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Lethal Monkeypox Virus Infection of CAST/EiJ Mice Is Associated with a Deficient Gamma Interferon Response

Patricia L. Earl, Jeffrey L. Americo, and Bernard Moss

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA

Monkeypox virus (MPXV) is endemic in Africa, where it causes disease in humans resembling smallpox. A recent importation of MPXV-infected animals into the United States raises the possibility of global spread. Rodents comprise the major reservoir of MPXV, and a variety of such animals, even those native to North America, are susceptible. In contrast, common inbred strains of mice, including BALB/c and C57BL/6, are greatly resistant to MPXV. However, several inbred strains of mice derived from wild mice, including CAST/EiJ, exhibit morbidity and mortality at relatively low inoculums of MPXV. Elucidating the basis for the susceptibility of CAST/EiJ mice could contribute to an understanding of MPXV pathogenicity and host defense mechanisms and enhance the value of this mouse strain as a model system for evaluation of therapeutics and vaccines. Here we compared virus dissemination and induced cytokine production in CAST/EiJ mice to those in the resistant BALB/c strain. Following intranasal infection, robust virus replication occurred in the lungs of both strains, although a relatively higher inoculum was required for BALB/c. However, while spread to other internal organs was rapid and efficient in CAST/EiJ mice, the virus was largely restricted to the lungs in BALB/c mice. Gamma interferon (IFN-γ) and CCL5 were induced in lungs of BALB/c mice concomitant with virus replication but not in CAST/EiJ mice. The importance of IFN-γ in protection against MPXV disease was demonstrated by the intranasal administration of the mouse cytokine to CAST/EiJ mice and the resulting protection against MPXV. Furthermore, C57BL/6 mice with inactivation of the IFN-γ gene or the IFN-γ receptor gene exhibited enhanced sensitivity to MPXV.

Monkeypox virus (MPXV) was isolated in 1958 from lesions on laboratory monkeys imported to Denmark from Singapore and was identified as a member of the *Orthopoxvirus* genus of the family *Poxviridae* (46). Human cases of monkeypox were first reported in the early 1970s in Africa, although it is likely they occurred earlier but were misdiagnosed as smallpox prior to the eradication of that disease (reviewed by Parker et al. [33]). MPXV is endemic in rain-forested regions of Africa and causes a disease that clinically resembles smallpox but with reduced morbidity and mortality. Two subgroups of MPXV with distinct genetic, clinical, and epidemiological characteristics have been described (6, 25, 47). Central African isolates can cause severe disease; they have limited human-to-human transmission and can cause up to 10% case fatality (4, 24, 33). In contrast, West African isolates are less virulent, with no reported human-to-human transmission. Sequencing studies have suggested several mutations that might account for the differences in virulence of MPXV strains.

The major reservoir of MPXV, in spite of its name, is believed to be rodents, particularly squirrels (10, 21, 36). The year 2003 outbreak of human monkeypox in the midwestern United States was caused by a West African strain carried by infected rodents imported from Ghana, highlighting the ease of transport and spread of MPXV to regions of the globe where the virus is not endemic (35). The susceptibility of North American rodents to MPXV highlights the potential of geographic spread of this virus. Moreover, MPXV like variola virus (VARV), the causative agent of smallpox, is a potential bioterrorist agent. Due to the cessation of routine vaccination more than 3 decades ago, much of the population is now at risk (17), and recent surveys have shown an increase in incidence of monkeypox in the Democratic Republic of Congo (37).

Numerous animal species with various degrees of susceptibility have been considered models for studying MPXV pathogenesis. Primates, such as cynomolgus and rhesus monkeys, have the advantage of their relationship to humans; however, high input doses are needed to achieve significant morbidity and mortality, and unnatural routes of infection are commonly used (16, 50). Small animals such as prairie dogs (12, 15, 20, 23, 48), ground squirrels (39, 44), and African dormice (40) are sensitive to infection with low doses of MPXV. However, these animals have several disadvantages. They are outbred and thus exhibit wide animal-to-animal variation. Prairie dogs and ground squirrels must be caught in the wild. There are no commercial sources of dormice. And there is a paucity of immunological reagents for any of these animals. Inbred strains of mice would be ideal small animal models, but common strains are relatively resistant to MPXV (2), leading to the use of immunodeficient C57BL/6 stat1$^{-/-}$ (42) and SCID-BALB/c (14, 30) mice and immunologically immature suckling white mice (27). By screening 38 different inbred mouse strains, we discovered that CAST/EiJ, PERA/EiJ, and MOLF/EiJ, were highly susceptible to MPXV-induced disease and death whereas the remainder were resistant (2). Interestingly, these three susceptible strains were independently derived from wild mice and are genetically quite distinct from the numerous classical varieties, which are closely related to each other (49). Compared to the classical varieties, the mouse strains derived from wild mice resemble other outbred rodent species in their susceptibility to MPXV. We chose the CAST/EiJ strain for our model because of

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Address correspondence to Patricia L. Earl, pearl@niaid.nih.gov.

