interferon response and the adaptive response. The most important outcome of this systemic response appears to be a tempering of the overall response to infection, avoiding immunopathology. In particular, the anti-inflammatory state observed, and the altered state of the vascular system appear to be important to preventing pathology and facilitating the ultimate clearance of the virus.

CONCLUSIONS

Our observations and analyses provide an experimental and computational framework for understanding the resistance of bats to filovirus infection. This framework and the data that we report and make public have the potential to aid in the development of new strategies to effectively mitigate and treat the effects of filovirus infections in humans.

Data

All data underlying the balloon plots is available as csv files on the filobat website (http://katahdin.girihlet.com/shiny/bat/). Additionally, a fasta file containing all the mRNA sequences used in our analysis is also available on the website. The raw sequencing reads will be deposited with GEO, and the filobat site has several tools for analysis and exploration of data.

MATERIALS AND METHODS

Experimental methods

Viruses. Recombinant wild-type EBOV, strain Mayinga, was recovered from the full-length clone and support plasmids in HEK 293T cells and passaged twice in Vero E6 cells for amplification[29]. Recombinant wild-type MARV, strain Uganda, was recovered similarly in BHK-21 cells[89] and passaged twice in Vero E6 cells for amplification.

Bat experimental protocol. All animal procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch at Galveston.

Adult ERBs were obtained from a commercial source and quarantined for 30 days under ABSL-2 conditions. Animals were implanted with microchip transponders for animal ID and temperature

data collection. For studies with EBOV and MARV, animals were transferred to the Galveston National Laboratory ABSL-4 facility. Animals were segregated into groups of three. Except for one MARV-infected male, all bats were female. Each group was housed in a separate cage for inoculation with the same virus. After acclimation to the facility, animals were anesthetized with isoflurane and infected subcutaneously in the scapular region with 10^5 focus forming units (FFU; titrated on Vero E6 cells) of EBOV or MARV. Every other day, animals were anesthetized by isoflurane, weighed, temperature was determined via transponder, and 100-150 µL of blood was collected from the propatagial vein. Blood was inactivated in 1 mL of TRIzol reagent (Thermo-Fisher Scientific). Samples were then removed from ABSL-4 containment, and RNA was extracted. Droplet-digital RT-PCR (ddRT-PCR) with primers specific to the nucleoprotein (NP) gene was used to detect viremia. If fewer than 10^6 MARV RNA copies/mL viremia were detected in a MARV-inoculated bat, the animal was observed for additional 2 days to allow the animal to reach a higher viral RNA load. All EBOV-inoculated bats were euthanized 48 hours after the first detection of viremia, regardless of viral RNA load. Animals were euthanized under deep isoflurane sedation via cardiac exsanguination confirmed by bilateral open chest. Tissues were collected (listed in Table S1) and immediately homogenized in an appropriate volume of TRIzol reagent and stored at -80°C. 1 cubic centimeter (cc) tissue sections were homogenized in minimal essential media (MEM) supplemented with 10% fetal bovine serum and stored at -80°C. Additional tissue sections were fixed in 10% neutral buffered formalin for histopathology. Tissues and PBMCs were also collected from three uninfected control animals.

Leukocyte isolation. Leukocytes were isolated using ACK lysis buffer (Gibco). Ten volumes of lysis buffer were added to whole blood, incubated for 2-3 minutes, and then neutralized with complete DMEM media containing 10% FBS. Following neutralization, samples were centrifuged at 250 g for 5 minutes at 4°C, after which the supernatant was decanted from the pellet. This process was repeated several times per sample until a white pellet of cells free of contaminating red blood cells remained. Because density gradient purification was not performed on these samples prior to or after red blood cell lysis, these leukocyte preparations were assumed to contain granulocytes in addition to PBMCs.

mRNA sequencing. Total RNA was isolated from bat tissues using Ambion's RNA isolation and purification kit. For most samples, polyA-tailed mRNA was selected using beads with oligo-deoxythymidine and then fragmented. A few samples with poor RIN (RNA Integrity Number) scores were treated with Ribominus (targeting human ribosomal RNA) to enrich for polyA-tailed mRNA before fragmentation. cDNA was synthesized using random hexamers and ligated with bar-coded adaptors compatible with Illumina's NextSeq 500 sequencer. A total of 88 samples were sequenced on the NextSeq 500, as 75 base pair single reads.

Analytical methods

Bat mRNA sequence database. The extant bat genomes are nowhere near completion and a comprehensive mRNA database does not exists. Thus, for this study, we constructed a custom non-redundant reference bat mRNA sequence database, which is available at https://katahdin.girihlet.com/shiny/bat/. We started with existing genome annotations[90]. The

complications arising from splice variants were avoided by keeping only the longest transcript for each gene. We added missing annotations/sequences (e.g., CYP11B2 and PLG) to our database by assembling reads from our own sequence data. These required custom scripts as there often was not enough reads covering a transcript, which precluded the use of standard assembly tools. The gene sequences were collected from different bat species, so error-free reads might not map perfectly to the transcripts in the database. The database has sequences of 18,443 bat mRNAs, and include EBOV and MARV sequences, the infectious agents used in our studies. The genes were identified by homology to mouse and human genes, 16,004 bat genes had high similarity to human or mouse homologues, as defined by BLASTn with default settings identifying matches spanning the length of the mRNA.

The set of remaining genes (2439) were labelled as divergent. Of these, 1,548 transcripts could be identified by increasing the sensitivity of BLASTn by reducing the word-size from 11 to 9, which is equivalent to matching at the protein level. Of the remaining 891 putative transcripts, homologues for 182 could be identified on the basis of partial homology and domain structure, while the remainder (709 sequences whose names start with UNDEF) belonged to one of four classes, 1) aligned to un-annotated transcripts in the human genome, 2) non-coding RNAs, 3) transcripts unique to bats, or 4) assembly errors. We use capitalizations to represent bat gene symbols, as in the human gene nomenclature.

