

COMPARATIVE PATHOLOGY OF EGYPTIAN ROUSETTE BATS (ROUSETTUS  
AEGYPTIACUS) EXPERIMENTALLY INFECTED WITH SOSUGA, KASOKERO AND  
MARBURG VIRUSES

by

SHANNON G.M. KIREJCZYK

(Under the Direction of Corrie C. Brown and Jonathan S. Towner)

ABSTRACT

Bats (Order: Chiroptera) are drawing increased attention as reservoirs for viruses that cause severe and sometimes fatal disease in humans but cause no apparent disease in bats. Marburg virus (family *Filoviridae*) is one such pathogen, for which the Egyptian rousette bat (ERB; family *Pteropodidae*) is the only known natural reservoir host. The ERB is host to several other RNA viruses, including Sosuga (SOSV) and Kasokero viruses (KASV), both of which are associated with systemic illness in humans, but no clinical disease in ERBs. Studies have shown that ERBs immunologically control MARV infection, but the mechanisms remain poorly understood. The goal of this work was to study ERB-host virus interactions within a spatial context using formalin-fixed paraffin-embedded tissues derived from experimental infection studies with MARV, KASV, and SOSV. By studying the pathology of three RNA viruses in a single host, the ERB, we aimed to uncover unique and/or potentially generalizable anti-viral mechanisms applicable to multiple virus families in this host, which could inform public health

preventative countermeasures in non-reservoir spillover hosts that suffer from severe systemic disease (i.e., primates).

In the first phase of this study, the histopathologic and immunohistochemical data showed that the small intestines and salivary glands are primary target tissues for SOSV in ERBs, and that the host immune responses may favor viral persistence (and shedding) in the intestines and clearance from the salivary glands. In the second phase, histopathological, immunohistochemical, and *in situ* hybridization studies showed that, while ERBs develop acute viral hepatitis following KASV infection, they possess immunologic mechanisms to quickly control and clear the infection. The final phase showed that recruitment of CD3<sup>+</sup> cells and mononuclear phagocytes to foci of hepatocellular infection and the early induction of apoptosis in monocyte-derived macrophages contribute to viral clearance and resolution of MARV- and KASV-induced hepatitis in ERBs. These data support long-standing evolutionary relationships between ERBs and MARV and SOSV that favor disease tolerance mechanisms and milder immune responses, whereas KASV is associated with a more robust immune response (i.e., disease resistance), and this tick-borne virus may be more limited in its ability to co-evolve with either host.

INDEX WORDS: Comparative pathology, pathogenesis, immunohistochemistry, *in situ* hybridization, apoptosis, Chiroptera, Egyptian rousette bats, Marburg virus, filovirus, Sosuga virus, paramyxovirus, Kasokero virus, nairovirus

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## DEDICATION

This work is dedicated to my family. I want to thank my Mom and Dad for instilling in me a life-long love for learning and curiosity, a thirst for knowledge, and the desire and skills to address problems that better the lives of animals and people. To my husband Ian – thank you for being my greatest supporter through so many phases and challenges of our lives. Without your support of my crazy endeavors over the last 15 years, none of this would have been possible. And finally, to my son, Owen – thank you for showing me what true joy is and for giving me motivation each day to make a difference.

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# CHAPTER 1

## INTRODUCTION AND LITERATURE REVIEW

### 1. INTRODUCTION

Bats (Order Chiroptera) comprise a diverse clade of placental mammals with more than 1,400 species occupying a variety of ecological niches and habitats around the globe.<sup>144</sup> Bats have been associated with numerous viruses that are highly pathogenic in humans, including Marburg, Hendra, Nipah, and rabies viruses, and the Severe Acute Respiratory Syndrome (SARS)-like coronaviruses.<sup>21,34,39,84,128,134</sup> Experimental and ecological studies have established the Egyptian fruit bat (ERB; *Rousettus aegyptiacus*; family *Pteropodidae*) as a reservoir host for Marburg and Ravn viruses (family *Filoviridae*) and Sosuga virus (family *Paramyxoviridae*), and as a suspected reservoir for Kasokero viruses (family *Nairoviridae*).<sup>14,16,120</sup> These viruses cause mild to severe, systemic disease in humans, but no overt illness in their bat hosts. While experimental infections of ERBs with MARV, SOSV, and KASV have demonstrated widespread virus dissemination, replication in multiple tissues, shedding into saliva, feces and urine, and seroconversion, comprehensive, tissue-based assessments of the associated ERB host responses have not been performed to date.<sup>14,16,120</sup> In fact, few studies have examined the tissues of bats infected with zoonotic viruses to determine the virus-associated lesions, tissue and cellular tropisms, and immune responses in the natural reservoir hosts. The goal of the studies presented herein was to elucidate host-virus interactions in ERBs experimentally infected with MARV, SOSV and KASV using archived formalin-fixed paraffin-embedded (FFPE) tissues derived from

experimental infection studies. A comparative pathology approach was employed, in which a standardized set of tissues was examined histopathologically for each virus-host relationship, immunohistochemistry (IHC) and *in situ* hybridization assays (ISH) were used to determine viral tissue and cellular tropisms, and a battery of immunohistochemical stains was applied investigate the role of mononuclear immune cells and apoptosis in the ERB anti-viral host responses.<sup>66</sup> Whenever possible, the IHC and ISH data were quantified using digital image analysis to conduct a fine scale analysis of ERB host responses over time. This chapter will provide an introduction to these studies by discussing the background, research problem, specific aims, objectives and research questions, the significance of the results and the limitations the studies, followed by an in-depth literature review of bats and viruses, mammalian and ERB-specific anti-viral immunity, and, finally, of MARV, SOSV, and KASV.

For the last several decades, the spillover of viruses from wild bat populations has garnered significant public and political attention. The pressing need to understand and mitigate these zoonotic threats has come to a head with the Severe Acute Respiratory Syndrome virus coronavirus-2 (SARS CoV-2) pandemic, as this and other betacoronaviruses are hypothesized to have originated in horseshoe bats (*Rhinolophus* spp.) in Asia.<sup>21,78</sup> Among the viruses isolated from or detected in bats, the most notorious for causing severe disease in humans are RNA viruses of the families *Filoviridae*, *Paramyxoviridae*, *Nairoviridae*, *Orthomyxoviridae*, and *Rhabdoviridae*.<sup>60</sup> The number of unique, freely available viral sequences deposited in the Database of Bat-Associated Viruses is currently 15,612, of which 8.2% are paramyxoviruses, 0.8% are filoviruses, and 0.2% are nairoviruses.<sup>1</sup>

Experimentally, studies of ERBs and other pteropid bats have elucidated many aspects of bat anti-viral immune responses. Morphologically, the ERB immune system shares several

features of other mammalian immune system, including primary and secondary lymphoid tissues. Genomic analyses have revealed unique features of ERB immunity which may have co-evolved with these viruses to prevent the development of severe disease. These features include expansions in the natural killer (NK) cell repertoire, major histocompatibility complex (MHC) I loci, and Type I interferons (IFNs), particularly IFN- $\omega$ , unusual structural aspects of the Fc receptor that might suggest an increased activation threshold, and the absence of genes encoding short pentraxins.<sup>75,104</sup> Transcriptomics have further demonstrated the induction of anti-viral gene pathways in key target tissues (liver, spleen, and skin at the inoculation site), in splenocytes from MARV-infected ERBs and in MARV-infected ERB-derived dendritic cells.<sup>55,111</sup> Collectively, these data suggest that the ERB immune system responds to MARV by mounting an immune response that effectively controls virus replication, limits tissue damage, and avoids the hyper-inflammatory state that is characteristic of filoviral-infected primates. These results are aligned with the concept of disease tolerance.<sup>87,89,91</sup>

*In vivo* and *in vitro* studies of MARV-, KASV- and SOSV-infected ERBs have demonstrated the induction of innate and adaptive immune responses following experimental infections.<sup>14,16,17,54,55,111,121</sup> However, studies of host-virus interactions within a spatial context have thus far been limited to routine histopathologic assessment of virus-associated lesions and tissue tropism for MARV only.<sup>66</sup> Preliminary histological review of SOSV-infected ERBs revealed mild lesions in the small intestines and salivary glands, tissues that supported a high level of virus replication in the experimental infection study.<sup>16</sup> Additionally, preliminary histopathologic assessments of tissues from MARV- and KASV-infected ERBs revealed mild to moderate hepatitis.<sup>66,120</sup> To date, no studies have characterized the immune cell populations in target tissues or the induction of apoptosis pathways, information that is crucial to begin to

understand how infected ERBs control MARV and KASV infection and avoid clinically relevant liver disease. The tissue and cellular tropism of KASV is also incompletely characterized. The biological relevance of genomics, transcriptomics and *in vitro* studies can only be elucidated in tissue-based studies such as those contained herein, where cell-cell interactions can be explored within a spatial context. Understanding how these viruses circulate in bats may help to inform public health preventive measures and lead to the development of viral countermeasures.

The over-arching goal of this dissertation work was to elucidate the host-virus interactions between ERBs and each of three zoonotic viruses – SOSV, KASV and MARV – within a spatial context using histopathology, advanced molecular pathology techniques and digital pathology. A comparative pathology approach was employed by examining a standardized set of ERB tissues for SOSV and KASV histopathologically, comparing the results to those previously published for MARV<sup>66</sup>, and performing an array of immunohistochemical assays (CD3, Iba1, and cleaved caspase 3) on the primary tissue targets, as determined by viral loads and IHC/ISH results, to characterize patterns of tissue alteration and immune cell responses which may be conserved across viruses families and/or tissues, or which may be unique to each virus-host relationship. To this end, a research plan was formulated with the following objectives:

1. Research objective 1 – Characterize ERB-SOSV host-virus interactions, including histological lesions in tissues with high viral loads as determined in the experimental infection study (small intestines and salivary glands), viral tissue tropism, and immune cell responses ERBs.

2. Research objective 2 – Characterize KASV-induced lesions, viral tissue and cellular tropism, and virus clearance from primary target tissues using *in situ* hybridization, immunohistochemistry and digital image analysis.
3. Research objective 3 – Conduct a side-by-side, direct, quantitative, and qualitative comparison of histopathologic lesions, apoptosis, and innate immune cell responses in the liver tissues of MARV- and KASV-infected ERBs.

These studies will contribute substantively to the growing body of knowledge on virus-bat host interactions, ERB immunology and bat pathology in general, fields that are rapidly evolving and have limited investigative tools. To our knowledge, our study of KASV pathogenesis in ERBs is the first to examine, in detail, bat-bunyavirus interactions within spatial and temporal contexts. The antibodies used to study CD3+ (T cells and/or natural killer T cells), mononuclear phagocytes and cleaved caspase 3 show cross-reactivity in a wide variety of mammalian and in some avian species, and thus, may be useful in studying immune responses in other bat species. The KASV ISH results highlight the sensitivity and usefulness of this technology in studying viruses that replicate at low levels in reservoir hosts, even in tissues that have been fixed for longer than the manufacturer's recommendation due to stringent inactivation protocols associated with the high-containment laboratory research environment. Finally, the application of computer-based digital image analyses to quantify immunohistochemical and *in situ* hybridization signals over time is an approach that may be applicable to a wide range of research questions in the fields of pathology and host-pathogen interactions.

These studies have a number of potential limitations. First, due to limited animal numbers in the CDC ERB breeding colony only three bats were included for each post-infection time

point for the experimental infections. Second, because the ERB breeding colony was founded from ERBs captured in Uganda, the bats are considered outbred, and as such, may have inherent differences in virus and/or disease susceptibility due to genetic, immunologic or other unknown factors. However, this could also be viewed as an advantage, in that the range of histologic lesions encountered in SOSV-, KASV- and MARV-infected ERBs could possibly be more representative of naturally occurring infections. Finally, due to prolonged formalin fixation for the SOSV-infected bats, initial *in situ* hybridization attempts were largely unsuccessful. As a result, the tissue tropism studies were restricted to immunohistochemical localization of SOSV antigen, and thus, quantitative and qualitative viral tropism comparisons for SOSV and KASV or MARV are limited.

In the first part of Chapter One, the context of these studies has been introduced, the research goal and objectives stated, and the value and limitations of these studies presented. In the remainder of this chapter, a comprehensive review of the literature as it pertains to bats and viruses, the natural history of ERBs, and all relevant epidemiological, ecological, immunological, virological and experimental aspects of MARV, SOSV, and KASV will be presented. In Chapter Two, an in-depth analysis of SOSV-ERB virus-host interactions will be presented, based on histopathologic lesions in SOSV-infected ERBs and immunohistochemical assays designed to elucidate virus tissue and cellular tropism and host responses. Chapter Three will provide an in-depth analysis of the pathogenesis of KASV in ERBs based on histopathology, morphometry, *in situ* hybridization, immunohistochemistry, and digital image analysis. In Chapter Four, these comparative pathology studies will culminate in a direct, side-by-side comparison of lesions and host innate immune responses in the liver of KASV- and MARV-

infected ERBs. Finally, in Chapter Five, the main conclusions of these studies will be summarized and future directions for research proposed.

## 2. BATS AND VIRUSES

Although the Order *Chiroptera* contains only about one-half the number of species compared to *Rodentia*, bats reportedly host more viruses per species than rodents.<sup>83</sup> Greater than 200 viruses have been isolated from or identified by nucleic acid sequence in bat hosts, representing 11 bat families, 37 bat genera, and 27 virus families.<sup>95</sup> The RNA viruses, which include viruses of the families *Bornaviridae*, *Bunyaviridae*, *Filoviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, and *Rhabdoviridae* have high plasticity traits such as cytoplasmic replication, and as a result, may be more likely to spill over into aberrant hosts.<sup>60</sup> Further, error-prone RNA polymerases, recombination, and reassortment are mechanisms that contribute to genetic variability, which may drive changes in host tropism, virulence, and pathogenesis for single-stranded RNA viruses, positive-sense RNA viruses, and segmented RNA viruses, respectively.<sup>140</sup>

Why do we observe high viral diversity in bats and how are the viruses maintained and transmitted in nature? The answers to these questions, among many others, remain elusive and are the subjects of intensive study. Ecologic, geographic, evolutionary, physiologic, anatomic, and immunologic factors likely factor into the diverse roles that bats play as zoonotic virus reservoirs.<sup>57,81</sup> Bats with smaller litter sizes, greater body mass, increased longevity, and those that reproduce more than once per year tend to host more zoonotic viruses.<sup>83</sup>

Following a brief introduction to bats and the natural history traits which make this diverse mammalian order unique, the remainder of this literature review will focus on three negative-sense RNA viruses for which the Egyptian rousette bat (ERB; *Rousettus aegyptiacus*;

family *Pteropodidae*) is a confirmed or putative natural reservoir host: Marburg virus (MARV; family *Filoviridae*), Sosuga virus (SOSV; family *Paramyxoviridae*), and Kasokero virus (KASV; family *Nairoviridae*).

#### **a. Phylogeny and Life History Traits of Bats**

Bats are placental mammals, which diverged from other mammalian lineages greater than 50 million years ago.<sup>95</sup> Bats are divided into two suborders, the Yangochiroptera, which includes most of the microbats, and the Yinpterochiroptera, which includes the megabats and five microbat families (*Rhinopomatidae*, *Rhinolophidae*, *Hipposideridae*, *Craseonycteridae* and *Megadermatidae*). Bats occupy diverse ecological niches and vary greatly in size from the 2g bumblebee bat (*Craseonycteris thonglongyai*) to the 1-2 kg flying foxes (*Pteropus* spp.).<sup>95</sup> Bats provide vital ecosystem services such as pollination, insect control and seed dispersal.<sup>20</sup> Bats have several life history traits that make them unique among mammals, including remarkable longevity compared to other mammals of similar size, the use of echolocation, flight, and diversity of social interactions.<sup>86</sup> Unlike the wings of birds and insects, bat wings (patagia) are quite thin, being comprised of two layers of stratified squamous epithelium supported by a thin septum of connective tissue. Their wing plasticity allows them to navigate diverse habitats.<sup>86</sup> Bats spend roughly half of their lives in their roosts, where they form aggregations ranging from only a few bats to millions of individuals depending upon the species, season and location of the roost.<sup>94</sup> The gregariousness nature of many bat species and the propensity to travel over large distances may facilitate pathogen transmission and maintenance among populations.<sup>109</sup>

#### **b. Egyptian Rousette Bats**

Egyptian rousette bats are broadly distributed throughout sub-Saharan Africa, in small parts of the Middle East, the eastern Mediterranean and on the Indian subcontinent. ERBs are

abundant and classified as least concern on the International Union on the Conservation of Nature's Red List.<sup>37</sup> ERBs are unique among pteropodid bats in that they echolocate, which allows them to inhabit caves or subterranean mines, where they aggregate in dense roosts often reaching numbers of over 100,000 individuals.<sup>134</sup> ERBs are nocturnal and frugivorous, leaving the roost at night to feed predominantly on the pulp and juice of soft, very ripe fruits. They are polygamous and can have up to two distinct breeding seasons, depending on the climate. During the breeding seasons, males and females separate, forming bachelor and maternity colonies. The gestation period is 3.5-4 months and females typically give birth to one pup each birthing cycle (twins are rare).<sup>71</sup>

### **3. MAMMALIAN ANTI-VIRAL HOST RESPONSES**

While many aspects of specific bat host-virus relationships remain unclear, there are features of mammalian antiviral immunity that tend to be conserved and can be extrapolated to bat antiviral responses. The mammalian host immune response to viral infections can be broken down into innate and adaptive components. In general, mechanisms that foster disease resistance tend to prevent infection and therefore fall under innate immunity. Mechanisms that promote disease tolerance typically do not prevent infection but tend to reduce tissue damage, and therefore may fall under either innate or adaptive immunity.

#### **a. Innate immunity**

The innate immune response constitutes the immediate and early host responses to a viral infection and serves three essential functions to protect the host: 1) to prevent, control or eliminate infection, 2) to eliminate damaged cells and initiate repair mechanisms, and 3) to stimulate the adaptive response.<sup>5</sup> Intrinsic antiviral immunity is a subset of innate immunity that

directly restricts viral replication and assembly, making a cell non-permissive to viral infections.<sup>146</sup> When a virus infects a cell, pathogen associated molecular patterns (PAMPs) such as single- and double-stranded viral RNA (ssRNA and dsRNA) engage with pattern recognition receptors (PRRs) including toll like receptors (TLRs) 3, 7, 8 and 9, the RNA helicases melanoma differentiation-associated protein 5 (MDA5) and retinoic-acid inducible gene I (RIG-I), among others. Pattern recognition receptors PRRs activate the transcription factors interferon regulatory factors (IRFs) 1, 3, and 7, which leads to the production of a variety of cytokines, including interferon (IFN), interleukin-6 (IL-6), tumor necrosis factor (TNF), pro-IL-1 $\beta$ , and IL-12).<sup>5</sup>

Interferons are key players in the host antiviral responses. Type I interferons (IFN- $\alpha$  and IFN- $\beta$ ) contribute to an antiviral state in a paracrine and autocrine manner through their binding of type I interferon receptors, which induces the transcription of interferon-induced genes such as protein kinase receptor (PKR), an inhibitor of viral transcription and translation, 2',5' oligoadenylate synthetase and RNase L, promoters of viral RNA degradation, and Mx GTPases, inhibitors of viral gene expression and virion assembly.<sup>5</sup> Type I interferons also promote sequestration of lymphocytes in lymph nodes via the upregulation of CD69 and downregulation of the sphingosine 1-phosphate receptor (S1PR1) on lymphocytes, thereby maximizing the probability of lymphocytes encountering viral antigens. Type I interferons enhance NK cell and CD8<sup>+</sup> T cell cytotoxicity and upregulate MHC-I expression, which increases the chances that infected cells will be recognized and killed by cytotoxic lymphocytes. Type I IFNs also elicit expression of TNF-related apoptosis inducing ligand (TRAIL) on NK cells, thereby providing a mechanism for killing infected target cells that is independent of NK cell activating receptors.

Immune cells known to play important roles in the innate antiviral defenses include macrophages, monocytes, dendritic cells and natural killer (NK) cells. NK cells are identified by

the expression of CD56 in humans.<sup>6</sup> NK cells are the primary producers of interferon- $\gamma$ , which contributes to activation of phagocytic cells and antigen presenting cells, maturation of dendritic cells from monocyte precursors, and shapes downstream T cell responses. Natural killer cells use the Fc $\gamma$ RIII to recognize immunoglobulin molecules on cell surfaces and to mediate antibody-dependent cellular cytotoxicity (ADCC); thus, their involvement in the antiviral response spans the innate and adaptive immune responses.<sup>48</sup>

### **b. Adaptive immunity**

The adaptive immune response generates a pathogen-specific response, achieved via the presentation of virus-derived antigen on class I and II major histocompatibility complexes (MHC) by antigen presenting cells (dendritic cells, Langerhans cells, macrophages and B cells) to T lymphocytes, thereby guiding the development of the T cell response (Th1, Th2, Th17, etc).<sup>3</sup> Adaptive immune responses lead to the development of antigen-specific antibodies, which facilitate a suite of host responses including neutralization, ADCC and antibody-dependent cellular phagocytosis (ADCP) and complement activation. Differences in responses between natural and non-natural hosts to viral infection may be related to small differences in the magnitude or timing of these responses rather than the presence or absence of specific signaling cascades.<sup>85</sup>

### **c. Apoptosis**

Apoptosis, or “cellular suicide” is an energy-dependent pathway to cell death that occurs in both physiological (i.e., during normal tissue development) and pathological contexts. Most viruses trigger apoptosis of infected cells, and both excessive and insufficient induction of apoptosis pathways can contribute to the pathogenesis of viral infections.<sup>115,116</sup> The pathways leading to apoptosis are divided into extrinsic and intrinsic, based on the location of the inciting

stimuli (extracellular versus intracellular, respectively). Accumulation of misfolded viral proteins in infected cells triggers an unfolded protein response that activate the intrinsic pathway of apoptosis. Binding of Fas ligand (FasL) to the Fas receptor (CD95) triggers the extrinsic pathway. CD95 is expression on activated CD4<sup>+</sup> and CD8<sup>+</sup> (cytotoxic) T cells, and binding of FasL to CD95 triggers a downstream signaling cascade that culminates in activation of the initiator caspases (8, 9 and 10) and finally the executioner caspases (3 and 7).<sup>4,35,139</sup> Activated caspases are endoproteases that dismantle cellular organelles in a controlled manner, thereby avoiding inflammation and unwanted damage to neighboring cells.<sup>90</sup>

Apoptosis can be observed visually in tissue sections using light microscopy via recognition of the characteristic morphologic changes in dying cells. Changes associated with apoptosis include cell shrinking, chromatin and cytoplasmic condensation, and nuclear and cell fragmentation into apoptotic bodies that typically exhibit phosphatidylserine (PS) on the outer cell membrane, an “eat me” signal that quickly induces their clearance by phagocytic cells.<sup>112</sup> In contrast, necrotic cell death results when the cellular energy stores are depleted, and as a result, the cell swells and releases its contents, including danger associated molecular patterns (DAMPs), into the extracellular milieu. In contrast to apoptosis, necrosis classically incites an inflammatory reaction, while apoptosis leads to reduced inflammation.<sup>112</sup>

Historically, the “gold standard” for confirming apoptosis in tissue sections has been direct visualization of characteristic chromatin and cytoplasmic changes via transmission electronic microscopy.<sup>79,132</sup> These changes include an electron-dense nucleus with marginalization of electron dense chromatin material in the early phase, nuclear fragmentation, an intact cell membrane even late in the cell disintegration phase, disorganized cytoplasmic organelles, large clear cytoplasmic vacuoles, and membrane blebs at the cell surface.<sup>141</sup> Simpler

alternatives are now readily available to detect apoptosis in tissue sections; these include the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, which detects fragmented DNA, and immunohistochemistry for cleaved caspase 3. Both of these methods have the added benefits of being quantifiable and providing spatial context to the lesion(s).

#### **d. The ERB Immune System**

Anatomically, ERBs have the typical mammalian lymphoid tissues required for a functional immune system including primary (thymus and bone marrow) and secondary lymphoid tissues [spleen, lymph nodes and mucosal associated lymphoid tissues (MALT) lymphoid tissues including gastrointestinal associated lymphoid tissues (GALT) in the stomach, distal small intestine and large intestine]. Peripheral lymph nodes are present bilaterally in the axilla, inguinal and submandibular regions, and single, elongate nodes are consistently present at the hila of the lungs and mesentery. Leukocyte subtypes that have been identified in ERB tissues morphologically and/or immunohistochemically include macrophages (immunopositive (+) for ionized calcium binding adaptor molecule 1; Iba1), dendritic cells (Iba1+), monocytes (Iba1+), T cells (CD3+), B cells (CD79a+), neutrophils, and eosinophils. Iba1+ resident tissue macrophages have been observed in a variety of tissues including the salivary glands, spleen, liver (Kupffer cells), brain (microglia), lamina propria of the gastrointestinal tract, and in the renal and adrenal interstitium.

Annotation of the ERB genome demonstrated the presence of 407 immune-related genes (2.75% of the ERB genome) and has uncovered several unique features of innate and adaptive immunity (Table 1.1).<sup>80</sup> Unique features of innate immune-related genes include expansion of the type I interferon gene family, particularly of interferon- $\omega$  genes, and of the natural killer (NK) cell receptor repertoire, as well as the presence of MHC class I genes that are outside the

typical chromosomal loci for these genes, which is suggestive of MHC class I gene dispersion and expansion.<sup>104</sup> Analysis of the ERB transcriptome recently revealed expression of a number of conserved immune-related genes, including pattern recognition receptors such as the toll-like receptors (TLRs) 1-9, RIG-1, MDA5, and LGP2, the crucial scaffold and signaling molecule MyD88, and numerous cytokines and chemokines involved in cell-cell signaling.<sup>80</sup>

**Table 1-1. Unique Features of Egyptian Rousette Bat Immunity**

|                          | <b>Feature</b>                                                                                                                                                                                                                                 | <b>Potential importance/implications</b>                                                                                                                                                  |
|--------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Innate Immunity</b>   | Expansion of Type I interferons, particularly the interferon- $\omega$ genes                                                                                                                                                                   | Possible increase in potency of early anti-viral defenses. <sup>103</sup>                                                                                                                 |
|                          | Expansion of the killer cell lectin like receptor type C (KLRC/NKG2) and killer cell lectin like receptor type D (KLRD/CD94) gene families                                                                                                     | NKG2 and CD94 receptors, which are associated with an inhibitory NK cell response in other animals, are expanded and may serve as the principal NK cell receptors in ERBs. <sup>103</sup> |
|                          | Expansion and dispersion of MHC I genes                                                                                                                                                                                                        | Natural killer cell receptors might interact with distinct MHC class I genes. <sup>103</sup>                                                                                              |
|                          | Absence of the short pentraxins C reactive protein and serum amyloid P                                                                                                                                                                         | May translate to a reduction in acute inflammation. <sup>75</sup>                                                                                                                         |
| <b>Adaptive Immunity</b> | Distinct features of the Fc $\gamma$ RI                                                                                                                                                                                                        | Inflammatory responses regulated by this receptor may necessitate a higher threshold of activation <sup>75</sup>                                                                          |
|                          | Unique features of the immunoglobulin heavy-chain locus <ul style="list-style-type: none"> <li>- Two putative functional <i>IGHE</i> genes and four functional <i>IGHG</i> genes</li> <li>- IgG1 highest expressed subclass in ERBs</li> </ul> | IgG1 has short half-life; might explain rapid waning of ERB anti-MARV antibodies <sup>75,123</sup>                                                                                        |

A number of distinct and functional IgE (2) and IgG (4) genes have been discovered in ERBs, in addition to the presence of Ig variable genes associated with protective human anti-viral antibody responses.<sup>75</sup> The finding of two IgE subtypes is unusual among mammals. Additionally, the complete absence of genes encoding the short pentraxins C reactive protein (CRP) and serum amyloid P (SAP) was noted.<sup>75</sup> The absence of these pentraxin-encoding genes has never been reported in mammals. C reactive protein is a strong acute phase reactant, and its absence suggests that ERB immune responses might favor lower levels of acute inflammation.<sup>43</sup>

Genomic analyses of the black flying fox (*Pteropus alecto*) indicate that this bat has a contracted type I interferon locus, with only three functional interferon- $\alpha$  genes compared to 7-18 functional interferon- $\alpha$  genes in other mammals, and in contrast to the marked expansion of type I interferons in ERBs and *Pteropus vampyrus*.<sup>70,147</sup> Further, Zhou et al. demonstrated constitutive and ubiquitous expression of interferon- $\alpha$  in tissues of three wild-caught in *P. alecto* bats, suggesting a heightened anti-viral baseline state in that species, which also contrasts with the induction of anti-viral genes observed in MARV infected ERBs.<sup>17,32,55</sup>

While *in vivo* and *in vitro* studies of captive bred ERBs have elucidated many aspects of the bat anti-viral immune responses, much remains unknown. The following sections will lay the foundation for three viruses – MARV, SOSV, and KASV – for which ERBs are a confirmed or putative natural reservoir host, and for which intensive research into host-virus interactions are ongoing.

## 4. MARBURG VIRUS

### A. Virus classification, structure, entry, and replication

The genus *Marburgvirus* (family *Filoviridae*) contains one species, *Marburg marburgvirus*, with two closely related viruses, Marburg virus (MARV) and Ravn virus (RAVV). These are the causative agents of Marburg virus disease (MVD). Marburgviruses have non-segmented, negative-sense, single-stranded RNA genomes.<sup>117</sup> The genome is approximately 19,111 nucleotides in length (GenBank accession #: NC\_001608) and encodes seven gene products, including, in order of transcription, the nucleoprotein (NP), VP35, VP40, glycoprotein (GP), VP30, VP24, and polymerase (L) proteins, with an overlap between the VP30 and VP24 genes. Ultrastructurally, MARV virions are pleomorphic, often appearing as long filamentous particles (hence the family name). Virions have also been described as branched, “6”-shaped, “U”-shaped, and circular.<sup>53</sup> Particles are typically 80 nm wide and lengths appear to be variable, typically ranging from 600-to-800 nm in length, with reports up to 14,000 nm.<sup>53,117</sup>

Glycoprotein peplomers coat the surface of MARV virions, and serve roles such as receptor binding and membrane fusion. Unlike ebolaviruses, marburgviruses do not produce a soluble glycoprotein.<sup>47</sup> Following micropinocytosis and entry into an endosome, filoviruses use the Niemann Pick C1 (NPC1) receptor in the membrane of late endosomes to enter the cytoplasm of susceptible host cells.<sup>117</sup> The NPC1 receptor is ubiquitously expressed in most human cell types, but is highly concentrated in hepatocytes, which may explain why the liver is a primary target for MARV. The protein products of the NP and VP30 genes function as the major and minor nucleoproteins, respectively. The L and VP35 proteins form the polymerase complex as the enzymatic component and co-factor, respectively; VP35 also acts as a type I IFN antagonist. VP40, the most abundant protein in the virion, is the major matrix protein, and

functions in virion assembly and budding. VP24 is the minor matrix protein, functioning in virion assembly and also interferon antagonism.<sup>59</sup>

## B. Emergence

Marburg virus was discovered in 1967 in Germany and the former Yugoslavia, when laboratory workers were exposed to infected, Ugandan-origin, African green monkeys (*Chlorocebus tantalus*).<sup>126</sup> It was the first filovirus described, and since 1967, there have been fourteen additional MARV outbreaks to date, all originating from Africa and having a wide range of case fatality rates (Table 1).<sup>59</sup> Large-scale outbreaks with extensive human-to-human transmission occurred in 1998-2000 centered on a gold mine in Durba, Democratic Republic of Congo and in Uige Province, Angola, West Africa.<sup>24,135</sup> The MARV outbreak in Angola had the highest case fatality rate recorded for any filovirus (90%).<sup>135</sup>

**Table 1-2. Details of the Fourteen Documented Marburg Virus Outbreaks.**

Adapted from: <https://www.cdc.gov/vhf/marburg/outbreaks/chronology.html>

| Year(s)   | Location(s)                        | Origin                           | Total Number of Cases and Case Fatality Rate (%) | Notes and References                                                                                            |
|-----------|------------------------------------|----------------------------------|--------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|
| 1967      | Germany and Yugoslavia             | Uganda                           | 31 (23%)                                         | Simultaneous outbreaks in laboratory worked handling African Green monkeys imported from Uganda. <sup>126</sup> |
| 1975      | Johannesburg, South Africa         | Zimbabwe                         | 3 (33%)                                          | Outbreak in a returning traveler and two contacts. <sup>38</sup>                                                |
| 1980      | Kenya                              | Mount Elgon National Park, Kenya | 2 (50%)                                          | Patient had recently visited Kitum Cave. <sup>127</sup>                                                         |
| 1987      | Kenya                              | Kenya                            | 1 (100%)                                         | Danish traveler had recently visited Kitum Cave. <sup>64</sup>                                                  |
| 1990      | Russia                             | Russia                           | 1 (100%)                                         | Laboratory-acquired infection. <sup>98</sup>                                                                    |
| 1998-2000 | Democratic Republic of Congo (DRC) | Durba and Watsa, DRC             | 154 (83%)                                        | Outbreak centered on a gold mine in Durba. <sup>24</sup>                                                        |

|           |             |                                                             |           |                                                                                                                                                                               |
|-----------|-------------|-------------------------------------------------------------|-----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 2004-2005 | Angola      | Uige Province, Angola                                       | 252 (90%) | Outbreak likely began in Uige Province and spread to several other Provinces. <sup>135</sup>                                                                                  |
| 2007      | Uganda      | Lead and gold mine in Kamwenge and Ibanda Districts, Uganda | 1 (25%)   | Patient-derived MARV sequences supported two separate introductions and matched very closely to sequences obtained from Egyptian rousette bats in the mines. <sup>7,134</sup> |
| 2008      | U.S.A.      | Maramagambo forest, Queen Elizabeth National Park, Uganda   | 1 (0%)    | Returning traveler who fully recovered. <sup>2</sup>                                                                                                                          |
| 2008      | Netherlands | Maramagambo forest, Queen Elizabeth National Park, Uganda   | 1 (100%)  | Returning traveler who had visited Python cave and had contact with bats. <sup>103,133</sup>                                                                                  |
| 2012      | Uganda      | Kabale, Ibanda, Mbarara and Kampala, Uganda                 | 15 (27%)  | Patient-derived MARV sequences were highly similar to sequences obtained from Egyptian rousette bats in 2008 and 2009 at nearby Python Cave. <sup>13</sup>                    |
| 2014      | Uganda      | Kampala, Uganda                                             | 1 (100%)  | Only 1 confirmed case. Eight contacts developed symptoms,                                                                                                                     |

|      |        |                |          |                                                                          |
|------|--------|----------------|----------|--------------------------------------------------------------------------|
|      |        |                |          | but all tested negative at the Uganda Virus Research Institute.<br>9,137 |
| 2017 | Uganda | Kween, Uganda  | 4 (75%)  | Outbreak in Eastern Uganda in a family cluster. <sup>101</sup>           |
| 2021 | Guinea | Guéckédou      | 1 (100%) | One case confirmed post-mortem.                                          |
| 2022 | Ghana  | Ashanti region | 3 (100%) | A single family of three.                                                |

### C. Marburg Virus Disease (MVD)

Human infection with MARV occurs when the virus enters the host through mucosal surfaces or breaks in the skin.<sup>92</sup> Following a 2-to-21-day incubation period, there is a sudden onset of fever, chills, headache, myalgia and variable degrees of maculopapular rash, nausea, abdominal pain, vomiting, and diarrhea. In fatal cases, MVD can progress to liver failure, pancreatitis, shock, delirium, coma, massive hemorrhaging and multi-organ failure. Marburg virus may persist in immunologically privileged sites such as the eye, central nervous system, and reproductive organs.<sup>92</sup>

#### i. Pathogenesis of MVD

Three mechanisms appear to drive severe MVD in primates, including humans: 1) rapid virus replication, 2) host immunosuppression induced by the virus, and 3) vascular dysfunction.