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their greater susceptibility, better commercial availability, and lower cost compared to the two other strains derived from wild mice. CAST/EiJ mice have a low 50% lethal dose (LD_{50}) for MPXV via both intranasal and intraperitoneal routes of infection. Importantly, they develop both humoral and cellular immunity to MPXV in response to nonlethal orthopoxvirus infection and can be used for evaluation of vaccines and therapeutics (2). Here, we compared BALB/c, C57BL/6, and CAST/EiJ mice and uncovered clues regarding the greater sensitivity of the last to MPXV infection.

MATERIALS AND METHODS

Cells. BS-C-1 cells were maintained at 37°C and 5% CO_2 in modified Eagle minimal essential medium (Quality Biologicals, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 U of penicillin/ml, and 10 mg of streptomycin/ml.

Viruses. A low-passage-number stock of MPXV-Z79-I-005 was obtained from I. Damon, CDC, Atlanta, GA. A clonal isolate, MPXV-Z79-CB2 (2), was used in all experiments. Purified virus was prepared using a modification of the protocols of Damon (unpublished) and Earl et al. (7) as previously described.

Animals. The following mice were obtained from Jackson Laboratories (Bar Harbor, ME): CAST/EiJ, C57BL/6, B6.129S7-IFNγ^{tm1ts} (gamma interferon [IFN-γ] knockout), and B6.129S7-IFNγr^{tm1agt} (IFN-γ receptor knockout) mice. BALB/c mice were obtained from Taconic Biotechnology (Germantown, NY). Mice were maintained in small, ventilated microisolator cages.

Inoculation of animals. MPXV was diluted in phosphate-buffered saline containing 0.05% bovine serum albumin just prior to inoculation. The concentration of each dilution of virus was verified by plaque assay. Animals were anesthetized by inhalation of isoflurane, and intranasal infections were performed by instillation of 10 μl of virus into one nostril. Virus diluent was used for mock infections. In experiments to determine susceptibility to MPXV, animals were weighed and observed for signs of disease daily for up to 2 1/2 weeks after infection. Animals that lost 30% of their starting weight were euthanized in accordance with NIAID Animal Care and Use protocols. All mouse experiments were performed in an animal biosafety level 3 (ABSL3) suite with approval of the NIAID Animal Care and Use Committee and the Centers for Disease Control and Prevention.

Kinetics of virus spread in CAST/EiJ and BALB/c mice. For the experiment described in detail in this report, 50 CAST/EiJ mice were inoculated with the doses of MPXV indicated below and sacrificed by cervical dislocation on predetermined days postinfection. Of these, 14 CAST/EiJ mice were inoculated with 10^6 PFU, and 4 were sacrificed on days 2, 4, and 6 and 1 on day 8. One mouse that was intended for sacrifice on day 8 died on day 6 and was included in the analysis of that group. Thus, the day 8 "group" consisted of a single animal. Sixteen mice were inoculated with 10^4 PFU, and groups of 4 were sacrificed on days 2, 4, 6, and 8. Twenty mice were inoculated with 10^2 PFU, and groups of 4 were sacrificed on days 2, 4, 8, 13, and 16. Fifty BALB/c mice were also inoculated with MPXV. Twenty-four and 26 mice were inoculated with 10^6 and 10^4 PFU, respectively. Four mice from each dosage group were sacrificed on days 2, 4, 6, 8, 13, and 16. An additional 2 mice from the 10^4-PFU group were sacrificed on day 16.

Immediately after sacrifice, major organs (lungs, liver, spleen, brain, and kidneys) were removed and placed in 2 to 3 ml of balanced salt solution containing 0.1% bovine serum albumin and stored at −80°C until use. For determining MPXV titers, organs were thawed and processed as follows. Tissues were homogenized with a GLH-1 mechanical grinder equipped with hard-tissue disposable probes and aerosol-proof caps (Omni International, Kennesaw, GA). After sonication three times for 45 s each in ice water, the homogenates were centrifuged for 10 s at 400 × *g* in a 4515 centrifuge (Eppendorf, Hauppauge, NY). Supernatants were aliquoted into fresh screw-cap tubes and either frozen or used for virus titration by plaque assay on BS-C-1 cells.

Quantitation of cytokines and chemokines in organ homogenates of mice infected with MPXV. Quantitation of 22 cytokines and chemokines (granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor [GM-CSF], IFN-γ, interleukin-10 [IL-10], IL-12, IL-13, IL-15, IL-17, IL-1a, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10 [CXCL10], KC [CXCL1], monocyte chemoattractant protein 1 [MCP-1], macrophage inflammatory protein 1α [MIP-1α], RANTES [CCL5], and tumor necrosis factor alpha [TNF-α]) in the lung and spleen homogenates of CAST/EiJ and BALB/c mice was performed with a Milliplex MAP mouse cytokine/chemokine immunoassay kit (Millipore Corp., Billerica, MA) using a BioPlex 200 Luminex (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Organ homogenates were thawed, and a cocktail of broad-spectrum protease inhibitors (Roche Applied Science, Indianapolis, IN) was added.