To identify genes within these divergent set that are relevant to our study, we then selected a subset of genes that had good expression (defined as tpm > 20) in at least one class of liver samples (MARV-, EBOV- or mock-infected) and responsive in either MARV- or EBOV- infected bat livers, which we defined as up- (log2 ratio > 0.6), or down- (log2 ratio < -0.6) regulated. We were left with 151 genes that are the foundation of our analyses of pathways involved in the response to filoviruses (Tables S3-S8).

Expression Analyses. To determine transcript expression levels, we used Kallisto, because this tool uses pseudo-alignments and is relatively more tolerant of errors/variants in reads[91], which we expect here because the reads and mRNA sequences in the database do not always come from the same species. Kallisto uses a parameter "k" while indexing the database to specify how sensitive it is to matches with smaller k values leading to more spurious hits. We empirically determined k=23 to be an appropriate parameter value with which to index the reference mRNA dataset. We used the transcripts-per-million (tpm) value as the transcript expression levels to determine changes in expression across samples.

We used viral transcripts to identify infected samples, which has previously helped us to identify and correct mistakes of annotation in some of the cell line data and also identified a problem with a published dataset[28], where all the naïve (uninfected) samples showed signs of viral infection. Furthermore, to ensure there was no mislabeling of tissue samples from different bats, we used single nucleotide variants in the sequenced reads to confirm that all tissue samples from an individual had the same set of variants.

Using clustering based on expression profiles and considering individual interferon responsive genes, it was clear that one non-infected control bat liver sample (labeled cb1 in the shiny tool) was

reacting to some stimulus (injury or infection) compared to the other two control samples (cb2 and cb3 in the shiny tool); Since we are interested in the innate response to infections, we had to exclude cb1 from the controls, but cb1 data are available for exploration in the filobat tool.

As such, most of our analyses concentrated on liver RNA transcripts since it had the strongest response and the genes indicated that a variety of cell types were involved in the response, capturing the systemic nature of the response. Liver function impacts a wide range of systems involving inflammation, iron homeostasis, and blood pressure. Other organs, such as kidney and spleen provide additional support for what is observed in the liver. For some genes, we also used the transcriptional response in kidney (Renin) and/or spleen (STING) in order to understand the regulation of pathways (e.g., Renin is expressed in kidney and regulates the blood pressure system).

Tools for data exploration and interrogation. To allow exploration of the data across various samples on a gene-by-gene basis, as well as analysis of viral expression in the samples, we developed a browser-based tool, filobat, using Shiny in R (http://katahdin.girihlet.com/shiny/bat/). Samples can also be compared using scatter plots and hierarchical clustering.

Statistics. Large changes in expression profiles were readily detected by comparing averages across replicates, since such changes are less affected by noise; however, subtle changes (less than 2-fold) were difficult to reliably detect due to lack of power in the sample size and variability between samples and are mostly not considered.

Pathway analyses. A fundamental assumption underlying our study is that bats are mammals that possess innate and adaptive responses to infections that roughly mirror those seen in humans. The data from comparative filovirus infections in human and bat cell lines supports this assumption[26]. To identify pathways of interest from particular genes, we used GO/pathways annotations of the human counterparts[92] and grouped them into functions that provided themes in the dataset. Using these themes, we identified other differentially expressed genes sharing these themes, identified by the GO annotations for human and mouse genes. This allowed us to build a picture of the pathways triggered by filovirus infections and delineate the ways in which the systemic bat responses differs from those seen in humans.

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Figures

Underlying data and tools for exploring the data available at http://katahdin.girihlet.com/shiny/bat/