<sup>59</sup> Mononuclear phagocytes (tissue macrophages, monocytes, and dendritic cells) play key roles in the pathogenesis of filoviral infections for several reasons. These cell types support high levels

of virus replication, disseminate the virus from the site of infection, secrete high levels of inflammatory cytokines that promote immune dysregulation, and express tissue factor on their cell surface, which promotes end-stage disseminated intravascular coagulation (DIC) and loss of vascular integrity.<sup>59</sup> The immune dysregulation observed in fatal cases of MVD in humans is multifaceted, owing to the failure of appropriate dendritic cell maturation and lack of T cell co-stimulation<sup>30</sup>, widespread bystander apoptosis of lymphocytes<sup>29,41</sup>, and inhibition of the Type I Interferon system through a variety of mechanisms involving the viral proteins VP35 and VP24.<sup>59</sup> Vascular dysfunction is likely the result of endothelial cell activation due to stimulation of the clotting cascade by tissue factor expressed on macrophages and possibly also decreased clotting factor synthesis due to widespread hepatocellular necrosis. Direct infection of adrenocortical cells and necrosis of the adrenal cortex may also contribute to the development of a shock-like state and multi-organ failure in fatal cases due to acute adrenal insufficiency.<sup>117</sup>

Filoviruses have broad tissue and cellular tropisms. In some fatal human cases, filovirus-infected cells, especially hepatocytes, develop prominent cytoplasmic inclusion bodies that are visible by light microscopy, although the presence of hepatic inclusions appears to be more consistent ebolaviruses than MARV.<sup>10,41,117</sup> In fatal MVD cases, there is typically little to no inflammatory cell response despite widespread virus replication in the liver.<sup>10,41</sup>

## **ii. MARV-induced Cell Death: Apoptosis and Necrosis**

Studies of experimentally MARV-infected NHPs indicate that infected cells, including macrophages, dendritic cells, hepatocytes, and endothelial cells, likely undergo necrotic rather than apoptotic cell death, as evidenced by ultrastructural changes such as vacuolation and swelling.<sup>51,102</sup> Filoviruses are known to interfere with antiviral signaling by blocking RIG-I-like receptor (RLR) and Type I interferon signaling pathways, but it is unknown whether or not

filoviral proteins directly interfere with induction of apoptosis pathways.<sup>23,102</sup> By immunohistochemistry, hepatocellular cleaved caspase 3 expression was elevated above baseline for NHPs experimentally infected with a variety of marburgviruses, suggesting that apoptosis may contribute to the pathogenesis of liver disease in MVD.<sup>29</sup>

#### **D. Animal Models of MVD**

Animal models have contributed significantly to the body of knowledge regarding the pathogenesis of MVD. Four models utilize immunocompetent animals; these include nonhuman primates (NHPs; cynomolgus and rhesus macaques, common marmosets, squirrel monkeys, African green monkeys and baboons), hamsters, guinea pigs, and mice.<sup>25</sup> NHPs are considered to be the “gold standard” animal models of MVD given their close evolutionary relationships to humans. Cynomolgus macaques, rhesus macaques and African green monkeys develop similar MVD overall following experimental infection with MARV.<sup>52,125</sup> NHPs are susceptible to infection with wild type MARV and succumb to severe and often fatal MARV infection which recapitulates many clinicopathologic, gross and histologic findings of fatal human MVD, including thrombocytopenia, lymphopenia followed by neutrophilia, immune dysregulation, lymphocyte apoptosis, disseminated intravascular coagulation (DIC) with subsequent thrombocytopenia and hemorrhage, severe liver and kidney lesions, maculopapular rash, and neurological symptoms.<sup>10,29,40,52</sup> In macaques, MARV exhibits cellular tropism for macrophages, monocytes, hepatocytes, hepatic sinusoidal lining cells (Kupffer cells and endothelial cells), fibroblasts, adrenocortical cells, cells of the male and female reproductive tracts including Sertoli cells in the testis, granulosa and theca cells of the ovary, smooth muscle cells of the uterine wall, multiple cell types within the eye, and neurofibroblasts and Schwann cells within peripheral

nerves.<sup>10,36,41,52,125</sup> The presence of MARV in NHP tissues is often associated with fibrin deposition and necrosis.<sup>10,40</sup>

## **E. Ecology**

Ecological and epidemiological field investigations in Uganda<sup>13,15,134</sup>, Kenya<sup>74</sup>, the Democratic Republic of Congo<sup>131</sup>, Gabon<sup>110,134</sup>, Sierra Leone<sup>12</sup>, Zambia<sup>33,68</sup>, and South Africa<sup>105</sup> have identified ERBs as the primary natural reservoir for MARV. Since 2007, more than 90 MARV sequences and 24 virus isolates, many of which are closely related, have been obtained from tissues of wild-caught ERBs, demonstrating widespread circulation and homology with every major human MARV strain that has spilled over into the human population.<sup>12</sup> No direct MARV spillover events have been reported in Sierra Leone, Zambia, South Africa, or Gabon, despite the detection of MARV in ERBs in those locations.<sup>12,33,68,105,110</sup>

Longitudinal field studies in Kitaka Mine and Python Cave, Uganda, following the 2007 and 2008 outbreaks found a 2-5% overall prevalence of MARV and RAVV infection in ERBs.<sup>13</sup> ERBs in these locations showed a clear age bias for active MARV infection, with juvenile bats (~ 6-month-old) having distinct biannual peaks of active infection (peak = 30/341 bats, 12.4% prevalence), which coincided temporally with influxes of susceptible juvenile bats from the previous birthing season (occurring twice-yearly) and 83% of historically reported spillover events to the human population. In contrast, the prevalence of active MARV infection in younger juvenile (< 3 months) and adult ERBs has been consistently lower at 2-3% (8/301 bats) and 2-5% (33/1467 bats), respectively, regardless of the season. These observations suggest a predominant pattern of horizontal MARV transmission in ERBs and that maternal antibodies are somewhat protective for young juveniles. Maternal antibodies were shown to be protective

against MARV infection in juvenile bats up to 4-5 months of age in one experiment.<sup>130</sup>

Transmission during mating may contribute to MARV transmission, as males bite females during or following copulation and some bats shed high levels of MARV in their oral secretions.<sup>14</sup> A mathematical model suggested that the virus was able to persist in ERBs given the following criteria: 1) a biannual pulse of susceptible juveniles, 2) a latent period of greater than or equal to 21 days, 3) and a host population size of at least 20,000 individuals.<sup>13,60,123,134</sup> Field studies indicate that arthropod vectors, including argasid ticks, are not involved in the natural transmission of MARV between ERBs.<sup>119</sup>

## **F. The ERB-MARV Virus-Host Relationship**

ERBs do well in captivity and the establishment of captive ERB colonies has made possible experimental infection studies, which have elucidated many aspects of this host's immune system and responses to viral infections. The sections that follow will summarize the current state of knowledge regarding the viral dynamics, immune response and pathogenesis in ERBs experimentally infected with MARV.

### **i. Viral Dynamics, Transmission and Shedding**

ERBs inoculated subcutaneously with a low-passage, bat-derived MARV isolate (GenBank accession # FJ750958) exhibit low-level viremia, with widespread dissemination of the virus, and replication in multiple tissues, but infected bats do not get sick.<sup>14</sup> Virus is shed in oral secretions and in the feces and urine, indicating that contact with MARV-infected bat fluids and/or biting can facilitate in bat-to-bat and bat-to-human MARV transmission.<sup>14,107</sup> Although one study failed to demonstrate experimental bat-to-bat transmission of the human-derived Hogan strain of MARV, a more recent study demonstrated bat-to-bat transmission of bat-derived

MARV between co-housed naïve and experimentally infected bats.<sup>106,121</sup> The latter study also demonstrated the possibility of a supershedder phenomenon, given that 20% of the inoculated bats were responsible for the vast majority of (95.4%) of oral MARV shedding.<sup>121</sup>

## **ii. Pathogenesis**

While ERBs experimentally infected with MARV remain apparently healthy, virus replication in the liver is associated with mild histopathologic lesions and transient, mild increases in the hepatocellular leakage enzyme ALT.<sup>66</sup> Similar to wild-caught MARV-infected ERBs, experimentally infected bats have variable numbers of randomly distributed foci of mononuclear cell inflammation in the liver, which occasionally contain necrotic or apoptotic hepatocytes and rare neutrophils.<sup>66</sup> Immunohistochemically, viral antigen is localized to hepatocytes and histocytes within the foci of inflammation. Apart from the liver, MARV antigen is contained within histiocytes at the MARV inoculation site, macrophages in the splenic red pulp, in the sinuses and paracortical regions of lymph nodes, and within mesenchymal cells of the oral submucosa.<sup>66</sup>

## **iii. Immune Responses**

### **1. Innate Immunity**

*In vitro* studies using ERB cell lines, including immortalized kidney cells (RoNi/7.1 cells) and bone marrow-derived dendritic cells (DC) have elucidated some of the complexity of the ERB-MARV host-virus relationship, and how the responses to MARV infection differ among cell types. Marburg virus-infected ERB kidney cells permitted high levels of virus replication with a lack of anti-viral gene induction, presumably due to the interferon antagonism of VP35.<sup>17</sup> In contrast, MARV-infected ERB DCs supported sustained, low-level virus transcription and strongly upregulated type I interferon-related antiviral genes. Further, DC

maturation genes were dysregulated or downregulated, and proinflammatory genes such as the chemokine CCL8 and IL-33, an alarmin, were downregulated.<sup>111</sup>

*In vivo* transcriptomic studies of MARV-infected ERBs using a custom RNA code set have demonstrated the induction of several canonical antiviral genes including ISG15, IFIT1, and OAS3 in several target tissues (liver, spleen, skin at the inoculation site) beginning at 2 dpi, emphasizing the importance of early host responses in controlling MARV infection.<sup>55</sup> Additionally, MARV-infected ERBs showed a lack of hyper-induction of cytokines (IL-6, CCL8, and TNF) that are characteristic of the dysregulated immune response that is seen in severe MVD in primates.<sup>55</sup>

## **2. Adaptive Immunity**

Experimental inoculation and simulated “natural” infections of naive contact bats confers long-term protective immunity (> 2 years) despite rapidly diminished antibody levels.<sup>123</sup> However, another study showed that previous exposure to MARV was not completely protective in ERBs, with bats that were re-infected with the same virus isolate having low-level viremia and virus replication limited to the liver and spleen, but no shedding; hence, reinfection does not appear to be a driver of virus maintenance in nature.<sup>130</sup> While virus neutralization does not appear to be a major function of MARV-specific antibodies in ERBs, antibodies may serve other effector functions such as opsonization and phagocytosis and antibody-dependent cellular cytotoxicity.<sup>122</sup>

## 5. SOSUGA VIRUS

### A. Virus classification, structure, entry and replication

Bats are hypothesized to be the ancestral hosts for paramyxoviruses, a diverse viral family that includes members of the genera *Henipavirus* (Nipah and Hendra viruses), *Morbillivirus*, and *Rubulavirus*.<sup>42</sup> Sosuga virus is classified within the family *Paramyxoviridae* and clusters with several other bat-derived zoonotic rubulaviruses (genus *Pararubulavirus*) including Menangle virus, Achimota viruses 1 and 2, Hervey virus, Teviot virus, Tioman virus, and Tuhoko virus 1, 2, and 3.<sup>19,22,31,65,77,108,136,145</sup> While *Pteropus* spp. flying foxes are known to be the hosts for Nipah and Hendra viruses, two novel, yet un-named paramyxoviruses related to the henipaviruses were recently isolated from ERBs in Kenya.<sup>96,99,109,142</sup>

Sosuga virus virions are enveloped and pleomorphic, and have a 15,480 base, linear, negative-sense RNA genome similar to that of most paramyxoviruses, with 6 genes encoding 7 viral proteins, including the nucleocapsid (N), phosphoprotein (P), hemagglutinin-neuraminidase (HN), polymerase (L), and the matrix (M), fusion (F), and V proteins (V).<sup>8</sup> Paramyxovirus attachment entry into host cells is mediated by the HN glycoprotein. A structural and functional investigation of the SOSV receptor binding protein suggests that SOSV utilizes a host cell entry pathway independent of sialic acid and ephrin-b2.<sup>129</sup>

### B. Emergence

Sosuga virus was first identified in 2012, when a wildlife biologist who had conducted field work in Africa became sick after returning to the United States.<sup>8</sup> The biologist had been trapping and sampling bats and rodents in South Sudan and Uganda (hence the name), and after an uncertain incubation period of several weeks, she developed systemic symptoms that included

fever, fatigue, headache, generalized muscle and joint pain, neck stiffness, a metallic taste and mild splenic enlargement, followed by a maculopapular rash, oropharyngeal ulceration, and diarrhea.<sup>8</sup> The patient's recovery was prolonged, with significant symptoms persisting for several months, and some symptoms lingering for years. Retrospective PCR analyses of rodent and bat liver/spleen samples from fieldwork locations revealed that ERBs were the likely source of SOSV spillover.<sup>11</sup>

### **C. Paramyxovirus Immune Evasion Strategies**

Paramyxoviruses have evolved multiple strategies to overcome the host IFN response.<sup>18</sup> Within *Paramyxoviridae*, host immune evasion mechanisms are remarkably diverse across viral genera and even between species within the same genus.<sup>18</sup> The V protein encoded by members of the subfamily *Paramyxovirinae* interacts with the cytoplasmic RIG-I-like receptor RNA helicase melanoma differentiation-associated protein 5 (MDA5), which recognizes long viral double-stranded RNAs in the cytoplasm of infected cells in which viral replication has begun.<sup>27</sup> The V protein of numerous paramyxoviruses binds to and inactivates the MDA5 by blocking its interaction with mitochondrial antiviral-signaling protein (MAVS), thereby interfering with downstream signaling including cytokine and interferon expression.<sup>113,27</sup> While henipaviruses do not appear to interact directly with MDA5, the W protein inhibits TLR-3-dependent phosphorylation of IFN regulatory factor 3 (IRF3). Additionally, several rubulaviruses inhibit cytokine signaling by direct interference with signal transducers and activators of transcription (STAT)1 and STAT2 signaling.<sup>100,72,73</sup>

## **D. Apoptosis**

Members of the family *Paramyxoviridae* have been shown to both interact with a variety of apoptosis pathways.<sup>82</sup> Sendai virus (genus *Respirovirus*) stimulates apoptosis through an IRF3-dependent mechanism that activates caspase 3 and 8, but despite the induction of the cell death pathway, viral progeny are still able to mature and bud.<sup>61,28</sup> These findings nicely demonstrate how apoptosis can be advantageous to the virus or host, or both. In the case of Sendai virus, apoptosis may be advantageous to both the host and virus by limiting the development of an inflammatory cell response induced by the leakage of cellular contents from dying cells and permitting the ongoing release of viral progeny, respectively. Measles virus (genus *Morbillivirus*) induces apoptosis in infected cells, which is thought to facilitate release of the virus from infected cells, which would be advantageous to the virus but not necessarily to the host.<sup>44,49</sup>

## **E. The ERB-SOSV Relationship**

### **i. Viral Dynamics and Shedding**

A recent experimental study confirmed that ERBs are a competent host for SOSV and the likely source of spillover for the index human case.<sup>11,16</sup> In that study, subcutaneously inoculated ERBs became viremic (peak = 5 DPI) and SOSV replicated in several tissues, particularly the small intestines, lymphoid tissues and salivary glands.<sup>16</sup> Compared to MARV, the feces appears to be the major route of SOSV shedding. Like MARV-infected ERBs, super-shedding was also noted, in that one SOSV-infected bat intermittently shed virus in the feces for the duration of the study.<sup>16,121</sup>

## **ii. Pathology**

There are few reports describing the histopathologic lesions associated with experimental paramyxovirus infections in their bat hosts, and these are largely limited to the henipaviruses. Hendra virus-infected pregnant grey-headed flying foxes (*Pteropus poliocephalus*) reportedly had a vasculopathy in the gastrointestinal tract, kidney spleen, placenta and lungs at 10 and 21 DPI, despite remaining asymptomatic.<sup>93,142,143</sup>

A range of histopathologic lesions were observed in Nipah virus-infected bats, including subacute eosinophilic inflammation in the sublingual salivary ducts at 3 DPI and vasculitis of a small intestinal mucosal blood vessel at 22 DPI.<sup>93</sup> However, in that study, while NIPV was isolated from the urine of one bat in that study, lesions were not present in the kidney or urinary bladder and the vascular lesions could not be definitively attributed to NIPV infection due to the lack of viral antigen on immunohistochemical stains. Mild but histologically notable lesions in the small intestines and salivary glands in a subset of the SOSV-infected ERBs prompted the in-depth histopathological and immunohistochemical investigations that are detailed in Chapter 3 of this dissertation.

## **iii. Immune response**

The innate immune response to SOSV in experimentally infected ERBs has been poorly characterized. While all SOSV-infected ERBs euthanized at 21 days post-infection seroconverted, ERB serum antibodies to SOSV were recently shown to be non-neutralizing *in vitro*.<sup>16,122</sup>

## **6. KASOKERO VIRUS**

### **A. Introduction**

Bats have been implicated in the transmission and spillover of several arboviruses (Order *Bunyavirales*), including viruses of the families *Flaviviridae*, *Reoviridae*, and *Togaviridae*.<sup>45</sup> The ERB is implicated in the maintenance or transmission of several arboviruses, including Kasokero<sup>69</sup> and Yogue viruses (genus *Orthonairovirus*; family *Orthobunyaviridae*), and *Bukakata orbivirus* (family *Reoviridae*, genus *Orbivirus*).<sup>46</sup> Additionally, a novel bunyavirus, Wolkberg virus (genus *Orthobunyavirus*), was recently isolated from wingless bat flies (*Eucampsipoda africana*) that live and feed on ERBs in Wolkberg cave South Africa, suggesting that this virus may also infect ERBs.<sup>63</sup> Serosurveys of ERBs in Africa have identified neutralizing antibodies to Crimean-Congo Hemorrhagic Fever Virus (CCHFV; genus *Nairovirus*), unspecified flaviviruses, and Rift Valley fever virus (RVFV; genus *Phlebovirus*; family *Bunyaviridae*), although their role, if any, in the enzootic cycle of these viruses remains uncertain.<sup>67,97</sup>

### **B. Virus classification, structure, entry and replication**

The genus *Nairovirus* contains bunyaviruses that are transmitted via a tick vector. The genus contains a variety of bunyaviruses of public health and veterinary importance including KASV, Yogue virus (YOGV), Leopards Hill virus (LPHV; a virus isolated from giant leaf-nosed bats, *Hipposideros gigas*, in Zambia), CCHFV and Nairobi sheep disease virus (NSDV).<sup>138</sup> Phylogenetic analysis of KASV has demonstrated that it is most closely related to YOGV and LPHV.

KASV has a negative-sense, segmented RNA genome. The genome is comprised of three segments, including the S (1.7 kb; Genbank accession MT279043), M (4.5 kb; Genbank

accession MT279044), and L (12.0 kb; Genbank accession MT279045) segments, which encode the nucleoprotein, glycoprotein and RNA-dependent RNA polymerase, respectively.<sup>138</sup>

Nairoviruses are distinguished from other bunyaviruses by their large “L” genes.<sup>26</sup>

Nairoviruses form enveloped, spherical virions ranging from 80-120 nm in diameter. The envelope is studded with glycoprotein dimers (Gn + Gn). Entry into host cells occurs via glycoprotein binding and receptor-mediated endocytosis. Viral replication takes place in the cytoplasm. Virus particles bud into vesicles at the Golgi apparatus cisternae and particles are released from the cell by fusion of the intracellular vesicles with the cell membrane.<sup>27,76</sup>

### **C. Immune Evasion**

While immune evasion mechanisms specific to KASV have not been investigated, CCHFV, a closely related nairovirus, and a distantly related bunyavirus, Haantan virus (family *Hantaviridae*), have been shown to evade RIG-I-mediated cytokine/chemokine activation by cleavage of negative-strand 5' triphosphates, which prevents binding to RIG-I.<sup>56</sup>

### **D. Apoptosis**

*In vitro* studies of CCHFV (genus *Nairovirus*) using a hepatocyte cell line demonstrated engagement of both the intrinsic and extrinsic pathways of apoptosis via ER stress and engagement of the death receptor pathway, respectively.<sup>114</sup> Interestingly, infection of tick cells with a related virus, Hazara nairovirus, failed to induce apoptosis whereas it induced apoptosis in adrenal epithelial (SW13) cells, indicative of distinct and opposing cellular responses in mammalian and tick cell lines.<sup>50</sup>

## E. Emergence

Kasokero virus was first recognized as a zoonotic pathogen following the occupational exposure of four people at the Uganda Virus Research Institute. Three individuals were working with KASV while the fourth case had access to the laboratory where KASV characterization assays were being performed, which is suggestive of human-to-human transmission.<sup>69</sup> The exposed individuals developed mild to severe systemic illness, with fever, headache, abdominal pain, diarrhea, and muscle and joint pain. The KASV isolate associated with the laboratory-acquired infections was obtained from the blood of a wild-caught ERB collected at Kasokero Cave, Uganda.<sup>69</sup>

## F. Ecology

In addition to the blood of ERBs, KASV has also been isolated from *Ornithodoros (Reticulinasus) faini* ticks collected at a large ERB roosting site in western Uganda (Kasokero Cave) and at Lanner Gorge Cave, South Africa, suggesting that KASV is maintained in an enzootic transmission cycle involving ERBs and ticks.<sup>118</sup> A KASV seroprevalence of 67.6% detected in ERBs at Kasokero Cave further supports their involvement in the enzootic transmission cycle. Additional studies are needed to determine if KASV is transmitted vertically and/or horizontally among *O. (R.) faini* ticks and if the virus has any negative effects on the tick vector.

Recently, the ability of ERBs to serve as competent host for KASV was assessed in an experimental infection study using colony-bred ERBs and a tick-derived inoculum of KASV (UGA-Tick-20170128; GenBank: MT309096.<sup>120</sup> In that study, ERBs were inoculated intradermally to recapitulate a tick bite, swabbed daily for viremia and shedding, and euthanized

in groups of 3 bats at 3-, 6-, 9-, 12-, 18-, and 20-days post-inoculation (DPI) to assess replication in tissues.<sup>120</sup> All bats became viremic, seroconverted by 14 DPI, and oral, fecal and urinary shedding was detected in inoculated bats by qRT-PCR. All bats remained apparently healthy, with no changes in weight, appearance or behavior for the duration, which is consistent with expectations for a natural reservoir host. The magnitude and duration of KASV viremia suggests that bat-to-tick transmission would be possible during feeding by *O. (R.) faini*. Additionally, the shedding data indicate that KASV is likely to be transmitted bat-to-bat and underscores the potential for spillover to humans or other vertebrate species via feces, oral secretions and urine.

## **F. Pathology**

All four known KASV-infected humans survived the infection, which is fortunate, but also, specific tropisms of this virus in humans remain conjectural. Hepatotropism and severe liver lesions are key pathologic features of other fatal bunyaviral infections in humans, including CCHFV and RVFV.<sup>26,58</sup> Rift Valley fever has complex transmission cycle involving mosquitoes, wildlife, livestock and humans.<sup>58</sup> The disease is most severe in young livestock, especially lambs. While RVFV infections in humans are typically mild, severe and even fatal disease does occur in a small percentage of infected people.<sup>58</sup> A retrospective study of the histologic lesions in sheep, cow, goat and human samples obtained during a large 2006-2007 Eastern African RVFV outbreak revealed that extensive hepatic necrosis without prominent inflammatory cell infiltrates was the most distinct histopathologic lesion across all species.<sup>124</sup> In severe RVFV, fibrinous vasculitis, sinusoidal fibrin deposition, and mineralization of necrotic hepatocytes may also occur in the liver. Eosinophilic intranuclear inclusion bodies are occasionally seen in degenerate

hepatocytes, although ultrastructurally these inclusions have been shown to be composed of filaments, not viral particles.<sup>88</sup>

Leopards Hill virus (LPHV), a nairovirus closely related to KASV, was identified in liver and lung (16/38 samples) of giant leaf-nosed bats (*Hipposideros gigas*) collected at Leopards Hill cave, Zambia.<sup>62</sup> Overt signs of illness were not reported in the bats from which the virus was isolated, and gross and histologic lesions were not reported. However, mice inoculated with one of the LPHV strains (11SB23) developed fatal disease characterized by severe hepatocellular degeneration and necrosis, intestinal hemorrhage, villus ulcerations, and splenic necrosis, lesions similar to those reported in fatal CCHFV infections.<sup>62</sup>

Experimental studies of bunyaviruses in bats are limited, as are descriptions of lesions in bats with confirmed infections. Preliminary histopathologic evaluation of KASV-infected ERBs tissues revealed inflammatory lesions in the liver, which was suggestive of virus replication; these findings prompted an in-depth analysis of KASV tissue tropism and clearance in ERBs using *in situ* hybridization, immunohistochemistry and digital image analysis; the results of these studies are detailed in Chapter 4 of this dissertation.

This chapter provides an overview of the relevant literature pertaining the unique natural history traits of bats which might favor them as viral hosts, with a focus on the ERB and details of the ERB immune response to Marburg virus, which thus far, aligns with the theory of disease tolerance. A brief overview of the mammalian immune response to viruses, much of which can be extrapolated to ERBs is provided. Finally, a comprehensive overview of the emergence, ecology, associated pathology, virology and virus order- or family-level immune evasion mechanisms for three negative sense RNA viruses – MARV, KASV, and SOSV – for which the ERB is a confirmed or putative reservoir host is presented as a necessary introduction to the

virus-host relationships which the foundation upon which the remainder of this dissertation was built.

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## CHAPTER 2

# HISTOPATHOLOGIC AND IMMUNOHISTOCHEMICAL EVALUATION OF INDUCED LESIONS, TISSUE TROPISM AND HOST RESPONSES FOLLOWING EXPERIMENTAL INFECTION OF EGYPTIAN ROUSETTE BATS (*ROUSETTUS AEGYPTIACUS*) WITH THE ZOONOTIC PARAMYXOVIRUS, SOSUGA VIRUS<sup>1</sup>

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<sup>1</sup> Kirejczyk SGM, Amman BR, Schuh AJ, Sealy TK, Albariño CG, Zhang J, Brown CC and Towner JS. 2022 *Viruses*. 14(6), 1278.

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## ABSTRACT

Ecological and experimental infection studies have identified Egyptian rousette bats (ERBs; *Rousettus aegyptiacus*: family Pteropodidae) as a reservoir host for the zoonotic rubula-like paramyxovirus Sosuga virus (SOSV). A serial sacrifice study of colony-bred ERBs inoculated with wild-type, recombinant SOSV identified small intestines and salivary gland as major sites of viral replication. In the current study, archived formalin-fixed paraffin-embedded (FFPE) tissues from the serial sacrifice study were analyzed in depth—histologically and immunohistochemically, for SOSV, mononuclear phagocytes and T cells. Histopathologic lesion scores increased over time and viral antigen persisted in a subset of tissues, indicating ongoing host responses and underscoring the possibility of chronic infection. Despite the presence of SOSV NP antigen and villus ulcerations in the small intestines, there were only mild increases in mononuclear phagocytes and T cells, a host response aligned with disease tolerance. In contrast, there was a statistically significant, robust and targeted mononuclear phagocyte cell responses in the salivary glands at 21 DPI, where viral antigen was sparse. These findings may have broader implications for chiropteran–paramyxovirus interactions, as bats are hypothesized to be the ancestral hosts of this diverse virus family and for ERB immunology in general, as this species is also the reservoir host for the marburgviruses Marburg virus (MARV) and Ravn virus (RAVV) (family *Filoviridae*).

**KEYWORDS:** Sosuga virus; zoonoses, paramyxovirus, *Rousettus aegyptiacus*, Egyptian rousette bat, natural reservoir, histopathology, immunohistochemistry

## INTRODUCTION

Bats (Order: Chiroptera) are drawing increased attention as natural reservoirs for viruses of public health importance, many of which are fatal for humans but cause little to no clinical disease in their bat hosts. These viruses include lyssaviruses such as rabies virus (family *Rhabdoviridae*), Hendra and Nipah viruses (NiV; family *Paramyxoviridae*), and the marburgviruses MARV and RAVV (family *Filoviridae*) [1–11]. Bats are hypothesized to be ancestral reservoirs of paramyxoviruses and coronaviruses, two virus families known for promiscuity among hosts [12–15]. Members of the family *Paramyxoviridae* comprise a diverse group of single-stranded, negative-sense RNA viruses and account for 1254 (9.0%) of the 13,973 publicly available genomic sequences derived from bats; 47 (3.7%) of these bat-derived paramyxovirus sequences are full length [16,17].

Within the family *Paramyxoviridae*, the subfamily *Rubulavirinae* comprises a diverse group of viruses infecting a variety of mammalian hosts, including humans, nonhuman primates, pigs, dogs and bats. In 2012, a novel rubula-like paramyxovirus, later named Sosuga virus (SOSV), was identified when a wildlife biologist became ill after performing field work on bats and rodents in South Sudan and Uganda, hence the name (South Sudan Uganda) [18].

Retrospective quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) testing of pooled bat liver and spleen samples collected from bats captured at the fieldwork sites revealed that Egyptian rousette bats (ERBs) were the likely source of SOSV spillover [5]. The ERB is widely distributed throughout Africa, small parts of the Middle East, the Eastern Mediterranean and the Indian subcontinent. In Equatorial Africa, ERBs live in large, dense colonies, sometimes in excess of 100,000 individuals [10]. Ecological, epidemiological and experimental infection studies have identified ERBs as the primary source of marburgvirus spillover to primates,

including humans [6,7,10,19–21]. ERBs have been associated with a number of novel viruses, including diverse, yet unnamed rubulaviruses and Bukakata orbivirus, two paramyxoviruses related to the henipaviruses, Achimota viruses 1 and 2 and Kasokero virus (KASV; family *Nairoviridae*), which have known zoonotic potential [22–26].

Experimental infection studies in colony raised ERBs, using recombinant SOSV confirmed that this bat species is susceptible to infection, as evidenced by viremia, viral replication in multiple tissues, seroconversion and shedding in the feces, saliva and urine, all in the absence of overt clinical disease [27]. Tissue viral loads were highest in the small intestines, viral shedding events were most frequent in the feces and a subset of SOSV-infected bats had small intestinal lesions [27]. Acute inflammation of the parotid salivary gland ducts was an unusual lesion in one 6 DPI bat that also had a high viral load in the salivary gland tissue and shedding into the oral secretions prior to necropsy [27]. These microscopic observations prompted a more in-depth investigation into the small intestines and salivary glands as target tissues, sources of shedding into the environment, and potential sites of localized immune responses to SOSV infection. Therefore, the goals of the current study were to describe and score the spectrum of histopathologic lesions in the small intestines and salivary glands to determine the tissue and cellular tropism of SOSV using immunohistochemical methods targeting tissues with high viral loads from the serial sacrifice study (small and large intestines, salivary gland, spleen and lymph nodes), and to characterize and quantify immune cell populations in the small intestines and salivary glands lesions using immunohistochemistry and whole-slide digital imaging analysis.

## **MATERIALS AND METHODS**

### **A. Tissue Samples**

The ERB tissues used in the present study were obtained during a previously published serial sacrifice study performed under biosafety level 4 (BSL-4) conditions in accordance with safety regulations at the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) [27]. The experimental infections were conducted with approval from the CDC Institutional Animal Care and Use Committee (IACUC protocol 2874TOWBATC), and in accordance with the animal welfare guidelines set forth in the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, National Research Council of the National Academies 2011) [27]. Husbandry, housing conditions and diet for the duration of the experiment were previously described [27]. Briefly, food consisted of a variety of fresh fruit dusted with a protein vitamin supplement (Lubee Bat Conservancy, Gainesville, FL, USA). Water was provided ad libitum. The SOSV isolate was a recombinant virus genetically identical to that obtained from the index human case (GenBank ID: KF774436.1) [18]. Bats were euthanized under anesthesia via cardiac exsanguination at days 3, 6, 9 and 21 post-inoculation (DPI). Three control bats were mock-inoculated and euthanized at 21 DPI. Following tissue collection, the bat carcasses were immersed in 10% neutral buffered formalin (10% NBF) for a minimum of 7 days, with a complete change in formalin after day 3. Tissues were collected for histology within 3–4 weeks.

### **B. Histology**

Representative sections from the following formalin-fixed tissues were prepared from bats at all time points and controls for histopathology: brain, heart, lung, trachea, thymus, liver, stomach, at least 13 cross-sections of small intestine (duodenum with pancreas, jejunum, and

ileum), large intestine, spleen, kidney, adrenal gland, salivary gland, submandibular lymph node, axillary lymph node, skin from the inoculation site, skin from the chest region and pectoral muscle. Tissue sections were routinely processed, embedded in paraffin, sectioned at 4–5 µm thickness, mounted on glass slides and stained with hematoxylin and eosin (H&E).