IFN-γ protection of mice. Recombinant mouse IFN-γ (CYCHGT VIES LESLNNYFNS SGIDVEEKSL FLDIWRNWQK DGDMKILQSQ II SFYLRLFE VLKDNQAISN NISVIESHLI TTFFSNSKAK KDAFMSIAKF EVNNPQVQRQ AFNELIRVVH QLLPESSLRK RKRSR) was obtained from Gibco. After reconstitution at a concentration of 1 mg/ml, it was stored in aliquots at −80°C until use. Three groups of CAST/EiJ mice were established. One group of 8 animals received 5×10^3 units of IFN-γ (26) by intranasal instillation at 8 a.m. daily for 5 consecutive days. This and another group of 8 animals were infected intranasally with 2×10^4 PFU of MPXV at noon on the first day of IFN-γ administration. A third group of 2 animals was given a daily dose of IFN-γ only. Animals were observed for signs of disease and weighed daily.

Four weeks after the initial infection with MPXV, all surviving animals were rechallenged with 10^6 PFU of MPXV by the intranasal route. They were followed for signs of disease and weight loss for 2 weeks.

Two groups of 6 mice were infected with 2×10^4 PFU of MPXV in the presence or absence of IFN-γ as described above. Signs of disease and weight loss were followed daily. Six days postinfection all animals were sacrificed by cervical dislocation, lungs were removed and frozen, and MPXV titers were determined as described above.

MPXV enzyme-linked immunosorbent assay (ELISA). All surviving mice from the IFN-γ protection study were bled from the mandibular plexus 3 weeks after infection. Serum IgG titers in response to MPXV were determined as follows. Microtiter plates were coated overnight with 10^6 PFU per well of sucrose cushion-purified MPXV and then fixed with 2% paraformaldehyde for 10 min at 4°C. Plates were blocked with phosphate-buffered saline containing 5% nonfat dry milk and 0.2% Tween 20 for 1 h at 37°C. Twofold serial dilutions of heat-inactivated sera in block buffer were added, and plates were incubated for 1 h at 37°C, followed by washing. Then anti-mouse IgG–peroxidase (POD) (Roche Applied Science, Indianapolis, IN) was added for 1 h at 37°C. After a washing, BM Blue substrate (Roche Applied Science, Indianapolis, IN) was added for 30 min at room temperature. Absorbance at 370 and 492 nm was measured with a Spectramax M5 using Softmax Pro software (Molecular Devices).

RESULTS

Kinetics of virus spread in CAST/EiJ and BALB/c mice. We previously demonstrated that CAST/EiJ and BALB/c mice have profoundly different susceptibilities to intranasal infection with MPXV (2). The disease course in CAST/EiJ mice is severe, with weight loss of 20 to 30% at doses of 10^3 PFU or higher, and an LD_{50} of 680 PFU was calculated. In contrast, BALB/c mice exhibit only transient weight loss and no mortality, even at 10^6 PFU (2, 13). In order to investigate the basis for this difference, we performed a serial sacrifice of both strains of mice and determined the titers of the virus in various organs. CAST/EiJ mice were infected intranasally with lethal doses of 10^6 or 10^4 PFU or a sublethal dose of 10^2