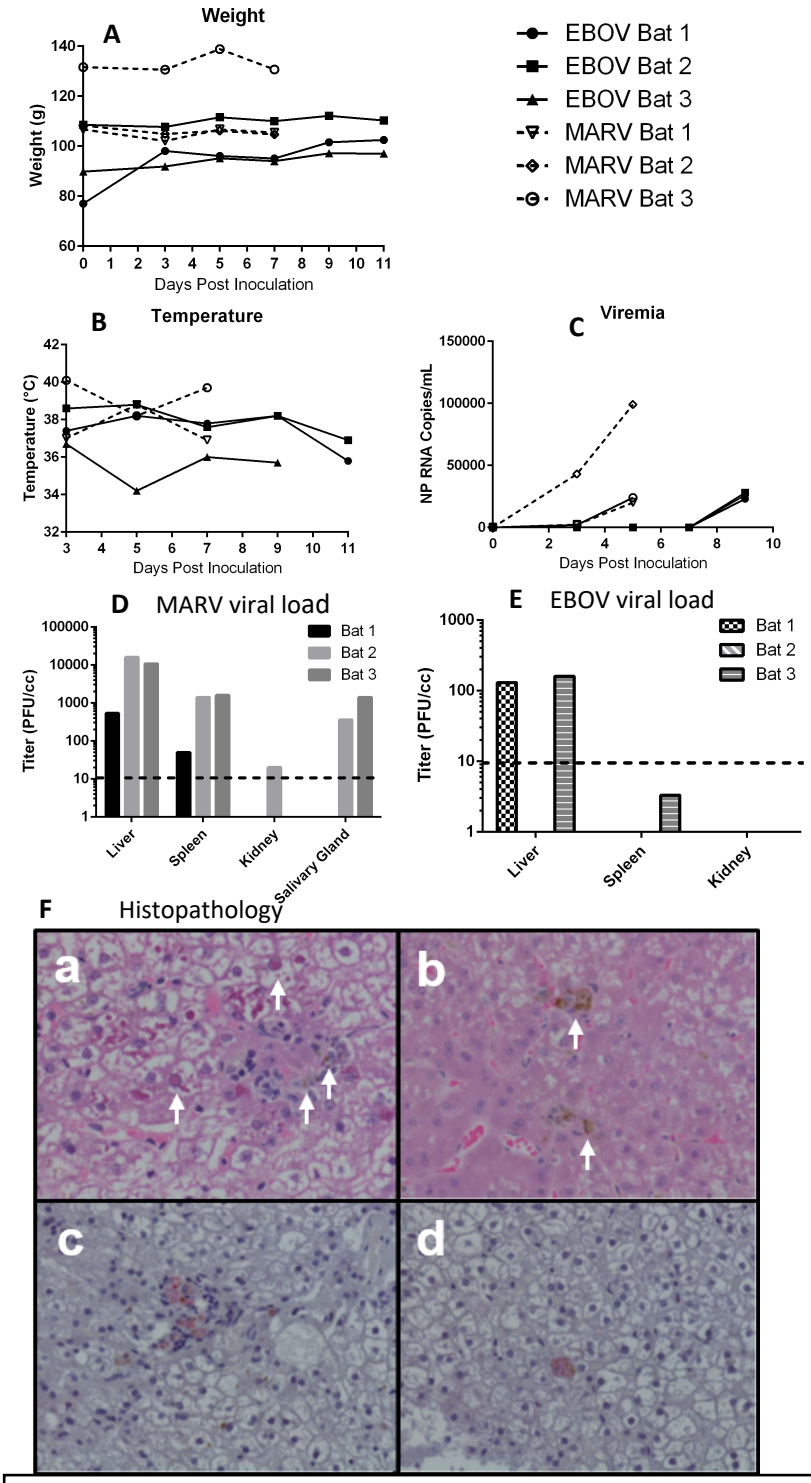

Figure 1: Bat infection with filovirus, MARV and EBOV. Time course after infection for A) Weight**, B**) temperature and **C)** viremia (MARV Bat 2 sensor failed). Viremia measured in total RNA extracted from whole blood via ddRT-PCR targeting the viral NP gene. Animals were euthanized 48 hours after last viremic timepoint. **Tissue viral loads (D and E)** were determined by conventional plaque assay on Vero E6 cells. **F) Histopathology in EBOV infected livers showing F.a)** EBOV Bat 1 liver with marked histopathological changes, including cytoplasmic and nuclear inclusions (arrows)**, F. b.)** EBOV Bat 2 liver displaying a less dramatic presentation compared to Bat 1, **F.c)** IHC detection of filovirus antigen in EBOV Bat 1 liver, and **F.d.)** IHC detection of EBOV VP40 in EBOV Bat 1 liver. Controls for the IHC images are shown in **Fig. S1.**