### C. Immunohistochemistry (IHC)

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded (FFPE) sections of small intestine, large intestine, spleen, axillary lymph node and salivary gland using a purified rabbit monoclonal antibody targeting the SOSV nucleoprotein (NP) (final dilution 1:2000; GenScript, Piscataway, NJ, USA). Tissue sections were heated at 60 °C and deparaffinized by washing in Hemo-De® (Fisher Scientific, Pittsburgh, PA, USA). Antigen retrieval was achieved by applying an unmasking preheat solution (1× Antigen Unmasking solution; Vector Laboratories, Burlingame, CA, USA) and microwaved at power level 10 for 2 min, followed by an additional 20 min of heating at power level 1. Slides were washed and then treated with a 1:10 dilution of Power Block Universal Blocking Reagent 10X (BioGenex Laboratories, San Ramon, CA, USA). The anti-SOSV NP antibody was applied and slides were incubated overnight at 4 °C. Primary Antibody Enhancer (Thermo Scientific, Waltham, MA, USA) was applied the following day to each slide for 20 min at room temperature. An alkaline phosphatase-based polymer detection system (Thermo Scientific, Waltham, MA, USA) with Fast Red chromogen was used. The slides were counterstained with hematoxylin (Leica, Richmond, IL, USA) and cover slipped with Permount for a permanent record. A formalin-fixed paraffin-embedded cell pellet created from a SOSV-infected Vero cell monolayer was used as a positive control. Tissues from the three non-infected ERBs were used as negative controls.

Immunohistochemical stains for ionized calcium-binding adapter molecule 1 (Iba1), a pan-macrophage and dendritic cell marker, and CD3, a T cell marker, were performed on sections of small intestine and salivary gland using an automated stainer (Nemesis 3600, Biocare Medical, Concord, CA, USA) at the University of Georgia Histology Laboratory. For the Iba1 protocol, a rabbit polyclonal antibody directed against Iba1 (1:8000 final dilution; Wako, Richmond, VA, USA.; catalog # 019-19741) was incubated on the tissue for 60 min. Antigen retrieval was performed using Antigen Retrieval Citra Solution 10X (BioGenex) at a 1:10 dilution for 15 min at 110 °C. For the CD3 protocol, a rabbit polyclonal antibody targeting the CD3 epsilon subunit (1:1000 final dilution; Dako, Carpinteria, CA, USA.; catalog # A0452) was incubated on the tissue for 60 min. For the cleaved caspase 3 protocol, a rabbit polyclonal antibody directed against activated (cleaved) caspase 3 (1:8000 final dilution; Biocare Medical, Concord, CA, USA.; catalog # CP229B) was incubated on the tissue for 60 min. Antigen retrieval was performed using Antigen Retrieval Citra Solution 10X (BioGenex) at a 1:10 dilution for 15 min at 110 °C. A biotinylated rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) was used to detect the antibody targets, and the immunoreactions were visualized using a 3,3-diaminobenzidine substrate (DAB; Dako, Santa Clara, CA, USA) for 12 min. The slides were counterstained with hematoxylin and cover slipped. Positive control tissues included canine brain and lymph node for Iba1 and CD3, respectively. Each slide also contained internal controls (various lymphoid tissues). Immunostains were not performed on the salivary gland tissue of one bat (289953) due to tissue limitations.

#### D. Histopathological Lesion Scoring

Histopathological lesions associated with SOSV infection in the small intestines and salivary glands were scored. Semi-quantitative scoring systems were designed and applied to the

lesions in each tissue based on principles previously described in order to fully capture the spectrum of lesions observed and to allow for comparison between individual bats and time points [27]. At least 13 sections of small intestine (average = 18.4; range = 13–28) from each bat were reviewed in an unblinded manner to determine lesions of interest in SOSV-infected bats. Sections were then reviewed in a blinded manner without knowledge of time point or infection status and assigned a score for each of the lesions observed: villus epithelial discontinuity or ulceration (0 = absent, 1 = focal, 2 = multifocal, 3 = frequent); accumulation of eosinophilic fluid in the lamina propria (0 = absent, 1 = minimal to mild, 2 = moderate, 3 = marked); hypercellularity of the lamina propria (0 = none, 1 = minimal to mild, 2 = moderate, 3 = marked); epithelial cell apoptosis and/or tingible body macrophages in the lamina propria (0 = absent, 1 = rare, 2 = multifocal, 3 = frequent); and villus fusion (0 = absent, 1 = present). A composite score was generated for each bat by adding the individual criterion scores. The percentage of small intestinal sections affected for each bat was calculated by dividing the number of affected sections by the total number of sections examined.

For the salivary glands, sections were reviewed in a blinded manner without knowledge of time point or infection status and assigned a score for each of the lesions observed: sialodochitis (0 = absent, 1 = minimal to mild, 2 = moderate, 3 = marked); sialadenitis (0 = absent, 1 = minimal to mild, 2 = moderate, 3 = marked); duct epithelial cell apoptosis (0 = absent, 1 = rare, 2 = multifocal, 3 = frequent); and epithelial syncytia (0 = absent, 1 = present). A composite score was generated for each bat by adding the individual criterion scores.

#### E. SOSV Immunohistochemical Scoring

Tissues subjected to the anti-SOSV NP immunohistochemical procedure were scored as follows: - = no staining; + = <1 cell per 400× field; and ++ = >2 cells per 400× field.

Immunostaining on intestinal tissue from bat 283936 (3 DPI) and salivary gland tissue from bats 290040 (21 DPI) and 290631 (control) was not performed due to tissue limitations.

#### F. Whole-Slide Digital Image Analysis (CD3 and Iba1 Staining Quantifications)

Tissues subjected to immunostaining for CD3 and Iba-1-immunostained scanned at 200× magnification using a whole-slide scanner (Aperio ScanScope XT, Aperio Technologies, Inc., Vista, CA, USA) and imported into a freely available open-source bioimage analysis software (QuPath version 0.3.0, Queen's University Belfast, Belfast, Northern Ireland, UK) [29]. A region of interest (ROI) was manually generated around each of the small intestine and salivary gland sections. Immunolabeled cells were characterized by DAB binding and hematoxylin counterstaining. Labeled cells were segmented from negative cells and background staining by creating a threshold for each slide (DAB threshold range = 0.14–0.56) and using the other default parameters in the Create Threshold tool (gaussian sigma = 1.5  $\mu$ m, background radius = 15  $\mu$ m, cell detection threshold = 0.1, and detection object diameter = 25 pixels). False positives based on size and morphology of the cells were removed, as was background staining and artifact.

The Fast Cell Counts tool was applied to each ROI to generate a cell count for CD3. For CD3, cell counts were normalized by dividing the total cell count by the area for each ROI in microns squared to obtain the number of cells per micron squared. A manual assessment of immunopositive cell morphology was performed for each slide as a means of quality control. For Iba1, % pixel positivity was obtained for each ROI using the Create Threshold tool, since the morphology (numerous cell processes) and occasional dense clustering of immunolabeled cells created difficulty in establishing accurate cell counts in some ROIs.

## G. Statistical Analyses

Statistical analyses were performed in Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). For the histologic scores and the quantified Iba1 and CD3+ immunostaining, values at each time point (n = 3 per time point) were compared to those of the control bats using Kruskal–Wallis’ one-way analysis of variance (ANOVA) test followed by Dunnett’s multiple comparison test if the ANOVA demonstrated significant differences between groups ( $p < 0.05$ ). Correlations between histologic scores and time points were analyzed using a simple linear regression.

## RESULTS

### A. Histopathology

Representative histologic sections of brain, heart, lung, trachea, thymus, liver, stomach, large intestine, spleen, kidney, adrenal gland, submandibular lymph node, axillary lymph node, pectoral muscle and skin from the inoculation site, wing and chest region were the same for SOSV-infected bats as the controls, with the following few exceptions. Four SOSV-infected bats ( $n = 1$  at 3, 6, 9 and 21 DPI) had minimal to mild numbers of perivascular inflammatory cells in the lungs, one bat (6 DPI) had hyperplasia of the bronchus-associated lymphoid tissue (BALT) in the lungs, and one bat (3 DPI) had mild lymphohistiocytic interstitial nephritis.

#### i. *Small Intestines*

Lesions in SOSV-infected bats ( $n = 12$ ; Figure 2-1) included villus epithelial ulcerations (8/12 bats; 75%), expansion of the lamina propria by eosinophilic fluid (8/12; 75%), lamina propria hypercellularity (10/12; 83%), increased apoptotic cells in the epithelium or just beneath the epithelium in the lamina propria (6/12; 50%), villus fusion (12/12; 100%) and epithelial syncytia (2/12; 17%). Lesions were randomly distributed throughout the representative intestinal

segments (duodenum, jejunum, and ileum). Although the percentage of affected sections increased slightly over time from 17.8 % at 3 DPI to 39.4 % at 21 DPI, the association was not statistically significant.

The first detectable tissue alteration at 3 DPI was the expansion of the lamina propria by a paucicellular eosinophilic fluid that elevated the epithelium and compressed the cellular core. Cellularity of the lamina propria varied between bats and was typically minimal to mild, although a few bats including one 6 DPI bat exhibited moderate hypercellularity with a predominantly mononuclear cell population that included numerous tingible body macrophages. Accumulation of fluid in the lamina propria resulted in expansion of the villus tips, and this was most evident in the 9 and 21 DPI bats (Figure 1B,C,G,H). Villus tip ulcerations were accompanied by degenerative changes in enterocytes adjacent to the epithelial defect, including loss of nuclear polarity, cytoplasmic hypereosinophilia and vacuolation and nuclear karyolysis or pyknosis (Figure 1C). Increased apoptotic enterocytes or subepithelial tingible body macrophages were most numerous in two of the 9 DPI bats (287014 and 287253; Figure 1E, F).

At 21 DPI, villus ulceration was extensive, with spilling of fluid from the lamina propria into the gastrointestinal lumen (Figure 1G, H). Rare epithelial syncytia were observed within the lamina propria at the villus tips or within the gastrointestinal lumen (Figure 1I). Beginning with the 9 DPI bats, crypt epithelial cells exhibited the following changes indicative of a regenerative response: lengthening of the crypt, increased cytoplasmic basophilia and increased mitotic figures. Villus fusion was seen multifocally in all SOSV-infected bats and in one control bat that also had a mild to moderate multifocal eosinophilic and lymphocytic enteritis and mild hepatitis as background lesions. The remaining control bats had no lesions in the small intestines.

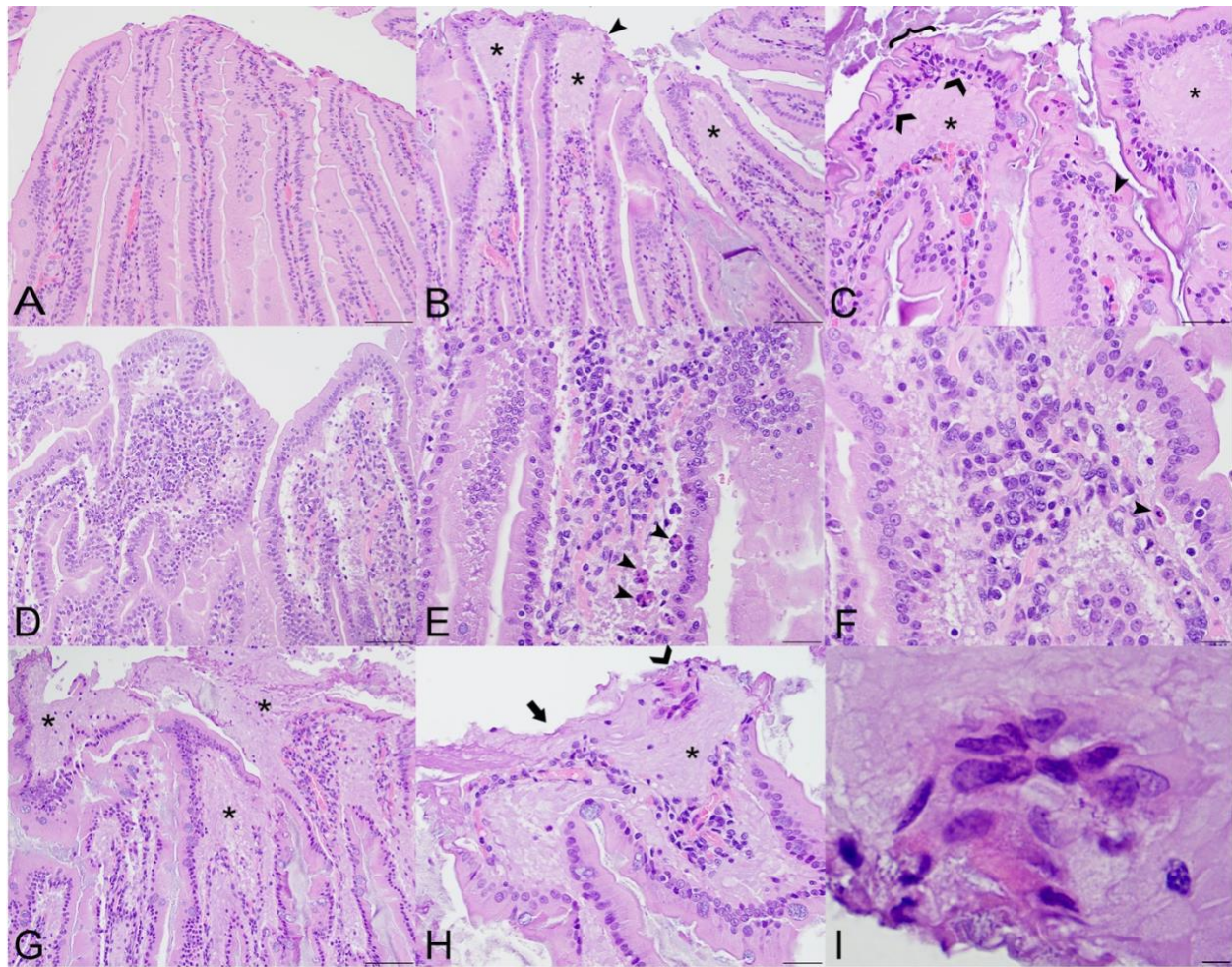


**Figure 2-1.** Histopathologic lesions in the small intestines of Sosuga virus (SOSV)-infected Egyptian rousette bats euthanized at 9 and 21 days post-inoculation (DPI). (A) Jejunum, control bat 290195. The intestinal villi are tall and slender with thin lamina propria cores. HE stain; original magnification = 200 $\times$ ; bar = 50  $\mu$ m. (B) Jejunum, 9 DPI bat 287756. The villus tips are swollen with an accumulation of a paucicellular, eosinophilic fluid (\*) that elevates the epithelium and compresses the lamina propria core. A villus tip is focally ulcerated (arrowhead). HE stain; original magnification = 200 $\times$ ; bar = 50  $\mu$ m. (C) Jejunum, 9 DPI bat 287756. Enterocytes lining villus tips show a range of degenerative changes, including piling of nuclei, loss of cell borders and nuclear polarity (†) and nuclear fragmentation (closed arrowhead). Several mononuclear cells are present just beneath the epithelium (open arrowheads) and the lamina propria is expanded by eosinophilic fluid (\*). HE stain; original magnification = 400 $\times$ ; bar = 20  $\mu$ m. (D) Jejunum, 9 DPI bat 287014. The villi are multifocally fused and the lamina propria is congested and mildly hypercellular. HE stain; original magnification = 200 $\times$ ; bar = 50  $\mu$ m. (E) Jejunum, 9 DPI bat 287014. The hypercellular lamina propria contains numerous tingible body macrophages beneath the epithelium (arrowheads). Inset: higher magnification of a tingible body macrophage from another but similarly affected villus showing phagocytosed cellular and nuclear debris. HE stains; original magnifications = 400 $\times$ ; bar = 20  $\mu$ m. (F) Jejunum, 9 DPI bat 287014. Higher magnification image of the same bat demonstrating a congested and mildly hypercellular lamina propria containing a mixed inflammatory cell

population composed of rounded to elongate macrophages with abundant cytoplasm, lymphocytes, and rare eosinophils and plasma cells. HE stain; original magnification = 600×; bar = 20 µm. **(G)** Duodenum, 21 DPI bat 287953. The villus tips are obscured by large ulcerations, accumulated eosinophilic fluid in the lamina propria (\*) and multifocal villus fusion. Eosinophilic fluid spills into the lumen at the top of the image (\*). HE stain; original magnification = 200×; bar = 50 µm. **(H)** Duodenum, 21 DPI bat 287953. The villus tip is folded over and has a focally extensive area of ulceration (arrow). At the top right of the image, a raft of syncytial epithelial cells (open arrowhead) is surrounded by eosinophilic fluid. HE stain; original magnification = 400×; bar = 20 µm. **(I)** Duodenum, 21 DPI bat 287953. High magnification image of the epithelial cell syncytium showing hypereosinophilic granular cytoplasm and karyolytic or pyknotic nuclei. HE stain; original magnification = 1000×; bar = 10 µm.

## ii. *Salivary Glands*

The spectrum of salivary gland lesions in SOSV-infected ERBs ( $n = 12$ ; Figure 2-2) included the presence of apoptotic cells within the interlobular duct epithelium (10/12; 83.3%), sialadenitis (7/12; 58.3%), sialodochitis (5/12 bats; 41.2%) and syncytial cells (2/12; 17%). Eleven of the 12 SOSV-infected bats (91.7%) had at least one of the aforementioned lesions and the only SOSV-infected bat without salivary gland lesions was a 3 DPI bat.

A mixed inflammatory cell infiltrate targeting the interlobular ducts (sialodochitis) was first observed in a 6 DPI bat (284899), where it was widespread and associated with epithelial cell degeneration, necrosis and apoptosis and rare syncytia formation (Figure 2-2B). Sialodochitis was minimal to mild in the 9 and 21 DPI bats and it was occasionally associated with epithelial hyperplasia, piling of epithelial cell nuclei, loss of nuclear polarity and rare epithelial syncytia (Figure 2-2C, D). At 9 and 21 DPI, there were also scattered aggregates of mononuclear cells within the lobular interstitium and rare acinar cell degeneration (Figure 2-2E, F). Intraductal cells exhibiting microscopic features consistent with apoptosis (shrunken cells with rounded cytoplasmic borders and pyknotic and/or fragmented nuclei) were first observed within the interlobular duct epithelia at 3 DPI, more frequently in ducts with inflammatory cell infiltrates but also in the absence of overt inflammation on the HE stain. Apoptotic cells were

most numerous in two 21 DPI bats including the supershedder bat who shed SOSV periodically into the saliva during the serial sacrifice study (bat 289953; Figure 2-2D bottom left inset).

Intraductal apoptotic bodies exhibited moderate diffuse cytoplasmic immunopositivity for cleaved caspase 3 (Figure 2-2D top left inset). One control bat had a small focus of lymphocytic inflammation adjacent to a sialolith. The remaining control bat salivary glands were histologically unremarkable.

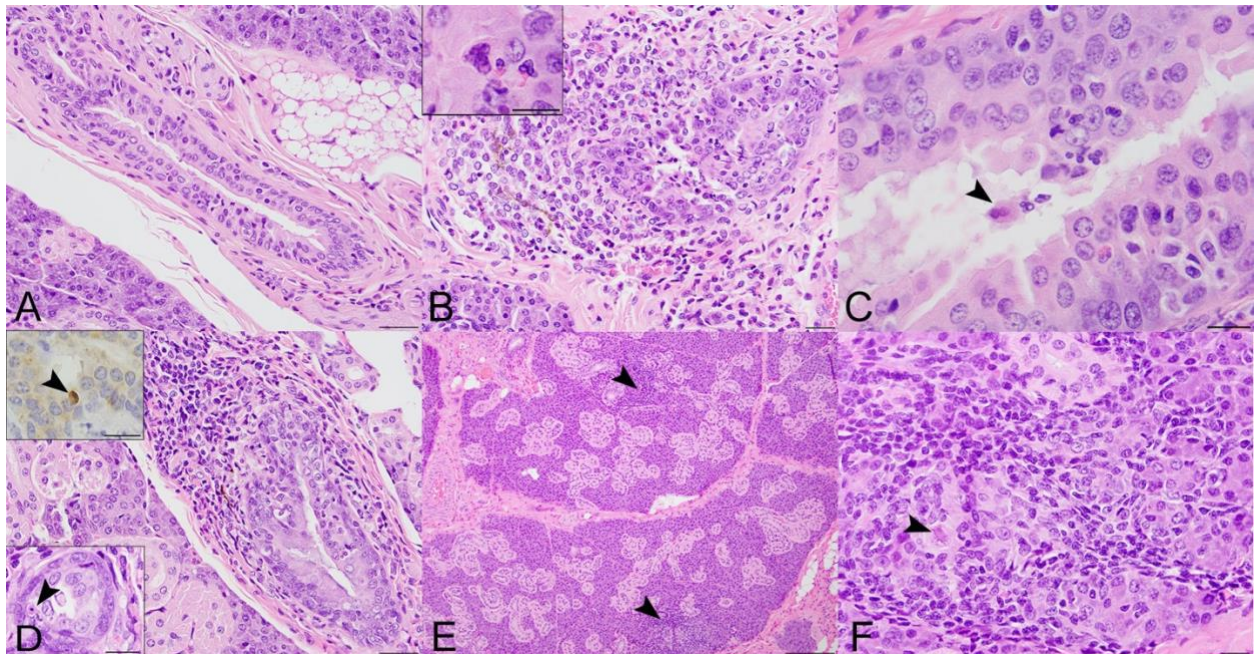


**Figure 2-2.** Histopathologic lesions in the salivary glands of SOSV-infected Egyptian rousette bats euthanized at 6, 9 and 21 DPI. **(A)** Salivary gland, control bat 290195. A normal interlobular duct is 1–3 cell layers thick and contains very low numbers of intraepithelial and peri-ductular resident immune cells. HE stain; original magnification = 400×; bar = 20 µm. **(B)** Salivary gland, 6 DPI bat 284899. Acute sialodochitis characterized by a robust infiltrate of mononuclear and granulocytic inflammatory cells that expands the duct wall and surrounding connective tissue. Granulocytic cells, mononuclear cells and cellular debris fill and partially occlude the duct lumen. Inset: The luminal aspect of a different, but similarly affected duct contains an epithelial syncytium and several granulocytes. HE stains; original magnifications = 400× (main image; bar = 20 µm) and 1000× (inset; bar = 10 µm). This bat was qRT-PCR positive for SOSV in the salivary gland at necropsy and had SOSV RNA in the oral swabs on days 5 and 6 PI. **(C)** Salivary gland, 9 DPI bat 287014. The duct is multifocally infiltrated by low numbers of mononuclear and granulocytic cells. The lumen contains low numbers of neutrophils and a binucleated cell with abundant granular cytoplasm (suspected syncytium). HE stain; original magnification = 400×; bar = 20 µm. **(D)** Salivary gland, 21 DPI bat 289338. An interlobular duct is focally infiltrated and expanded by a mixture of mononuclear and granulocytic inflammatory

cells. Bottom left inset: The wall of a smaller but similarly affected duct contains an apoptotic body that is shrunken and rounded with distinct cell borders and a pyknotic nucleus (arrowhead). HE stains; original magnifications = 400× (main image; bar = 20 µm) and 1000× (inset; bar = 10 µm). Top left inset: An apoptotic body in the wall of the same duct as the lower left inset has diffuse, moderate cytoplasmic immunoreactivity for cleaved caspase 3 (arrowhead). Immunohistochemical stain with 3-3'-Diaminobenzidine (DAB; brown) chromogen to visualize cleaved caspase 3 antigen and hematoxylin counterstain. Original magnification = 600×; bar = 20 µm. (E) Salivary gland, 21 DPI bat 290040. Low magnification image of salivary gland acinar tissue showing multiple foci of mononuclear cells which expand the interstitium and focally obscure the acini. HE stain; original magnification = 200×; bar = 50 µm. (F) Salivary gland, same 21 DPI bat as previous image. Higher magnification of one hypercellular focus showing interstitial expansion with numerous mononuclear inflammatory cells. One intralobular duct epithelial cell exhibits cytoplasmic swelling, peripheralization of nuclear material and accumulation of eosinophilic material within the nucleus suggestive of intranuclear inclusion material. HE stain; original magnification = 400×; bar = 20 µm.

### iii. *Histopathological Lesion Scores*

The composite lesion scores for the small intestinal and salivary gland lesions are presented for each bat in Figure 2-3. For the small intestines, histologic lesion scores were similar for the 3 and 6 DPI. Compared to the controls, the mean scores were increased for the 9 and 21 DPI bats; however, these differences were not statically significant, and the scores did not correlate with time (Figure 3A). Salivary gland scores were positively correlated with time ( $r^2 = 0.55$ ;  $F = 16.51$ ;  $p = 0.0013$ ).

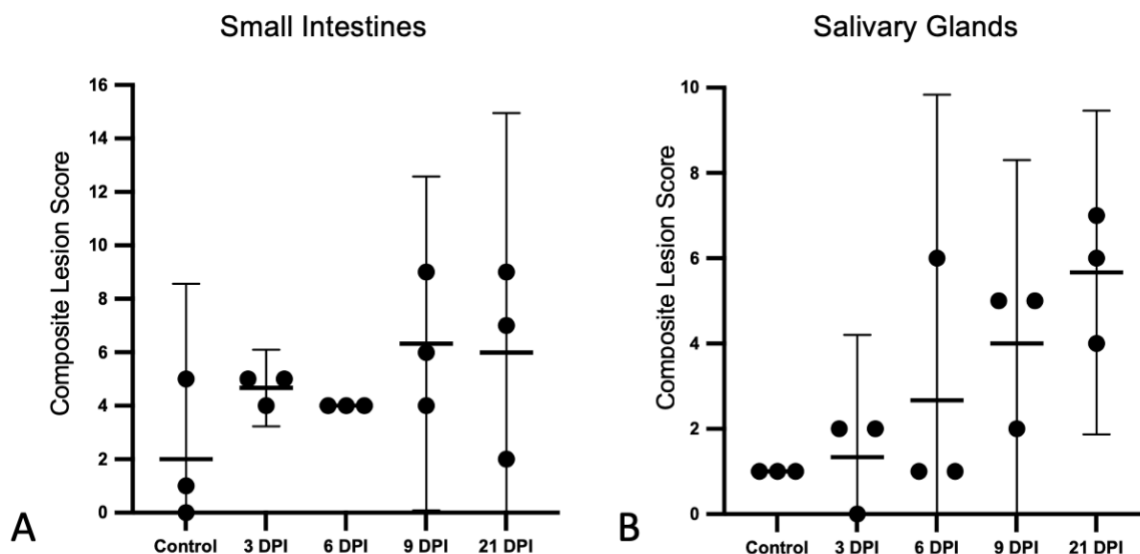


**Figure 2-3.** Histopathologic lesion scores in the small intestines and salivary glands of SOSV-infected Egyptian rousette bats. Individual groups are represented on the x axis and each dot represents the score for a single bat. Bars represent the mean and lines represent the  $\pm 95\%$  confidence interval. Control bats were euthanized at 21 DPI. **(A)** Criteria used to generate a composite small intestine lesion score for each bat included: villus epithelial discontinuity or ulceration, accumulation of eosinophilic fluid in the lamina propria, hypercellularity of the lamina propria, epithelial cell apoptosis and/or tingible body macrophages in the lamina propria and villus fusion. **(B)** Salivary gland scores were positively correlated with time. Criteria used to generate a composite salivary gland lesion score for each bat included: sialodochitis, sialadenitis, duct epithelial cell apoptosis and epithelial syncytia.

## B. Immunohistochemistry

### i. *SOSV Tissue tropism*

Immunohistochemical signal for SOSV NP antigen was detectable in one or more of the following tissues for 9 of the 12 (75%) SOSV-inoculated bats: small and large intestines, salivary gland, axillary lymph nodes and spleen (Table 2-1). In the intestines, viral antigen was localized to the cytoplasm of mononuclear cells in the lamina propria ( $n = 3$ ; Figure 2-4A), within fluid in the gut lumen ( $n = 2$ ) and in the gastrointestinal-associated lymphoid tissue ( $n = 2$ ). In the salivary glands, viral antigen was observed within or surrounding the interlobular ducts ( $n = 2$ ; Figure 2-4B). Of the tissues tested, viral antigen was most frequently detected in the

axillary lymph nodes ( $n = 7$ ); it was present in two of the three 21 DPI bats and in at least one bat at all other time points. In the lymph nodes, viral antigen was localized to the cytoplasm of histiocytic cells within the sinuses, germinal centers and paracortical regions (Figure 2-4C). In the spleen, antigen was present in the cytoplasm of macrophages within the red pulp ( $n = 4$ ; Figure 2-4D). Viral antigen was not detected in tissues from the three control bats.

**Table 2-1.** Distribution of Sosuga virus nucleoprotein antigen in selected tissues from SOSV-infected Egyptian rousette bats euthanized at 3, 6, 9 and 21 DPI.

| DPI | Bat ID  | Small Intestines | Salivary Gland | AxLN | Spleen | Large Intestinal GALT |
|-----|---------|------------------|----------------|------|--------|-----------------------|
| 3   | 283936  | NP               | -              | +    | -      | NP                    |
|     | 284049  | -                | -              | +    | -      | -                     |
|     | 284459  | -                | -              | -    | -      | -                     |
|     | 284509  | -                | -              | ++   | -      | -                     |
| 6   | 284899  | +                | +              | +    | ++     | -                     |
|     | 285146  | -                | -              | -    | -      | -                     |
|     | 287014  | -                | -              | -    | -      | -                     |
|     | 287253  | -                | -              | -    | +      | -                     |
| 9   | 287756  | +                | -              | +    | +      | +                     |
|     | 289338  | -                | +              | -    | -      | -                     |
|     | 289953* | +                | -              | +    | +      | +                     |
|     | 290040  | -                | -              | +    | -      | -                     |
| 21  | 290195  | -                | -              | NP   | NP     | -                     |
|     | 290494  | -                | -              | -    | -      | -                     |
|     | 290631  | -                | -              | -    | -      | -                     |
|     | Control | -                | -              | -    | -      | -                     |

Abbreviations: DPI = days post-inoculation, AxLN = axillary lymph node, GALT = gastrointestinal-associated lymphoid tissue, NP = not performed due to tissue limitations.

IHC Scores: - = no staining; + = rare staining (<1 cell per 400× field); ++ = moderate staining (>2 cells per 400× field).

\* Indicates the supershedder bat identified during the serial euthanasia study [27].

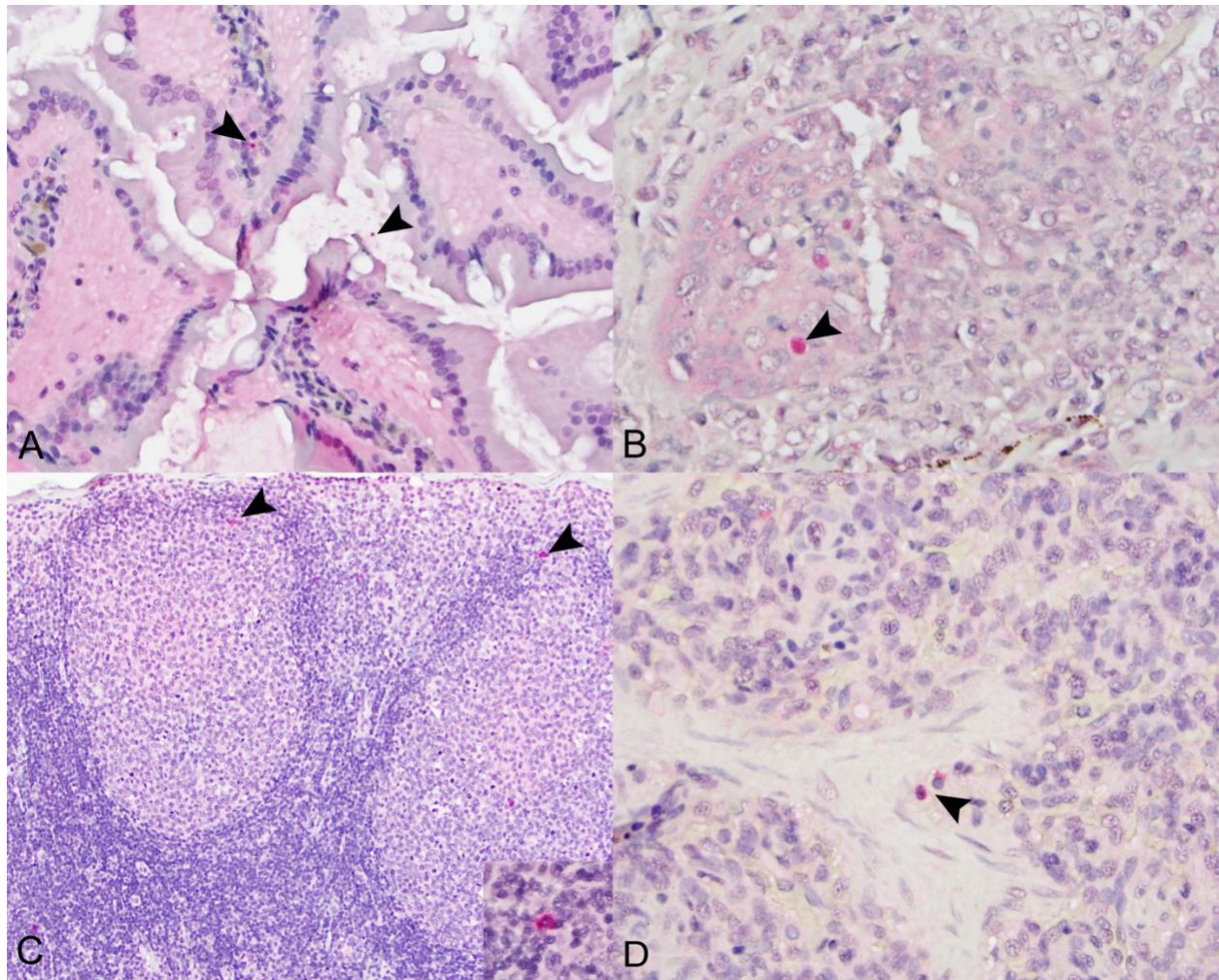


**Figure 2-4.** Immunohistochemical localization of SOSV nucleoprotein (NP) antigen in experimentally infected Egyptian rousette bats euthanized at 6, 9 or 21 DPI. **(A)** Small intestines, 21 DPI bat 289953. Granular, intracytoplasmic NP antigen is present within the villus lamina propria core and within the lumen (arrowheads). Immunohistochemical stain with Fast Red chromogen and hematoxylin counterstain; original magnification = 400×. **(B)** Salivary gland, 6 DPI bat 284899. Multifocal cytoplasmic immunolabeling for NP antigen within the wall of an inflamed interlobular salivary duct (arrowhead). Immunohistochemical stain with Fast Red chromogen and hematoxylin counterstain; original magnification = 400×. **(C)** Axillary lymph node, 6 DPI bat 284509. Viral nucleoprotein antigen (arrowheads) is present within the cytoplasm of histiocytic cells in the cortical germinal centers and in the paracortical regions (arrowheads and inset). Immunohistochemical stain with Fast Red chromogen and hematoxylin counterstain; original magnifications = 200× (main image) and 600× (inset). **(D)** Spleen, 9 DPI bat 287014. NP antigen (arrowheads) is present within the cytoplasm of few histiocytic cells in

the red pulp. Immunohistochemical stain with Fast Red chromogen and hematoxylin counterstain; original magnification = 600×.

ii. *Quantification and Spatial Analyses of Iba1- and CD3-Immunopositive Cell*

*Populations*

The subcellular localization for the Iba1 and CD3 antibodies was appropriate for both markers (cytoplasmic for Iba1 and membranous to cytoplasmic for CD3).

a. Small Intestines

There were no statistically significant changes in Iba1+ positive pixel of CD3+ cell counts in the small intestines. There was a very mild (1.69-fold) increase in the percentage of Iba1+ pixels when comparing SOSV-infected bats euthanized at 21 DPI ( $n = 3$ ) to the controls (Figure 2-5A). In both infected and non-infected bats, Iba1+ cells were located predominantly within the lamina propria (Figure 2-6A–C). In SOSV-infected bats, Iba1+ cells occasionally formed small, dense clusters near the villus tips, particularly in fused villi (Figure 2-6B, C). In SOSV-infected bats, low numbers of Iba1+ cells were also present just beneath the epithelium, within the accumulated lamina propria fluid, and in the gut lumen, suggesting that these cells are responding to the tissue changes observed (Figure 2-6B, C). The morphology of Iba1+ cells within the lamina propria core was either mesenchymal (slender, elongate cells with long, branching cytoplasmic processes) or amoeboid (round to oval with large cell bodies and several, short cytoplasmic processes). Iba1+ cells within the lamina propria fluid of SOSV-infected bats often exhibited the amoeboid morphology (Figure 2-6C inset).

