Persistent Infection with Ebola Virus under Conditions of Partial Immunity

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Ebola hemorrhagic fever in humans is associated with high mortality; however, some infected hosts clear the virus and recover. The mechanisms by which this occurs and the correlates of protective immunity are not well defined. Using a mouse model, we determined the role of the immune system in clearance of and protection against Ebola virus. All CD8 T-cell-deficient mice succumbed to subcutaneous infection and had high viral antigen titers in tissues, whereas mice deficient in B cells or CD4 T cells cleared infection and survived, suggesting that CD8 T cells, independent of CD4 T cells and antibodies, are critical to protection against subcutaneous Ebola virus infection. B-cell-deficient mice that survived the primary subcutaneous infection (vaccinated mice) transiently depleted or not depleted of CD4 T cells also survived lethal intraperitoneal rechallenge for ≥25 days. However, all vaccinated B-cell-deficient mice depleted of CD8 T cells had high viral antigen titers in tissues following intraperitoneal rechallenge and died within 6 days, suggesting that memory CD8 T cells by themselves can protect mice from early death. Surprisingly, vaccinated B-cell-deficient mice, after initially clearing the infection, were found to have viral antigens in tissues later (day 120 to 150 post-intraperitoneal infection). Furthermore, following intraperitoneal rechallenge, vaccinated B-cell-deficient mice that were transiently depleted of CD4 T cells had high levels of viral antigen in tissues earlier (days 50 to 70) than vaccinated undepleted mice. This demonstrates that under certain immunodeficiency conditions, Ebola virus can persist and that loss of primed CD4 T cells accelerates the course of persistent infections. These data show that CD8 T cells play an important role in protection against acute disease, while both CD4 T cells and antibodies are required for long-term protection, and they provide evidence of persistent infection by Ebola virus suggesting that under certain conditions of immunodeficiency a host can harbor virus for prolonged periods, potentially acting as a reservoir.

Ebola virus causes severe hemorrhagic fever in humans and is associated with a high rate of mortality. The virus appears periodically in central and western Africa, and major outbreaks in the central African countries of Zaire (1976 and 1995) and Uganda (2000) were associated with mortality rates of up to 88% (29, 30). Since 1994, four outbreaks of Ebola virus infection (Ebola virus subtype Gabon) have been reported from Gabon (17), with the latest occurring in 2002 (26, 38). The most recent outbreak was first reported from the Cuvette Ouest Region of the Republic of the Congo (northwestern Congo, on the border with Gabon) on 4 January 2003 (31). This outbreak, with its close geographic proximity to Gabon’s Ogoue-Ivindo Province and to the Democratic Republic of the Congo, suggests ongoing circulation of the virus, with repeated introductions into human populations (17, 25, 26, 38). Interestingly, in the most recent outbreak in Gabon (2002), nonhuman primates (20 gorillas and four chimpanzees) were found dead near a village where human cases occurred, and several of the animals were positive for Ebola virus (26, 38).

The natural reservoir(s) of Ebola virus is unknown, but reports of deaths attributable to Ebola virus among nonhuman primates in the same locations as human outbreaks and the linkage of several infected hunters to consumption of meat from or exposure to dead nonhuman primates point to these animals as a potential source for introduction of the virus into human populations (16, 17, 26, 38). There is clearly an urgent need to identify the natural reservoir of Ebola virus in order to better understand the epidemiology of the infection and the ecology of the virus.

Ebola hemorrhagic fever, the disease caused by Ebola virus infection in humans, usually begins abruptly with nonspecific symptoms such as nausea, fever and chills, headache, and abdominal pain (11). These symptoms rapidly progress as viremia is established, and the virus spreads throughout the body, involving multiple tissues and organs. The late stages of severe infections are often accompanied by hemorrhagic manifestations with evidence of dysregulation of coagulation, which results in conjunctival hemorrhage, easy bruising, and the presence of blood in urine and feces (11). At this stage, the disease progresses rapidly to a shock-like state, with multisystem failure culminating in death (30).

Previous reports suggest that one of two outcomes occurs
with Ebola virus infection in humans and nonhuman primates: either the virus produces an overwhelming infection that rapidly leads to death of the host, or it is cleared by a vigorous immune response that results in complete recovery of the host (3, 10). Thus, Ebola hemorrhagic fever is characteristically an acute illness, and the outcome usually becomes apparent fairly early in the course of infection; a prolonged course of infection has not been reported.