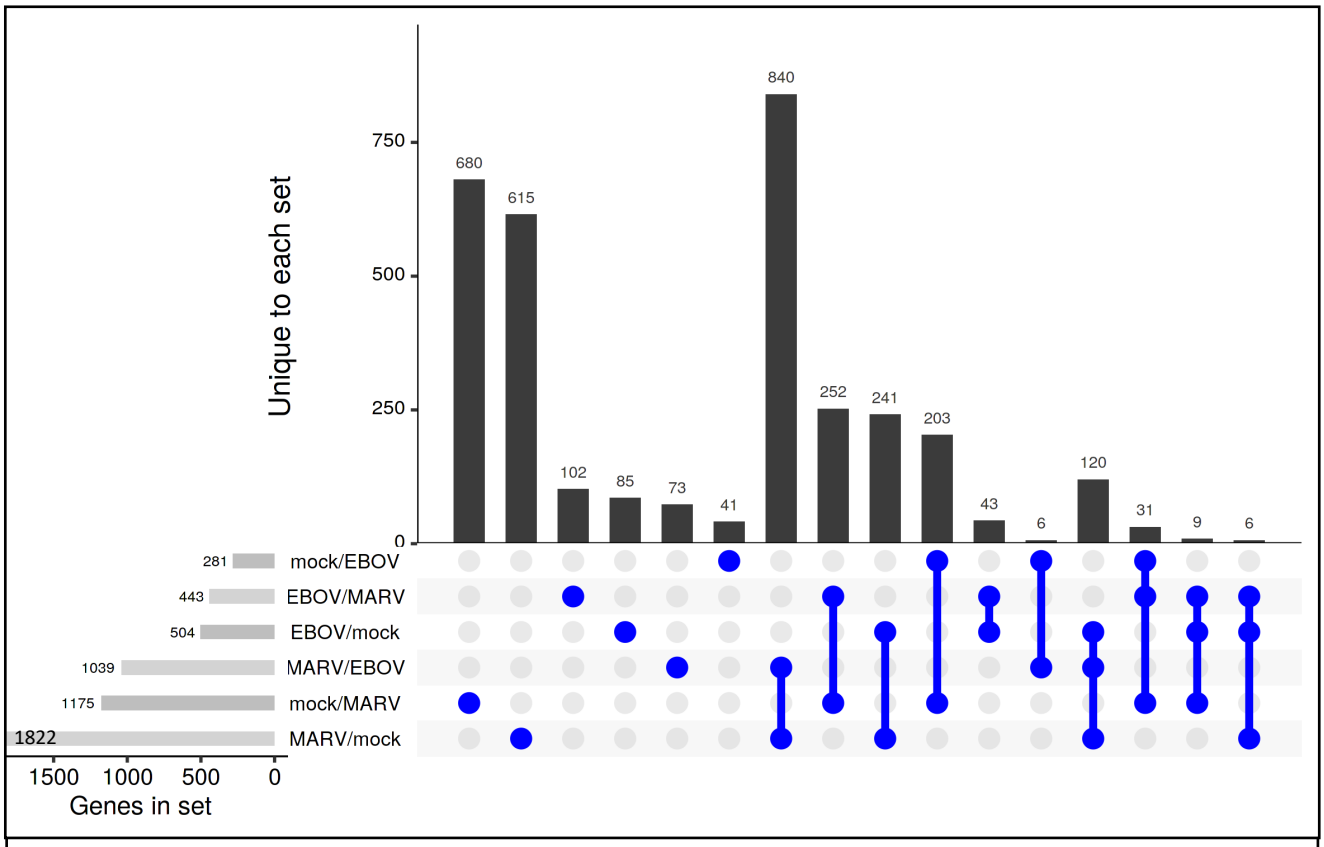

Figure 2: Broad response of bat liver genes to filoviral infection. Many genes in the liver respond to filoviral infections, with MARV having a bigger impact compared to EBOV (840 genes that are responsive to MARV alone, compared to the 43 specific to EBOV alone). The EBOV-specific (*EBOV/MARV)* and MARV-specific (*MARV/EBOV)* genes are likely host responses specific to the viral VP40, VP35 and VP24 genes. In the plot, *mock* refers to mock-infected bats, *EBOV* to EBOV-infected bats, and *MARV* to MARV-infected bat livers. Each row in the lower panel represents a set, there are six sets of genes based on various comparisons, e.g., *EBOV/mock* is the set of genes at least 2-fold up regulated in EBOV infection, compared to the mock samples. The gray bars at the lower left representing membership in the sets. The vertical blue lines with bulbs represent set intersections, e.g., the last bar is the set of genes common to *EBOV/MARV*, *EBOV/mock* and *MARV/mock,* so the genes in this set are up 2-fold in EBOV compared to the *mock* and *MARV* samples, and at least 2-fold up in *MARV* compared to *mock*. The main bar plot (top) is number of genes unique to that intersection, so the total belonging to a set, say mock/EBOV, is a sum of the numbers in all sets that have mock/EBOV as a member (41+203+6+31=281).

