SUPPLEMENT ARTICLE

Filovirus Infection of STAT-1 Knockout Mice

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We evaluated the susceptibility to Ebola and Marburg virus infection of mice that cannot respond to interferon (IFN)–α/β and IFN-γ because of deletion of the *STAT-1* gene. A mouse-adapted *Zaire ebolavirus* (ZEBOV) caused rapidly lethal disease; wild-type ZEBOV and *Sudan Ebolavirus* and 4 different Marburg virus strains produced severe, but more slowly progressive illness; and *Reston Ebolavirus* caused mild disease that was late in onset. The virulence of each agent was mirrored by the pace and severity of pathologic changes in the liver and lymphoid tissues. A virus-like particle vaccine elicited strong antibody responses but did not protect against mouse-adapted ZEBOV challenge.

The filoviruses, Marburg virus and Ebola virus, cause sporadic epidemics of hemorrhagic fever in central Africa, with high case fatality rates. Because few data have been obtained from patients, the pathogenesis of these diseases is understood principally from studies in laboratory animals. Nonhuman primates provide the most accurate model of the human illness, because all filoviruses produce severe hemorrhagic fever when introduced by a variety of routes. In contrast, the same viruses do not cause disease when inoculated into immunocompetent adult mice [1]. A murine model of lethal infection by Ebola virus from the 1976 Zaire epidemic (ZEBOV-76) has been developed, but it required repeated animal-to-animal passage before a virulent variant (mouse-adapted ZEBOV) was isolated [2]. Normal mice can be rendered susceptible to wild-type ZEBOV-76 by treating them with antibodies to interferon (IFN)–α/β, suggesting that the critical factor in susceptibility is the inability of wild-type filoviruses to suppress murine type I interferon responses [1].

To determine if the loss of IFN function would render mice susceptible to the entire range of wild-type filoviruses, we obtained knockout (KO) mice that fail to express the signal transducer and activator of transcription (STAT)–1 protein, which signals the binding of IFN-α/β and IFN-γ to cell-surface receptors and is essential for type I and II IFN function [3]. We challenged cohorts of these mice with either mouse-adapted ZEBOV, wild-type ZEBOV from the 1976 or the 1995 outbreaks, *Sudan* or *Reston ebolavirus* (SEBOV or REBOV), or the Ravn or Musoke strains of Marburg virus (MARV). We then observed them for weight loss and signs of illness. We also examined tissues from 2 randomly chosen animals killed each day, beginning on day 2 after infection, for pathologic changes in major target organs of filovirus infection, the liver, spleen, and lymph nodes. To determine if STAT-1 KO mice could be protected against filovirus infection, despite their defect in IFN function, we immunized a cohort with a viruslike particle (VLP) vaccine, measured their antibody responses, then challenged them with mouse-adapted ZEBOV [4].

MATERIALS AND METHODS

Mice

Adult male and female STAT-1 KO mice (Taconic) were transferred to Biosafety Level 4 containment at the United States Army Research Institute of Infectious Diseases (USAMRIID), a facility that is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All research was conducted in compliance with the Animal Welfare Act

Potential conflicts of interest: none reported.

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and other federal statutes and regulations and adhered to the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996.

Viruses and Cells

Filoviruses from the USAMRIID collection consisted of tissue culture–passaged strains of ZEBOV-76 and ZEBOV-95, the Boniface strain of SEBOV, the AZ-1435 strain of REBOV, the mouse-adapted variant of ZEBOV, and tissue culture–passaged and guinea pig–adapted strains of Marburg Musoke and Marburg Ravn viruses [5]. The titer of each stock was determined by plaquing on Vero E6 cells.

Experimental Infections

Each group of 12 STAT-1 KO mice was inoculated intraperitoneally with 1000 plaque-forming units (PFU) of one of the filoviruses listed above. The animals were then observed daily and weighed as a group. Beginning on day 2 after infection, 2 animals were selected at random from each group and killed, and their tissues were examined and fixed in formalin for pathology studies.