The mechanisms underlying the pathogenesis of and the determinants of protective immunity to Ebola virus are not clearly defined. The virus replicates in a variety of cell types, including macrophages, epithelial cells, hepatocytes, and endothelial cells. Viral replication in these cells induces high levels of inflammatory chemokines and cytokines that may be responsible for the inflammatory pathology observed during the early phase of infection (18, 21, 37). However, alpha/beta interferon responses, which would normally inhibit viral replication and dissemination, are suppressed by the virus (4, 8, 18, 20). Binding of the Ebola virus viral glycoprotein to endothelial cells induces cytotoxic effects and increases vascular permeability, which may provide a mechanism for endothelial cell leakage in later stages of infection (41).

Evidence from human studies suggests that a poor cellular immune response (as measured by low levels of gamma interferon and CD8 T-cell activation markers in serum) and low levels of anti-Ebola virus immunoglobulin G are associated with fatal infections (3). In contrast, convalescent patients have high levels of anti-Ebola virus immunoglobulin G and typically clear the infection within 3 weeks after onset of symptoms (3, 24). Studies in animal models suggest that the presence of high titers of antibodies can protect the host from fatal infection (19, 36, 40). However, neither the mechanisms of protection during natural infection nor the elements of the immune response that are responsible for viral clearance are well understood.

A mouse model of Ebola virus infection has been developed for use in studying immune responses to the virus in an attempt to understand the correlates of protective immunity (9, 32, 36, 39). In this model, subcutaneous infection with an adapted Ebola virus results in a nonfatal infection associated with long-term immunity against lethal rechallenge (9). Using this model, we show that CD8 T cells play a crucial role in the initial clearance of the virus following primary and secondary (rechallenge) infections and that CD4 T cells and antibodies are not required for short-term protection. However, in the absence of B cells and antigen-specific CD4 T cells, Ebola virus establishes a persistent asymptomatic infection, with disease symptoms appearing only during the late stage of infection. These data indicate that short-term control of the virus is achieved by CD8 T cells alone, but long-term control requires the presence of antibodies and CD4 T cells.

**MATERIALS AND METHODS**

**Viruses and mice.** A mouse-adapted strain of Ebola virus was derived from Ebola virus Zaire 1976 by serial passage through progressively older suckling mice, followed by plaque purification as previously described (9). Virus was amplified to a titer of $5 \times 10^7$ PFU/ml by one passage in Vero E6 cells (monkey kidney cell line; American Type Culture Collection [ATCC], Manassas, Va.) that had been propagated in modified Eagle medium containing 2% fetal bovine serum, 2 mM glutamine, 100 µg of streptomycin per ml, and 100 U of penicillin per ml (Invitrogen Life Technologies, Carlsbad, Calif.). All infected samples and animals were handled under maximum containment in the biosafety level 4 laboratory at the Centers for Disease Control and Prevention, Atlanta, Ga. All samples from the biosafety level 4 laboratory were gamma-irradiated ($5 \times 10^6$ rads) before further processing under biosafety level 2 and 3 conditions.

Mice deficient in B cells (μMT-/-, referred to as B-cell−/−), β2-microglobulin (B2M-/-), T cell receptor β (TCRβ-/-), or CD4 cells (CD4-/-) and their background control C57BL/6 mice, which were matched for sex and age (6 to 8 weeks), were obtained from a commercial supplier (Jackson Laboratory, Bar Harbor, Maine). All mice were maintained under pathogen-free conditions and allowed to acclimate to the biosafety level 4 laboratory for 3 to 4 days before use in experiments.

**Infection protocol.** Lethal Ebola virus infections were produced by intraperitoneal inoculation of $10^7$ or $10^6$ PFU of mouse-adapted Ebola virus in 0.2 ml of phosphate-buffered saline (PBS) as previously described (9). Vaccinations consisted of a single subcutaneous inoculation of $10^6$ PFU of mouse-adapted Ebola virus in 0.2 ml of PBS (19). Three weeks after subcutaneous infection, vaccinated mice were intraperitoneally challenged with $10^6$ PFU of Ebola virus in 0.2 ml of PBS. After infection, mice were caged in groups of five, weighed daily or on alternate days, and observed for survival.