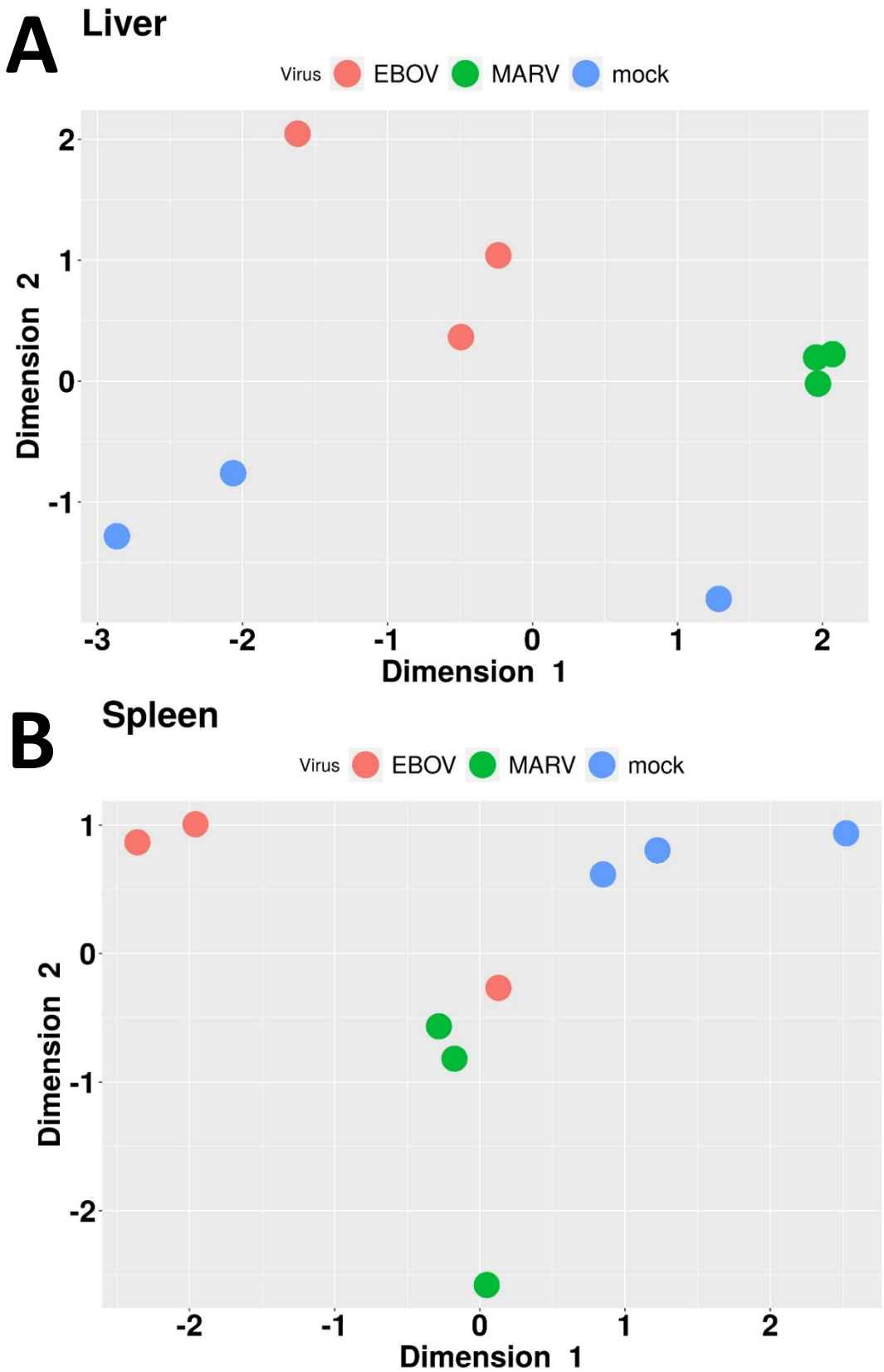

Figure 3: MDS plots along the two leading dimensions (the x and y axis respectively). There is clear separation between different infections (MARV and EBOV) and the mock-infected **A**) Liver and **B**) Spleen samples. Despite the paucity of viral transcripts, Spleen and other tissues (PBMC, kidney, salivary gland, lung, large and small intestine, **Fig. S2**) also exhibit virus-specific signatures, implying the response to filovirus infections extends to the whole bat. The top panel shows a mock-infected liver sample (the blue dot in lower right) that seems to be different from the other two mock-infected samples. This bat seems to be reacting to some stimulus, either an infection or injury, and has been excluded from our analysis.

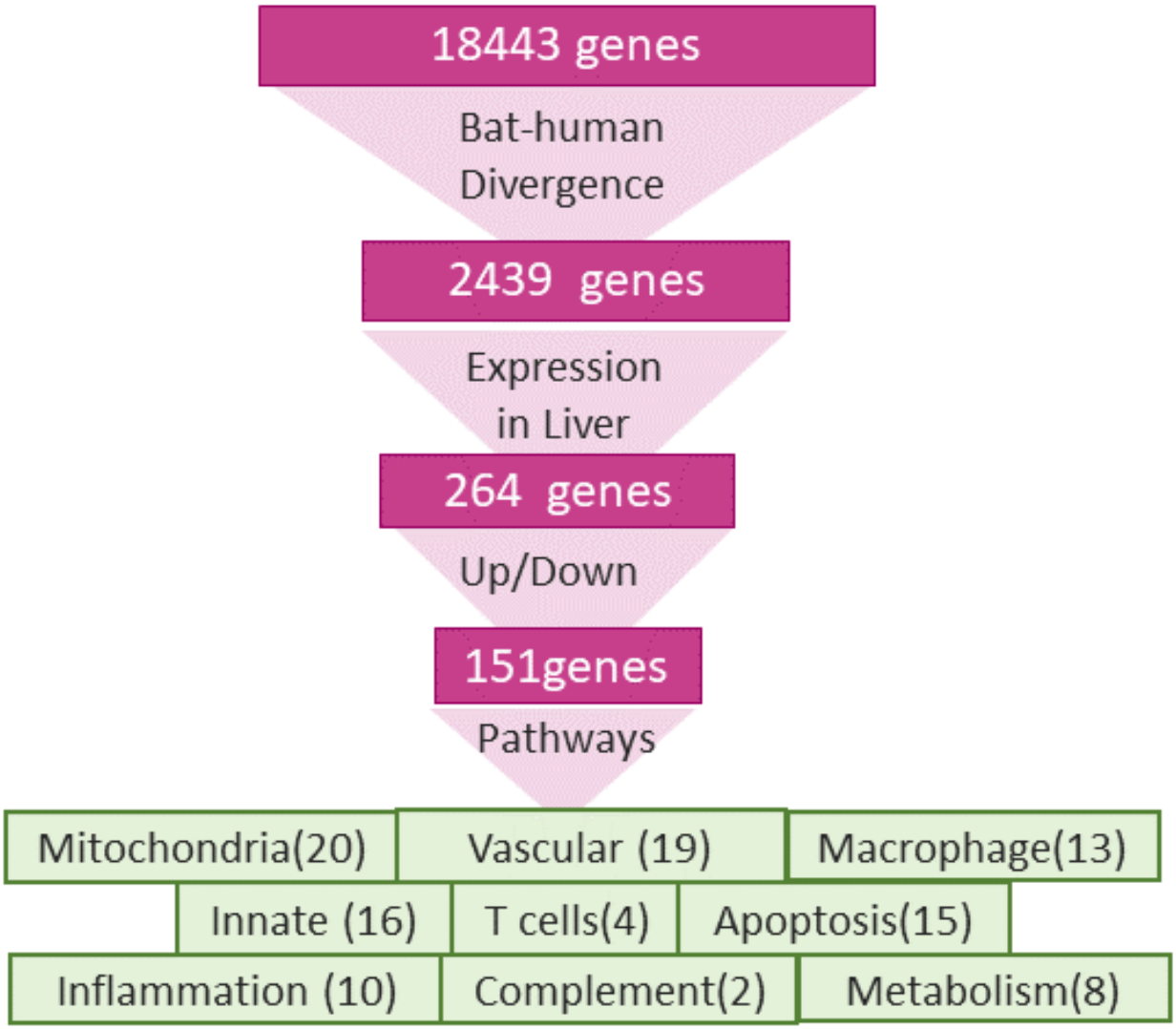

Figure 4. Pathways from mRNA-seq. The process used in the paper to identify pathways relevant to the bat's resilience in the face of filoviral infection. It is assumed that most homologous genes perform similar functions in bats and humans. Bat genes evolutionarily divergent from their human homologs have a greater probability of having altered functions, Of these, those responsive in liver to filovirus infection were identified. The pathways they influence were explored to evaluate the systemic response to filovirus infections in bats and identify key differences from human responses. The vascular system (Blood pressure, Coagulation and Iron homeostasis) was a prominent pathway. Glycolysis, which is controlled by Hypoxia, shifts the balance between M1 and M2 states of macrophage activation. These changes create an anti-inflammatory state that modulate the response and allows the adaptive immune system to clear the infection. The complement system is not fully activated, likely compromising the antibody response, but T cells are active and play a major role in clearing the infection The pathways are interconnected, as shown in **Fig. 6**.