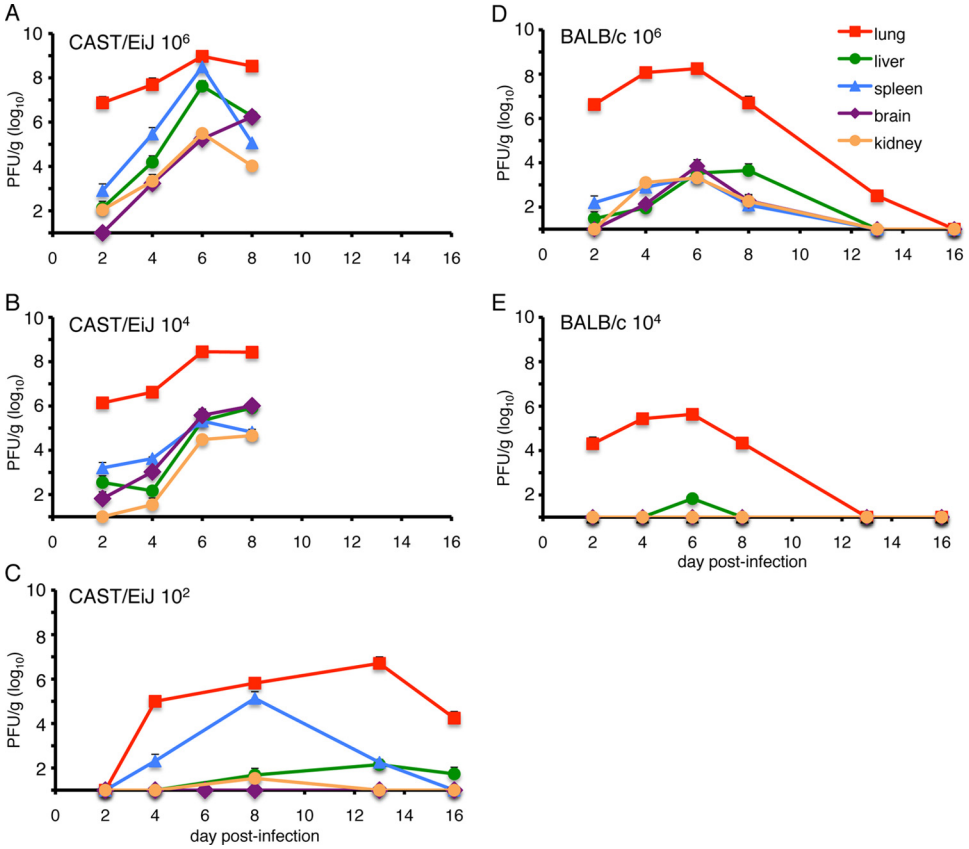

FIG 1 Virus titers in internal organs of CAST/EiJ and BALB/c mice infected with MPXV. Mice were infected intranasally with the indicated doses of MPXV and sacrificed on days 2, 4, 6, 8, 13, and 16 postinfection. Major organs were excised and weighed, and titers were determined by plaque assay. (A) CAST/EiJ mice infected with 10^6 PFU. (B) CAST/EiJ mice infected with 10^4 PFU. (C) CAST/EiJ mice infected with 10^2 PFU. (D) BALB/c mice infected with 10^6 PFU. (E) BALB/c mice infected with 10^4 PFU. All groups had 4 animals except that only one CAST/EiJ mouse infected with 10^6 PFU survived to day 8. Error bars indicate standard deviations.

PFU; BALB/c mice were infected intranasally with either of the two higher doses, both of which were sublethal. On days 2, 4, 6, 8, 13, and 16 postinfection, mice were sacrificed, lungs, spleen, liver, brain, and kidneys were removed, and the MPXV infectivity titers were determined. The kinetics of virus spread at each dose is shown in Fig. 1. In all groups, MPXV replicated in the lungs to high titer prior to other sites; virus was detected at 2 days in the lungs and peaked at 6 days (except for the prolonged course after the 10^2-PFU dose infection of CAST/EiJ mice). In CAST/EiJ mice, the MPXV titer on day 2 was higher for the 10^6-PFU dose (7.5×10^6 PFU/g of lung) than for the 10^4-PFU dose (1.4×10^6 PFU/g of lung) but both exceeded 10^8 PFU/g of lung by the time of death. Surprisingly, the lung titers in BALB/c mice infected with 10^6 PFU were similar to the titers in CAST/EiJ mice, although the BALB/c mice all survived. In BALB/c mice infected with 10^4 PFU, the kinetics of virus replication in the lungs was similar to that for the higher dose but the titers were lower. With both doses, the BALB/c mice cleared the virus by day 16.

The major difference between the nonsurviving CAST/EiJ mice and the surviving BALB/c mice correlated with the virus spread to other organs (Fig. 1). At the lethal doses in CAST/EiJ mice, the organ titers were between approximately 10^5 and 10^8 PFU/g of tissue, whereas in the BALB/c mice the titers were less than 10^4 PFU/g of tissue at the higher dose and barely detectable at the lower.

Induction of cytokines in the lungs and spleens of infected mice. We previously reported that CAST/EiJ mice can make robust antibody and cytotoxic T cell responses upon nonlethal infection with vaccinia virus (VACV) and that this immune response was protective against a subsequent infection with MPXV (2). This result, together with the rapid onset of morbidity and mortality in unvaccinated MPXV-infected CAST/EiJ mice, suggested a defect in the kinetics or quality of the innate immune response. To investigate this possibility, we compared the levels of 22 cytokines and chemokines in the lungs and spleens of individual CAST/EiJ and BALB/c mice before and after infection. The majority of cytokines showed little or no increase. However, after infection with 10^6 PFU of MPXV, IFN-γ, CCL5 (RANTES), and CXCL10 (IP-10) increased in BALB/c mice and interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF), CXCL1 (KC), and CXCL10 increased in CAST/EiJ mice (Fig. 2). The same pattern was observed in lungs of CAST/EiJ mice infected with 10^4 PFU albeit at lower levels (Fig. 2). Only very small amounts of any cytokine were detected in the lungs of CAST/EiJ mice infected with 10^2 PFU and BALB/c mice infected with 10^4 PFU except for CXCL10 (Fig. 2). The low IFN-γ and CCL5 responses in the lungs and the elevations of the other cytokines, except for IL-6, were recapitulated in two additional experiments in which CAST/EiJ mice were inoculated intranasally with 2×10^5 PFU of MPXV (data not shown). Thus, while both CAST/EiJ and BALB/c mice