**T-cell depletion.** The role of cellular immunity in protection was evaluated by depleting vaccinated mice of CD4 or CD8 T cells with a single intraperitoneal dose of $500 \mu g$ of protein A-purified GK1.5 (anti-CD4 monoclonal antibody; cell line purchased from ATCC) or L2.43 (anti-CD8 monoclonal antibody; cell line purchased from ATCC), or both antibodies 1 day before they were challenged with $10^6$ PFU of Ebola virus per mouse. Depletion of >97% of CD4 and CD8 T cells was confirmed in splenocytes 4 days after infection by flow cytometry.

**Viral antigen titers.** Levels of circulating Ebola virus antigens in serum and tissues were estimated by using a capture enzyme-linked immunosorbent assay (ELISA) as described (23). Briefly, Flexplates (Falcon, Atlanta, Ga.) were coated overnight at 4°C with a mixture of seven monoclonal antibodies raised against viral protein 40, glycoprotein, and nucleoprotein from Ebola virus Zaire 1976 and Ebola virus Sudan 1976. Tissue and serum samples were serially diluted (fourfold) in 5% PBS skim milk with 0.1% Tween 20 (blocking buffer) and incubated at 37°C for 1 h. Captured antigen was detected by polyclonal anti-Ebola virus Zaire serum produced in rabbits and then with a goat anti-rabbit immunoglobulin-horseradish peroxidase conjugate and 2,2'-azino-bis(3-ethylbenzthiazoline-sulfonate) (ABTS) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). The optical density at 410 nm (OD410) of each sample in control wells (coated with normal mouse ascitic fluid) was subtracted from the OD of the same sample in the corresponding test well (coated with anti-Ebola virus monoclonal antibodies) to derive a corrected OD. The highest dilution of sample that resulted in a corrected OD of 0.1 was designated the viral antigen titer of the sample. The limit of detection of antigen titers in tissue homogenates and serum varied from 1.20 to 1.40.

**Viral plaque assays.** Virus was titrated by an immunoplaque assay on the Vero E6 cell line. Briefly, virus-containing Vero E6 supernatants or samples were serially diluted (1:10) and incubated for 1 h at 37°C on confluent monolayers of Vero E6 cells in 48-well plates. Then 400 µl of 2% Basal Eagle’s medium (Life Technologies, Gaithersburg, Md.) supplemented with 5% fetal bovine serum, antibiotics, HEPES, and L-glutamine mixed 1:1 with 2% carboxymethylcellulose (CMC) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). The optical density at 410 nm (OD410) of each sample in control wells (coated with normal mouse ascitic fluid) was subtracted from the OD of the same sample in the corresponding test well (coated with anti-Ebola virus monoclonal antibodies) to derive a corrected OD. The highest dilution of sample that resulted in a corrected OD of 0.1 was designated the viral antigen titer of the sample. The limit of detection of antigen titers in tissue homogenates and serum varied from 1.20 to 1.40.

**Histopathology and immunohistochemistry.** Routine hematoxylin- and eosin-stained sections were examined on formalin-fixed tissues from representative mice in each group. Immunohistochemistry assays were performed with a labeled streptavidin-biotin technique described previously for the detection of Ebola virus antigens (42).

**RESULTS**

CD8 T cells mediate immunity against acute Ebola virus infection. Previous reports suggested that SCID mice infected subcutaneously with Ebola virus were unable to control the
virus and succumbed to the infection (8). In this study, we
determined if T-cell immunity by itself was sufficient to control
Ebola virus infection and which T-cell subpopulations were
responsible for protection against acute Ebola virus infection.
We used B-cell−/−, TCRβ−/−, β2m−/−, and CD4−/− mice in our exper-
iments. The mice were infected subcutaneously with 100 PFU
of Ebola virus, and animals from each group were sacri-
cified on postinfection days 3 to 16 and assayed for viral antigen levels in
spleen, liver, kidney, and serum. Viral antigen titers in
B-cell−/− and CD4−/− mice peaked on day 7 and were below
the limit of detection by day 14 after infection, a result similar
to that in the control group (Fig. 1A). Interestingly, TCRβ−/−
and β2m−/− mice had viral antigen titers 2 to 3 logs higher
than those for control, B-cell−/−, and CD4−/− mice (1:105
versus 1:102) on day 7 or 8 after infection, and their titers
remained high until day 16 postinfection (Fig. 1A).