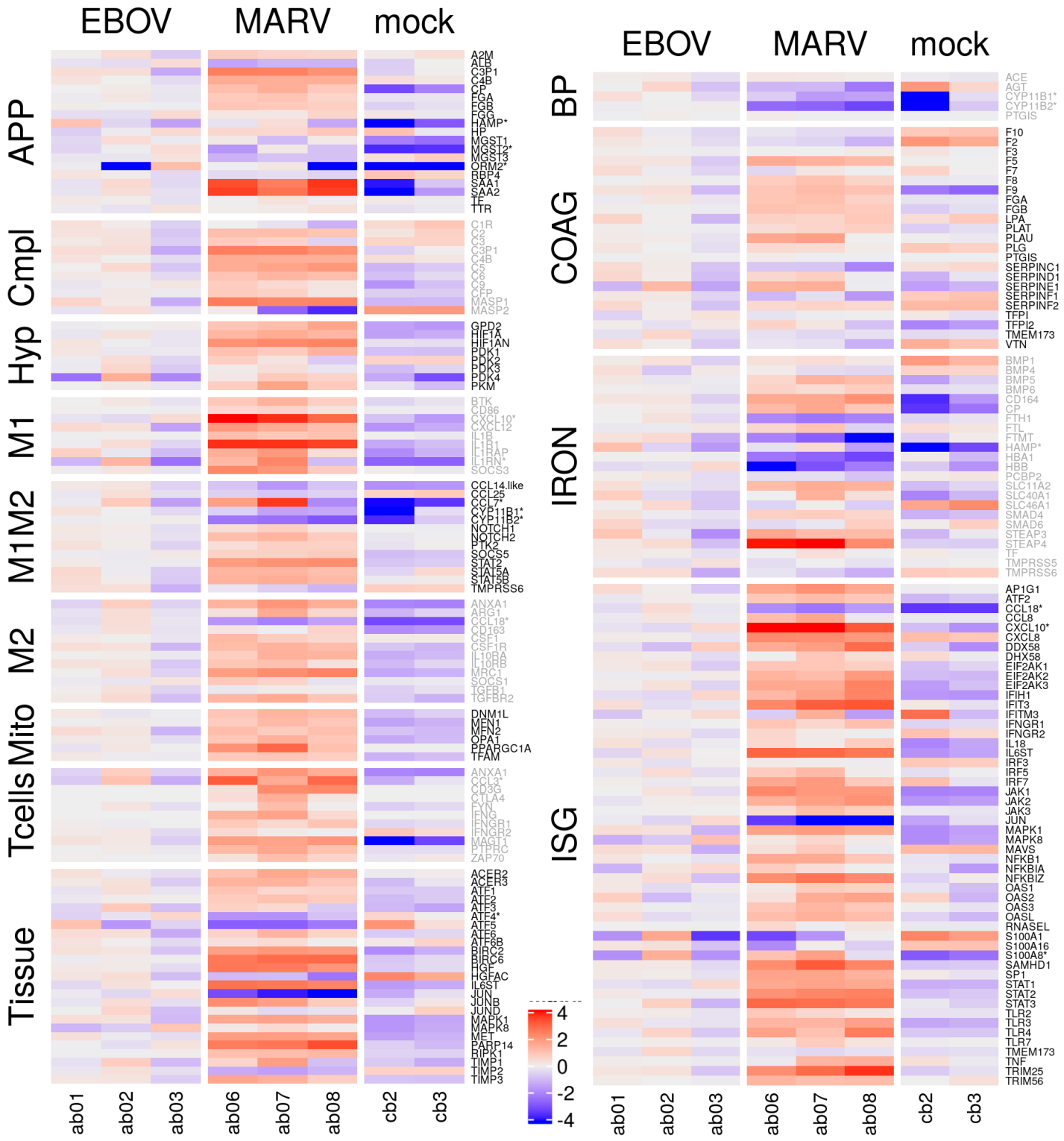

Figure 5. Regulation of pathways by MARV and EBOV infections in liver. On the left are genes in Acute Phase Response (APP), Complement (Cmpl), Hypoxia (Hyp), Tissue regeneration/apoptosis (Tissue), and genes specific to macrophages in the M1 state (M1), M2 state (M2) or both (M1M2). On the right are genes for Blood Pressure(BP), Coagulation (COAG), iron homeostasis (IRON) and Interferon stimulated genes (ISG). The columns show the three liver samples from EBOV-infected bats, and MARV-infected bats, as well as two un-infected samples. The values are log2 of the fpm values, with the mean value of the EBOV samples subtracted out. Broadly, majority of the genes are in a quiescent, low expression state in the mock infected samples, and get stimulated upon infection, with large effects in the case of MARV and intermediate effects in the case of EBOV. A * after a gene name signifies the bat version is diverged from its human counterpart. **Fig. S11A** and **S11B** show corresponding figures for kidney and spleen.