Histology and Immunohistochemistry

Formalin-fixed tissues were embedded in paraffin and sectioned at 5–6 μm for hematoxylin and eosin staining. Sections for apoptosis staining were treated with 30% hydrogen peroxide in methanol, then with proteinase K, before processing with a TACS2 TdT In Situ apoptosis detection kit (Trevigen). Immunohistochemical staining was performed as previously described, using a rabbit polyclonal antibody to ZEBOV at a dilution of 1:1500 or a cocktail of 2 monoclonal antibodies to MARV at a concentration of 1:100 [6]. Tissue controls consisted of known filovirus-infected and uninfected samples, and normal mouse serum was used as a control antibody.

Vaccination and Challenge

Five STAT-1 KO mice were inoculated intramuscularly 3 times, at 3-week intervals, with 10 μg of Ebola Zaire VLPs [4]. The VLPs were prepared as previously described, except that they contained viral nucleoprotein in addition to GP and VP40. The animals were bled 28 days after the last vaccination, and immunglobulin M (IgM) and immunoglobulin G (IgG) antibody titers were determined by enzyme-linked immunosorbent assay, using inactivated ZEBOV as the antigen, as previously described [4]. Forty days after the last vaccination, the mice were inoculated intraperitoneally with 1000 PFU of mouse-adapted ZEBOV.

RESULTS

Course of Illness

All 9 filoviruses caused visible illness (slowed activity and ruffled fur) in the STAT-1 KO mice, but the groups differed in the time of disease onset and the rapidity of progression. Mice inoculated with mouse-adapted ZEBOV or with SEBOV appeared ill beginning on day 3 after infection, but the other groups displayed normal activity until day 5. For each group, the progression of disease was accompanied by a loss in mean body weight (Figure 1, *A* and *C*). Mice infected with mouse-adapted ZEBOV did not survive past day 4. In contrast, no deaths were observed through day 5 of mice infected with wild-type ZEBOV-76, SEBOV, MARV Ravn, or MARV Musoke virus. All mice infected with ZEBOV-95 lived through day 6, and those infected with REBOV or with guinea pig–adapted MARV Musoke virus survived through day 7.

Necropsy Findings

Mice that showed no signs of illness at the time of euthanasia were grossly normal at necropsy. When animals appeared ill at the time of euthanasia, visible changes in the viscera were limited to the liver and spleen. The livers of sick mice were soft to the touch and yellow-brown to red-brown in color, with a reticulated pattern, while the spleens were softened and pale, often with multiple small grayish foci.

Histopathology and Immunohistochemistry

The principal histopathologic abnormalities in filovirus-infected mice were seen in the liver and lymphoid tissues (Figure 1, *B* and *D*, and Figure 2, *A*–*D*). From day 3 onward, all animals except those infected with REBOV showed hepatocellular degeneration and necrosis, with positive histochemical staining for viral antigen in hepatocytes and Kupffer cells; similar changes began on day 5 in REBOV-infected mice. The severity of hepatocellular degeneration and necrosis was scored on a range of 1–5 for each animal and averaged for each group for each day. For the Ebola viruses, hepatic injury and positive staining for viral antigen developed most rapidly in mice infected with mouse-adapted ZEBOV, followed by ZEBOV-76 and SEBOV, then by ZEBOV-95 and REBOV (Figure 1*B*). For the Marburg viruses, the tissue culture–passaged and guinea pig–adapted strains of Marburg Ravn virus produced earlier and more severe hepatic injury than the 2 Marburg Musoke strains (Figure 1*D*).