We also assessed survival and disease symptoms in a sepa-
rate observation group that included animals from each group.
Mice deficient in B cells and CD4 T cells survived the infection
and did not show any sign of illness during the observation
period (Fig. 1B). However, both TCRβ−/− and β2m−/− mice
appeared ill from postinfection day 12 onward, and all died by

FIG. 1. Survival and viral kinetics in wild-type (+/+), TCRβ−/−,
B-cell−/−, β2m−/−, and CD4−/− mice infected with Ebola virus.
(A) Viral kinetics in mice subcutaneously challenged with 100 PFU of
Ebola virus. Viral antigens were assayed in the spleen (○), liver (□),
kidney (▲), and serum (●) from days 3 to 16 after infection (n = 3
mice per group per time point). Antigen was assayed by capture
ELISA and plotted as mean ± standard error. The detection limit of
the assay is indicated by dotted lines. Wild-type, CD4−/−, and
B-cell−/− mice efficiently controlled the viral load, whereas viral anti-
gen levels were 2 to 3 logs higher in TCRβ−/− and β2m−/− groups than
in controls. (B) Survival of wild-type (n = 10; ○), TCRβ−/− (n = 5; □),
B-cell−/− (n = 10; ■), CD4−/− (n = 4; ▲), and β2m−/− (n = 13; ●) mice
challenged subcutaneously with 100 PFU of Ebola virus. All
β2m−/− and TCRβ−/− mice died by day 16 or 22 after infection, while
all CD4−/− and B-cell−/− mice survived the infection.
Together, these data demonstrate that CD8 T cells play an important role in viral control and protection and that antibodies and CD4 T cells are not required for protection against acute Ebola virus infection.

**T cells alone are sufficient for protection against lethal Ebola virus rechallenge.** Previous studies have shown that vaccinated wild-type mice (i.e., mice inoculated subcutaneously with 10^2 PFU of mouse-adapted Ebola virus in 0.2 ml of PBS) survive lethal intraperitoneal rechallenge with the virus, whereas naive mice die from the infection (9). Our results showed that B-cell^-/-^ mice survived the subcutaneous infection and controlled viral replication as efficiently as wild-type mice (Fig. 1). We next determined whether these vaccinated B-cell^-/-^ mice were protected against the lethal Ebola virus challenge in the absence of antibodies. For this purpose, naive and vaccinated mice were challenged intraperitoneally with 10^5 and 10^6 PFU of mouse-adapted virus, respectively, and observed for survival before samples were collected and assayed for viral antigen levels.

Among vaccinated mice, all wild-type and B-cell^-/-^ mice survived intraperitoneal challenge, whereas naive wild-type and B-cell^-/-^ mice died from the intraperitoneal infection (Fig. 2A). We determined viral antigen levels in tissues on days 2, 4, and 6 after infection to confirm that death was due to viral infection. As expected, both naive wild-type and B-cell^-/-^ groups had high viral antigen titers (≥10^5) on day 6 after infection (Fig. 2B), whereas vaccinated B-cell^-/-^ mice (similar to control mice) had viral antigen titers below the level of detection by day 6 after infection (Fig. 2B). These data suggest that T cells are sufficient to protect mice against lethal reinfection with Ebola virus in the absence of antibodies.