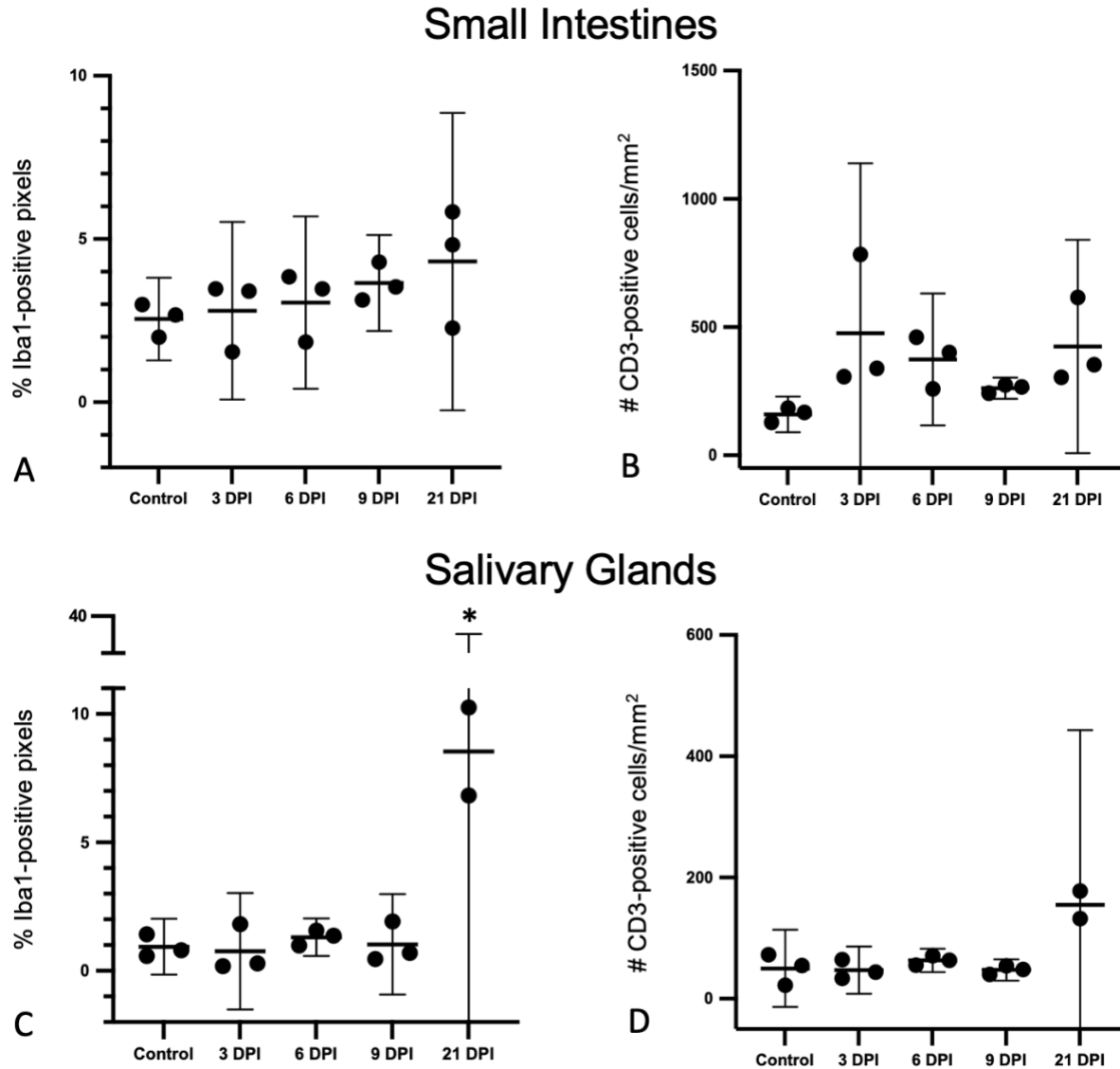


**Figure 2-5.** Iba1+ pixel percentages and CD3+ cell counts obtained by immunohistochemistry and whole-slide digital image analyses of the small intestines and salivary glands from SOSV-infected Egyptian rousette bats. Individual groups are represented on the x axis and each dot represents the score for a single bat. Bars represent the mean and lines represent the  $\pm 95\%$  confidence interval. Control bats were euthanized at 21 DPI. **(A)** Percentage of Iba1-immunopositive pixels in the small intestines. **(B)** Number of CD3-immunopositive cells in the small intestines per mm<sup>2</sup> tissue. **(C)** Percentage of Iba1-immunopositive pixels in the small intestines. The Iba1% positive pixel count was significantly increased in the salivary glands of SOSV-infected bats at 21 DPI relative to the controls (\*). **(D)** Number of CD3-immunopositive cells in the small intestines per mm<sup>2</sup> tissue.

CD3+ cell counts were highly variable between individual bats and although there were no statistically significant differences between groups, SOSV-infected bats tended to have higher

CD3+ cell counts in the intestines compared to the control bats. There were also spatial differences in the distribution of CD3+ cells relative to controls (Figure 2-5B). In the control bats, the majority of CD3+ cells were distributed within the epithelium, with fewer immunopositive cells in the villus core (Figure 2-6D). In SOSV-infected bats, the lamina propria core contained slightly increased numbers of CD3+ cells. Immunopositive cells were also present in the accumulated lamina propria fluid in low numbers (Figure 2-6E, F).

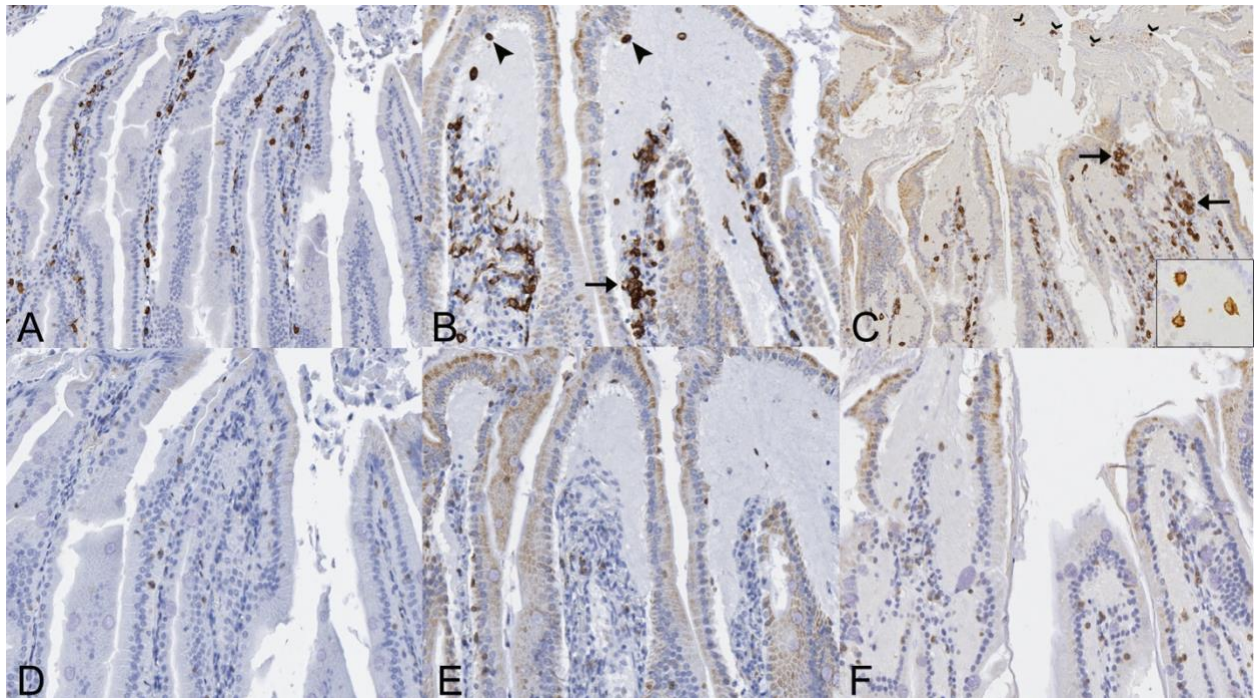


**Figure 2-6.** Distribution of Iba1- and CD3-immunolabeled cells in the small intestines of SOSV-infected Egyptian rousette bats euthanized at 9 and 21 DPI. (A) Iba1 immunohistochemistry (IHC), jejunum, control bat 290195. Iba1-immunolabeled cells (brown) are present in moderate numbers throughout the lamina propria core of the small intestinal villi. Original magnification = 200×. (B) Iba1 IHC, jejunum, 9 DPI bat 287756. Moderate numbers of Iba1-immunolabeled cells are present within the lamina propria core, occasionally in small clusters (arrow). Low numbers of Iba1+ cells with an amoeboid morphology are scattered within the lamina propria fluid and just beneath the epithelium at the villus tip (arrowheads). The small intestines of this bat had the highest viral load of all tissues tested during the serial sacrifice study. Original magnification = 200×. (C) Iba1 IHC, jejunum, 21 DPI bat 289953. The villus core and lamina propria contain moderate numbers of Iba1+ cells, occasionally clumped in small aggregates (arrows). Several villus tips have epithelial defects through which fluid spills from the lamina propria into the lumen. This luminal fluid contains several Iba1+ cells (open arrowheads). Inset: Higher magnification image demonstrating the amoeboid phenotype of macrophages present within the lamina propria fluid. This bat intermittently shed SOSV in the feces throughout the

duration of the serial euthanasia study. Original magnifications = 100× (main image) and 600× (inset). **(D)** CD3 IHC, jejunum, control bat 290195. Low numbers of CD3+ cells (brown) are scattered throughout the epithelium. Rare CD3+ cells are present within the lamina propria core. Original magnification = 400×. **(E)** CD3 IHC, jejunum, 9 DPI bat 287756. Low numbers of CD3+ cells are scattered throughout the intestinal epithelium and occasionally present within the lamina propria fluid. Slightly increased CD3+ cells are present within the lamina propria core compared to the control. Original magnification = 400×. **(F)** CD3 IHC, jejunum, 21 DPI bat 289953. Scattered CD3+ cells are present just beneath the intestinal epithelium and within the lamina propria fluid. Slightly increased numbers of CD3+ cells are distributed throughout the lamina propria core compared to the control. Original magnification = 400×. All immunohistochemical stains were performed using 3-3'-Diaminobenzidine (DAB; brown) chromogen to visualize cellular antigen and hematoxylin counterstain.

#### b. Salivary Glands

There were distinctive differences in the quantity and distribution of Iba1+ and CD3+ cells in the salivary glands of SOSV-infected bats compared to the controls, consistent with a controlled immune response to infection (Figure 2-7). There was a statistically significant 4-fold increase in the % of Iba1+ pixels at 21 DPI ( $n = 2$ ) relative to the controls (Figure 5C; Dunnett's multiple comparison test;  $p < 0.0001$ ). There was a 3-fold increase in the number of CD3+ cells per mm<sup>2</sup> at 21 DPI ( $n = 2$ ) compared to the controls, but this was not statistically significant. There were no other statistically significant differences between groups. In the control bats, Iba1+ cells were distributed in low numbers within the duct epithelium and occasionally in small aggregates within the lobular interstitium (Figure 2-7A). There were no changes in the distribution of Iba1+ cells for the 3 DPI bats. The 6 DPI bat with sialodochitis exhibited the expansion of the periductal connective tissue with numerous Iba1+ cells and increased numbers of Iba1+ cells were also observed within the duct epithelium (Figure 2-7B). In the 9 and 21 DPI bats, Iba1+ cells were present in small clusters within the duct epithelium; these clusters were most numerous in the two 21 DPI bats analyzed (Figure 2-7C, D). Large aggregates of Iba1+ cells were

also distributed throughout the salivary lobules, where they separated and surrounded acinar cells and intralobular ducts (Figure 2-7I).

In the control bats, CD3<sup>+</sup> cells were distributed in comparatively low numbers within the duct epithelium and occasionally in small aggregates within the lobular interstitium. The 3 DPI bats did not show any changes in the quantity or distribution of CD3<sup>+</sup> cells. By 6 DPI, one bat with sialodochitis had increased numbers of CD3<sup>+</sup> cells surrounding the inflamed ducts (Figure 2-7F). Slightly increased numbers of CD3<sup>+</sup> cells were also present within and surrounding the affected ducts of 9 and 21 DPI bats (Figure 2-7G, H). There were increased interstitial CD3<sup>+</sup> cell aggregates in the 21 DPI bats, which expanded the interstitium, surrounded and infiltrated intralobular ducts in moderate numbers and colocalized with Iba1<sup>+</sup> cells (Figure 2-7J and inset).

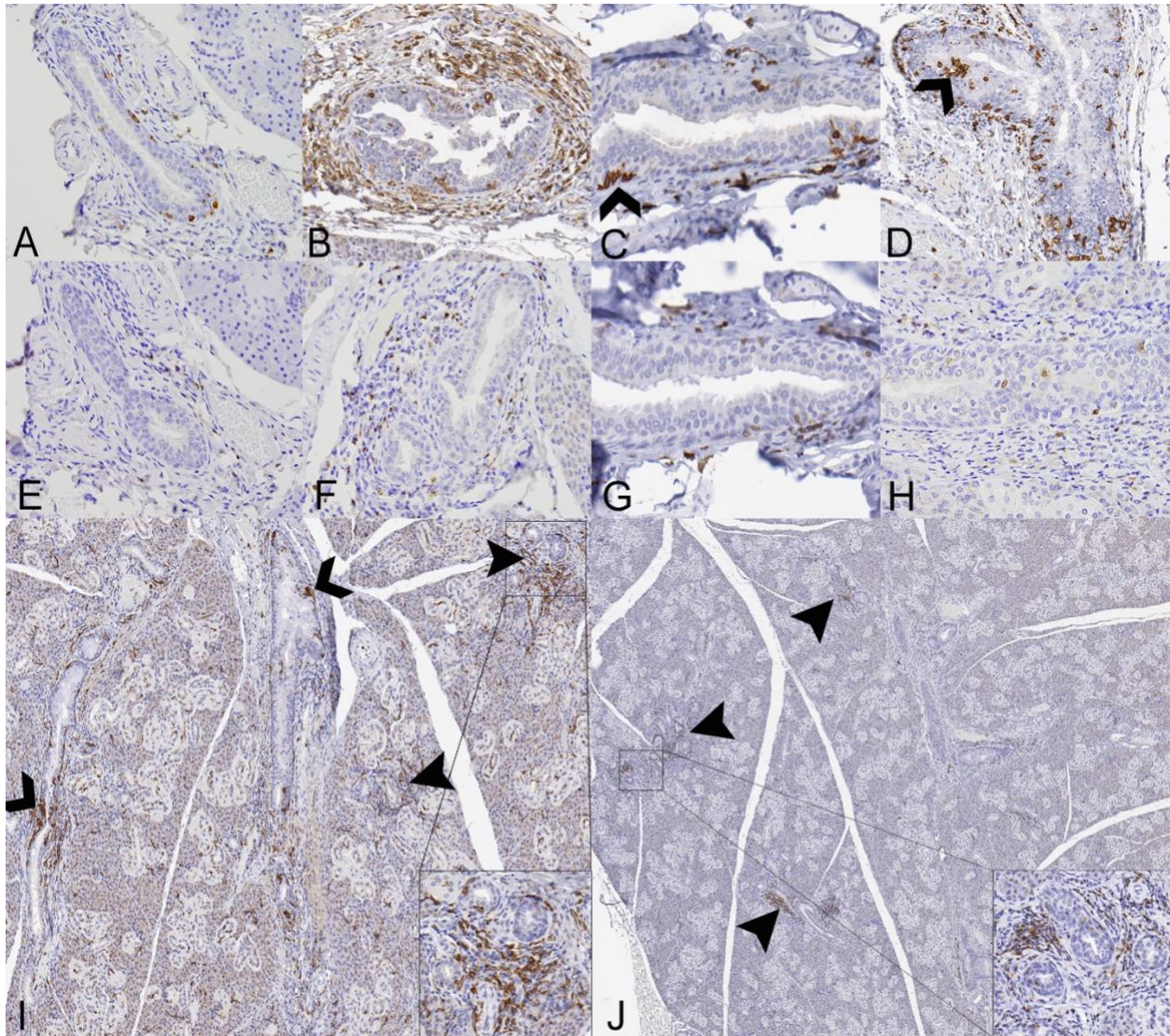


**Figure 2-7.** Distribution of Iba1- and CD3-immunolabeled cells in the salivary glands of SOSV-infected Egyptian rousette bats euthanized at 6, 9 and 21 DPI. **(A)** Iba1 immunohistochemistry (IHC), salivary gland, control bat 290195. Low numbers of Iba1+ cells (brown) are present within the salivary duct and surrounding connective tissue. Original magnification = 400× **(B)** Iba1 IHC, salivary gland, 6 DPI bat 284899. Numerous Iba1+ cells morphologically consistent with macrophages or dendritic cells infiltrate the salivary gland duct epithelium and expand the adjacent connective tissues. Original magnification = 400×. **(C)** Iba1 IHC, 9 DPI bat 287014. A small cluster of Iba1+ cells (open arrowhead) is present within a hyperplastic salivary duct. This bat had mild eosinophilic and neutrophilic sialodochitis, scattered apoptotic ductular epithelial cells and an epithelial syncytium. Original magnification = 350×. **(D)** Iba1 IHC 21 DPI bat 289338. Increased numbers of Iba1+ cells are distributed throughout a salivary duct, and multifocally in small clusters (open arrowhead). This bat had moderate eosinophilic sialodochitis, sialoadenitis and numerous apoptotic cells within interlobular ducts. Original magnification = 200×. **(E)** CD3 IHC, salivary gland, control bat 290195. Rare CD3+ cells (brown) are present within the salivary duct and surrounding connective tissue. Original

magnification = 400×. **(F)** CD3 IHC, salivary gland, 6 DPI bat 284899. Moderate numbers of CD3+ cells infiltrate the periductular connective tissues. Original magnification = 400×. **(G)** CD3 IHC, 9 DPI bat 287014. Very low numbers of CD3+ cells surround an interlobular duct. Original magnification = 350×. **(H)** CD3 IHC 21 DPI bat 289338. Mild to moderate numbers of CD3+ cells surround and infiltrate an affected salivary duct. This bat had moderate eosinophilic sialodochitis, sialoadenitis and numerous apoptotic cells within interlobular ducts. Original magnification = 200×. **(I)** Iba1 IHC, 21 DPI bat 289338. Low magnification image demonstrating widespread Iba1+ immunolabeling and aggregation of these cells within interlobular ducts (open arrowheads). Iba1+ cells also form loose aggregates multifocally within the interstitium (closed arrowheads), where they separate, surround and interdigitate with acinar and intralobular duct epithelial cells (inset is higher magnification of boxed region). Original magnifications = 80× (main image) and 300× (inset). **(J)** CD3 IHC 21 DPI bat 289338. Low magnification image demonstrating loose aggregates of CD3+ cells distributed multifocally throughout the interstitium (arrowheads), where they separate, surround and infiltrate intralobular ducts (inset is higher magnification of boxed region). Original magnifications = 80× (main image) and 300× (inset). All immunohistochemical stains were performed using 3-3'-Diaminobenzidine (DAB; brown) chromogen to visualize cellular antigen and hematoxylin counterstain.

## DISCUSSION

Our histopathological and immunohistochemical findings corroborate the virological data previously reported [27]. Histopathologically, SOSV infection was associated with villus tip ulcerations, lamina propria fluid accumulation and rare syncytia formation in the small intestines. The presence of viral antigen in the lamina propria and gut lumen demonstrates that shedding into the feces likely occurred via ulcerations at the villus tips or in shed enterocytes. The assumption that small intestines are a primary target tissue for SOSV replication and the likely principal source of virus shedding in experimentally infected ERBs was confirmed [27]. In the salivary glands, acute sialodochitis with epithelial cell apoptosis, necrosis, syncytia and intraepithelial viral antigen provides support for the salivary ducts as an additional site of SOSV replication and a source of shedding into the oral secretions. Average lesion scores in the small intestines and salivary glands increased over time, indicative of ongoing host responses to SOSV infection. Our findings in the tissues of SOSV-infected ERBs are aligned with the concept of

disease tolerance, in which cellular and molecular damage control mechanisms limit the development of severe lesions, thereby avoiding clinical disease and promoting individual bat survival, virus persistence and asymptomatic infections [9,27,30–36].

Spatial, quantitative and temporal analyses of the Iba1<sup>+</sup> and CD3<sup>+</sup> immune cell populations revealed tissue-specific host responses following experimental SOSV infection. In the salivary glands, a targeted and robust immune cell response was evident at 21 DPI. In contrast, in the small intestines, immune cell counts increased only mildly despite high viral loads and ongoing enterocyte damage and loss. These results suggest that interactions between infected or damaged host cells and macrophages or T cells with distinct immunologic properties and the presence of soluble mediators may be important factors in limiting the development of severe, life-threatening disease despite disruption of the intestinal epithelial barrier. Intestinal macrophages have evolved to produce anti-inflammatory cytokines such as interleukin (IL)-10 and transforming growth factor (TGF) beta, thereby promoting the expansion of regulatory T cells and establishing tolerance to food antigens and commensal bacteria [37–40]. The amoeboid morphology of macrophages within the lamina propria fluid in SOSV-infected ERBs may provide some insight into their immunological phenotype, as alternatively activated (M2) macrophages are capable of transitioning from a mesenchymal to amoeboid migratory mode in response to a fluid extracellular milieu [41]. Furthermore, some M2 macrophage subtypes promote wound healing and tissue repair, which would be beneficial for SOSV-infected ERBs. Given that MARV has a documented tropism for mononuclear phagocytes in ERBs and fecal MARV shedding has also been reported, the involvement of this cell lineage in the ERB host response to SOSV infection is of interest [7,9,20].

Ongoing apoptosis of enterocytes and salivary duct epithelial cells, the detection of viral antigen in several tissues at 21 DPI and fecal shedding of one bat throughout the serial sacrifice study raise the potential for persistent or prolonged ERB–SOSV interactions. Chronic infections have been reported for a number of paramyxoviruses, including parainfluenza virus 3, measles virus and canine distemper virus, although the molecular mechanisms driving these prolonged host–virus infections remain unclear [42]. Recrudescence of SOSV may also be a concern in ERBs, as this phenomenon was the only plausible explanation for Nipah virus shedding in a captive closed colony of Malaysian flying foxes (*Pteropus vampyrus*) following the seroconversion of two naïve contact bats [44]. Persistent antigen in the axillary lymph nodes of SOSV-infected ERBs may serve as a driver of the adaptive immune response [44]. While antibody-mediated virus neutralization does not contribute significantly to the control and clearance of SOSV, anti-SOSV antibodies may serve other functions such as opsonization and phagocytosis or antibody-dependent cellular cytotoxicity [45].

## CONCLUSIONS

The potential for persistent SOSV infection and a dampened or anergic immune response in the small intestines may have broader implications for host–paramyxovirus dynamics in chiropterans, a heterogeneous group of mammals who are hypothesized to be the ancestral hosts of this diverse virus family. We identified the small intestines and salivary glands as primary target tissues for SOSV in ERBs, and described the disparate host responses in these tissues, which are likely a function of the anatomy, immunophenotypes of tissue resident immune cell populations and tissue-specific intrinsic repair and renewal capacities [36]. Despite histologic evidence of both direct and indirect tissue damage, the lesions were overall limited in scope and

severity, and the lack of clinical disease supports the concept of disease tolerance for this model. In both tissues, immune responses were driven at least in part by mononuclear phagocytes and CD3<sup>+</sup> cells. In the small intestines, there was direct damage to the villi, and subsequent immunopathology was likely limited by the intrinsic hypo-responsiveness of the resident immune cell populations and the high repair capacity of this tissue. The same or related mechanisms may have contributed to substantial shedding from this tissue. While there was evidence of immunopathology in the salivary glands, there was variability between individual bats, and overall, the lesions, similar to those of the intestines, were mild to moderate without any major changes to tissue structure or detectable losses of function. The robust inflammatory cell infiltrates at 21 DPI in the salivary glands suggests the consistent recruitment of immune cells from the circulation, and possibly the local expansion of resident immune cells in this tissue, which supports the release of inflammatory mediators. However, the clinical implications of damage to this tissue would be minor compared to those of the small intestines, and so the development of salivary gland lesions may not be as critical in terms of host fitness [36]. Additional tissue-based studies that preserve the spatial relationships between immune cells and infected host cells will be necessary to further resolve the induction of pro- and/or anti-inflammatory pathways in target tissues and to explore the anatomical and physiological features which have made this bat species a suitable natural reservoir for Marburg virus, Ravn virus and Kasokero virus.

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## CHAPTER 3

# PATHOGENESIS OF KASOKERO VIRUS IN EXPERIMENTALLY INFECTED EGYPTIAN ROUSETTE BATS (ROUSETTUS AEGYPTIACUS)<sup>2</sup>

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## ABSTRACT

Egyptian rousette bats (ERBs; *Rousettus aegyptiacus*; family *Pteropodidae*) are associated with a growing number of bunyaviruses of public health importance, including Kasokero virus, which was first identified as a zoonosis in Uganda in 1977. In the study described here, formalin-fixed paraffin-embedded tissues from a previous experiment in which KASV infection was confirmed in 18 experimentally infected ERBs were utilized for an in-depth analysis using histopathology, *in situ* hybridization (ISH) for detection of virus, immunohistochemistry (IHC) to assess the mononuclear phagocyte system response, and quantitative digital image analysis to investigate virus clearance from the liver and spleen within a spatial context. Significant gross and histological lesions were limited to the liver, where KASV-infected bats developed mild to moderate lymphohistiocytic hepatitis, which was first observed at 3 DPI, peaked at 6 DPI, and was resolved by 20-DPI. A subset of bats had glycogen depletion (n=10) and hepatic necrosis (n=3), rarely with intralesional bacteria (n=1). Virus replication was confirmed by ISH in the liver, spleen, lymph nodes, and tongue. In the liver, KASV replicated in the cytoplasm of hepatocytes, to a lesser extent in mononuclear phagocytes, and rarely, in endothelial cells. The majority of KASV RNA, as detected by ISH, was cleared from the spleen and liver by 6 DPI. It is concluded that ERBs have effective mechanisms to respond to this virus *in vivo*, clearing it without evidence of clinical disease.

**KEYWORDS:** bats, Egyptian rousette, IHC, ISH, Kasokero virus, nairovirus, bunyavirus, pathogenesis, viral hepatitis, zoonosis

## INTRODUCTION

Bats (Order Chiroptera) are the natural reservoir hosts for several high-consequence viral pathogens of humans, including Nipah, Hendra, and Marburg viruses.<sup>23,25,27,32,33</sup> Additionally, a growing number of arboviruses have been associated with bats, including viruses of the families *Nairoviridae*, *Flaviviridae*, *Reoviridae*, and *Togaviridae*.<sup>11</sup> Within the family *Nairoviridae*, the genus *Orthonairovirus* contains tick-borne viruses such as the human-pathogenic Crimean-Congo Hemorrhagic Fever virus (CCHFV), the ovine-pathogenic Nairobi Sheep Disease orthonairovirus (NSDV), along with eight other known orthonairoviruses, including Ahun, Kasokero, and Issyk-Kul viruses, which have been associated with bats based on molecular detection or virus isolation (from bats and/or their ectoparasites).<sup>1,10,11,17,20,22,34,35</sup>

Orthonairoviruses constitute an emerging group of viruses of public health importance, several of which cause severe systemic disease in humans, whereas it is unclear if bats develop orthonairovirus-associated disease. The initial description of Ahun virus, identified by RNA sequencing from the lungs of a whiskered bat (*Myotis mystacinus*) and common pipistrelle (*Pipistrellus pipistrellus*) in France, indicates that the two infected bats were moribund prior to tissue collection; however, histopathology was not included in that report to confirm or refute virus-associated pathology or other underlying disease(s) or trauma.<sup>10</sup> Tissue-based assessments of bats infected with orthonairoviruses are critical in order to begin to understand how bats respond to these viruses, if and how they avoid clinical disease, and to inform viral countermeasure development in anticipation of spillover events.

Ecological and experimental studies have linked the Egyptian rousette bat (ERB; *Rousettus aegyptiacus*), a common, cave-dwelling African bat, with two closely related orthonairoviruses, Kasokero and Yogue viruses (KASV and YOGV), as well as the human-

pathogenic Marburg and Ravn (MARV and RAVV; family *Filoviridae*), and Sosuga viruses (SOSV; family *Paramyxoviridae*).<sup>2,4,3,5,18,20,30,32,33</sup> Kasokero virus was first isolated from the sera of ERBs at Kasokero Cave, Uganda, in 1977.<sup>20</sup> Since its initial discovery, KASV has been collected from argasid ticks (*Ornithodoros [Reticulinasus] faini*) in the rock crevices of Lannar Gorge Cave, South Africa and Python Cave, Uganda, where large colonies of ERB are present.<sup>8,20</sup> The fact that KASV has been isolated from *O. (R.) faini* ticks and from ERBs upon which they feed suggests that this virus is naturally maintained in an ERB-*O. (R.) faini* enzootic life cycle. An outbreak of KASV infection in humans occurred following the occupational exposure of four individuals who were either working with the virus or had access to the laboratory where virus characterization assays were performed at the Uganda Virus Research Institute.<sup>20</sup> During the outbreak, symptoms of KASV infection ranged from a mild, febrile illness to severe, systemic disease with fever, headache, abdominal pain, diarrhea, and muscle and joint pain; all individuals made a full recovery.<sup>20</sup>

Orthonairovirus-associated pathology in vertebrate hosts is understudied, and descriptions have thus far been limited to humans naturally infected with Crimean-Congo Hemorrhagic Fever virus (CCHFV), murine and nonhuman primate models of CCHF, sheep experimentally and naturally infected with Nairobi Sheep Disease virus (NSDV), and mice experimentally infected with the 11SB23 strain of Leopards Hill virus (LPHV), a virus isolated from a giant leaf-nosed bats (*Hipposideros gigas*) in Zambia.<sup>9,13,16,17</sup> Gross and/or histological lesions in severe or fatal orthonairovirus infections include hepatopathy or hepatic necrosis, often with minimal inflammation, enteritis and/or gastrointestinal hemorrhage, and splenic lymphoid depletion and/or necrosis.<sup>9,16,17</sup> Severe systemic orthonairoviral disease can progress to widespread hemorrhaging, typified by CCHF in humans, and is hypothesized to be driven by a

dysregulated immune response and strong tropism of these viruses for endothelium and cells of the mononuclear phagocyte system.<sup>9</sup> In contrast, very little is known about how bat-derived orthonairoviruses interact with their suspected natural reservoir hosts, including KASV in ERBs.

Egyptian rousette bats experimentally infected with KASV remained apparently healthy despite developing high-level, transient viremias (peak viral loads = 5.1–5.9 log<sub>10</sub>TCID<sub>50</sub>eq/mL) with virus replication in multiple tissues including spleen, lymph nodes, and skin at the inoculation site, and shedding of virus into the oral secretions, feces, and urine.<sup>29</sup> The present study aimed to build on these findings by using formalin-fixed paraffin-embedded ERB tissues derived from the previous experiment to conduct a comprehensive tissue-based analysis of KASV-ERB interactions within a spatial context. To accomplish this goal, we used histopathology, morphometry, *in situ* hybridization for detection of virus, immunohistochemistry for co-localization of KASV RNA with cells of the mononuclear phagocytic system, and quantitative image analysis to investigate virus clearance from the spleen and liver. To our knowledge, this is the first study to investigate the pathogenesis of an orthonairovirus in a bat host. Understanding more about how these viruses circulate within their bat hosts could help inform prevention and control strategies for other emerging viral zoonosis of bats.

## **METHODS**

### **A. Tissue sample collection, processing, and virus origin**

The ERB tissues used in the present study were obtained from an experimental infection study which was performed under biosafety level 4 (BSL-4) conditions in accordance with safety regulations at the Centers for Disease Control and Prevention (CDC, Atlanta, GA, U.S.A.).<sup>29</sup> The experimental infections were conducted with approval from the CDC Institutional Animal Care and Use Committee (IACUC), and in accordance with the animal welfare guidelines set forth in

the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, National Research Council of the National Academies 2011). Husbandry, housing conditions and diet for the duration of the experiment were previously described.<sup>4</sup> Briefly, food consisted of a variety of fresh fruit dusted with a protein vitamin supplement (Lubee Bat Conservancy, Gainesville, FL). Water was provided *ad libitum*.