All of the filoviruses produced a similar sequence of histologic changes in the spleen and lymph nodes, but they differed in the rate at which the changes progressed. Mouse-adapted ZEBOV produced the most rapid and extensive alterations, while REBOV showed the slowest course and the smallest amount of change (Figure 2, *E*–*H*). The first observed abnormality was lymphoid hyperplasia, with secondary follicle formation, which was most prominent in mice infected with the more virulent viruses. This was followed by the development of lymphocytolysis, with increasing numbers of apoptotic lymphocytes and extensive neutrophilic infiltrates. Numerous cells with typical features of apoptosis were positive by TUNEL staining (not shown). These changes were evident on day 3 in mice infected

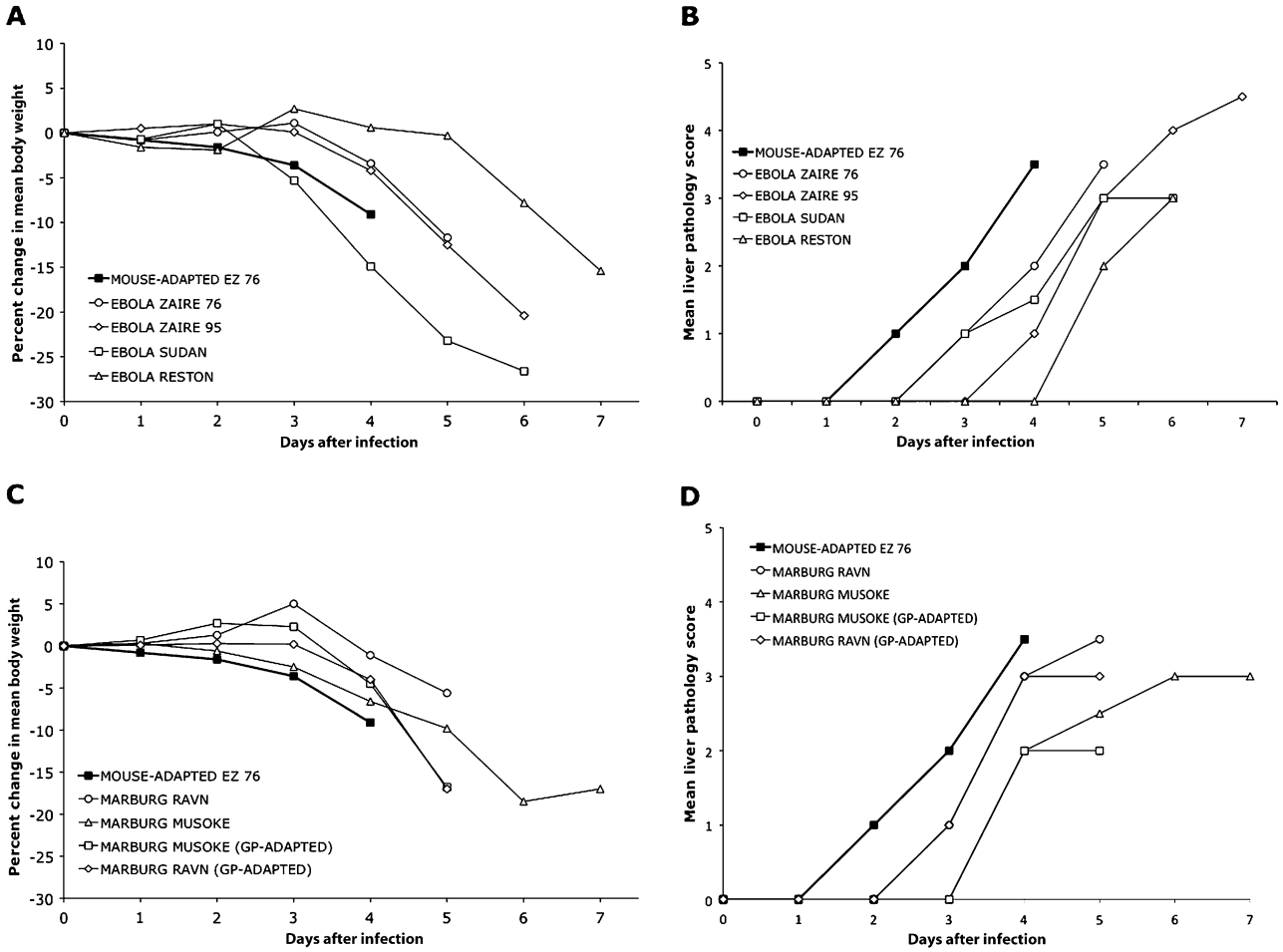

Figure 1. Groups of 12 STAT-1 knockout mice were infected with various filoviruses and weighed as a group on each day after infection, and the percentage of change in mean weight relative to day 0 was calculated. Tissues collected at necropsy were examined by light microscopy for severity of hepatocellular degeneration and necrosis and scored, with 0 = no change, 1= minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe. The scores were averaged for each group for each day. (*A*) Change in mean body weight for mice infected with Ebola viruses. (*B*) Liver scores for the same groups. (*C*) Change in mean body weight for mice infected with Marburg viruses. (*D*) Liver scores for the same groups. To facilitate comparison, results for mice infected with mouse-adapted *Zaire ebolavirus* (EZ) are included in each graph.