**Primed CD8 T cells alone control viral replication and protect mice against lethal rechallenge.** To identify the T-cell subset that mediates protective immunity in vaccinated B-cell^-/-^ mice, we depleted CD4 T cells, CD8 T cells, or both from the vaccinated B-cell^-/-^ mice at the time of intraperitoneal Ebola virus challenge. We found that primed CD8 T cells alone could control viral replication and protect against lethal Ebola virus disease (Fig. 3B). All mice depleted of CD4 T cells survived and controlled the infection (viral antigen titer of <40), whereas all CD8 T-cell-depleted mice died and had high levels of virus in all tissues tested (viral antigen titers of 10^7 to 10^8; Fig. 3A, B). The control groups showed the expected results: untreated wild-type and B-cell^-/-^ vaccinated mice (i.e., no cell depletion) survived and controlled the infection, whereas the vaccinated B-cell^-/-^ mice depleted of both CD4
FIG. 3. Memory CD8 T cells protect B-cell–/– mice against lethal Ebola virus challenge. (A) Vaccinated B-cell–/– mice undepleted (○) or depleted of CD4 T cells (◆), CD8 T cells (▲), or both CD4 and CD8 cells (◇) were challenged intraperitoneally with 10⁶ PFU of Ebola virus and observed for survival (five mice per group). (B) Memory CD8 T cells are required for viral control in vaccinated B-cell–/– mice. Vaccinated B-cell–/– mice (undepleted or depleted of CD4, CD8, or both CD4 and CD8 T cells) were challenged with 10⁶ PFU of Ebola virus intraperitoneally 21 days after vaccination. Viral antigen levels were determined by antigen capture ELISA in the spleen (spl), liver (liv), kidney (kid), and serum (ser) on day 4 after infection (three mice per group per time point). Both undepleted and CD4 T-cell-depleted B-cell–/– mice survived and efficiently controlled viral replication (similar to wild-type mice), whereas CD8-depleted and CD4- and CD8-depleted groups had high viral antigen titers on day 4 and died by day 8 after infection. Data are plotted as mean ± standard error.
and CD8 T cells died with high levels of virus (Fig. 3A, B). Viral antigens were detected in wild-type, B-cell−/− (titers, 10 to 40), and CD4 T-cell-depleted B-cell−/− mice (titers, 40 to 160) on postinfection day 2; thereafter, levels remained below the limit of detection.

Ebola virus persists under certain conditions of immunodeficiency. Having established that CD8 T cells can provide protection and control viral replication and that CD4 T cells do not play a significant role in protection against acute Ebola virus infection, we next investigated the role of CD4 and CD8 T cells in long-term protection in vaccinated mice in the absence of antibodies. Vaccinated B-cell−/− mice (undepleted or CD4 T-cell depleted) that survived the intraperitoneal infection were observed for survival, illness, and reappearance of the virus for up to 7 months after intraperitoneal challenge. For this, we used weight loss, ruffling of the fur, and reduced activity as surrogates of illness in the mice. Vaccinated wild-type mice that survived the intraperitoneal infection and vaccinated B-cell−/− mice were included as controls.

There was no apparent illness in any of the three control groups of mice (i.e., vaccinated and rechallenged wild-type mice, B-cell−/− mice, and vaccinated B-cell−/− mice) during 7 months of observation. However, we found that CD4 T-cell-depleted B-cell−/− mice started losing weight about 50 to 70 days after intraperitoneal infection. These mice became progressively more ill, and six died between 51 and 203 days after intraperitoneal infection (Fig. 4B); however, all vaccinated and rechallenged wild-type mice and vaccinated B-cell−/− mice survived for the 7-month observation period (Fig. 4A). In a separate observation group, some of the undepleted vaccinated and rechallenged B-cell−/− mice also died, but they did so later than mice in the CD4-depleted group. Surprisingly, vaccinated B-cell−/− mice that were not rechallenged had viral antigen titers (10² to 10⁵) in the liver and spleen more than 368 days after infection.

To confirm that Ebola virus was responsible for this late illness, we determined viral titers in tissues and serum from all of the experimental groups. As determined by nested PCR, no viral RNA (data not shown), viral antigen, or infectious virus (data not shown) was detected in the tissues of the vaccinated wild-type group (Fig. 4A). The B-cell−/− vaccinated group had low levels of virus: of two mice, one had detectable virus in serum (antigen titer 1:40 and viral titer 500 PFU/ml) on day 120 and the other had detectable virus (antigen titer 1:640; viral titer 900 PFU/ml) in liver homogenate and in serum (antigen titer 1:160) on day 150 after intraperitoneal challenge. In contrast, the vaccinated CD4 T-cell-depleted B-cell−/− group began showing signs of illness about 70 days after intraperitoneal challenge and had readily detectable levels of virus (antigen titer of 10² to 10⁴ and viral titer of 10³ to 10⁵ PFU/ml) in several tissues. Viral antigen was present in the spleen (six of eight mice), liver (eight of eight mice), and serum (seven of eight mice) on days 70, 120, and 150 after infection (Fig. 4A).