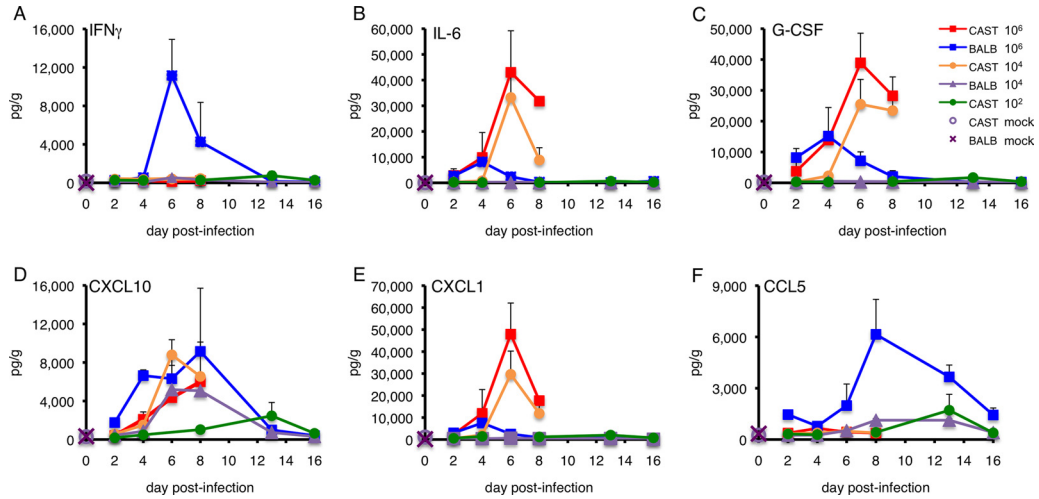

FIG 2 Cytokine levels in lung tissues of CAST/EiJ and BALB/c mice infected with MPXV. A Milliplex MAP mouse immunoassay kit was used to quantitate the amounts of 22 cytokines and chemokines in lung homogenates of CAST/EiJ and BALB/c mice after infection with MPXV. Six cytokines were elevated postinfection: IFN-γ, IL-6, G-CSF, CXCL10, CXCL1, and CCL5. All groups had 4 animals except that only one CAST/EiJ mouse infected with 10^6 PFU survived to day 8. Values in the key are inoculum titers (PFU). Error bars indicate standard deviations.

had high virus titers in the lungs, IFN-γ and CCL5 were elevated only in the latter, suggesting a crucial difference in the cytokine response to MPXV infection.

In contrast to results for the lungs, the 6 cytokines, including IFN-γ, were increased in spleens of CAST/EiJ mice and, with the exception of CCL5, were higher than in spleens of BALB/c mice (Fig. 3).

Protection of CAST/EiJ mice from MPXV by exogenous IFN-γ. Because IFN-γ is known to be important for control of virus infections, including poxviruses (11, 19, 22), we considered that its failure to be induced in the lungs of CAST/EiJ mice might be responsible for morbidity and mortality. Therefore, we tested whether exogenously administered mouse IFN-γ would compensate for the failure to induce this cytokine following MPXV infection in CAST/EiJ mice. Two groups of CAST/EiJ mice were infected with a lethal dose of MPXV. One of these groups also received IFN-γ intranasally on 5 consecutive days beginning 4 h prior to infection. The animals that did not receive IFN-γ were lethargic by day 4, began showing signs of morbidity, including weight loss (Fig. 4A), hunched posture, and ruffled fur, beginning on day 5, and became moribund or died by days 8 to 10 (Fig. 4B). In contrast, similar signs of disease were delayed and markedly less pronounced in the animals that received IFN-γ, and these animals fully recovered by day 14 (Fig. 4A and B). No toxic effects of IFN-γ were noted in control, uninfected mice (Fig. 4A and B).