The KASV isolate was derived from an argasid tick (*O. [R.] faini*) collected from an ERB roost, Python Cave, Uganda and passaged twice in Vero E6 cells.<sup>30</sup> To mimic a tick bite, as described in the original study, all bats were inoculated intradermally in the ventral abdominal region with 4 log<sub>10</sub> TCID<sub>50</sub> KASV diluted in phosphate-buffered saline (PBS); 3 control bats were mock inoculated the same volume of PBS. Bats were euthanized at 3, 6, 9, 12, 18, and 20 DPI, along with three controls (n=3 for each time point and controls). Blood and selected tissue samples were collected for viral load determination by qRT-PCR at the time of euthanasia, with multiple tissues testing positive for KASV by qRT-PCR. Hepatic hemorrhage was noted in the liver of one 9 DPI bat at the time of tissue collection. Following tissue collection, all bat carcasses were immersed in 10% neutral buffered formalin (10% NBF) for a minimum of 7 days, with a complete change of formalin after day 3, prior to removing the carcasses from the laboratory. Carcasses were transferred to 70% ethanol within 2 weeks and further dissected for histological analyses as detailed below.

#### B. RNA Extraction and qRT-PCR

RNA extraction and qRT-PCR were performed as previously described.<sup>29</sup> KASV qRT-PCR cycle threshold (C<sub>T</sub>) values were normalized using ERB beta-2-microglobulin (B2M) qRT-PCR C<sub>T</sub> values to account for any differences in tissue specimen weights.

### C. Gross Pathology and Histopathology

The carcasses, once removed from ethanol, had major organs intact *in situ*, with some pieces removed for qRT-PCR testing. A more rigorous examination of the tissues was done at this time, with removal and extensive slicing of various organs for observation of lesions which might be only visible on cut section. A full set of tissues from each carcass was collected, including representative sections from the brain, eye, heart, lungs, trachea, thymus, esophagus, thyroid glands, liver (4 non-contiguous sections including gall bladder), stomach, small intestines (duodenum with pancreas, jejunum, and ileum), large intestines, spleen, kidney, adrenal glands, salivary gland, submandibular lymph node, tongue, axillary, inguinal, sublumbar and mesenteric lymph nodes, skin from the inoculation site, patagium, skin from the chest region and pectoral muscle. Tissue sections were placed into cassettes, immersed in 70% ethanol and processed routinely for histological analyses at the University of Georgia (UGA) Histology Laboratory. Slides were reviewed in a blinded manner without knowledge of time point or infection status. A subset of livers from 6-, 9-, 12-, and 18-DPI, and control bats were stained with Perl's Prussian Blue and Periodic Acid-Schiff reaction stains at the UGA Histology Laboratory to identify the accumulated pigment within macrophages within the foci of inflammation.

#### i. Morphometric Analysis of H&E-stained Liver Sections

Liver slides were scanned at 200x using an Aperio Slide Scanner (Aperio ScanScope XT, Aperio Technologies, Inc., Vista, CA, USA) and loaded into the open source bioimage analysis software QuPath (ver 0.3.2.).<sup>7</sup> Foci of mononuclear cells, defined as clusters of at least 3 mononuclear cells either located within the sinusoids, distorting the hepatic cords, or in the perivascular space, were manually annotated. The total liver area (mm<sup>2</sup>) was measured by tracing

each section of liver using the wand tool and summing the individual annotations. The sum of the area for all foci was digitally quantified and divided by the total liver area to obtain the percentage of liver tissue affected (% liver affected).

#### ii. Assessment of Hepatocellular Glycogenosis

Hepatocellular glycogenosis is a normal finding in frugivorous bats and in our colony of ERBs.<sup>12</sup> Semi-quantitative hepatocellular vacuolation scores were determined for all bat livers. At least 4 non-contiguous H&E-stained liver sections were reviewed and assigned a score from 1 to 4 according to the following criteria: 0 = normal hepatocellular glycogenosis; 1 = minimal, focal depletion or condensation of hepatocellular cytoplasmic vacuoles, typically restricted to hepatocytes surrounding the foci of inflammation; 2 = mild, zonal depletion of vacuoles; 3 = moderate, occasionally coalescing and/or bridging depletion of vacuoles; 4 = moderate to marked, diffuse depletion of vacuoles.

### D. RNAScope<sup>®</sup> *in situ* Hybridization (ISH)

#### i. RNAScope<sup>®</sup> (Tissue tropism)

To localize KASV RNA in tissues, we performed *in situ* hybridization using the RNAScope<sup>®</sup> 2.5 HD Reagent Kit—RED (Advanced Cell Diagnostics, Newark, CA, USA) and followed the manufacturer's instructions to visualize the signal in formalin-fixed paraffin-embedded (FFPE) liver, spleen, and lymph nodes for bats euthanized at 3, 6, 9, 12, 18 and 20 DPI, and in the 3 control bat tissues. A custom anti-sense probe targeting the complementary RNA sequence of the nucleoprotein gene of KASV obtained from the published genome was designed (GenBank Accession # MT309090).<sup>30</sup> To confirm adequate RNA integrity of the FFPE samples, a medium-expressing housekeeping gene (peptidylprolyl isomerase B, PPIB) probe was

applied to a subset of samples (3 infected and 2 control bats) as a positive control (*R. aegyptiacus* GenBank Accession # XM\_016141088.1). A probe targeting the *dapB* gene (Advanced Cell Diagnostics, Newark, CA, USA) was used as a negative control. For each bat, 4 sections of liver, 2 sections of spleen and one cross section of lymph node were evaluated on a single slide. A subset of other tissues from one 3- and one 6-DPI bat with histological lesions that were suggestive of virus-induced tissue injury and/or with high tissue viral loads were subjected to ISH to investigate potential sources of shedding and replication in the subcutis at the inoculation site; these tissues included small intestines, kidneys, tongue, salivary gland, and skin at the inoculation site.

ii. Co-detection of KASV with Mononuclear Phagocytes

Following the identification of KASV in cells morphologically consistent with mononuclear phagocytes in the primary target tissues (liver, spleen, and lymph nodes), a combination ISH-IHC protocol was developed to investigate KASV interactions with cells of the mononuclear phagocyte system using the pan-macrophage/monocyte/dendritic cell marker, ionized calcium binding adapter molecule (Iba-1). RNAScope<sup>®</sup> was performed first using the protocol described in previous section. Subsequently, the immunohistochemical protocol was initiated by applying a universal blocking reagent (Background Punisher, Biocare Medical, Pacheco, CA), incubating at room temperature for 10 minutes, then washing the slides (1X TBST, 2x5 minutes). A solution containing the anti-Iba1 antibody (FujiFilm, catalog # 019-19741) diluted in co-detection antibody diluent (Advanced Cell Diagnostics, Newark, CA, USA, catalog # 323160; final antibody dilution = 1:8000) was applied to each slide, and the slides were incubated overnight at 4° C. The next day, slides were washed (1X TBST, 3x5 minutes), incubated for 15 minutes at RT with the MACH 3 Rabbit Probe (Biocare Medical, Pacheco, CA),

and washed again (1X TBST, 2x5 minutes). Slides were then incubated for 15 minutes with the MACH 3 Horseradish-Peroxidase (HRP) Polymer (Biocare Medical, Pacheco, CA) and washed (1X TBST, 3x5 minutes). Finally, one drop of the HRP Chromogen (Biocare Medical, Pacheco, CA) was applied and incubated for 5-7 minutes at RT, and the slides were washed (1X TBST, 3x5 minutes), counterstained with hematoxylin (Leica, Richmond, IL) and coverslipped with Permount for a permanent record. Appropriate immunostaining was verified using internal controls (tissue macrophages).

#### E. Semi-Quantitative and Quantitative Analysis of KASV Positive Hybridization Signal

ISH slides containing four non-contiguous sections of liver tissue, two spleen sections and one section of lymph node were scanned at 400x using an Aperio Slide Scanner (Aperio ScanScope XT, Aperio Technologies, Inc., Vista, CA, USA) and imported in to the open-source bioimage analysis software QuPath ver. 0.3.2.<sup>7</sup> Positive KASV hybridization was characterized by punctate to saturated, bright red staining within the cytoplasm of target cells. The liver, spleen, and inguinal lymph nodes were assigned semi-quantitative scores based on the degree of ISH signal observed as follows: + = focal signal, less than 2-3 cells per 40x high-powered field (hpf); ++ = scattered signal in multiple foci throughout section, less than 2-3 cells per hpf; +++ = multifocal signal throughout section; 3-5 positive cells per hpf; ++++ = multifocal signal throughout section, 5-10 cells per hpf; and +++++ = multifocal signal throughout section, frequent saturated signal, 5-10 cells per hpf. KASV was detected in the axillary, but not the inguinal lymph node of one bat; the results are presented for the former tissue in that case.

The liver and spleen sections were further analyzed to quantify the KASV signal within spatial and temporal contexts. Tissue areas of at least 95 mm<sup>2</sup> (range = 95-231 mm<sup>2</sup>) and 5 mm<sup>2</sup>

(range = 5-22 mm<sup>2</sup>) were analyzed for liver and spleen, respectively. Positive pixels were segmented from negative regions and background staining by thresholding; the threshold was customized for each slide (range = 0.15-0.18). The other default parameters in the Create Threshold tool (gaussian sigma = 1.5 mm and background radius = 15 mm) were used. False positives based on morphology and location were removed, as was background staining and artifact.

#### F. Statistical Analyses

Statistical analyses were performed using Prism 9.3.1 (GraphPad Software, LLC, La Jolla, CA). For the % liver affected values obtained via digital image analysis, from KASV-infected bats at each time point (n = 3) were compared with those of the control bats (n = 3) using a one-way analysis of variance (ANOVA) test, followed by Dunnet's multiple comparison test if the ANOVA yielded a statistically significant result ( $p < 0.05$ ). Correlations between the viral loads and the % positive pixels in the liver and spleen at 3 and 6 DPI were analyzed using the Spearman rank test.

#### G. Data availability

The data analyzed in this study are available as Supplemental Materials.

## RESULTS

#### A. Necropsy Findings and Histology

Gross lesions were observed in a subset of KASV-infected bats. The most frequently observed lesion was hemorrhage in the liver (n=4). Hemorrhage was observed in the liver of one 9-DPI bat at the time of tissue collection.<sup>29</sup> Variable pinpoint hepatic hemorrhages were identified during dissection of 3 additional formalin-fixed carcasses (two 6-DPI bats and one 9-

DPI bat). Hepatic hemorrhages were generally minimal to mild, pinpoint, and few, except for one 6-DPI bat which had numerous pinpoint hemorrhages distributed throughout all liver lobes (Fig. 3-1b-c). The formalin-fixed carcass of the 9-DPI bat for which hemorrhages were observed at the time of tissue collection had a regionally extensive focus of pallor, which extended into the hepatic parenchyma on cut section (Fig. 3-1d). All animals had moderate to abundant abdominal and subcutaneous adipose tissue stores. There were no other significant gross lesions in any animal.

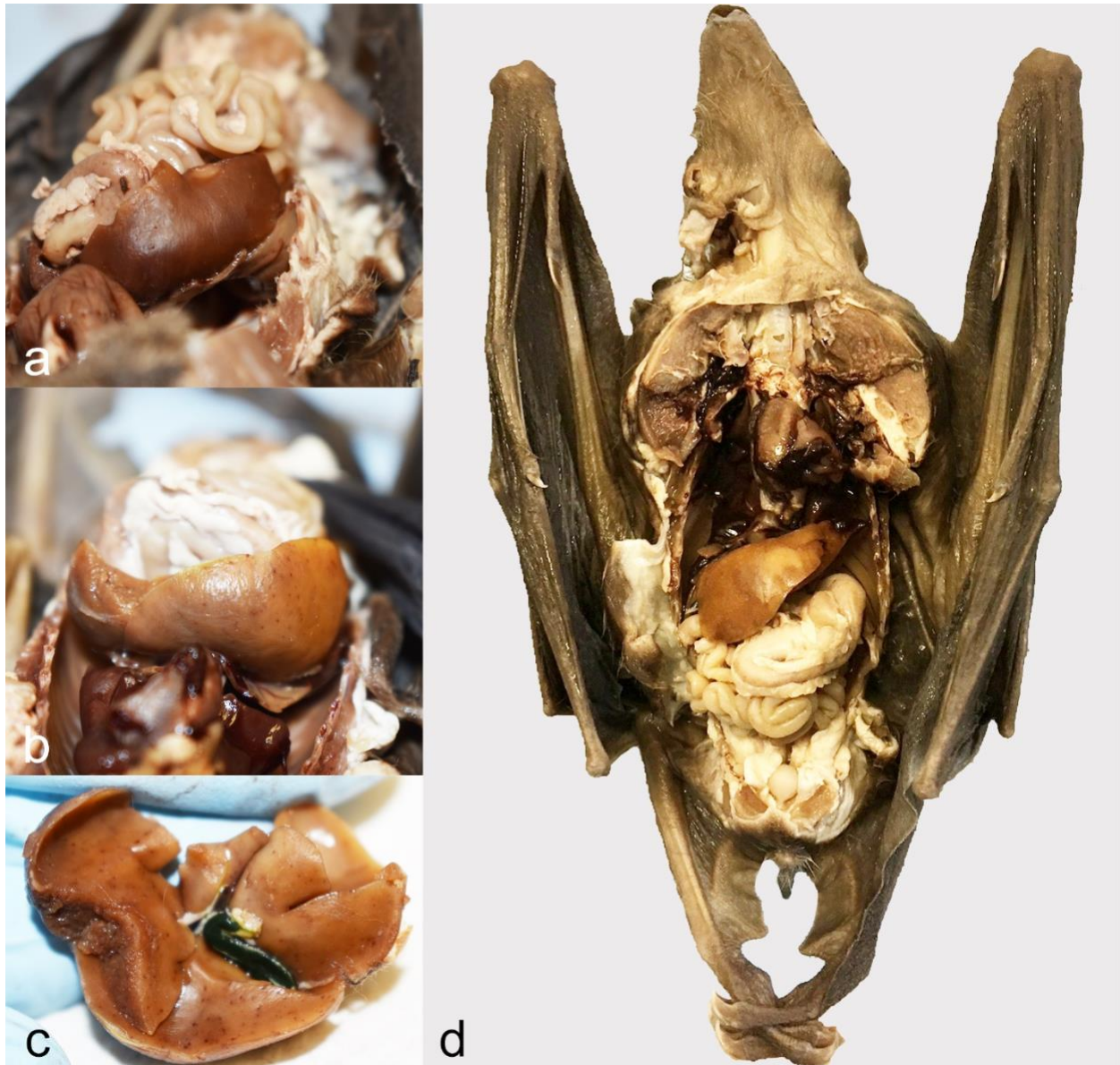


**Figure 3-1.** Kasokero virus infection, liver, Egyptian fruit bat, 6 and 9 days post-infection. (a) Formalin-fixed carcass of a control bat with the organs *in situ*, demonstrating normal color of the liver in this species. Liver color is variable, depending on the glycogen content of hepatocytes. Hepatic glycogenosis is a normal finding in ERBs, and in fruit bats in general. (b) Formalin-fixed carcass of an ERB experimentally infected with Kasokero virus and euthanized at 6-DPI. The liver contains numerous pinpoint hemorrhages distributed throughout all lobes. (c) Liver from the same 6-DPI bat as in (b), removed to demonstrate the widespread distribution of pinpoint hemorrhages on the ventral surface. The gall bladder is grossly normal. (d) Formalin-fixed carcass of a KASV-infected ERB euthanized at 9-DPI. There are ill-defined areas of hemorrhage and a focally extensive region of pallor (necrosis).

The livers of all KASV-infected bats (n=18), but not control bats, contained multifocal to coalescing, randomly distributed, variably sized inflammatory cell foci containing numerous macrophages and lymphocytes, and rare granulocytes (Fig. 3-2a-b). At 3- and 6-DPI, foci frequently contained degenerating, apoptotic or necrotic hepatocytes, especially at the junction with normal parenchyma, and hemorrhage (Fig. 3-2a-f). Hepatocytes immediately adjacent to inflammatory foci in one 3-DPI bat contained numerous eosinophilic to amphophilic, round variably sized cytoplasmic inclusions (Fig. 3-2b). That same 3-DPI bat had a focally extensive area of capsular to subcapsular fibrin exudation and hemorrhage, and a small focus of hepatic necrosis with low numbers of mononuclear inflammatory cells and apoptotic cells (Fig. 3-2c).

In the 6-DPI bats, the inflammatory cell foci frequently coalesced, tended to have more hemorrhage, and often contained numerous hemosiderin-laden macrophages (Fig. 3-2d-e). Round, variably sized, eosinophilic cytoplasmic inclusions were rarely observed in hepatocytes along the periphery of inflammatory foci in two 6-DPI bats (Fig. 3-2e). Foci contained increased numbers of small mononuclear cells morphologically consistent with lymphocytes compared to the 3-DPI bats and were more clearly delineated from the surrounding hepatic cords (Fig. 3-2e-f). Hepatocytes with morphologic features consistent with apoptosis (shrunken and rounded cell borders, and pyknotic nuclei) were present within and along the periphery of some foci (Fig. 3-2f).

At 9-DPI, inflammatory foci were less numerous and comprised primarily of brown pigment-laden macrophages with fewer lymphocytes and rare apoptotic hepatocytes (Fig. 3-2g-h). The cytoplasmic pigment was interpreted to be a mixture of hemosiderin and lipofuscin based on the mixed staining on Perl's Prussian Blue and Periodic Acid-Schiff reaction stains. In addition to foci of mononuclear cells, two of the 9-DPI bats had foci of hepatic necrosis with

sinusoidal fibrin accumulation, necrotic and apoptotic cells debris, and hemorrhage. In one 9 DPI bat, at least two 1-2 mm bacilli were evident in an area of necrosis, consistent with sepsis (Fig. 3-2i). Foci continued to decrease in size and frequency at 12-DPI, and by 18- and 20-DPI, only few small foci, consisting predominantly of hemosiderin-laden macrophages were present, either in the sinusoids or in the periportal or centrilobular connective tissues (Fig. 3-2j-k).

In addition to mononuclear inflammatory cell infiltrates and occasional hepatic necrosis, most KASV-infected bats (10/18; 56%) exhibited some degree of hepatocellular glycogen depletion that was not restricted to hepatocytes immediately surrounding the foci of mononuclear inflammatory cells. The distribution of glycogen depletion varied among bats from mild centrilobular or portal depletion, to bridging swaths of depletion, and rarely to widespread or global depletion. In addition to hepatocellular glycogen depletion, one 12-DPI bat exhibited multifocal hepatocellular degeneration, characterized by hepatocellular hypereosinophilia and the accumulation of 1-2 round cytoplasmic vacuoles (Fig. 3-2l); the distribution of degenerating hepatocytes was centrilobular to random.

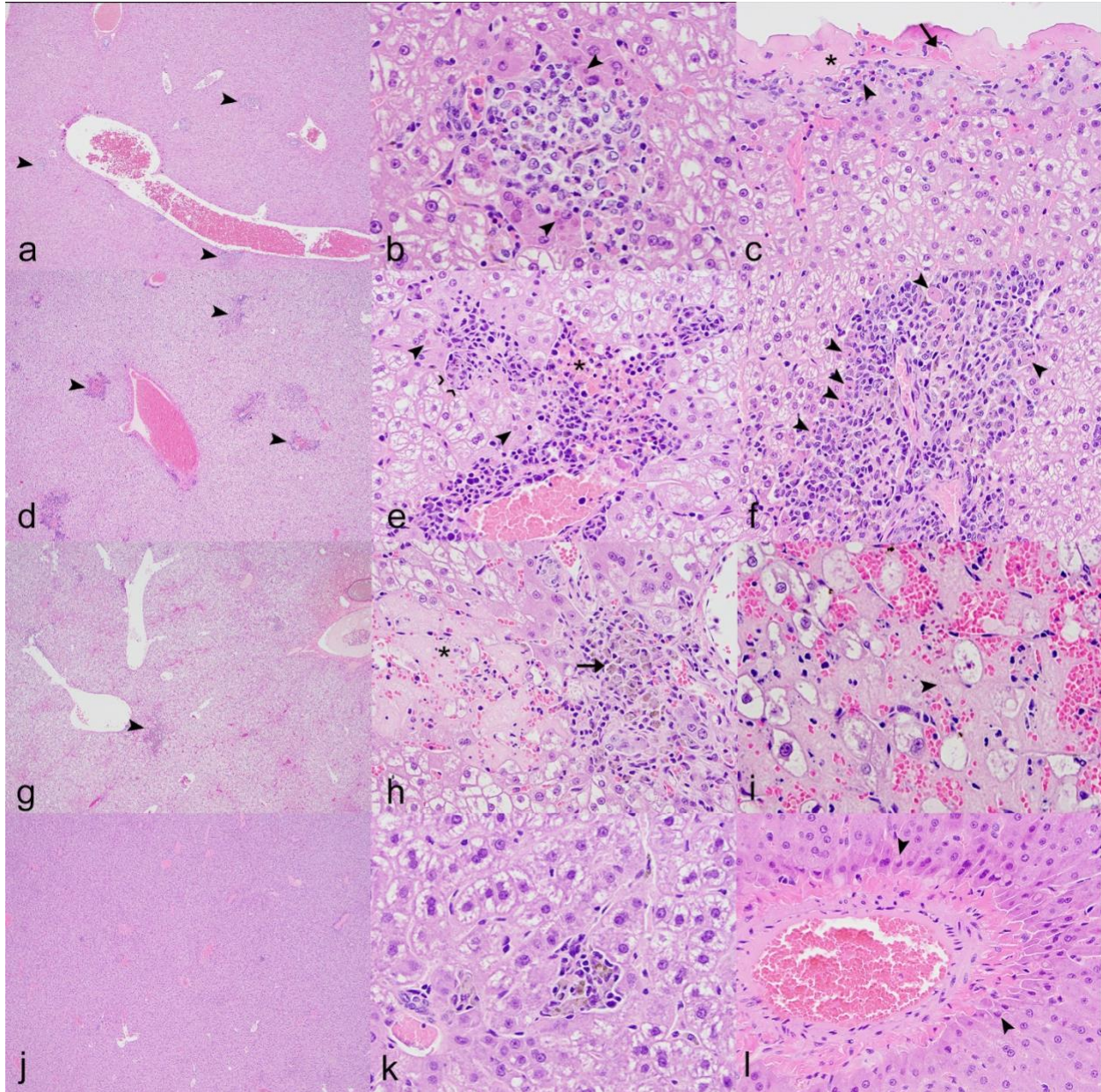


**Figure 3-2.** Kasokero virus infection, liver, Egyptian rousette bats, 3-, 6-, 9-, and 12-days post-infection. (a) Multifocal, randomly distributed mononuclear inflammatory cell infiltrates (arrowheads), 3-DPI bat. (b) An aggregate of lymphocytes and histiocytic cells is poorly demarcated from the surrounding hepatic parenchyma. Along the periphery of the inflammatory cell focus, hepatocytes frequently contain one or more irregularly round, 2–4-micron diameter, eosinophilic cytoplasmic inclusions (arrowheads), and rare apoptotic or necrotic cells are present. (c) Fibrinous capsulitis (\*) with focal hemorrhage (arrow) and mild lymphohistiocytic inflammation within the subjacent hepatic parenchyma (arrowhead). (d) Multifocal to coalescing, randomly distributed mononuclear inflammatory cell foci with hemorrhage (arrowheads), 6-DPI bat. (e) Perivascular lymphocytes and histiocytes coalesce with several smaller foci of mononuclear cell inflammation and there is moderate hemorrhage (\*). The inflammatory cell foci are frequently well demarcated from the surrounding hepatic cords (open arrowheads) and

low numbers of hepatocytes along the periphery contain eosinophilic cytoplasmic inclusions (arrowheads), 6-DPI bat. (f) Several hepatocytes along the periphery of the inflammatory cell focus are rounded with pyknotic or karyorrhectic nuclei (arrowheads) in a 6-DPI bat. (g) Foci of mononuclear cells (arrowhead) are sparse in the liver of a 9-DPI bat. (h) Focal necrosis and hemorrhage (\*), and a peri-portal accumulation of lymphocytes and hemosiderin-laden macrophages (arrow). (i) Focally extensive necrosis and hemorrhage with two intralesional bacilli (arrowhead) in the liver of a 9-DPI bat. (j) Diffuse decrease in hepatocellular vacuolation and two small foci of hypercellularity in a 12-DPI bat. (k) A small focus of sinusoidal mononuclear cells contains few lymphocytes and hemosiderin-laden macrophages in a 12-DPI bat. (l) There is a lack of hepatocellular glycogenosis and several centrilobular hepatocytes contain one or more round cytoplasmic vacuoles (arrowheads). HE stains.

#### i. Morphometric Liver Analyses and Hepatic Viral Loads

At 3 and 6 DPI, there were statistically significant increases in the % of liver tissue affected compared to the negative control bats (Dunnett's multiple comparison test;  $p = 0.0419$  and  $0.0148$ , respectively). From 6- to 12-DPI, the mean % liver affected decreased over time, and by 18-DPI, inflammatory cell foci were rare.

The detailed qRT-PCR results for blood, oral, rectal and urine swabs, and tissues collected at the time of euthanasia were previously reported.<sup>29</sup> Viral loads obtained from the liver are summarized here for correlation with the hepatic morphometrics (Fig. 3-3). Viral loads in the liver peaked at 3 DPI (mean =  $4.3 \log_{10} \text{TCID}_{50} \text{ eq/g}$ ) and KASV RNA was detected by qRT-PCR in all liver samples but one at 20 DPI.





degeneration of villus tip enterocytes, with focal ulceration of a villus tip, and mild accumulation of eosinophilic fluid in the lamina propria with few neutrophils (data not shown). One 6-DPI and two 9-DPI bats had multifocal small intestinal villus fusion, and two of these bats had mild, multifocal accumulations of eosinophilic fluid in the villus lamina propria (data not shown). No significant lesions were observed in any other tissues.

#### B. KASV Tissue and Cellular Tropism

The KASV nucleoprotein gene was detected by *in situ* hybridization (ISH) in the liver and secondary lymphoid tissues (spleen and lymph nodes) using a custom anti-sense RNA probe (Figs. 3-5 & 3-6). In the liver of the 3 DPI bats, much of the positive hybridization signal was localized to discrete, randomly distributed foci of infection throughout all liver lobes. Within these foci, viral RNA was present within the cytoplasm of hepatocytes, mononuclear phagocytes, and rarely in endothelial cells (Fig. 3-5). Foci of hepatocytes containing KASV RNA were often adjacent to the portal veins found in the portal triads (Fig. 3-5a-c). Occasionally, elongate cells (presumptive endothelial cells) lining central veins (Fig. 3-5c-d) and Iba1+ macrophages within the vessel walls contained viral RNA (Fig. 3-5d). Within individual hepatocytes, the extent of the hybridization signal varied from low (single or few dots) to high with filling of the entire cytoplasmic space and saturation of the ISH signal (Fig. 3-5b-f). In one 3 DPI bat, infection of contiguous hepatocytes along individual or adjacent hepatic cords was observed (Fig. 3-5e). Extracellular, intravascular signal was occasionally observed in the sinusoids adjacent to foci of infected hepatocytes (Fig. 3-5e). Hepatocytes along the periphery of the foci of mononuclear inflammatory cells contained abundant KASV RNA, as did fragmented cells, numerous Iba1+ mononuclear phagocytes within the cellular foci, and occasional Kupffer cells (Fig. 3-5f).

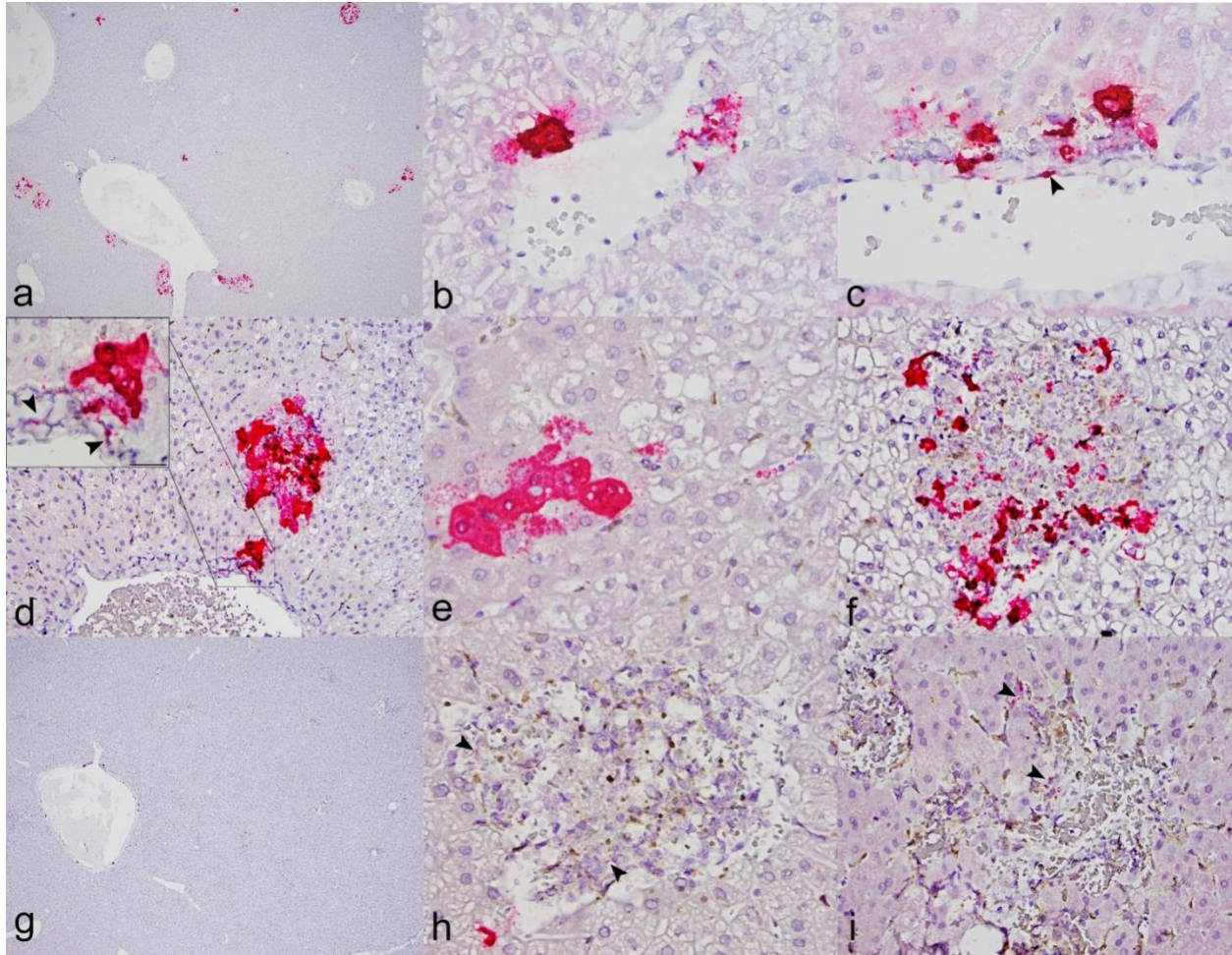


**Figure 3-5.** Kasokero virus infection, liver, Egyptian rousette bats, 3-, 6-, and 9-days post-infection. RNAScope *in situ* hybridization assay designed with an anti-sense probe targeting the KASV nucleoprotein gene. (a) Randomly distributed foci with intense KASV ISH signal are present in the liver, 3 DPI bat. (b) Intense KASV ISH signal within hepatocytes surrounding a small blood vessel, 3 DPI bat. (c) Hepatocytes and endothelial cells surrounding a blood vessel contain KASV RNA, 3 DPI bat. (d) Dual KASV ISH (red) and Iba-1 (brown) IHC stain demonstrating focal colocalization of KASV with Iba-1+ cells in the wall of a large artery (arrowheads), and in a large focus of hepatocellular infection, 3 DPI bat. (e) Dual KASV ISH (red) and Iba-1 (brown) IHC stain demonstrating KASV RNA in several contiguous hepatocytes and in the sinusoids, 3 DPI bat. (f) Dual KASV ISH (red) and Iba-1 (brown) IHC stain demonstrating KASV within Iba-1+ cells in a large focus of mononuclear cell inflammation, 3 DPI bat. (g) Low magnification image demonstrating the scarcity of KASV RNA in a 6 DPI bat. (h) High magnification image of a dual KASV ISH (red) and Iba-1 (brown) IHC stain demonstrating rare KASV RNA within Iba-1+ cells in the focus of inflammation (arrowhead) and in a hepatocyte along the periphery of the focus, 6 DPI bat. (i) High magnification image of a dual KASV ISH (red) and Iba-1 (brown) IHC stain demonstrating rare KASV RNA within Iba-1+ cells and along the periphery of a focus of inflammation (arrowheads), 9 DPI bat. Red chromogen with hematoxylin counterstain (a, b, c & g). Dual ISH/IHC for KASV nucleoprotein (red) and Iba-1 (brown) with hematoxylin counterstain (d, e, f, g & h).

Positive hybridization signal was sparse in the liver of bats euthanized at 6 DPI (Fig. 3-5g), where it was localized to the cytoplasm of few hepatocytes along the periphery of inflammatory foci and in the cytoplasm of scattered mononuclear phagocytes within the foci (Fig. 3-5h). Rare KASV RNA was detected by ISH in two of the 9-DPI bat livers, where it was localized to the cytoplasm of mononuclear phagocytes within the inflammatory cell foci (Fig. 3-5i), and rarely, within hepatocytes. No KASV RNA was detected by ISH in any of the 12-DPI bats. Of the two 18-DPI bats tested by ISH, one bat had focal positive hybridization signal in the cytoplasm of a single hepatocyte. Foci of necrosis in the two 9-DPI bats did not contain KASV RNA on the ISH assays.

Abundant KASV hybridization signal was detected in the spleen of the 3 DPI bats by ISH. Most of the ISH signal was localized to the walls of the sheathed red pulp capillaries. The signal varied greatly among individual capillaries, with some capillaries having 1-3 punctate dots and others having saturated ISH signal with partial to complete circumferential staining of the capillary wall (Fig. 3-6a). Using the Iba1+ antibody to co-detect KASV RNA and mononuclear phagocytes, positive hybridization signal was observed both within the cytoplasm of endothelial cells and within Iba1+ macrophages that interdigitate and cuff the sheathed capillaries (Fig. 3-6b). Viral RNA was also multifocally present within the white pulp, in the cytoplasm and branching cytoplasmic processes of Iba1+ cells morphologically consistent with macrophages or dendritic cells (Fig. 3-6c). At 6 DPI, scattered Iba1+ cells containing minimal positive hybridization signal were observed in the red pulp (Fig. 3-6d-e). No KASV RNA was detected in the splenic white pulp of the 6 DPI bats by ISH. At 9 and 12 DPI, only one bat from each time

point had focal positive hybridization in the red pulp. At 18 DPI, KASV RNA was not detectable in the spleens by ISH.