It should be noted that these mice had initially controlled the acute infection, and viral antigen levels were below the limit of detection 5 days after intraperitoneal challenge (Fig. 3B).

Of importance, we isolated Ebola virus from serum samples collected 120 days after infection from both B-cell−/− and CD4 T-cell-depleted B-cell−/− mice by culture on Vero E6 cells (data not shown), confirming the presence of replicating virus in both groups of mice. Furthermore, naive mice (five in each group) were challenged intraperitoneally with viruses isolated from these persistently infected mice and, like the animals challenged with the parent virus, all died. These data suggest that although the B-cell−/− mice initially controlled the infection, they could not maintain long-term control. Furthermore, the absence of CD4 T cells accelerated the reappearance of virus in B-cell−/− mice that had persistent infection.

Viral persistence was confirmed in vaccinated CD4 T-cell-depleted B-cell−/− mice by histopathologic examination and immunohistochemistry of liver samples obtained on day 70. The samples were then compared with liver samples obtained from naive B-cell−/− mice on day 5 after intraperitoneal Ebola virus infection, when viral antigen titers were 10⁵ to 10⁶. Different patterns of tissue inflammation were found in the persistently versus acutely infected mice. In intraperitoneally infected naive mice, there was a prominent neutrophil infiltration and necrosis of hepatocytes on day 5 (Fig. 5B), whereas in persistently infected mice there was granulomatous inflammation with mononuclear cell infiltrates (Fig. 5D), and necrosis of hepatocytes was rare.

Immunohistochemical staining of the liver for Ebola virus viral antigen showed that the virus was associated with histocytes within the granulomas in persistently infected mice with high viral antigen titers (>10⁵; Fig. 5C): in contrast, in naive infected mice, viral antigen was observed in hepatocytes and sinusoidal lining cells, particularly in Kupffer’s cells (Fig. 5A). However, persistently infected mice with lower viral antigen titers (<10⁴) also showed liver immunohistochemistry patterns similar to those in mice with acute infection, in that virus was associated with Kupffer’s cells and hepatocytes (data not shown). Thus, in the late stages of infection, both the histology and the target cells infected are different during acute versus chronic Ebola virus infection.

**DISCUSSION**

Recent data from other investigators showed that adoptive transfer of CD8 T cells primed by DNA vaccination could protect against an Ebola virus challenge, but the study did not rule out contribution of the recipient’s antibodies in protection (39). To our knowledge, our data provide the first direct evidence for the important role of CD8 T cells in protective immunity against Ebola virus: in the absence of CD8 T cells, the other arms of the immune response (e.g., CD4 T cells or antibodies) could not control the virus. Our rechallenge experiments further demonstrate that CD8 T cells by themselves, in the absence of antibodies and CD4 T-cell help, can protect against fatality and initially control the virus after a lethal rechallenge. This is an interesting observation in the context of the role of antibodies in protection against cytopathic viruses. For most cytopathic viruses, viral clearance is thought to be antibody dependent (7, 12, 35); the critical role of CD8 T cells in Ebola virus clearance is unexpected and suggests that this virus does not follow a general paradigm of antiviral immunity. These data also imply that the ability to induce effective CD8 T-cell responses against viral antigens will be a desirable feature in the design of effective vaccines.