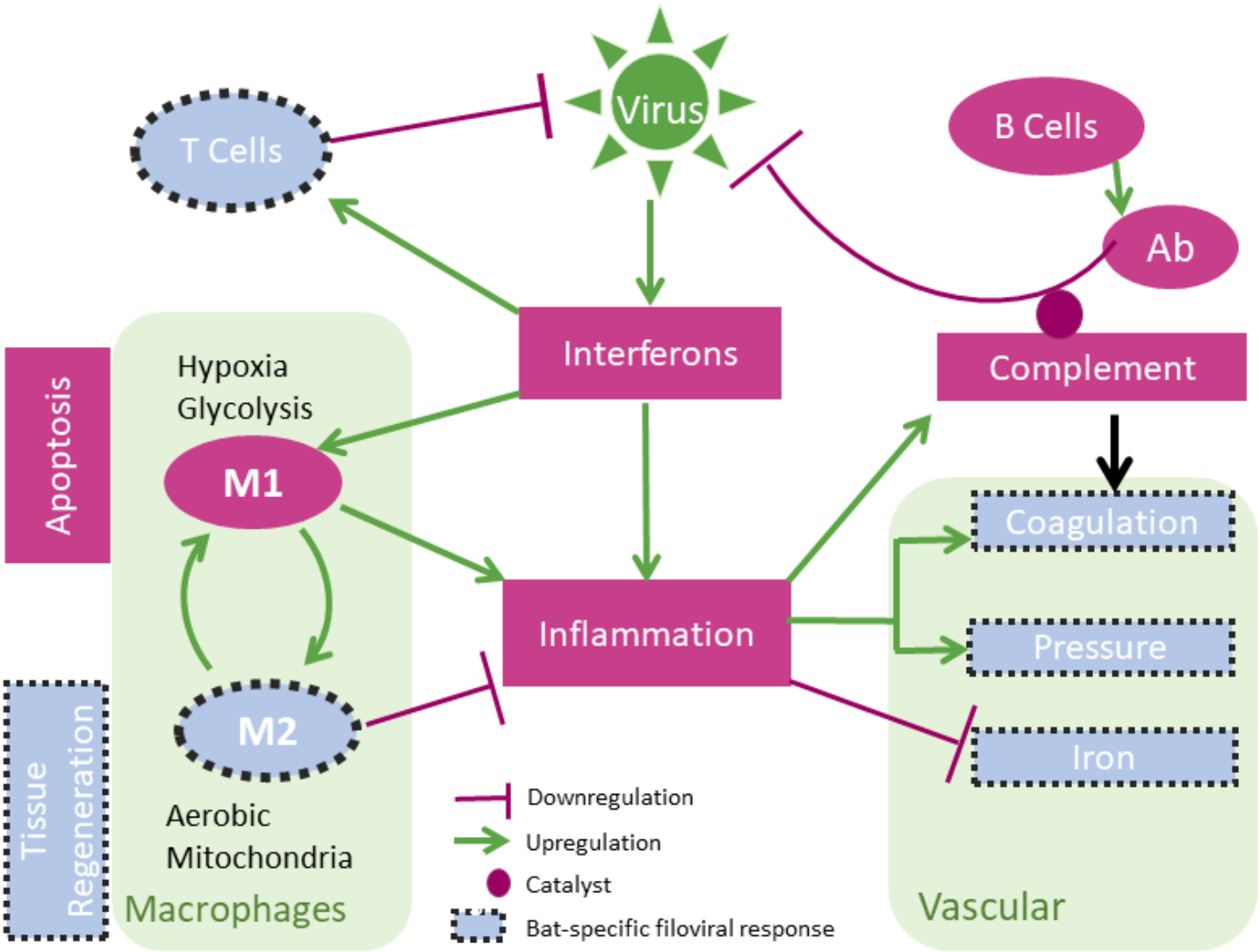

Figure 6: Overview of the response to filovirus replication. Interferon stimulated genes (ISG **Fig. 5,S3,S4**) cause inflammation, which triggers an acute phase response (APR, **Table 1, Fig. 5**), leading to a cascade of vascular events, affecting regulation of HAMP (iron, **Fig. 5,S8**), coagulation (**Fig. 5,S10**) blood pressure (**Fig. 5,S9**) and M1 macrophage stimulation (**Fig. 5,S5,S6**). The inflammatory M1 macrophages are stimulated by infection and phagocytize infected cells and promote apoptosis. Over the course of MARV or EBOV infection, they get converted to anti-inflammatory M2 macrophages. Fatty acid oxidation and mitochondrial activity are up, which are hallmarks of M2 macrophages (**Fig 5,S5,S6,S7A**). The complement system is incompletely stimulated by the acute phase response, leading to potentially restricted antibody activity (**Fig. 5,S7B).** Blood pressure (**Fig. 5,S9**) and coagulation (**Fig. 5,S10**) are down regulated in MARV and EBOV infection, while iron levels (**Fig. 5,S8**) are high, especially in EBOV infection (contrary to the levels of HAMP). T cell (CD8+) activity (**Fig. S7C**) is also upregulated, leading to the infection being cleared. Dotted boundaries show functions that likely distinguish the response of the bats from the human response to filovirus infections.

Tables

Positive APPs	MARV (fold change)	EBOV (fold change)	Mock (tpm)
Serum Amyloid A 1 (SAA1)	21X	3X	6858
Serum Amyloid A 2 (SAA2)	39X	5X	440
Ceruloplasmin (CP)	10X	5X	129
HAMP*	8.5X	10X	211
Orosomucoid 2 Alpha1-Acid glycoprotein (ORM2*)	34X	47X	14
Microsomal Glutathione S-Transferase MGST1	4X	4X	277
MGST2*	11X	16X	5.5
MGST3	0.4X	0.7X	461
Fibrinogen (FGA)	2X	1X	1277
Fibrinogen (FGB)	2X	1X	9007
Fibrinogen(FGG)	1X	1X	6070
C4B	2X	1X	1015
C3P1	6X	1X	31
Haptoglobin (HP)	1.1X	0.7X	15906
Alpha2-Macroglobulin (A2M)	1.3X	1X	409
C-reactive protein (CRP)	N/A	N/A	N/A
Negative APPs			
Albumin (ALB)	0.6X	1.2X	51400
Transferrin (TF)	1X	1X	22856
Transthyretin (TTR)	2X	2X	1
Retinol Binding protein (RBP4)	0.5X	0.6X	3107