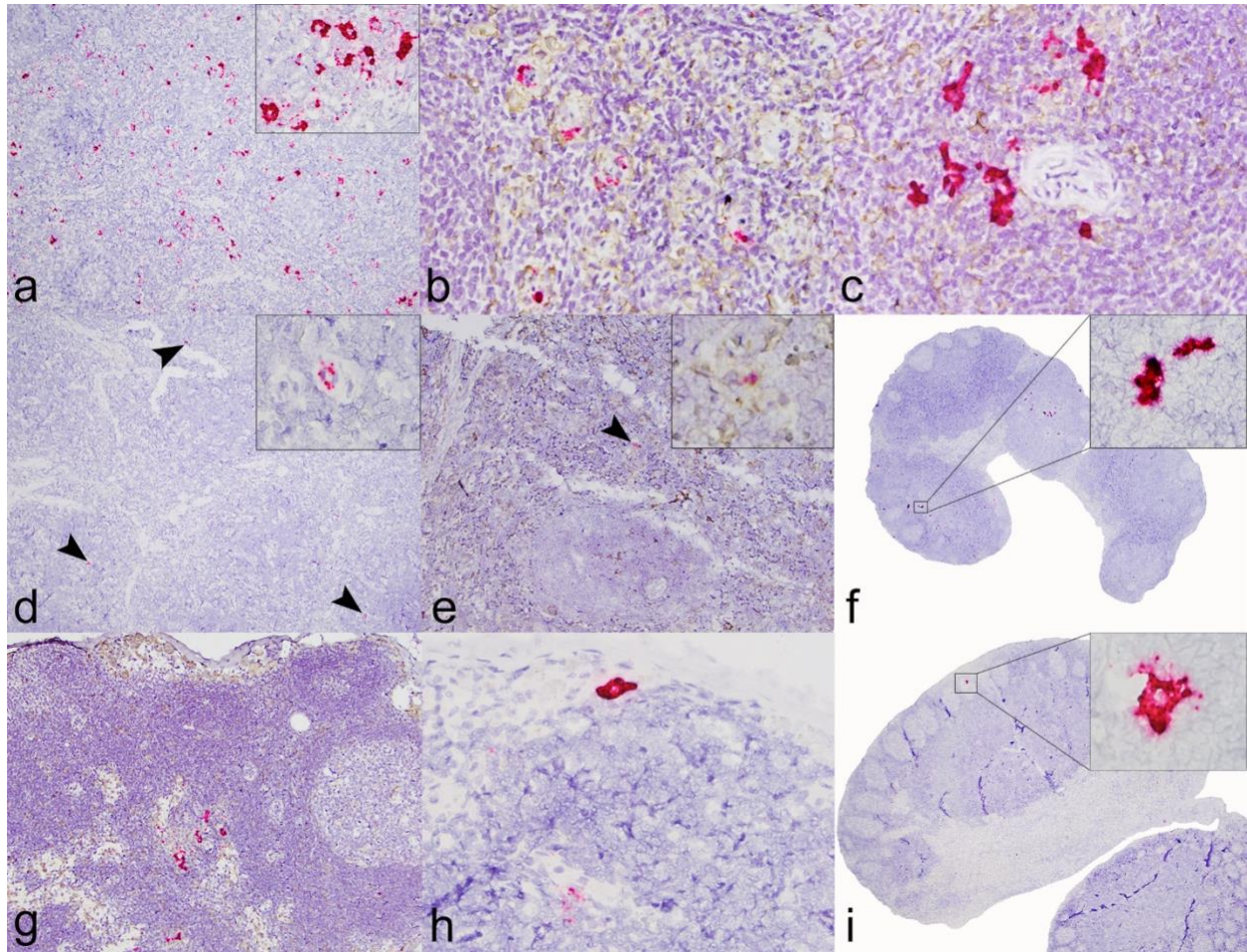


**Figure 3-6.** Kasokero virus infection, secondary lymphoid tissues, Egyptian rousette bats, 3 and 6 days post-infection. RNAScope *in situ* hybridization assay designed with an anti-sense probe targeting the KASV nucleoprotein gene. (a) Extensive positive ISH signal is distributed throughout the red pulp, primarily within the walls of red pulp capillaries, 3 DPI bat. (b) Dual KASV ISH (red) and Iba-1 (brown) immunohistochemical stain demonstrating multifocal colocalization of KASV with cells of the mononuclear phagocyte system and within endothelial cells of red pulp capillaries, 3 DPI bat. (c) Dual KASV ISH (red) and Iba-1 (brown) IHC stain demonstrating multifocal colocalization of KASV with dendritiform Iba-1<sup>+</sup> cells in the white pulp, 3 DPI bat. (d) Rare KASV ISH signal is localized to the walls of scattered red pulp capillaries, 6 DPI bat. (e) Dual KASV ISH (red) and Iba-1 (brown) IHC stain demonstrating focal colocalization of KASV with an Iba-1<sup>+</sup> cell in the red pulp, 6 DPI bat. (f) Multifocal KASV ISH signal throughout the inguinal lymph node paracortex and sinuses, 6 DPI bat. Inset is higher magnification of boxed area. (g) Multifocal positive KASV ISH signal (red) throughout the paracortical and medullary sinuses within Iba-1<sup>+</sup> macrophages (brown), 6 DPI bat. (h)

Within the subcapsular sinus of an inguinal lymph node, the cytoplasm of a large macrophage or dendritic cell is saturated with KASV ISH signal (red), 6 DPI bat. (i) Within a germinal center of an axillary lymph node, the cytoplasm of a cell with follicular dendritic cell morphology is saturated with KASV ISH signal, 6 DPI bat. Inset is higher magnification of boxed area. Red chromogen with hematoxylin counterstain (a, d, f, h & i). Dual ISH/IHC assay for KASV nucleoprotein (red) and Iba-1 (brown) with hematoxylin counterstain (b, c, e & g).

KASV RNA was detected by ISH in the lymph nodes (LNs) for most of the KASV-infected bats at 3 and 6 DPI, and in two 12-DPI and one 18-DPI bat. At 3 DPI, the majority of KASV RNA was present within Iba-1+ macrophages or dendritic cells within the subcapsular, paracortical and medullary sinuses (Fig. 3-6f). A smaller amount of KASV RNA was present within the paracortex and deep cortex, also in cells morphologically consistent with macrophages (Fig. 3-6g). At 6 DPI, KASV RNA was present in large mononuclear cells (macrophages and/or dendritic cells) within subcapsular sinuses (Fig. 3-6h) and occasionally in the germinal centers. In one bat at 6 DPI, the cytoplasm of a cell morphologically compatible with a follicular dendritic cell (FDC) was saturated with positive hybridization signal (Fig. 3-6i). Rare signal was also present within macrophages in the deep cortex and medullary sinuses. No KASV RNA was detected in any of the 9 DPI bat LNs tested by ISH. Two out of 3 of the 12 DPI bats had focal positive hybridization in the paracortex. At 18 DPI, one bat had scattered positive signal in the germinal centers. Semi-quantitative ISH scores for the liver, spleen, and lymph nodes are presented in Table 3-1.

**Table 3-1.** Semi-quantitative analysis of KASV nucleoprotein RNA, as detected by RNAScope *in situ* hybridization in the liver, spleen, and lymph nodes of KASV-infected Egyptian rousette bats. Abbreviations and symbols: DPI = days post-infection; NP = not performed; \* = ISH was performed on inguinal lymph nodes for all bats, except for bat 9; multiple peripheral lymph nodes were analyzed for this individual, and ISH signal was detected in the axillary, but not in the inguinal lymph node; \*\* = tissue washed off during processing. ISH staining was scored as follows: + = focal signal, less than 2-3 cells per 40x high-powered field (hpf); ++ = scattered signal in multiple foci throughout section, less than 2-3 cells per hpf; +++ = multifocal signal throughout section; 3-5 positive cells per hpf ++++ = multifocal signal throughout section, 5-10 cells per hpf; and +++++ = multifocal signal throughout section, frequent saturated signal, 5-10 cells per hpf.

| DPI           | Bat ID | Liver | Spleen | Lymph node |
|---------------|--------|-------|--------|------------|
| Neg. Controls | 1      | -     | -      | -          |
|               | 2      | -     | -      | -          |
|               | 3      | -     | -      | -          |
| 3             | 4      | ++++  | +++++  | +++        |
|               | 5      | ++++  | ++++   | -          |
|               | 6      | +++   | +++    | +++        |
| 6             | 7      | +     | +      | -          |
|               | 8      | +     | +      | ++         |
|               | 9      | +     | +      | +          |
| 9             | 10     | -     | -      | -          |
|               | 11     | +     | +      | -          |
|               | 12     | +     | -      | -          |
| 12            | 13     | -     | -      | +          |
|               | 14     | -     | +      | +          |
|               | 15     | -     | +      | -          |
| 18            | 16     | +     | -      | +          |
|               | 17     | -     | -      | -          |
|               | 18     | NP    | NP     | NP         |

In the oral tissues, KASV RNA was detected deep within the tongue, within the cytoplasm of elongate interstitial cells morphologically consistent with macrophages (Fig. 3-7). Skin sections from the inoculation site of one 3-DPI and one 6-DPI bat, both of which had prominent histiocytic inflammatory cell infiltrates, were negative for KASV by ISH.

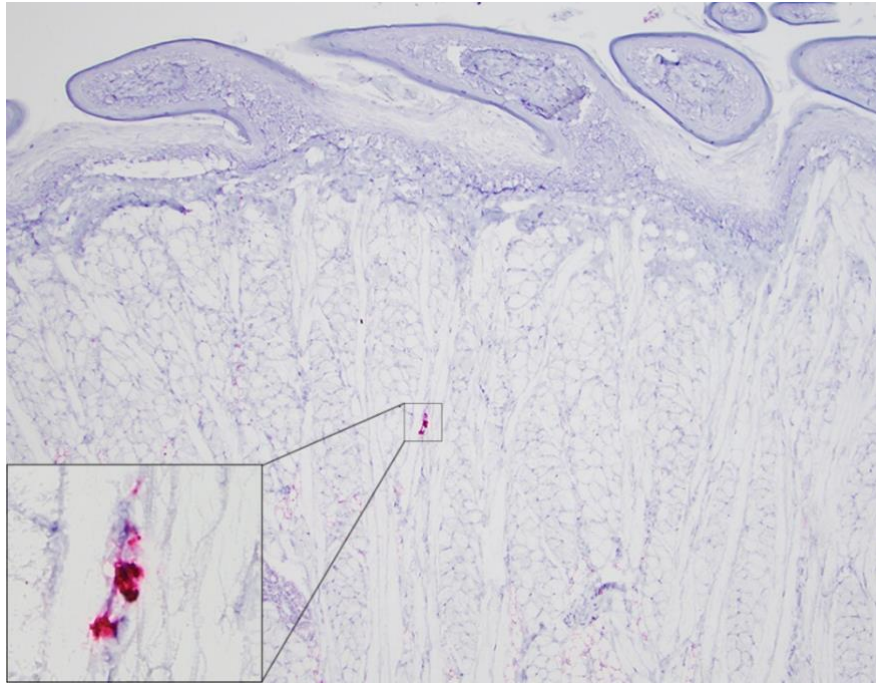


**Figure 3-7.** Kasokero virus infection, tongue, Egyptian rousette bat, 6 days post-infection. Intense KASV ISH signal is present within the connective tissue of the tongue. RNAScope *in situ* hybridization assay with an anti-sense probe targeting the KASV nucleoprotein gene. Red chromogen with hematoxylin counterstain.

### C. Quantitative Analysis of KASV Hybridization Signal in the Liver and Spleen

#### i. Liver

The average KASV nucleoprotein gene signal peaked in the liver at 3 DPI (% PP = 0.269%) and decreased 15-fold from 3 to 6 DPI (Figure 3-8). The 3- and 6-DPI %PP values correlated with the viral loads obtained via qRT-PCR for these time points (Spearman  $r = 0.81$ ;  $p = 0.036$ ). After 6 DPI, KASV RNA hybridization signal was minimally detectable by ISH ( $<$

0.01 %) in a subset of the 9-, 12-, and 18-DPI bat livers (2/3, 1/3, and 1/2, respectively), and was undetectable by ISH and digital image analysis in the remaining 18-DPI bat. KASV RNA was not detected in any of the control bat livers (n = 3).

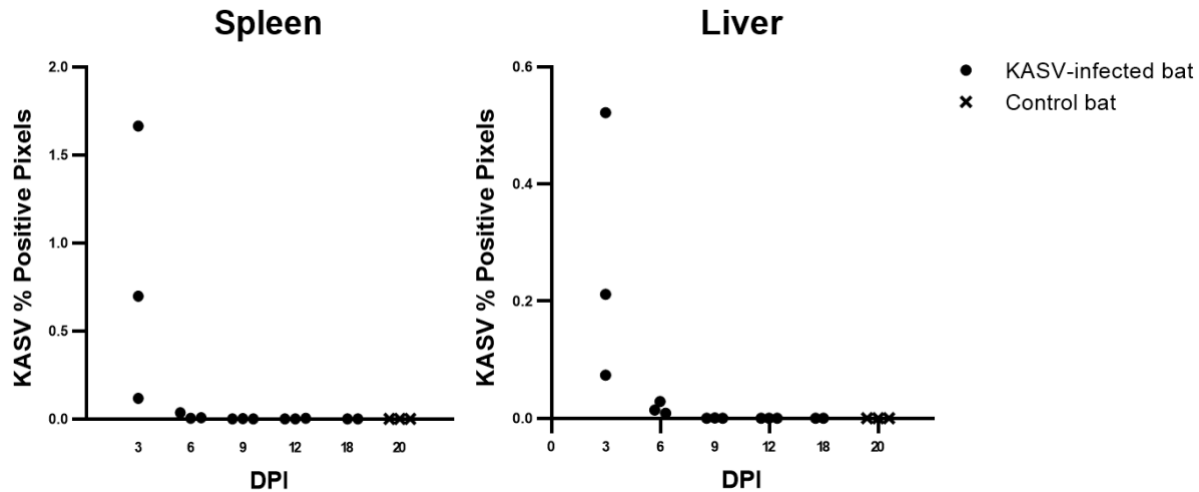


**Figure 3-8.** Quantification of KASV *in situ* hybridization signal in the liver and spleen of KASV-infected Egyptian rousette bats at 3-, 6-, 9-, 12-, and 18-DPI as a percentage of total area using digitized slides. Negative control bats (x) were euthanized at 20 DPI. Each dot represents an individual bat (n=3 per time point except 18-DPI [n=2]).

## ii. Spleen

In the spleen, the average %PP also peaked at 3 DPI with an average % pixel positivity of 0.826% (Figure 3-8). The average %PP decreased 52-fold from 3 to 6 DPI. At 3 and 6 DPI, the %PP values correlated with the qRT-PCR values previously reported for each bat (Spearman  $r = 0.78$ ;  $p = 0.032$ ).<sup>29</sup> KASV RNA signal was minimally detectable by ISH at 6 dpi, with all bats having %PP values  $< 0.03$  (2/3 bats were  $< 0.001$ ). KASV RNA was undetectable by ISH in 2/3 of the 9-DPI bats, and in the 18-DPI bats tested (2/2). KASV RNA was not detected in the control bat spleens (n = 3) by ISH.

## DISCUSSION

The findings presented herein are relevant to the pathogenesis of KASV, an emerging zoonotic bunyavirus capable of causing severe systemic disease in humans, and which is thought to be maintained in an enzootic transmission cycle involving Egyptian rousette bats and the argasid tick *O. (R.) faini*.<sup>20,29,30</sup> Experimentally infected ERBs remained asymptomatic and KASV-induced tissue damage was limited to the liver, where lesions were consistent with acute viral hepatitis.<sup>29</sup> In the liver, hepatocellular KASV replication was associated with cytopathic effects, cell death, mononuclear inflammatory cell recruitment, hemorrhage, and necrosis in a subset of infected bats. The liver lesions were mostly resolved by 20 DPI, highlighting the remarkable ability of this bat species to mount a rapid and localized immune response in this tissue following experimental KASV infection. Hepatic and splenic viral replication, as detected by ISH, peaked at 3 DPI, and the vast majority of KASV RNA was cleared from these tissues by 6 DPI.

In comparison to studies in which ERBs were infected with MARV using very similar protocols, the hepatic damage and immune cell response in this study was similar in character (i.e. lymphohistiocytic) but of increased severity for KASV.<sup>19,20</sup> The ISH results demonstrate that KASV is hepatotropic, a feature shared by several bunyaviruses of public health and veterinary importance including CCHFV, NSDV and Rift Valley Fever virus.<sup>9,16,28,31</sup> The observed minor tropism for endothelial cells and Kupffer cells may promote more advanced liver disease due to direct and/or indirect effects of viral infection; it may also predispose to bacterial infection, as rare bacilli were observed in one 9-DPI bat with hepatic necrosis. Hepatocellular glycogen depletion suggests that KASV infection may be associated with increased metabolic disturbances compared to MARV.<sup>19</sup> Whether, and to what extent, the 3 KASV-infected bats with hepatic

necrosis would have proceeded to develop clinically significant liver disease at later post-infection time points remains uncertain.

Quantitative analysis of the ISH slides permitted evaluation of virus clearance from the liver and spleen within a spatial context. The scarcity of viral RNA at 6 DPI indicates that the host responses are effective at controlling and clearing KASV early during the course of infection. Replicating KASV frequently co-localized with cells of the mononuclear phagocyte lineage in the liver, spleen, and lymph nodes, suggesting a prominent role for this diverse cell lineage in the dissemination of virus, in driving the innate and adaptive immune response(s), in the clearance of infected cells from the blood and liver, and in resolution of the liver lesions. Replicating KASV was detected in the cytoplasm of a cell with follicular dendritic cell morphology in the axillary lymph node of one bat at 6 DPI. Follicular dendritic cells are long-lived, mesenchymal-origin cells that shape the antibody response and have been shown to harbor antigens for long periods of time.<sup>6,21,36</sup>

In ERB spleens, sheathed capillaries are numerous, particularly in the marginal zone and throughout the red pulp. These thick-walled sheathed capillaries, often termed ellipsoids, are a prominent feature of the spleens of several other Old-World fruit bat species.<sup>15</sup> Extensive hybridization signal within the ellipsoids coupled with the lack of overt cytopathic effects suggests that these structures contribute significantly to the clearance of viral RNA from the blood between 3 and 6 DPI, thereby limiting spread to other tissues.

Our spatial, quantitative, and temporal analyses underscore the importance of the liver and spleen in the early host responses to KASV infection and shed light on the variety of mechanisms by which these tissues may function to control viral replication and clear viral RNA. ERB host responses to KASV may, in part, be generalized responses that are effective at

controlling other RNA viruses with shared tissue tropisms such as MARV. The increased pathogenicity of this virus in ERBs compared to MARV, a host-virus relationship for which mechanisms of disease tolerance are hypothesized to limit tissue damage caused by both host and pathogen, make it an attractive experimental model for studying ERB immunology and virus-bat host interactions.<sup>14,24,26</sup>

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## **DECLARING OF CONFLICTING INTERESTS**

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## CHAPTER 4

# COMPARATIVE PATHOLOGY OF MARBURG AND KASOKERO-INDUCED VIRAL HEPATITIS IN EXPERIMENTALLY INFECTED EGYPTIAN ROUSETTE BATS (ROUSETTUS AEGYPTIACUS)<sup>3</sup>

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## ABSTRACT

The Egyptian rousette bat (ERB; *Rousettus aegyptiacus*; family *Pteropodidae*) is a natural reservoir host for the zoonotic filovirus Marburg virus (MARV; family *Filoviridae*) and the bunyavirus Kasokero virus (KASV; family *Nairoviridae*), which is thought to be maintained in an enzootic ERB-tick transmission cycle. While MARV- and KASV-infected humans experience mild to severe systemic illness, experimentally infected ERBs remain asymptomatic, and the specific mechanisms by which they control and clear these infections remain largely unknown. Herein, the ERB host responses to KASV and MARV were assessed side-by-side using formalin-fixed paraffin-embedded (FFPE) tissues obtained from experiments in which bats were euthanized at similar post-infection time points, focusing on the liver as a primary target tissue for both viruses where early host-virus interactions occur. Comparative histopathology, morphometrics and immunohistochemical analyses of MARV- and KASV-infected ERB livers revealed overall increased pathogenicity for KASV compared to MARV, and a peak severity of lymphohistiocytic hepatitis at 6 DPI and increased immunostaining for Iba1 and CD3 at early time points (3-9 DPI) for both viruses. Distinctive CD3<sup>+</sup>-cell-hepatocyte interactions suggestive of cell mediated cytotoxicity were observed in KASV-infected ERBs at 3 DPI. Further, cleaved caspase 3 immunostaining peaked at 3 DPI, with intense and widespread staining in mononuclear phagocytes, and rarely, in hepatocytes. The data suggest that for both KASV and MARV, the bat host uses a common strategy to resolve virus-induced hepatitis, namely, recruitment of cytotoxic CD3<sup>+</sup> cells and mononuclear phagocytes to foci of hepatocellular infection and the early induction of apoptosis in monocyte-derived macrophages. Further, immune cell-hepatocyte interactions may promote T cell tolerance as their adaptive immune responses evolve. These

mechanisms may be more broadly applicable to other viruses circulating in ERBs and possibly to other bat-virus relationships.

**KEYWORDS:** Marburg virus, filovirus, Kasokero virus, nairovirus, zoonoses, Egyptian roussette bat, natural reservoir, comparative pathology, viral hepatitis, immunohistochemistry, apoptosis

## INTRODUCTION

Bats (Order Chiroptera) are receiving increased attention as natural reservoir hosts for zoonotic viruses. Many of these viruses appear to circulate within bat populations without causing notable disease in their reservoir hosts. The Egyptian roussette bat (ERB; *Rousettus aegyptiacus*) is a confirmed reservoir host for Marburg and Ravn viruses (MARV and RAVV; family *Filoviridae*) and a suspected reservoir host for Sosuga virus (SOSV; family *Paramyxoviridae*) and Kasokero virus (KASV; family *Nairoviridae*).<sup>1,4,5,7,25,40-42</sup> Experimental infections of ERBs with MARV, SOSV and KASV have demonstrated that ERBs are a competent host for these viruses, with productive infections characterized by viremia, oral, fecal and urinary shedding, and replication in specific target tissues, all in the absence of overt clinical disease, showing only mild to moderate histological lesions that are limited to the liver for MARV and KASV and the small intestines and salivary glands for SOSV.<sup>6,8,26</sup>

MARV and KASV are hepatotropic in ERBs and hepatocellular infection is associated with the recruitment of mononuclear inflammatory cells, the majority of which are cleared by three-to-four weeks-post infection.<sup>24,27</sup> Marburg virus has a broad tissue and cellular tropism that is similar across susceptible species, including humans, nonhuman primates and ERBs.<sup>24,28,31</sup> However, innate immune mechanisms fail to clear MARV in humans and nonhuman primates

(NHPs), largely due to early, uncontrolled virus replication in mononuclear phagocytes resulting in their dysregulation. MARV becomes widely disseminated with and severe lymphoid depletion resulting from bystander lymphocyte apoptosis in secondary lymphoid tissues.<sup>3,15,18</sup> In susceptible non-bat hosts, replication in hepatocytes and Kupffer cells, hepatic necrosis, and significant liver dysfunction are characteristic of end-stage Marburg Virus Disease (MVD).<sup>3,19,30,31</sup> Apoptotic mononuclear cells and hepatocytes have been observed in the liver of macaques experimentally infected with MARV, although the mechanisms driving apoptosis in this tissue are unknown.<sup>13</sup> Much less is known about Kasokero virus, especially in susceptible non-bat hosts. Although KASV infection has been definitively diagnosed in clinically ill humans, there have been no experimental studies in animal hosts, other than those in ERBs, where it was shown to cause mild to moderate, acute viral hepatitis, but not cause overt clinical disease.<sup>27,35</sup> However, Crimean-Congo Hemorrhagic Fever Virus (CCHFV; family *Nairoviridae*), another tick-associated orthonairovirus closely related to KASV, has been more extensively studied, in naturally infected humans, in experimentally infected immunocompromised or humanized mice, and in NHPs, where it causes severe damage to the liver and significant mortality.<sup>17,21,44</sup> *In vitro* and *in vivo* experiments exploring ERB-MARV interactions support a strategy of immunoprotective disease tolerance for this virus-host relationship and there is considerable public health interest in uncovering the specific immunologic mechanisms by which ERBs avoid severe virus-induced disease.<sup>20,33,46</sup>

The purpose of the present study was to compare and contrast the ERB host responses to two diverse RNA viruses MARV and KASV, that are naturally hosted by the Egyptian rousette bat. Our approach used formalin-fixed paraffin-embedded (FFPE) tissues from previous experimental infection studies, focused on the liver, the primary site of parenchymal cell

replication for both viruses where virus replication is associated with acute lymphohistiocytic hepatitis.<sup>27,35</sup> To this end, the study had three aims. The first aim was to conduct fine-scale histologic and morphometric analyses of the liver lesions using FFPE tissues from ERBs infected with MARV or KASV according to similar protocols and euthanized at similar post-infection time points. The second aim was to identify and quantify the infiltrating immune cell types in the liver of MARV and KASV-infected ERBs using immunohistochemical protocols targeting ionizing calcium binding adaptor molecule 1 (Iba1; mononuclear phagocyte marker) and CD3 (T cell and/or natural killer T (NKT) cell marker). The final aim of this study was to determine the extent of programmed cell death in the ERB virus-host responses as a mechanism of virus clearance using an immunohistochemical protocol targeting cleaved caspase 3.<sup>23,24,31,34</sup> The rationale for this aim was based on the prominent role apoptosis plays in the pathogenesis of MARV infection in humans and NHPs, the role of apoptosis as an anti-viral mechanism in general, and the detection of hepatocytes with morphologic features of apoptosis in the livers of MARV- and KASV-infected ERBs. This study provides important insight into ERB immunology and the unique anti-viral responses of bats.

## **MATERIALS & METHODS**

### **A. Tissue sample collection, processing, and virus origins**

Archived FFPE liver tissues from ERBs used in the present study (n=39) were obtained from two serial sacrifice studies performed under biosafety level 4 (BSL-4) conditions in accordance with safety regulations at the Centers for Disease Control and Prevention (CDC, Atlanta, GA, U.S.A.).<sup>6,35</sup> The experimental infections were conducted with approval from the CDC Institutional Animal Care and Use Committee (IACUC), and in accordance with the animal

welfare guidelines set forth in the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, National Research Council of the National Academies 2011). Husbandry, housing conditions and diet for the duration of the experiment were previously described.<sup>8,35</sup> Briefly, food consisted of a variety of fresh fruit dusted with a protein vitamin supplement (Lubee Bat Conservancy, Gainesville, FL). Water was provided *ad libitum*. The MARV isolate was collected from a wild-caught ERB (MARV371; GenBank ID: FJ750958).<sup>40</sup> The KASV isolate (UGA-Tick-20170128; GenBank ID: MT309096) was collected from an argasid tick [*Ornithodoros* (Reticulinasus) *faini*] known to be involved in the life cycle of this arbovirus, and was collected at Python Cave in Uganda.<sup>37</sup> Both isolates were passaged twice in Vero E6 cells. For the inocula, MARV and KASV were diluted in 250  $\mu$ L of Dulbecco's modified Eagle's medium (DMEM) and 0.1 mL phosphate-buffered saline (PBS), respectively, to concentrations of  $4 \times 10^5$  tissue culture infective dose 50 (TCID<sub>50</sub>)/mL. MARV was inoculated subcutaneously in the ventral abdominal region while KASV was inoculated intradermally in the same region to simulate a tick bite. For each experiment, the control bats were inoculated with equal volumes of the respective dilution medium using the same site and route as the infected bats.

At the end of the studies, bats were euthanized under anesthesia via cardiac exsanguination at predetermined time points (days post-inoculation; DPI). Blood and selected tissue samples were collected for viral load determination by qRT-PCR at the time of euthanasia. RNA extractions, qRT-PCR, and normalization to account for sample-to-sample variation were performed as previously described.<sup>6,35</sup>

FFPE tissues from animals euthanized at similar time points during each study were selected for the current study (3-, 6-, 9-, 12-, 18- and 20-DPI for KASV; 3-, 6-, 9-, 12-, and 28-

DPI for MARV; n=3 for each time point). Three control bats were mock-inoculated and euthanized at the conclusion of each serial euthanasia study (day 20 and 28 for the KASV and MARV studies, respectively). Following tissue collection, the bat carcasses were immersed in 10% neutral buffered formalin (10% NBF) for a minimum of 7 days, with a complete change of formalin after day 7, prior to removing the carcasses from the BSL-4 laboratory and transferring the carcasses to 70% ethanol.

## B. Histology

A full set of tissues from each formalin-fixed bat, including brain, heart, lungs, trachea, thymus, liver, stomach, small intestines (duodenum with pancreas, jejunum, and ileum), large intestine, spleen, kidney, adrenal gland, salivary gland, submandibular lymph node, axillary lymph node, skin from inoculation site, skin from the chest region and pectoral muscle, was collected and reviewed histologically following previous studies.<sup>24,27</sup> Histologic lesions attributable to MARV- and KASV infections, consisted of multiple, randomly distributed foci of mononuclear inflammatory cells and necrotic or apoptotic hepatocytes, and was limited to the livers of infected bats. The archived FFPE blocks containing liver tissues from the MARV serial sacrifice study (n=18) were sectioned at 4-5  $\mu$ m thickness and stained with hematoxylin and eosin (H&E) for evaluation. H&E-stained sections from the KASV serial sacrifice study were re-analyzed for the current study. Unstained liver sections were prepared from all FFPE blocks (n=39) at 4-5  $\mu$ m thickness and mounted on glass slides for immunohistochemistry.

## C. Immunohistochemistry (IHC)

Immunohistochemical procedures for CD3, ionized calcium-binding adapter molecule 1 (Iba1), a marker of mononuclear phagocytes, and cleaved caspase 3 (ClvCasp3), a marker of

apoptosis were performed on 4 non-contiguous FFPE sections of liver for each bat (n=39).

Appropriate immunostaining was verified using internal controls for each stain (CD3+ and Iba1+ resident immune cells in the liver and/or spleen and ClvCasp3+ apoptotic bodies in the germinal centers of the spleen).

#### i. CD3 IHC

The FFPE tissue sections were heated at 65°C and deparaffinized by washing in Hemo-De® (Fisher Scientific, Pittsburgh, PA), three times for 10 minutes each. Slides were washed in 100% ethanol for 2 minutes, air dried and the tissue circled by a hydrophobic barrier PAP pen (Vector Laboratories, Burlingame, CA). Endogenous peroxidases were blocked by applying Peroxidazed 1 (Biocare Medical, Pacheco, CA) for 10 minutes and slides were washed (1X TBST, 2x5 minutes). Antigen retrieval was achieved by applying an unmasking preheat solution (1x Antigen Unmasking solution; Vector Laboratories, Burlingame, CA) which was preheated to boiling at power level 10 for 2 minutes and steaming the slide for 20 minutes at 100°C. Slides were washed and then incubated at room temperature (RT) with a universal blocking reagent (Background Punisher, Biocare Medical, Pacheco, CA) and washed (1X TBST, 2x5 minutes). A solution containing the anti-CD3 antibody (Dako, catalog # A0452) diluted in Renaissance Background Reducing Diluent (Biocare Medical, Pacheco, CA; final dilution = 1:1000) was applied to each slide, and slides were incubated overnight at 4°C. The next day, slides were washed (1X TBST, 3x5 minutes), incubated for 15 minutes at RT with the MACH 3 Rabbit Probe (Biocare Medical, Pacheco, CA), and washed (2x5 minutes). Slides were then incubated for 15 minutes with the MACH 3 Alkaline Phosphatase Polymer (Biocare Medical, Pacheco, CA), washed (1X TBST, 3x5 minutes) and finally, one drop of the Warp Red Chromogen (Biocare Medical, Pacheco, CA) was applied and incubated for 5-7 minutes at RT, and again

washed (1X TBST, 3x5 minutes). The slides were counterstained with hematoxylin (Leica, Richmond, IL) and coverslipped with Permount for a permanent record.

#### ii. Iba1 IHC

Immunohistochemistry was performed as described above for CD3, except for the primary antibody, a rabbit-anti-Iba1 antibody (FujiFilm, catalog # 019-19741) was applied at a final dilution of 1:8000 and the MACH 3 Horseradish-Peroxidase (HRP) Polymer detection kit (Biocare Medical, Pacheco, CA) was used.

#### iii. ClvCasp3 IHC

Immunohistochemistry was performed as described above for CD3, except for the primary antibody, a rabbit-anti-caspase 3 antibody (Biocare Medical, catalog # CP229B) was applied at a final dilution of 1:150.

### D. Quantitative Whole-Slide Image Analysis

#### i. Comparative Morphometric Analysis of KASV- and MARV-Infected ERB Livers

At least 4 non-contiguous, H&E-stained sections of liver tissue from each bat (n=39) were reviewed in a blinded manner by a veterinary pathologist (SK) without knowledge of time point or infection status. Slides were scanned at 200x magnification using an Aperio Slide Scanner (Aperio ScanScope XT, Aperio Technologies, Inc., Vista, CA, USA) and imported into a freely available open-source bioimage analysis software (QuPath version 0.3.0, Queen's University Belfast, Belfast, Northern Ireland, UK).<sup>9</sup> Foci of mononuclear inflammatory cells, defined as clusters of at least 3 mononuclear cells, either located within the sinusoids, distorting the hepatic cords, or in the centrilobular or periportal connective tissues, were manually

annotated in QuPath to generate a count of the total number of inflammatory foci. The total liver area was measured by tracing each section of liver and adding the individual tissue section areas ( $\text{mm}^2$ ). The number of foci per  $\text{mm}^2$  tissue for each bat was calculated by dividing total number of inflammatory foci by the total liver area.