In order to show that the mice that did not succumb to the

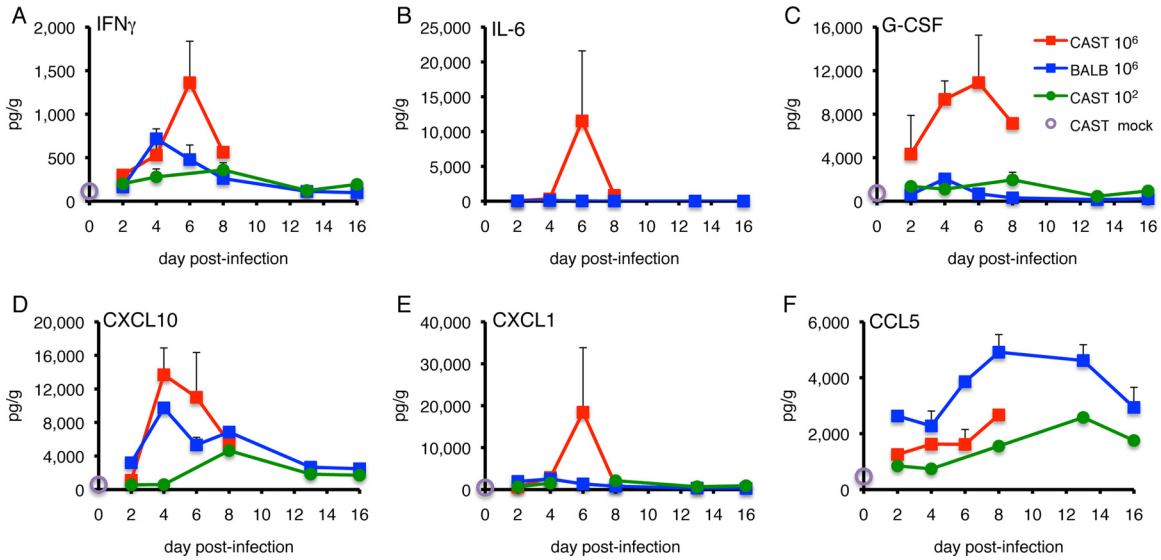

FIG 3 Cytokine levels in spleen tissues of CAST/EiJ and BALB/c mice infected with MPXV. A Milliplex MAP mouse immunoassay kit was used to quantitate the amount of 22 cytokines and chemokines in spleen homogenates of CAST/EiJ and BALB/c mice after infection with MPXV. Six cytokines were elevated postinfection: IFN-γ, IL-6, G-CSF, CXCL10, CXCL1, and CCL5. All groups had 4 animals except that only one CAST/EiJ mouse infected with 10^6 PFU survived to day 8. Values in the key are inoculum titers (PFU). Error bars indicate the standard deviations.

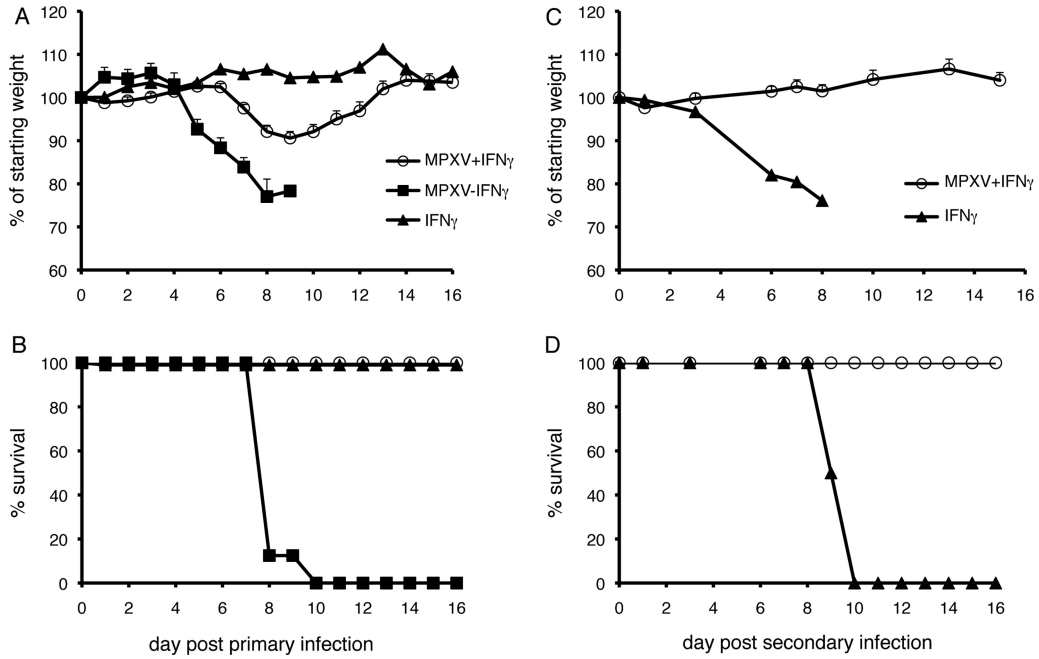

FIG 4 Weight loss and survival of CAST/EiJ mice infected with MPXV in the presence or absence of exogenous IFN-γ. Two groups of mice ($n = 4$) were infected intranasally with 2×10^4 PFU of MPXV. One group received recombinant IFN-γ daily for 5 days starting 4 h prior to infection. A control group ($n = 2$) received IFN-γ only. Surviving animals were rechallenged with 10^6 PFU of MPXV at 4 weeks after the initial infection. (A) Weight loss after the first infection. (B) Survival after the first infection. (C) Weight loss after rechallenge. (D) Survival after rechallenge. Error bars show standard deviations.