Table 1 Acute Phase Proteins (APPs) in livers respond strongly to inflammatory cytokines (IL-6, TNFα etc.). Inflammation upregulates positive APPs and downregulates negative APPs. Basal expression levels in tpm units are shown in the mock column. The fold change upon infection is shown in the EBOV and MARV columns. SAA1/2, CP are highly expressed in livers normally and also get highly up regulated by the filovirus (MARV more than EBOV). ORM2, MGST2 are highly upregulated, but from a low basal expression level. CRP, used as a marker for acute phase response in humans, is not expressed in these bats and might be absent in bats altogether. TF is highly expressed in all samples, but does not react to filoviral infection, while TTR is not expressed in any of the samples. There is similar inflammation in the liver upon both MARV and EBOV infection, despite the lack of viral transcripts in the liver of EBOV infected animals.* signifies the bat gene is divergent from its human homolog.

Table 2 Panel of genes used in a human study to classify survivors versus non-survivors (Liu *et. al.* 2017) . Our data from bats for this panel suggests that except for GBP1, which goes up 2-fold in EBOV and MARV infections and is highly expressed, most of these genes have low expression and do not exhibit significant response to filoviral infections in bats.

Gene	Log2(MARV-liver/mock-liver)	Log2(EBOV-liver/mock-liver)	mock-liver	Description
EOMES	0.5	0.3	0.3	Maybe important in differentiation of effector CD8+ T cells
TGFBI	0.7	0.2	13.2	Inhibits cell adhesion
DBN1	-0.4	-0.4	1.1	Cytoplasmic, actin-binding , role in Neuronal growth
HIST1H3J(H3C12)	NA	NA	NA	Core Component of nucleosome
GUK1	-0.3	0	290	Essential for recycling GMP and indirectly cGMP
ADGRL2/LPHN2	1.8	0.4	7.3	Regulates exocytosis
VCAM1	1.1	0.3	0.5	member of the Ig superfamily, encodes cell surface sialoglycoprotein expressed by cytokine-activated endothelium
HOPX	NA	NA	NA	homeodomain protein that doesn't bind DNA
PLPP/PPAP2B	2.0	0.7	14.8	Important in receptor-activated signal transduction mediated by phosphlipase D
GBP1	1	1	123	ISG, hydrolyzes GTP to GMP

Table 3 Genes analyzed using a Luminex multiplex assay (antibodies) on patients infected with Sudan virus (SUDV) in Gulu district of Uganda (McElroy *et al.* 2014) The authors classified the patients into 3 possible dichotomies (fatal/non-fatal, hemorrhaging/non-hemorrhaging, or high/low viremia) and identified genes that characterized these states. The first column provides the official names of the genes, while the last column shows the names used in the publication. The columns 2-4 list information provided by the human study (higher in fatal column implies fatal cases exhibit higher levels of the gene), and columns 5-7 show expression in infected and non-infected bat livers. Most genes are low-expressed and/or do not show much change under infection in bat livers. Acute phase response (APP) genes respond to inflammation induced by filoviral infection, except for the CRP gene, which is not expressed in these bats. Ferritin is lower in the MARV infected bats compared to the uninfected bats, which is consistent with the claim that Ferritin is higher is fatal human case.

Name	Fatal	Hemorrhage	Viremia	Log2(MARV/mock-liver)	Log2(EBOV/mock-liver)	Mock-liver	Gene
Inflammation							
IL1A	higher	-	-	-	-	-	interleukin 1A
IL1RN	higher	-	-	4.3	3.1	1.9	IL-1RA
IL6	higher	-	-	0.2	0	0	IL6
IL8	higher	-	-	-	-	-	IL8
IL10	higher	-	-	1.2	0.7	0.1	IL10
IL1B	-	-	-	1.6	0.1	0.6	IL-1B
CCL4	higher	-	-	-	-	-	MIP-1B
CCL2	higher	-	-	-	-	-	MCP-1
CSF1	higher	higher	-	1.1	-0.2	1	MCSF
CCL3	higher	higher	-	3.6	0.9	2.3	MIP-1A
Coagulopathy							
PLAT	-	-	higher	1.4	0.7	0.5	TPA
	higher	-	-	-	-	-	D-dimer(DIC)
THBD	higher	higher	-	0.4	0.3	0	thrombomodulin
FGA	-	-	-	0.9	0.2	1269	Fibrinogen
FGB	-	-	-	1.1	0.4	8954	Fibrinogen
F3			higher	0.1	0.1	0.3	Tissue Factor
APP							
CRP	-	-	-	N/A	N/A	N/A	C-Reactive Protein
SAA1	-	-	-	4.4	1.5	6812	SAA
SAA2				5.3	2.3	437	
ALB	-	-	-	-0.8	0.2	50742	Albumin
ICAM1	-	higher	-	2.4	0.2	3.6	soluble intracellular adhesion molecule 1
FTL	higher	higher	-	-1.9	-0.1	3596	ferritin
FTH1	higher	higher	-	-1.4	0.3	7878	ferritin
CD40LG	higher non-fatal	-	-	0	0	0	sCD40L
CXCL10	-	higher	-	5.2	1.5	1.5	IFN-G inducible protein 10 (IP-10)
Complement/adaptive							
SELE	-	-	-	0	0	0	soluble E-selectin
FASLG	-	-	-	-0.2	-1	3.5	soluble Fas ligand
TNF	-	-	-	1.2	-0.2	0.7	tumor necrosis factor A
VEGFA	-	-	-	-1.1	-0.2	180	VEGF-A
CCL5	-	-	-	1.3	1	1.1	RANTES