with mouse-adapted ZEBOVand SEBOV, and first appeared on day 4 in mice infected with the other viruses, with the exception of REBOV-infected mice, in which apoptotic lymphocytes were not seen until day 6 (Figure 2, *E–H*)). All groups of mice showed positive staining for viral antigen in macrophages by day 3 (not shown); the quantity of antigen was greatest for the viruses that caused the most rapidly progressive disease.

Vaccination and Challenge

Twenty-eight days after the conclusion of a 3-dose regimen, the 5 mice immunized with ZEBOV viruslike particles showed a mean reciprocal IgM titer of 784 ± 453 and a mean IgG titer of 38 912 ± 12 288 against ZEBOV. These values were comparable to those obtained previously in immunocompetent mice that were solidly protected when challenged with mouse-adapted ZEBOV [4]. However, all 5 vaccinated STAT-1 KO mice became ill and died after challenge. In contrast to unvaccinated animals, which did not survive past day 4 (Figure 1*A*), the immunized mice died on days 6, 7, 10, 10, and 10, suggesting partial protection.

DISCUSSION

The STAT-1 protein is responsible for triggering the transcription of multiple genes following the binding of IFN-α/β or IFN-γ to its cell-surface receptor. Its loss therefore has profound consequences for innate immune responses to microbial pathogens. Mice from which the *STAT-1* gene has been deleted can be lethally infected by viruses such as Crimean-Congo hemorrhagic fever virus that fail to cause disease in normal animals [3, 7]. Similarly, because humans homozygous for defective forms of STAT-1 lack normal type I IFN responses, they