initial MPXV challenge were truly infected, all surviving mice, i.e., those receiving MPXV plus IFN-γ or IFN-γ only, were bled 3 weeks after the start of the experiment and serum IgG MPXV titers were determined by ELISA. The animals that were infected with MPXV and treated with IFN-γ mounted a strong antibody response, while the controls receiving only IFN-γ did not (Table 1). A week later (4 weeks after the initial infection), both groups were challenged with a high dose (10^6 PFU) of MPXV. The animals that had been infected in the presence of IFN-γ and developed MPXV antibodies showed no signs of disease, while the previously uninfected controls that received only IFN-γ became morbid by day 5 and succumbed by day 10 postinfection (Fig. 4C and D).

To further investigate the role of exogenous IFN-γ, we repeated the previous experiment depicted in Fig. 4A and B but sacrificed the mice on day 6. At this time, animals that did not receive IFN-γ had lost weight and become lethargic, while those that received IFN-γ remained healthy (Fig. 5A). A 22-fold reduction in virus titer was found in the lungs of IFN-γ recipients in comparison to those that did not receive IFN-γ (Fig. 5B). Thus, protection by IFN-γ therapy correlated with a reduction in virus load in the lungs.

TABLE 1 ELISA antibody titers in serum from CAST/EiJ mice infected with MPXV

Treatment	Animal designation	Reciprocal endpoint titer
MPXV + IFN-γ	C294 LP	200,000
	C294 NP	400,000
	C295 LP	200,000
	C296 LP	400,000
	C296 NP	400,000
	C297 LP	200,000
	C297 NP	400,000
IFN-γ only	C302 NP	<800
	C303 LP	<800

Susceptibility of IFN-γ and IFN-γ receptor mutants to MPXV. To further evaluate the role of IFN-γ in protection from MPXV, we infected C57BL/6 mice and derivative strains homozygous for targeted mutations of IFN-γ or IFN-γ receptor genes with 2×10^4 PFU or 10^6 PFU of MPXV. CAST/EiJ mice were infected with 2×10^4 PFU as positive controls. The C57BL/6 mice behaved much like BALB/c mice following infection with MPXV (2, 14); there was an initial dose-dependent weight loss but then full recovery (Fig. 6A and B). However, both mutant strains lost significantly more weight and recovered more slowly than the parental strain ($P < 0.05$ on days 7 through 11) but did not succumb to the infection, as did the CAST/EiJ mice (Fig. 6A and B). Thus, mice lacking IFN-γ or IFN-γ receptor display a phenotype intermediate between that of their resistant parent and that of susceptible CAST/EiJ mice, supporting the importance of IFN-γ in resistance to MPXV but indicating that other host factors are also involved.

DISCUSSION

We previously showed that inbred CAST/EiJ mice derived from wild mice, in contrast to classical inbred strains, are highly susceptible to lethal MPXV infection. In order to investigate the host responses that underlie sensitivity and resistance, we first compared kinetics of virus replication in CAST/EiJ and BALB/c mice. The intranasal route was used to partially mimic upper respiratory transmission of MPXV and VARV. High levels of virus replication were detected within 2 days in the lungs of both strains of mice. In

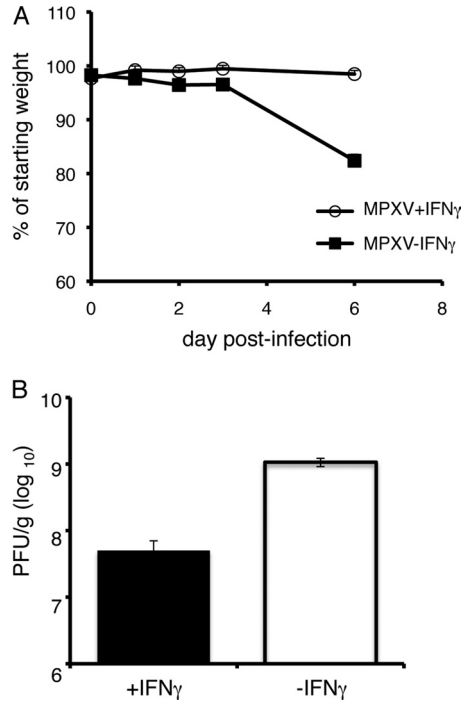

FIG 5 Effect of IFN-γ on virus replication in the lungs of CAST/EiJ mice infected with MPXV. Groups of CAST/EiJ mice ($n = 4$) were infected with 2×10^4 PFU of MPXV in the presence or absence of IFN-γ. On day 6 postinfection, mice were euthanized, lungs were excised, and virus titers were determined. (A) Weight loss. (B) MPXV titers in lungs. Error bars indicate standard deviations.