In all animal models studied to date, Ebola virus infection has an extremely rapid course, with widespread viral replica-
FIG. 4. Ebola virus persistence in mice with partial immunity. (A) The indicated groups of vaccinated mice were challenged intraperitoneally with $10^6$ PFU of Ebola virus, and animals were euthanized on days 5, 57, 70, 120, and 150 after infection. Viral antigen in the indicated tissues was quantitated by capture ELISA (bars), and infectious virus was measured by plaque assay (diamonds). Each pair of symbols (bar and diamond) represents an individual mouse. The detection limit of the antigen assay was 1:40, and for plaque assay it was 400 PFU/ml. (B) Survival of mice under different immunodeficiency conditions following Ebola virus infection. Vaccinated wild-type (+/+; n = 5; ○) or undepleted vaccinated B-cell $^+/−$ mice challenged intraperitoneally with $10^6$ PFU of Ebola virus (n = 4; ●) or vaccinated B-cell $^+/−$ mice depleted of CD4 T cells (n = 6; ■) 1 day prior to intraperitoneal challenge were observed for survival. Vaccinated B-cell $^-/-$ mice (n = 3; □) not challenged intraperitoneally were also included. All mice in the three groups (i.e., vaccinated B-cell $^+/−$, vaccinated and intraperitoneally challenged wild-type, and B-cell $^-/-$ mice) survived the infection, and none died during the 210-day observation period, whereas CD4 T-cell-depleted vaccinated B-cell $^-/-$ mice died between 50 and 210 days after intraperitoneal challenge.
tion and damage to multiple organs. Because of this, it was remarkable to discover that virus could survive in a host for a long period of time without causing obvious illness or death. The striking novel observation in our study was the persistence of Ebola virus in mice deficient in B cells (i.e., in the absence of antibodies). Reemergence of virus was seen in some vaccinated B-cell mice on day 70 after infection were examined for the presence of viral antigen by immunohistochemistry and for liver pathology by hematoxylin and eosin staining and found to have comparable viral antigen titers ($10^5$ to $10^6$) in liver homogenates. Insets show high-power views of the same sections, demonstrating infected hepatocytes (small arrow) and Kupffer's cell (large arrow) in an acutely infected mouse (A) and infected histiocytes (arrows) in a chronically infected mouse (C). Hematoxylin and eosin staining shows infiltrating neutrophils (arrows) in the day 5 liver section from a naive mouse (B) and a granuloma with histiocytes (arrows) in the day 70 liver section from a vaccinated CD4 T-cell-depleted B-cell mouse (D).

In these experiments, CD4 T-cell depletion was done at the time of the intraperitoneal Ebola virus challenge by treating the mice with anti-CD4 monoclonal antibody. This treatment results in a transient depletion of CD4 T cells, and in these mice, CD4 T-cell numbers had returned to essentially normal levels within a month (data not shown). Thus, despite the recovery of CD4 T-cell numbers, these mice were unable to exert long-term control of the Ebola virus infection. These results suggest that lack of long-term virus control is likely due to a deficiency in the Ebola virus-specific CD4 T cells and antibodies, not simply to a reduction in the total number of CD4 T cells. Similar results have been reported in other models of chronic infection where it has been shown that antigen-specific CD4 T cells are necessary to sustain CD8 T-cell function.

In B-cell vaccinated mice transiently depleted of CD4 T cells, it is possible that the absence of antigen-specific primed CD4 T cells results in failure of a sustained CD8 T-cell response, resulting in the reappearance of Ebola virus after a period of apparent control. These studies highlight the different but complementary roles played by the various arms of the immune system in protection against this highly virulent pathogen. The two T-cell subsets, CD8 and CD4 cells, played distinct roles in this protection. Memory CD8 T cells were remarkably effective on their own against the acute Ebola virus infection, whereas memory CD4 T cells by themselves were of little value in protection against acute infection 3 weeks after subcutaneous infection.

Failure of viral clearance could result from a number of
factors related to the virus or to the host immune response. Mutations in the virus could result in emergence of escape mutants, which may have attenuated growth potential and pathogenicity. The fact that intraperitoneal inoculation of Ebola virus isolates from persistently infected mice produced acute disease and mortality similar to that produced by the parent virus argues against biological differences in the virus itself. However, detailed studies are required to correlate sequence changes with the ability of the virus to replicate and persist long term. Host factors, such as the role of anti-Ebola virus antibodies, CD8 function, and CD4 T-cell help for CD8 function, are also important for clearance of the virus and may be identified by passive transfer of antibodies and cell populations in these mice. Detailed studies to address these issues are under way.

Ebola virus appears to persist in humans during convalescence after acute infection. In one study, virus was isolated from semen samples 39 and 61 days after onset of illness (14). In another study, Ebola virus was isolated from a patient 82 days after recovery (34). It was not known in either of these two studies whether the infected individuals were immunocompromised. Areas of central Africa where outbreaks of Ebola hemorrhagic fever occur have a high prevalence of human immunodeficiency virus infection (1, 15). Immune suppression is also associated with other infections prevalent in nonhuman primates in living conditions as a type I IFN antagonist. Proc. Natl. Acad. Sci. USA 96:4649–4654. 2001. Passive control of Sindbis virus replication in vitro. Virology 284:20–25.

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