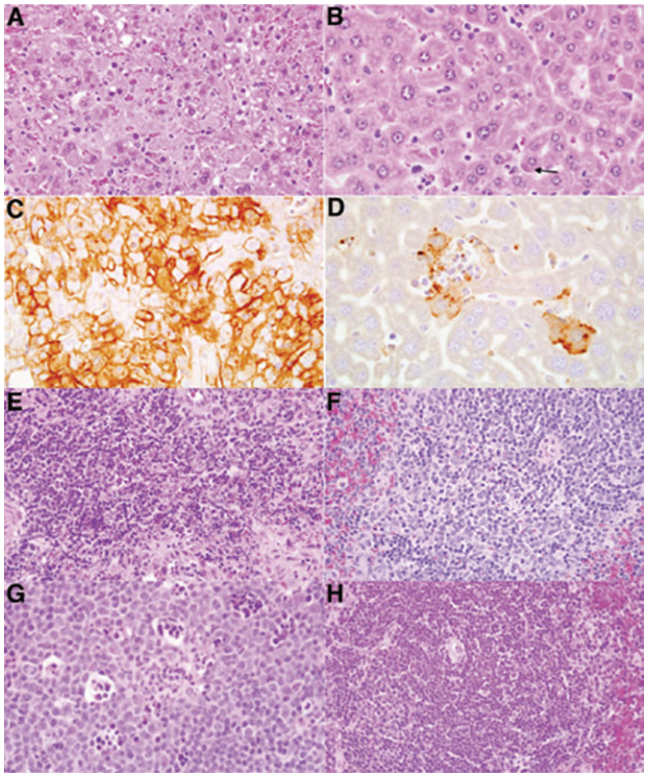

Figure 2. Spleen and liver of STAT-1 knockout mice on day 4 after infection. *A*, *B*, and *E–H* are stained with hematoxylin and eosin; *C* and *D* are immunohistochemical stains for viral antigen. All images ×40. (*A*) Liver, mouse-adapted *Zaire ebolavirus* (ZEBOV), showing moderate random degeneration and necrosis of hepatocytes with infiltration by a few neutrophils. (*B*) Liver, Ebola Sudan virus, showing focal, mild hepatocellular degeneration with inflammatory cells within vascular sinusoids. Arrow: cytoplasmic inclusion body. (*C*) Liver, mouse-adapted ZEBOV, showing diffuse hepatocellular cytoplasmic immunoreactivity. (*D*) Liver, Marburg Musoke virus, showing multifocal hepatocellular cytoplasmic immunoreactivity. (*E*) Spleen, mouse-adapted ZEBOV, showing widespread lymphocytolysis in follicles and parafollicular areas. (*F*) Spleen, ZEBOV-76, showing moderate lymphocytolysis within the periarteriolar lymphoid sheath, with infiltration by moderate numbers of neutrophils. (*G*) Spleen, Marburg Musoke virus, showing multifocal lymphocytolysis within the periarteriolar white pulp. (*H*) Spleen, Ebola Reston virus, within normal limits.

are highly susceptible to a range of viral infections, while the loss of type II IFN responses also renders them sensitive to bacterial pathogens [8, 9].

We found that deletion of the *STAT-1* gene rendered mice susceptible to 3 different species of Ebola virus and 4 different Marburg virus strains, but there were major differences in the severity of infection. Mouse-adapted ZEBOV caused the most severe and rapidly progressive disease; wild-type ZEBOV, SEBOV, and MARV caused an illness that was slower to develop, but was still fatal within 7 days; and REBOV, which is apparently avirulent for humans, caused a mild illness, in which weight loss did not begin until day 6 after infection. These differences correlated with the pace of development and extent of pathologic changes in the liver and lymphoid tissues.

An especially interesting finding was the presence of lymphocyte apoptosis in all infected mice, as it had previously been described only for mice infected with mouse-adapted ZEBOV [6]. Apoptotic lymphocytes were most prominent in animals infected with mouse-adapted ZEBOV and SEBOV, and less evident in groups with slower disease progression. Such "bystander" apoptosis of lymphocytes, apparently induced by proinflammatory mediators, is also seen in ZEBOV-infected nonhuman primates and in humans dying of filoviral hemorrhagic fever, bacterial sepsis, and other severe infections [10–12]. Activated, virus-specific $CD4^+$ and $CD8^+$ lymphocytes are also observed in the spleen and lymph nodes of normal mice infected with mouse-adapted ZEBOV toward the end of the disease course, reflecting the development of an adaptive immune response [13]. We did not specifically identify such reactive lymphoblasts in lymphoid tissues of our STAT-1 KO mice, but it would be of interest to determine whether a virus-specific cell-mediated immune response occurs in these animals in the absence of type I and II IFN function.

There is evidence that adaptive immune responses remain functional in the absence of the STAT-1 protein, as shown by the fact that STAT-$1^{-/-}$ humans are able to clear rhinovirus and parainfluenza virus infections and can develop an antibody response to live poliovirus vaccine [9]. Similarly, STAT-1 KO mice immunized against rotavirus resisted a subsequent challenge [14]. In the present study, we found that KO mice immunized with a ZEBOV VLP vaccine developed IgM and IgG responses comparable to those in normal mice [4]. However, despite this strong humoral response, the mice developed lethal illness following challenge with mouse-adapted ZEBOV, showing only a brief delay in time to death compared with unvaccinated animals. It is possible, however, that STAT-1 KO mice could be vaccinated successfully against less virulent filoviruses, such as wild-type ZEBOV-76 or MARV Musoke. The response to vaccination of STAT-1 KO mice will be characterized in future experiments.

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