## ii. Quantitative Analysis of CD3, Iba1 and Cleaved Caspase 3 Immunostaining

The CD3, Iba1, and ClvCasp3-immunostained slides were scanned at 400x magnification using a whole slide scanner (Aperio Technologies, Inc., Vista, CA, USA) and imported into QuPath. A region of interest (ROI) was manually annotated around each of the liver sections. Immunolabeled cells were visualized with DAB (Iba1) or Fast Red (CD3 and ClvCasp3) chromogens. Labeled cells were segmented from negative cells and background staining by creating a threshold that was customized for each slide (threshold ranges for Iba1, CD3 and ClvCasp3 were 0.2-0.55, 0.2-0.81, and 0.2-0.8, respectively). The other default parameters in the Create Threshold tool (Gaussian sigma = 1.5  $\mu\text{m}$ , background radius = 15  $\mu\text{m}$ , cell detection threshold = 0.1, and detection object diameter = 25 pixels) were used. The percent pixel positivity (%PP) was measured for each ROI to remain consistent across samples and antibodies. A manual assessment of detected immunopositive cell morphology was performed for each slide as a means of quality control.

## E. Statistical Analyses

Statistical analyses were performed using Prism 9.3.1 (GraphPad Software, LLC, La Jolla, CA). For the Iba1, CD3 and ClvCasp3 %PP, values from KASV- and MARV-infected bat livers at each time point ( $n = 3$ ) were compared with those of the control bats ( $n = 3$ ) using a one-way analysis of variance (ANOVA) test, followed by Dunnet's multiple comparison test if the

ANOVA demonstrated significant differences between groups ( $p < 0.05$ ). Differences between the morphometric data (# foci/mm<sup>2</sup>) for each virus (MARV and KASV) at each time point were analyzed using nonparametric Wilcoxon rank sum tests. Correlations were analyzed using the nonparametric Spearman rank test.

#### F. Data Availability

The data analyzed in this study are available as Supplemental Materials.

## RESULTS

### A. Comparison of Gross and Histologic Lesions in the Livers of MARV- and KASV-infected ERBs

#### i. Gross lesions

No gross lesions were observed in the MARV-infected ERBs included in the current study.<sup>24</sup> Four KASV-infected bats had gross lesions limited to the liver, which were previously reported.<sup>27,35</sup> These lesions consisted of hepatic hemorrhages (two 6-DPI and two 9-DPI bats) and a regionally extensive focus of pallor corresponding to hepatic necrosis (n=1; one of the 9 DPI bats with hemorrhages).

#### ii. Histopathology

The livers of all ERBs experimentally infected with KASV (n=18) or MARV (n=15) were histologically notable for the presence of randomly distributed, variably sized foci of mononuclear inflammatory cells. Inflammatory cell foci were composed of a majority of histiocytes and lymphocytes, and rare neutrophils. Starting at 3 DPI, inflammatory cell foci in MARV-infected ERBs were rare; they were ill-defined from the surrounding parenchyma and occasionally incorporated degenerating hepatocytes which lacked normal glycogen-type

cytoplasmic vacuolation (Fig. 4-1a). One MARV-infected bat at 3-DPI had a focally extensive accumulation of histiocytes and lymphocytes and apoptotic or necrotic hepatocytes adjacent to a large branch of the hepatic artery (Fig. 4-1b). Compared to MARV-infected bats, KASV-infected bats euthanized at 3-DPI had larger and more frequent foci, which often contained hemorrhage and degenerating, apoptotic or necrotic hepatocytes (Figs. 4-1c-d). One KASV-infected bat euthanized at 3-DPI had numerous 2-5- $\mu$ m-diameter, round eosinophilic cytoplasmic inclusions within hepatocytes along the periphery of the inflammatory foci (Fig. 4-1d). Another KASV bat euthanized at 3-DPI had fibrinous capsulitis with mild, multifocal hepatic necrosis. By contrast, no hepatocellular inclusions were observed in any MARV-infected bat.

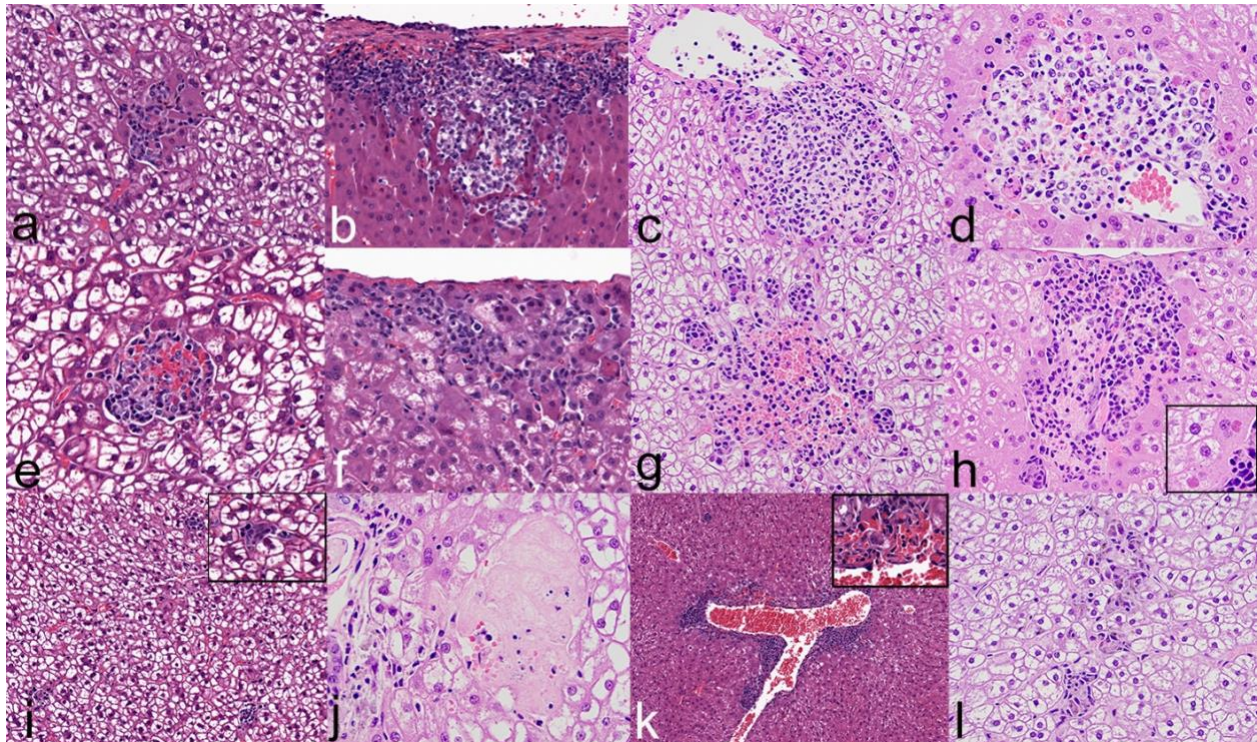


**Figure 4-1.** Comparative histopathology of Egyptian rousette bats experimentally infected with Marburg virus (MARV) or Kasokero virus (KASV), and euthanized at 3-, 6-, 9-, and 20 or 28-days post-infection (DPI). **a)** Mild focal lymphohistiocytic hepatitis with hepatocellular degeneration in a MARV-infected ERB, 3 DPI. Hematoxylin and eosin (HE). **b)** Focally extensive lymphohistiocytic hepatitis subjacent a large branch of the hepatic artery in a MARV-infected ERB, 3 DPI. HE. **c)** Moderate lymphohistiocytic hepatitis with hepatocellular

degeneration in a KASV-infected ERB, 3 DPI. HE. **d)** Moderate lymphohistiocytic hepatitis with hepatocellular degeneration, necrosis and/or apoptosis, hemorrhage, and hepatocellular inclusions in a KASV-infected ERB, 3 DPI. HE. **e)** Moderate, multifocal lymphohistiocytic hepatitis with hemorrhage in a MARV-infected ERB, 6 DPI. HE. **f)** Mild capsulitis and lymphohistiocytic hepatitis and hepatocellular apoptosis or necrosis in a MARV-infected ERB, 6 DPI. HE. **g)** Marked, multifocal to coalescing lymphohistiocytic hepatitis and hemorrhage in KASV-infected ERB, 6 DPI. HE. **h)** Moderate subcapsular lymphohistiocytic hepatitis in a KASV-infected ERB, 6 DPI. HE. Inset: Different area from the same bat showing low numbers of round, eosinophilic cytoplasmic inclusions within hepatocytes adjacent to an inflammatory focus. **i)** Minimal, multifocal lymphohistiocytic hepatitis in a MARV-infected ERB, 9 DPI. HE. **j)** Hepatic necrosis and periportal hepatitis in a KASV-infected ERB, 9 DPI. HE. **k)** Moderate, focally extensive perivascular mononuclear cell infiltrates and acute hemorrhage (inset) in a MARV-infected ERB, 28 DPI. HE. **l)** Multifocal sinusoidal hemosiderosis in a KASV-infected ERB, 20 DPI. HE.

At 6 DPI, hepatic inflammatory cell foci were numerous in both MARV- and KASV-infected bats, often containing acute hemorrhage and low numbers of hemosiderin-laden macrophages (Figs. 4-1e-f), incorporating degenerating, necrotic or apoptotic hepatocytes (Figs 4-1f-h), and occasionally extending to and involving the capsule (Figs. 4-1f & 1h). Foci tended to be well demarcated from the surrounding hepatic parenchyma (Figs. 4-1e, 1g & 1h) and adjacent foci frequently coalesced for KASV-infected bats (Figs. 4-1g-h). In general, inflammatory foci contained more hemorrhage in KASV-infected bats compared to MARV-infected bats at 6-DPI (Figs. 4-1e & g). The three KASV-infected bats euthanized at 6-DPI rarely had 1-3- $\mu$ m-diameter, round eosinophilic cytoplasmic inclusions within hepatocytes immediately adjacent to the inflammatory cell foci (Fig. 4-1h, inset). No hepatocellular inclusions were observed in any MARV-infected bat at 6 DPI.

At 9 DPI, the inflammatory cell foci of KASV-infected bats at 9-DPI contained numerous hemosiderin-laden macrophages and two KASV-infected bats had randomly distributed foci of hepatocellular necrosis (Fig. 4-1j), one of which contained at least two bacilli. Hepatic necrosis was not observed in any MARV-infected bat, and at 9-DPI, inflammatory cell foci were

fewer and smaller compared to 6-DPI (Fig. 4-1i). While inflammatory cell foci were rare in the MARV-bats euthanized at 28 DPI, one bat had a focally extensive mononuclear cell infiltrate adjacent to a large vein, with acute peri-venular hemorrhage (Fig. 4-1k). In the KASV-infected bats euthanized at 20 DPI, inflammatory cell foci were also rare, and those that remained consisted primarily of hemosiderin-laden macrophages with scattered lymphocytes (Fig. 4-1l). The livers of control bats from both experiments contained few small foci of mononuclear inflammatory cells randomly scattered throughout the hepatic parenchyma.

#### B. Comparison of liver morphometrics and MARV and KASV viral loads in experimentally infected ERBs

Overall, compared to MARV-infected bats, mononuclear cell foci appeared earlier in the liver of KASV-infected bats and were more numerous from 3 to 12 DPI (Fig. 4-2A-B). The average number of foci per mm<sup>2</sup> peaked at 6 DPI for both experiments and correlated over time ( $r=-.4757$ ,  $p=0.0460$ ). For KASV- and MARV-infected bats at 20- and 28-DPI, respectively, the average number of foci per mm<sup>2</sup> was similar to that of the controls, indicating resolution of the inflammatory response. The number of foci per mm<sup>2</sup> liver tissue for the MARV-infected bats correlated with the semi-quantitative liver lesion scores previously reported for these animals ( $r=0.6137$ ;  $p=0.0034$ ).<sup>24</sup>

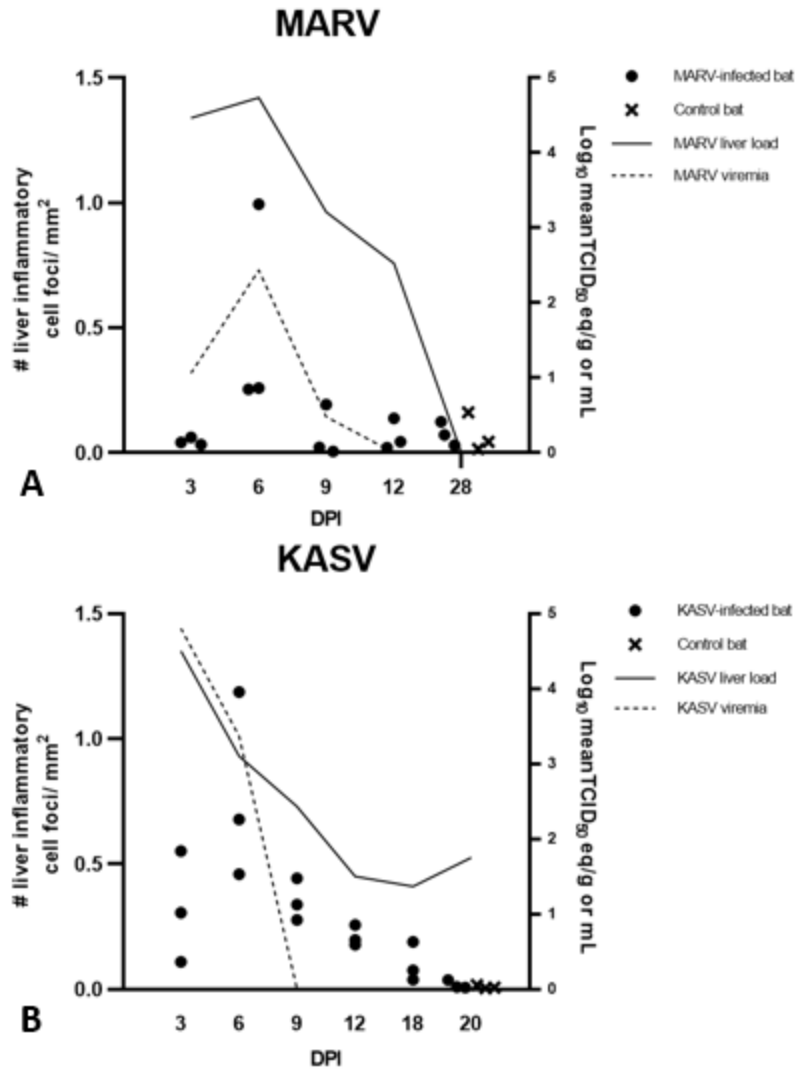


**Figure 4-2.** Comparative analysis of the hepatic morphometric data, hepatic viral loads and viremias from ERBs experimentally infected with Marburg virus (MARV) or Kasokero virus (KASV). **A)** Liver morphometrics for each bat (# of inflammatory foci per mm<sup>2</sup>), and average liver MARV loads and viremia per time point. **B)** Liver morphometrics for each bat (# of inflammatory foci per mm<sup>2</sup>), and average liver KASV loads and viremia per time point. Each time point is grouped on the x axis and each dot represents the # of foci per mm<sup>2</sup> in the liver of a single bat obtained by whole slide imaging and digital analysis. Control bats were euthanized at 20 DPI (KASV study) or 28 DPI (MARV study), and each “x” represents a single control bat. The solid and dashed lines represent the mean liver viral loads and viremias (log<sub>10</sub> meanTCID<sub>50</sub> eq/g or mL) per time point, respectively, obtained at necropsy and previously reported.<sup>6,35</sup> Controls tested uniformly negative by qRT-PCR for each virus at necropsy (data not shown). There was a statistically significant increase in the # of foci per mm<sup>2</sup> at 6 DPI, compared to controls, in the KASV study.

Although the mean peak liver viral loads for KASV and MARV, as reported in the previous experiments, was similar (4.5 and 4.73 log<sub>10</sub> TCID<sub>50</sub> eq/g, respectively), the peak replication for KASV occurred earlier compared to that for MARV (3 and 6 DPI, respectively; Fig. 4-2).<sup>6,35</sup> Additionally, there was a steep decrease in the KASV viral load from 3 to 9 DPI, whereas for MARV, there was a more gradual decrease in the liver viral load. There were also distinctive differences in the viremia kinetics for KASV and MARV. During the serial sacrifice study, the average KASV viremia, measured at necropsy, peaked at 3 DPI, sharply declined, and was undetectable after 9 DPI (Fig 4-2). Compared to KASV, the average viremic peak for MARV, measured at necropsy, was of smaller magnitude (3.3 versus 5.6 log<sub>10</sub>TCID<sub>50</sub> eq/mL), and occurred later (6 versus 3 DPI; Fig. 2). The average duration of viremia was longer for KASV (7.7 days) than it was for MARV (3 days; data not shown).<sup>6,35</sup>

#### C. Quantitative analysis of CD3 and Iba1 immune cell markers and cleaved caspase 3 immunostaining in the liver of MARV- and KASV-infected ERBs

The subcellular localization was appropriate for both markers (cytoplasmic for Iba1 and membranous to cytoplasmic for CD3), as was the cell morphology for each. Both MARV and KASV infections were associated with increases in the average percentage of Iba1-immunopositive pixels (Iba1 %PP) in the liver of ERBs, consistent with recruitment of mononuclear phagocytes to the liver early during the course of both infections (Fig. 4-3A). The average increases in % Iba1 %PP were higher for KASV-infected bats euthanized at 3- and 12-DPI compared to MARV-infected bats at these same time points. The increases in % Iba1 %PP at 6- and 9-DPI were of similar magnitude for MARV- and KASV-infected bats. A few individual KASV-infected bats euthanized at 3-, 9-, and 12-DPI (n=1 per time point) had 2-to-3-fold increases in the Iba1 %PP compared to their corresponding controls. However, there were

no statistically significant differences when comparing the %PP for each time point to controls nor when comparing the same time points between viruses. The KASV-infected bat with the overall highest Iba1 %PP at 3-DPI (044997) had the most numerous inflammatory cell foci for that time point.

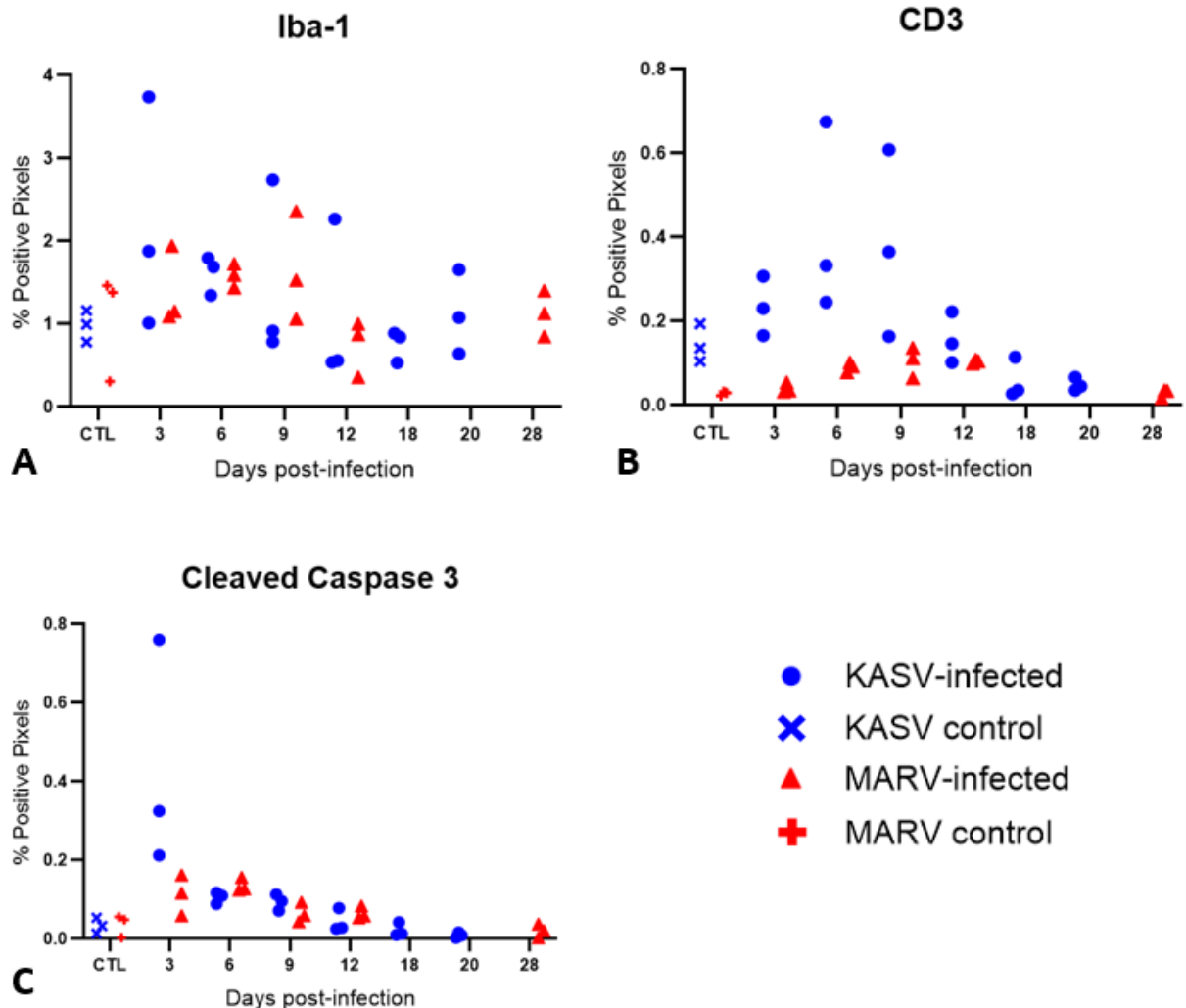


**Figure 4-3.** Quantitation of Iba1, CD3 and Cleaved Caspase 3 immunopositivity in the liver of ERBs experimentally infected with Marburg virus or Kasokero virus and euthanized at 3-, 6-, 9-, 12- and 18/20- or 28-DPI. Individual time points are represented on the x-axis and each dot represents the percentage of positive pixels for a single bat obtained by whole-slide imaging and quantitative analysis. Control bats (CTL) were euthanized at 20 and 28 DPI during the KASV and MARV studies, respectively. **A)** Hepatic Iba1 immunostaining. **B)** Hepatic CD3 immunostaining. **C)** Hepatic cleaved caspase 3 immunostaining.

MARV and KASV infections were associated with increases in the average percentage of CD3-immunopositive pixels (CD3 %PP) in the liver of ERBs (Fig. 4-3B), but compared to Iba1, CD3+ pixels overall comprised a smaller percentage of positive pixels in the livers of MARV- and KASV-infected ERBs. At each time point through 12 DPI, CD3+ cells comprised a higher average %PP of liver tissue for KASV-infected bats compared to MARV-infected bats. However, there were no statistically significant differences between the corresponding time points for KASV- and MARV-infected bats. At 9- and 12-DPI, MARV-infected bats showed a mild but statistically significant increase in the CD3+ %PP compared to control bats from the MARV experiment. Two individual KASV-infected bats euthanized at 6- and 9-DPI (bats 909535 and 909659) had 4.7- and 4.2-fold increases, respectively, in the CD3 %PP compared to the average CD3 %PP for the control bats. The KASV-infected bat with the overall highest CD3 %PP at 6-DPI (909535) had the most frequent inflammatory cell foci of all bats in that study. For KASV-infected bats, the CD3 %PPs correlated with the Iba1 %PP values ( $r=0.4662$ ;  $p=0.0166$ ), indicating the recruitment and resolution of these inflammatory cell populations along a similar time course.

KASV- and MARV-infected bats euthanized at 3-DPI, there was a 13-fold and 3-fold increase, respectively, in the percentage of pixels immunopositive for cleaved caspase 3 (ClvCasp3 %PP; apoptosis marker) relative to their respective controls (Fig. 4-3C). While this increase was statistically significant on the ANOVA ( $p=0.0119$ ), it was not significant on the multiple comparisons test ( $p=0.098$ ). Relative to control values, there were mild increases in the ClvCasp3 %PP at 3- and 6-DPI for MARV-infected bats, and at 6- and 9-DPI for KASV-infected

bats; these increases were not statistically significant. The ClvCasp3 %PP values correlated with the Iba1 %PP values for both KASV- and MARV-infected bats.

D. Qualitative assessment of CD3 and Iba1 immune cell markers and cleaved caspase 3 immunopositivity in the liver of MARV- and KASV-infected ERBs

Iba1+ Kupffer cells were widely distributed in the sinusoids of MARV- and KASV-infected bats at all timepoints and in the control bats. At 3 DPI, the few inflammatory foci present in the livers of MARV-infected bats contained scattered Iba1+ cells, whereas those in KASV-infected bats contained numerous, densely packed Iba1+ cells with abundant cytoplasm (Figs. 4-4a-b). Frequently, mild to moderate numbers of intravascular Iba1+ cells (monocytes) were present in the hepatic blood vessels of both groups. In KASV-infected bats at 3-DPI, there was frequent margination of Iba1+ monocytes within blood vessels, and in the centrilobular hepatic parenchyma, these cells frequently formed large peri-vascular aggregates (Fig. 4-4b). Overt mononuclear cell intravascular margination was not a prominent feature in the liver of MARV-infected ERBs.

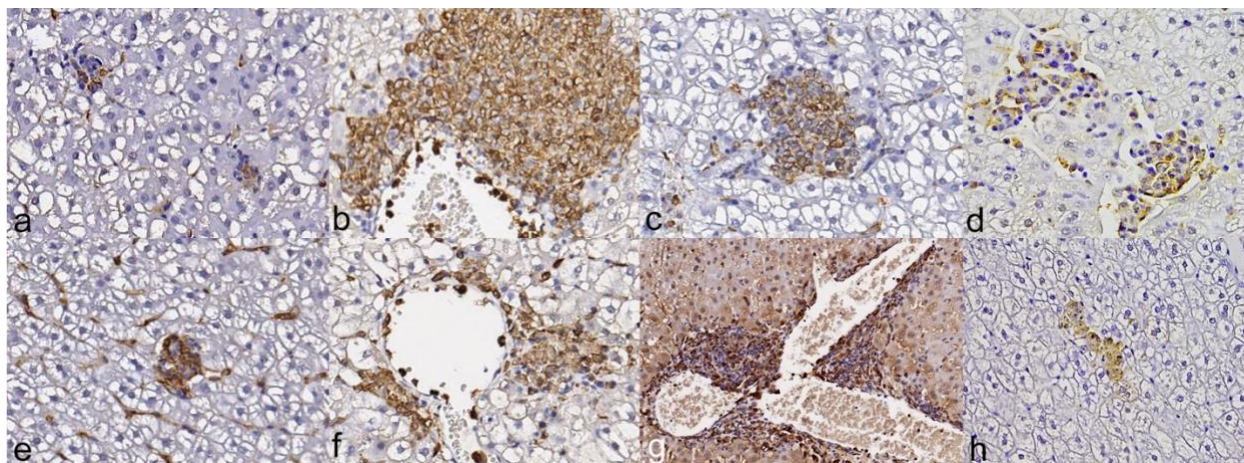


**Figure 4-4.** Comparative immunohistochemical analysis of the mononuclear phagocyte cell (Iba1) responses in the livers of Marburg virus- and Kasokero virus-infected ERBs euthanized at 3-, 6-, 9-, and 20- or 28-DPI. **a)** Two small clusters of Iba1+ cells a MARV-infected ERB, 3 DPI. **b)** Extensive monocyte margination and recruitment of Iba1+ cells to a large peri-vascular focus of inflammation in a KASV-infected ERB, 3 DPI. **c).** Foci of inflammation are comprised largely of Iba1+ cells in a MARV-infected ERB, 6 DPI. **d)** Adjacent foci of inflammation coalesce and contain moderate numbers of Iba1+ cells as well as several Iba1-immunonegative mononuclear cells in a KASV-infected ERB, 6 DPI. **e).** A small, solitary focus of Iba1+ cells in a MARV-infected ERB, 9 DPI. **f).** Monocyte margination and accumulation of perivascular Iba1+ cells in a KASV-infected ERB, 9 DPI. **g)** Focally extensive perivenular accumulation of Iba1+ cells mixed with other Iba1-immunonegative mononuclear cells in a MARV-infected ERB, 28 DPI. **h)** An elongate cluster of pigment-laden Iba1+ macrophages in a KASV-infected ERB, 20 DPI. All images show Iba1+ cells labeled with the 3,3'-Diaminobenzidine (brown) chromogen and counterstained with hematoxylin.

At 6-DPI in MARV-infected bats, inflammatory cell foci tended to be larger than those at 3-DPI and contained numerous Iba1+ cells (Fig. 4-4c); foci were more numerous compared to 3-DPI and were typically discrete. In contrast, in KASV-infected bats at 6-DPI, adjacent foci often coalesced but Iba1+ cells comprised a subjectively smaller overall proportion of the cells within individual foci compared to that observed in the MARV-infected bats and compared to KASV-infected bats at 3-DPI (Fig. 4-4d). In the livers of MARV-infected bats at 9-DPI, inflammatory foci were few and contained several Iba1+ cells (Fig. 4-4e). In KASV-infected bats at 9-DPI, clusters of Iba1+ cells were adjacent to both central and portal veins, and often contained brown

granular cytoplasmic pigment consistent with hemosiderin (Fig. 4-4f). At the final time points for both studies (28 for MARV and 20 DPI for KASV), inflammatory foci containing Iba1+ cells were rare. One 28-DPI MARV-infected bat (same bat as in Fig. 4-1k) had a focally extensive, perivascular accumulation of mixed mononuclear cells (Fig. 4-4g). In the KASV-infected bats euthanized at 20-DPI, Iba1+ immunostaining was limited to Kupffer cells and small clusters of hemosiderin- or lipofuscin-containing macrophages (Fig. 4-4h).

In the livers of control bats, in addition to Kupffer cells, positive Iba1 immunostaining (Iba1+) was present in small, slender cells (macrophages) within the walls of large blood vessels and bile ducts, and within the hepatic capsule. Occasionally, control bats from both experiments had small foci of mononuclear inflammatory cells randomly scattered throughout the hepatic parenchyma. These foci typically contained 2-10 Iba1+ cells and few other Iba1-immunonegative mononuclear cells.

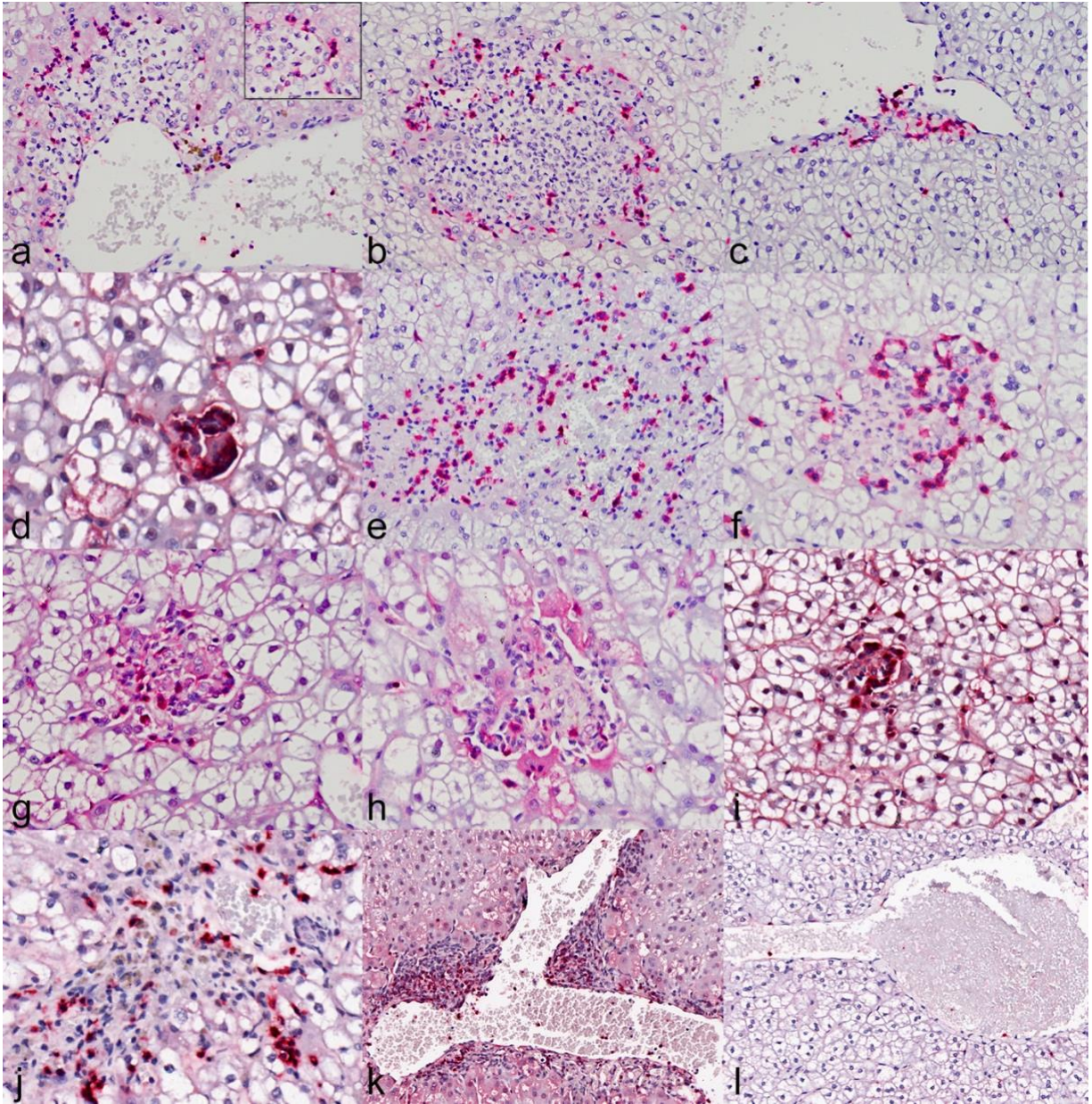
In addition to overall increases in CD3-immunostaining in the livers of MARV- and KASV-infected bats at early time points (3 to 9 DPI), there were distinctive spatial relationships between CD3+ cells and hepatocytes at the periphery of the foci, particularly for KASV-infected bats euthanized at 3- and 6-DPI, but also for MARV-infected bats euthanized at 6-DPI. In KASV-infected bats euthanized at 3-DPI, CD3+ cells were frequently located along the periphery of the inflammatory foci, immediately adjacent to and sometimes overlapping with degenerating hepatocytes, and occasionally forming short chains of closely associated CD3+ cells (Figs. 4-5a-b). CD3+ cells often formed dense subvenular aggregates adjacent to blood vessels (Fig. 4-5c). While there was margination of CD3+ cells within blood vessels adjacent to perivascular inflammatory cell foci, margination of CD3+ cells was not as robust as it was for Iba1+ monocytes in KASV-infected bats at 3-, 6-, and 9-DPI. In contrast, while inflammatory

cell foci were rare in MARV-infected bats euthanized at 3-DPI, CD3<sup>+</sup> cells were scattered throughout these foci in low numbers (Fig. 4-5d).

In MARV- and KASV-infected bats euthanized at 6 DPI, numerous CD3<sup>+</sup> cells were distributed throughout the foci of infection (Figs. 4-5e-h). Some inflammatory foci had uniformly distributed CD3<sup>+</sup> cells (Fig. 4-5e), while others had more CD3<sup>+</sup> cells concentrated along the periphery of the focus (Figs. 4-5f-g), similar to the distribution noted in KASV-infected bats at 3 DPI. In 6 DPI MARV-infected bats, rarely were CD3<sup>+</sup> cells observed overlapping with hepatocytes or within the cytoplasm of hepatocytes at the periphery of inflammatory foci (Fig. 4-5h).

The mild but statistically significant increase in CD3 %PP for MARV-infected bats at 9- and 12-DPI corresponded spatially to slightly increased numbers of CD3<sup>+</sup> cells distributed throughout the sinusoids, but not in discrete inflammatory cell foci as in the earlier timepoints (Fig. 4-5i). By comparison, in KASV-infected bats euthanized at 9 DPI, CD3<sup>+</sup> cells were mixed with hemosiderin-laden macrophages in periportal and centrilobular regions, and multifocally within the sinusoids. By the final time point, CD3<sup>+</sup> staining had returned to control-like levels (Figs. 4-5k-l). However, one MARV-infected bat euthanized at 28-DPI (same bat as in Figs. 4-1k and 4-4g) had numerous CD3<sup>+</sup> cells surrounding a central vein (Fig. 4-5k).

In the control bats, CD3<sup>+</sup> cells were distributed in low numbers throughout the sinusoids, in periportal connective tissue and in bile duct epithelia. The vast majority of CD3<sup>+</sup> cells morphologically resembled small lymphocytes. Occasionally, CD3<sup>+</sup> cells within sinusoids had a more elongate morphology.



**Figures 4-5.** Comparative Immunohistochemical Analysis of the CD3<sup>+</sup> Immune Cell Responses in the Livers of ERBs Experimentally Infected with MARV or KASV and Euthanized at 3-, 6-, 9- and 20- or 28-DPI. **a)** CD3<sup>+</sup> cells are concentrated at the periphery of a centrilobular inflammatory cell focus, adjacent to hepatocytes that were previously shown to contain abundant KASV RNA on ISH, KASV-infected ERB, 3 DPI. Inset: CD3<sup>+</sup> cells form a short chain along a degenerating hepatocyte at the periphery of the focus of inflammation. **b)** Numerous CD3<sup>+</sup> cells are present along the periphery of a large inflammatory focus in a KASV-infected ERB, 3 DPI. Fewer CD3<sup>+</sup> cells are scattered throughout the center of the focus. **c)** Moderate numbers of CD3<sup>+</sup> cells are present between and within the wall of the central vein, and few CD3<sup>+</sup> cells are being recruited from the circulation in a KASV-infected ERB, 3 DPI. **d)** Moderate numbers of CD3<sup>+</sup> cells are present within a focus of inflammation in a MARV-infected ERB, 3 DPI. **e)**

Abundant CD3<sup>+</sup> cells are distributed throughout a large focus of inflammation and hemorrhage in a KASV-infected ERB, 6 DPI. **f)** CD3<sup>+</sup> cells are distributed throughout the focus of inflammation and concentrated at the periphery at the top and on the right side of the focus in a KASV-infected ERB, 6 DPI. **g)** Mild numbers of CD3<sup>+</sup> cells are concentrated at the periphery of an inflammatory focus, at the junction with the hepatic parenchyma in a MARV-infected ERB, 6 DPI. **h)** Mild numbers of CD3<sup>+</sup> cells are distributed throughout the focus of inflammation. At the bottom of the focus, three small CD3<sup>+</sup> round cells are present and either overlapping a hepatocyte or within the cytoplasm of a hepatocyte in a MARV-infected ERB, 6 DPI. **i)** Mild numbers of CD3<sup>+</sup> cells are distributed throughout the focus of inflammation and present in slightly increased numbers throughout the sinusoids in a MARV-infected bat, 9 DPI. **j)** Moderate numbers of CD3<sup>+</sup> cells are distributed throughout a focus of perivascular hemosiderin-laden macrophages in a KASV-infected bat, 9 DPI. **k)** A focally extensive, perivascular infiltrate of CD3<sup>+</sup> cells in a MARV-infected bat, 28 DPI. **l)** Normal distribution of CD3<sup>+</sup> cells in a KASV-infected bat, 20 DPI. All images show CD3<sup>+</sup> cells labeled with the Fast Red (red) chromogen and counterstained with hematoxylin.

At multiple time points in both MARV- and KASV-infected bats, mononuclear cells within the hepatic inflammatory cell foci exhibited strong, diffuse cytoplasmic immunostaining for cleaved caspase 3 (Fig. 4-6). These ClvCasp3<sup>+</sup> cells corresponded to Iba1-immunolabeled cells and were morphologically consistent with macrophages. Of all the bats in this study, ClvCasp3 immunostaining was most intense and widespread in the inflammatory cell foci of KASV-infected bats euthanized at 3 DPI (Figs. 4-6a-c). In those 3 DPI bats, ClvCasp3<sup>+</sup> mononuclear cells were also present in mild to moderate numbers multifocally within blood vessels and within the sinusoids (Fig. 4-6a, inset). There was a spectrum of ClvCasp3 intensity within the inflammatory foci for the 3-DPI bats; the bat with widespread hepatocellular inclusions and the most frequent inflammatory cell foci had the lowest intensity and overall ClvCasp3 immunostaining for this time point (Figs. 4-6b-d). The CD3<sup>+</sup> cells noted along the periphery of the inflammatory foci in KASV-infected bats euthanized at 3-DPI were immunonegative for ClvCasp3 (Fig. 4-6b). Few hepatocytes along the periphery of the foci had minimal to mild cytoplasmic ClvCasp3 immunoreactivity (Figs. 4-6c-d). In MARV-infected bats

euthanized at 3-DPI, intense ClvCasp3 immunostaining was present within the cytoplasm of macrophages within the foci and in scattered mononuclear cells within the sinusoids (Fig. 4-6e).

Although the average ClvCasp3 %PP values were similar for KASV- and MARV-infected bats at 6-DPI, there were distinctive differences in the distribution of immunostaining. In KASV-infected bats euthanized at 6-DPI, ClvCasp3<sup>+</sup> mononuclear cells were sparse within the inflammatory cell foci and throughout the sinusoids, and there were scattered immunopositive hepatocytes with morphologic features of apoptosis (cell shrinkage and pyknosis; Fig. 4-6f). In MARV-infected bats at 6 DPI, ClvCasp3 was strongly expressed in the cytoplasm of macrophages within the inflammatory cell foci, in mononuclear cells throughout the sinusoids and in occasional degenerating hepatocytes along the periphery of the inflammatory foci (Figs. 4-6g-h).

At 9 DPI, the distribution of ClvCasp3 immunostaining was similar to that at 6 DPI for both groups, with overall less immunopositivity (Fig. 4-6i-j). Although inflammatory cell foci were sparse in the livers of MARV-infected bats euthanized at 9- and 28-DPI, the foci that did remain contained mild to moderate numbers of mononuclear phagocytes strongly immunopositive for ClvCasp3 (Figs. 4-6j-k). KASV-infected bats euthanized at 20-DPI showed minimal ClvCasp3 immunostaining, which was similar to that of the control bat livers (Fig. 4-6l). In the control bats, low numbers of ClvCasp<sup>+</sup> mononuclear cells and hepatocytes were scattered throughout sinusoids and parenchyma, respectively.

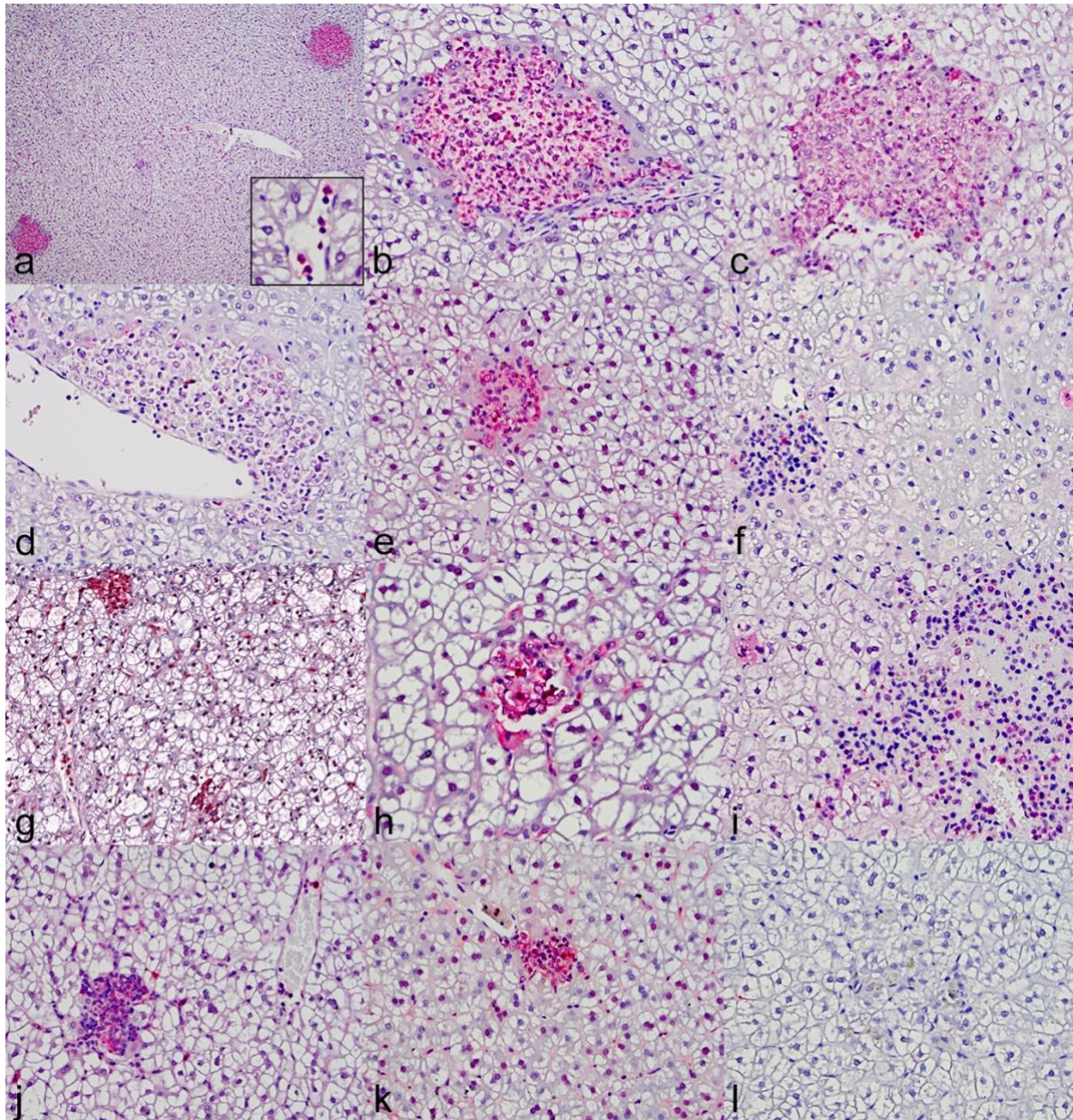


**Figure 4-6.** Comparative Immunohistochemical Analysis of Cleaved Caspase 3 Expression in the Livers of ERBs Experimentally Infected with MARV or KASV and Euthanized at 3-, 6-, 9- and 20- or 28-DPI. **a)** Strong cytoplasmic immunopositivity for ClvCasp3 is evident in macrophages within the foci of inflammation, in mononuclear cells within blood vessels, and multifocally within the sinusoids in a KASV-infected ERB, 3 DPI. Inset: High magnification demonstrating strong immunopositivity in circulating mononuclear cells. **b)** High magnification image of a peri-vascular focus of inflammation demonstrating strong diffuse cytoplasmic immunopositivity for ClvCasp3 in macrophages in a KASV-infected ERB, 3 DPI. Hepatocytes surrounding the focus and the CD3+ cells along the periphery (Fig. 25) are immunonegative. **c)** High magnification image from a different bat showing strong immunopositivity within macrophages and intravascular mononuclear cells and minimal to mild hepatocellular immunopositivity in a KASV-infected ERB, 3 DPI. **d)** High magnification image of liver from

the bat with the highest hepatic viral load from the serial sacrifice study (044997) demonstrates weak cytoplasmic immunoreactivity within macrophages in a peri-vascular focus of inflammation and minimal, multifocal staining in adjacent hepatocytes. KASV-infected ERB, 3 DPI. **e)** Strong cytoplasmic immunopositivity for ClvCasp3 is present in macrophages within the focus of inflammation and in low numbers of mononuclear cells in the sinusoids in a MARV-infected ERB, 3 DPI. **f)** Low numbers of mononuclear cells in the focus of inflammation and scattered hepatocytes exhibit mild to moderate cytoplasmic immunopositivity for ClvCasp3 in a KASV-infected ERB, 6 DPI. **g)** Strong cytoplasmic immunopositivity for ClvCasp3 is evident in macrophages within multiple foci of inflammation, in mononuclear cells within blood vessels, and multifocally within the sinusoids in a MARV-infected ERB, 6 DPI. **h)** High magnification image of an inflammatory cell focus demonstrates strong immunopositivity within macrophages and a degenerating hepatocyte at the bottom of the focus in a MARV-infected ERB, 6 DPI. **i)** A solitary hepatocyte and low numbers of mononuclear cells within a focus of inflammation exhibit mild to moderate cytoplasmic immunopositivity for ClvCasp3 in a KASV-infected ERB, 9 DPI. **j)** Strong cytoplasmic immunopositivity for ClvCasp3 is evident in numerous mononuclear cells within a focus of inflammation and few intravascular and intrasinusoidal mononuclear cells in a MARV-infected bat, 9 DPI. **k)** Macrophages within a small peri-vascular mononuclear inflammatory cell focus exhibit strong cytoplasmic immunoreactivity for ClvCasp3 in a MARV-infected bat, 28 DPI. **l)** An ill-defined focus of hemosiderin-laden macrophages lacks immunoreactivity for ClvCasp3 in a KASV-infected bat, 20 DPI. All images show ClvCasp3+ cells labeled with Fast Red (red) chromogen and counterstained with hematoxylin.

## DISCUSSION

In this study, the host responses in the livers of Egyptian rousette bats experimentally infected with Marburg virus or Kasokero virus, two zoonotic and hepatotropic RNA viruses that share this common African fruit bat as a reservoir host were characterized histologically and immunohistochemically. While similar inflammatory cell populations (i.e. CD3+ lymphocytes and histiocytes) were recruited to the liver at early post-infection time points (3 and 6 DPI), the response was slightly delayed for MARV compared to KASV, and comparatively increased pathogenicity was observed in the livers of KASV-infected ERBs. This increase in pathogenicity may be related to a variety of factors, including individual ERB differences (genotype and immunological phenotypes), virus-specific traits (viral kinetics and cellular tropisms), viral life cycles (i.e. direct transmission of MARV between ERBs vs an ERB-*O. (R.) faini* enzootic

transmission cycle and/or direct transmission between ERBs for KASV), and evolutionary pressures affecting the hosts and viruses.<sup>6,35,45</sup> The experimental infections demonstrated distinctive differences in the viral kinetics for KASV and MARV in ERBs, with the KASV viremic peak occurring earlier (3 versus 6 DPI), and being of greater magnitude (5.6 versus 3.3 log<sub>10</sub>TCID<sub>50</sub> eq/mL) and longer duration, on average, compared to MARV.<sup>6,35</sup> While a more robust and prolonged viremia may facilitate the transmission of KASV from ERBs to *O. (R.) faini* ticks in nature, this likely also promotes enhanced pathogenicity via early and widespread seeding of the liver with KASV, increased hepatocellular replication and cytopathic effects, and a more pronounced inflammatory response.<sup>27,35</sup> Additionally, the genetic diversity of marburgviruses observed in wild populations of ERBs supports a long-standing evolutionary relationship in which decreased pathogenicity may favor host survival and virus persistence, whereas KASV, in having a life cycle that involves two hosts (ERBs and *O. (R.) faini*), may be limited in its ability to co-evolve with either host.<sup>40,43,45</sup>

The concentration of CD3<sup>+</sup> cells at the leading edge of the hepatic inflammatory foci at early time points (3 and 6 DPI) supports a strong cell mediated cytotoxic CD3<sup>+</sup> cell response directed at KASV-infected hepatocytes. Given that ERBs have an expanded NK cell receptor gene repertoire and recent analyses indicate that the dominant circulating T cell populations are NKT-like cells expressing CD4, CD8, and a variety of NK cell markers, these CD3<sup>+</sup> cells may represent novel NKT-like cells type with potent anti-viral effector cell capabilities.<sup>16,32</sup> While these distinctive CD3<sup>+</sup>-hepatocyte interactions were not observed in MARV-infected bats, this may be due to a variety of factors including the presence of fewer and smaller inflammatory cell foci for most MARV-infected bats and the fact that hepatocytes containing MARV antigen are characteristically sparse in naturally and experimentally infected ERBs.<sup>24,40</sup> Mild increases in

circulating CD3<sup>+</sup> cells within the sinusoids in MARV-infected bats at 9- and 12- DPI may represent low-level T cell recruitment at these time points, possibly due to circulation of MARV-specific CD8<sup>+</sup> cells or regulatory T cells.<sup>2</sup> Finally, cell-in-cell structures involving a hepatocyte and multiple CD3<sup>+</sup> cells along the edge of an inflammatory focus in a MARV-infected bat at 6-DPI provide an additional link between MARV infection and host mechanisms such as emperiopolesis, which have been associated with the promotion of immune homeostasis and tolerance.<sup>10,38</sup>

Distinctive spatial and temporal patterns of cleaved caspase 3 expression in the livers of KASV- and MARV-infected ERBs support a prominent role for apoptosis in shaping the ERB early host responses to these pathogens. In contrast to bystander lymphocyte apoptosis, which characteristically occurs in the secondary lymphoid tissues and peripheral blood of MARV-infected NHPs and humans and contributes to lymphoid depletion, apoptosis of mononuclear phagocytes recruited to the foci of hepatic infection would be advantageous for ERBs in terms of limiting virus replication and virus-induced immunopathology, and in promoting the resolution of liver lesions.<sup>3,13,15,23</sup> Further, it may have downstream implications for the host adaptive immune response, including the induction of T cell tolerance.<sup>2,39</sup> Minimal, multifocal hepatocellular cleaved caspase 3 expression was noted occasionally for MARV- and KASV-infected bats from 3 to 12 DPI, but was not consistently associated with inflammatory cell foci nor was it a significant contributor to the overall immunostaining for either virus at any time point.

In addition to hepatocytes, MARV and KASV are tropic for mononuclear phagocytes in ERBs, and the results presented herein support the multifaceted involvement of this diverse cell lineage in shaping the ERB innate and adaptive immune responses, and in resolution of the liver

lesions over time.<sup>24,27</sup> These data support the hypothesis that ERBs and marburgviruses have a long-standing co-evolutionary relationship, one in which mononuclear phagocyte responses have evolved to control and clear virus, and to resolve virus-induced lesions, as opposed to the dysregulated, maladaptive mononuclear phagocyte responses that occur in MARV-infected primates.<sup>11,12,22,40</sup>

The presence of an acute perivascular lesion in the liver of one MARV-infected bat at 28 DPI raises the possibility of MARV persistence in ERBs. MARV persistence has been documented in the testes of experimentally infected NHPs and experimental transmission studies of MARV in ERBs have raised the suspicion that MARV may exhibit prolonged latency or establish a persistent infection in this host, thereby contributing to its maintenance in wild populations of ERBs.<sup>14,36</sup>

The study of bats as reservoir hosts for other viruses with the potential for spillover is currently expanding. Given the similarities in the host responses in the livers of KASV- and MARV-infected ERBs, these responses may be generalizable to other viruses infecting ERBs and possibly to other bat hosts that harbor pathogens like Ebola and Nipah viruses. These immunohistochemical studies lend further support to the hypothesis that the lack of clinical disease observed in MARV-infected bats is due to an overarching strategy of immunoprotective disease tolerance, which limits virus-induced tissue pathology and promotes resolution of lesions.<sup>20,29</sup> In contrast, the ERB host response to KASV appears to favor disease resistance mechanisms (i.e. a more robust immune response), likely due to the high-level viremia that is required for efficient ERB-to-tick transmission and associated increased seeding of the liver which drives increased pathogenicity compared to MARV in ERBs.

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## **DECLARING OF CONFLICTING INTERESTS**

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## CHAPTER 6

### CONCLUSIONS

The goal of these studies was to investigate bat host-virus interactions within a spatial context using formalin-fixed paraffin embedded tissues from Egyptian rousette bats infected with three negative-sense zoonotic RNA viruses from the families *Paramyxoviridae*, *Nairoviridae*, and *Filoviridae* (Sosuga, Kasokero, and Marburg viruses, respectively), for which the ERB is the confirmed or suspected reservoir host. This comparative pathology approach allowed us to identify unique ERB-virus interactions and mechanisms which may be more generalizable to viruses from other families or to other related bats. Histopathological, immunohistochemical (IHC), *in situ* hybridization (ISH), and digital pathology methods were employed to observe host responses in ERB tissues from animals euthanized at similar post-infection time points. This chapter will summarize the key findings and discuss whether or not they support or refute current theories pertaining to ERBs-virus interactions and ERB immunology in general. Finally, the limitations of these studies and possibilities for future research will be discussed.

In Chapter 2, the small intestines and salivary glands were identified as the primary target tissues and confirmed as the principal sources of virus shedding into the environment in SOSV-infected ERBs. A hyporesponsive or anergic immune response to SOSV replication in the small intestines despite villus ulceration and lamina propria fluid accumulation may promote virus shedding into the feces and limit virus-induced immunopathology and clinical disease in the host. In contrast, a targeted and robust mononuclear phagocyte response to SOSV infection of

the interlobular salivary duct epithelium likely limits virus replication and shedding into the saliva. From an evolutionary standpoint, sialodochitis would not have as significant an impact on overall host fitness as would severe intestinal inflammation. Finally, the presence of lesions and SOSV antigen in the small intestines and salivary glands at 21 days raised the possibility of SOSV persistence in ERBs, which has been proposed for other bat-paramyxovirus systems, further supporting the ancestral relationship between bats and paramyxoviruses.<sup>6,7,10</sup>

In Chapter 3, histopathological, ISH, IHC, and digital pathology investigations provided novel insights into bat-nairovirus interactions within a spatial context. The liver and spleen were identified as primary tissue targets for KASV. In the liver, KASV replicated primarily in hepatocytes, and to a lesser extent in Kupffer cells and endothelial cells. In contrast to the lesions observed in the liver of humans infected with the related nairovirus Crimean-Congo Hemorrhagic Fever virus (CCHFV)<sup>5</sup>, foci of KASV hepatocellular infection were frequently associated with the recruitment of circulating mononuclear inflammatory cells from the circulation in ERBs, and the majority of virus was cleared by 6 days-post infection (DPI). Disruption of the hepatic cords was associated with hemorrhage and hepatic necrosis in a subset of infected animals, possibly due to virus-induced endothelial damage and/or sepsis. KASV RNA localized with cells of the mononuclear phagocyte system in the liver, spleen and lymph nodes, highlighting the importance of this diverse cell lineage in dissemination of the virus to target tissues, in controlling virus replication and clearing the virus, and in resolution of the liver lesions. In the spleen, despite extensive replication of KASV in the sheathed capillaries of the marginal zone and red pulp, there were no observable lesions at the light microscopic level, and the vast majority of virus was cleared by 6 DPI. While lymphoid depletion and focal necrosis were observed in the spleen of humans naturally infected with CCHFV<sup>5</sup>, splenic pathology was

not a feature of KASV infection in ERBs. These data support the theory that ERBs, and perhaps other bats, have evolved a defensive-type spleen with specialized microvasculature and a mononuclear phagocyte system that are effective at clearing viruses, in addition to other pathogens, particulates and senescent erythrocytes, from the circulation.<sup>13,19,12</sup> The mechanisms by which KASV replication is controlled and viral RNA is cleared from the spleen requires further study.

The observation that KASV was strongly hepatotropic and induced acute viral hepatitis in ERBs prompted a systematic comparison of KASV and MARV liver lesions and host-virus interactions to uncover anti-viral mechanisms which might be applicable to other RNA viruses and perhaps other bat-virus systems. These comparative histopathological and immunohistochemical studies, which investigated the roles of T cells, mononuclear phagocytes and programmed cell death in KASV and MARV infections in ERBs, were presented in Chapter 4. Mononuclear phagocytes were the predominant immune cell type recruited to foci of infection. The rapid induction of apoptosis in these cells appears to play a prominent role in controlling KASV and MARV replication, clearing viral RNA from the tissue, and in resolving the lesions of acute viral hepatitis. This might be advantageous to the host in terms of limiting virus replication and promoting resolution of the liver lesions, but the precise mechanisms driving monocyte apoptosis in the liver of KASV- and MARV-infected ERBs remain undetermined. Spontaneous apoptosis in human monocytes recruited to sites of inflammation has previously been reported.<sup>8</sup> Fewer CD3<sup>+</sup> cells than monocytes were recruited to foci of hepatocellular viral replication, but in the KASV-infected bats euthanized at 3 DPI (day of peak virus replication), early and distinctive interactions between CD3<sup>+</sup> cells and hepatocytes at the leading edge of replication foci were suggestive of cellular cytotoxicity. The aforementioned

CD3<sup>+</sup> cells are of particular interest given the recent identification of two large subsets of circulating Natural Killer T-like (NKT-like) cells in the peripheral blood of ERBs, both of which have transcriptomic profiles indicative of potent cytotoxic capabilities.<sup>9</sup> Recruitment of similar cell types (i.e. mononuclear phagocytes and CD3<sup>+</sup> cells) to the liver of MARV- and KASV-infected ERBs suggests that similar chemokines, cytokines and effector cell activities may drive the clearance of these viruses from the liver. Further, the very mild inflammatory response in the liver of MARV-infected ERBs suggests that MARV is highly adapted to replicating in this tissue, inducing minimal cytopathic effects in infected hepatocytes, and recruiting low numbers of monocytes and CD3<sup>+</sup> cells to foci of infection, thereby limiting immunopathology, both in this tissue and at the organism level. Viral innate immune evasion mechanisms, which are characteristic of filoviruses in spillover hosts, may also factor into this observed delay of immune cell recruitment to the liver of MARV-infected ERBs, as these mechanisms are likely to have evolved in the natural reservoir host.<sup>4</sup>

In addition to the fine-scale analyses of each virus-host relationship, a comparative pathology approach afforded big-picture insights into evolutionary relationships between these viruses and ERBs. The small intestines of SOSV-infected ERBs had the highest viral loads by qRT-PCR out of all the tissues examined, yet SOSV was the least immunopathogenic of the three viruses in ERBs, perhaps underscoring the tolerance of this tissue to SOSV replication. Data from the KASV ISH study indicate that early innate immune mechanisms (< 6 days post-infection) are important in clearing KASV from infected ERBs, but virus clearance may be less important for SOSV-infected ERBs. This observation supports a long-term association between SOSV and ERBs, and between bats and paramyxoviruses in general.<sup>15</sup> In contrast, despite the liver being traditionally regarded as a tolerogenic organ, the host innate immune cell responses in

the liver of KASV- and MARV-infected ERBs appear to favor virus clearance from this tissue. Compared to MARV, a more rapid and robust viremic peak for KASV likely permits its transmission to the argasid tick vector *Ornithodoros (Reticulinasus) faini* during a bloodmeal, but likely also promotes enhanced pathogenicity via early and widespread seeding of the liver, with increased hepatocellular replication, cytopathic effects, and a more pronounced inflammatory response.<sup>2,14,17</sup> The genetic diversity of marburgviruses observed in wild populations of ERBs supports a long-standing evolutionary relationship in which host adaptation and decreased pathogenicity may favor host survival and virus persistence, whereas KASV, in having a life cycle that involves two hosts (ERBs and *O. (R.) faini*), may be limited in its ability to co-evolve with either host.<sup>21-23</sup>

In contrast to the storage type spleen of dogs and cats, which is adapted for blood storage, ERBs, like mice, rats, rabbits, and humans, appear to have a defensive type spleen that prioritizes the immune response to circulating pathogens over blood storage.<sup>20</sup> In ERBs, like that in rats, and mice to a lesser extent, the marginal zone is well developed.<sup>11</sup> B cells, T cells and mononuclear phagocytes comprise the bulk of the splenic parenchyma, but the specific cell types, particularly marginal zone B cells, which contribute significantly to T-cell independent humoral immune responses, and macrophage subsets are yet to be described in ERBs. The sheathed capillaries (ellipsoids) are likely to play significant role(s) in the rapid clearance of KASV RNA from the circulation. While the detection of viral antigen (SOSV) and viral RNA (KASV) in the secondary lymphoid tissues provides a clear linkage to the adaptive immune system, the role of antibodies in the ERB anti-viral immune response remains unclear. Anti-SOSV and ant-MARV antibodies were recently shown to be non-neutralizing *in vitro*; thus antibodies may serve other functions such as antibody dependent cellular phagocytosis (ADCP)

or antibody dependent cellular cytotoxicity (ADCC).<sup>18</sup> In the lymph node and splenic germinal centers, viral RNA within follicular dendritic cells may represent potential long-term virus repositories, as these cells are known to be capable of retaining infectious virus for months to years via immune complex cycling.<sup>1,3,16</sup>

Virus replication in ERBs tissues, namely the small intestines and salivary glands for SOSV, and liver for KASV and MARV, was associated with apoptosis in a variety of cell types. While apoptosis can be beneficial to both the virus and the host, the induction of apoptosis in monocytes recruited to foci of MARV and KASV replication in the liver may be advantageous to the host by limiting virus replication, limiting the severity of immunopathology and promoting the resolution of liver lesions. On the other hand, limitation of the inflammatory response in replication foci might also be advantageous to KASV and MARV, depending on the potency and/or effectiveness of virus clearance mechanisms. In SOSV-infected bats, apoptosis was limited to scattered salivary duct epithelial cells and enterocytes. Whereas induction of apoptosis in the liver of MARV- and KASV-infected ERBs may promote virus clearance, delivery of SOSV antigen in phagocytosed apoptotic epithelial cells in the small intestines and salivary gland ducts to the secondary lymphoid tissues may promote a tolerogenic response as the infection progresses.

These studies have several limitations that merit discussion. The tissue and cellular tropism studies for SOSV-infected bats were limited due to prolonged and or variable formalin-fixation between bats prior to trimming and processing the tissues. We first attempted *in situ* hybridization using an RNAScope® probe targeting the nucleoprotein and matrix genes but had little success in detecting signal in the majority of bats. As a result, we were limited to an immunohistochemical protocol using an anti-SOSV nucleoprotein antibody for those bats, which

is likely an underestimate of SOSV in the tissues. However, the KASV-infected bat tissues were more optimally fixed (10 days in formalin, followed by trimming and a switch to 70% ethanol). For KASV, RNAScope® was very useful in detecting minute amounts of viral RNA, particularly in the spleen, where single copies of KASV nucleoprotein RNA were often observed in the walls of splenic arterioles as punctate dots, and in investigating clearance of KASV RNA from the key target tissues, the liver and spleen, over time. While the skin is the presumptive site of initial virus-host engagement for KASV in intradermally inoculated ERBs, multiple attempts at localizing KASV RNA in the skin at the inoculation site for a subset of 3 and 6 DPI bats were unsuccessful, likely due to the fact that several sections of the inoculation site had been sampled for PCR and virus isolation. Finally, the availability of bats for the experimental infection limited the number of animals assigned to each time point to three, which likely limited statistical inferences from the data. Additionally, given the outbred nature of the ERB colony at the Centers for Disease Control and Prevention, variability in individual immune and healing responses are expected; this may be more of an asset than a limitation in terms of modeling natural infection systems.

There are several lingering questions about ERB host-virus interactions that merit further investigation. While we confirmed mononuclear phagocytes and CD3<sup>+</sup> as the primary innate immune cell responders in the liver of KASV- and MARV-infected ERBs, the phenotypic subtype(s) of the CD3<sup>+</sup> cells remain unclear, namely whether or not they represent NKT-like cells. The lymph nodes and splenic tissues provide invaluable material to investigate the evolution of the antibody response, from early post-infection time points, where marginal zone B cells begin to secrete IgM, to the germinal centers where somatic hypermutation and class switching occurs. Additionally, the existence of cellular repositories of virus (if any), particularly

in the lymph node and splenic germinal centers of virus infected ERBs requires further study. The salivary gland tissues of SOSV-infected ERBs which showed targeted mononuclear phagocyte responses in the salivary gland ducts may provide a good starting point for investigations of antibody-dependent cellular phagocytosis. Tissue-based research ISH or IHC studies will be important to address these questions within a spatial context.

In conclusion, the data presented herein demonstrate that while ERBs experimentally infected with SOSV, KASV and MARV do not show overt disease, there are mild to moderate, subclinical lesions in the primary target tissues, which are fertile ground to study host-pathogen interactions. The tissue changes and immune cell infiltrates support disease tolerance as a defense strategy by which ERBs respond to SOSV, KASV and MARV infections and limit virus-induced pathology. It is important to note that disease tolerance does not equate to an absence of lesions, but rather that virus-induced tissue damage is limited or mitigated by host repair mechanisms. The clinical significance of any given lesion is tissue-specific, and tissues that have a high intrinsic repair capacity (i.e., liver and small intestines) tend to be more tolerant to virus-induced lesions. ERBs use a variety of innate immune mechanisms to rapidly respond to infection with SOSV, MARV and KASV. Some of these mechanisms may be virus specific due to unique viral tissue and cellular tropisms, such as the salivary glands and small intestines for SOSV, while others may be part of more generalizable anti-viral innate host defense strategies, such as apoptosis and recruitment of CD3<sup>+</sup> innate immune cells (possibly NKT-like cells) and mononuclear phagocytes to target tissues, including the liver of MARV- and KASV-infected ERBs. Tissue-based studies of ERB host-virus interactions will continue to play an important role the fields of bat immunology and ERB virus-host interactions by providing spatial context to

*in vitro* genomic, transcriptomic and proteomic data, and by generating novel hypotheses derived from histopathological and/or ultrastructural observations and molecular pathology assays.

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