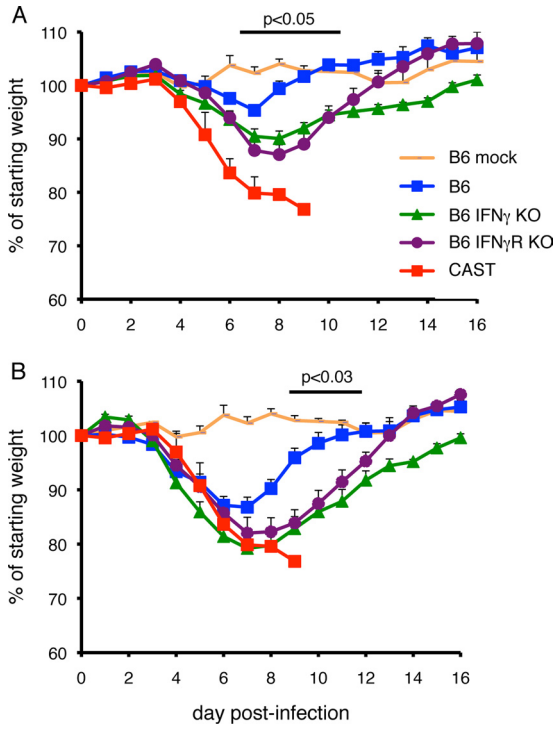

FIG 6 Weight loss of IFN-γ knockout and IFN-γ receptor knockout mice after infection with MPXV. C57BL/6 (B6), B6 IFN-γ knockout (KO), and B6 IFN-γ receptor (IFNγR) KO mice ($n = 5$) were infected intranasally with 2×10^4 PFU (A) or 10^6 PFU (B) of MPXV. A group of CAST/EiJ mice ($n = 4$) was also infected with 2×10^4 PFU and is included in both graphs. Days when the difference in weight loss between the parental and KO mice were significant are indicated with bars.

CAST/EiJ mice, virus spread quickly to other organs (liver, spleen, kidney, and brain), with high titers accumulating on days 6 to 8, the time of death. Surprisingly, kinetics of replication in the lungs of BALB/c mice paralleled that in CAST/EiJ mice. In both mouse strains, virus titers peaked on day 6, with those in BALB/c mice infected with 10^6 PFU similar to those in CAST/EiJ mice infected with lethal doses. However, replication in other organs differed markedly, with only minimal spread in BALB/c mice and subsequent full recovery.

CAST/EiJ mice have not been extensively used to study microbial infections. However, they have been reported to be more resistant than BALB/c mice to *Bordetella pertussis* (3) and more sensitive to *Coccidioides immitis* (9). We had previously shown that CAST/EiJ and BALB/c mice make similar CD8 T-cell and antibody responses following scarification with VACV, suggesting that CAST/EiJ mice are competent in their ability to mount an acquired immune response. We suspected that differences in the kinetics or magnitude of the innate immune response might underlie the sensitivity of CAST/EiJ mice to MPXV. Accordingly, we analyzed the induction of 22 different cytokines and chemokines in lung tissue following MPXV infection. Although most cytokines showed little change after infection of BALB/c mice with 10^6 PFU of MPXV, a sharp peak of IFN-γ was detected on day 6, coincident with the peak virus titer and preceding virus clearance. CCL5 and CXCL10, which are induced by IFN-γ, peaked on day 8 in BALB/c mice. Remarkably, neither IFN-γ nor CCL5 increased in the lungs of MPXV-infected CAST/EiJ mice after infection with either 10^6 or 10^4 PFU of MPXV, although there was some increase in CXCL10. Interestingly, CAST/EiJ mice mounted a higher IFN-γ response in the spleen following virus spread than BALB/c mice, indicating that production of this cytokine was not genetically impaired. Fierer et al. (9) demonstrated equivalent levels of IFN-γ mRNA in the lungs of BALB/c and CAST/EiJ mice after infection with *Coccidioides immitis* although they did not measure levels of the cytokine.

To determine whether the timely induction of IFN-γ in the lungs could have prevented disease in CAST/EiJ mice, we administered mouse IFN-γ intranasally just before and following infection. This treatment lowered the level of MPXV in the lungs, reduced morbidity, and completely prevented mortality, indicating that CAST/EiJ mice could effectively respond to IFN-γ. While this experiment demonstrated that exogenous IFN-γ protected CAST/EiJ mice, it remained to be determined whether the production of IFN-γ contributed to the protection of naturally resistant strains of mice. To evaluate this, IFN-γ and IFN-γ receptor gene knockout C57BL/6 mice were infected with MPXV. Both mutant strains were more sensitive to MPXV than the parent C57BL/6 mice though less sensitive than CAST/EiJ mice or STAT1-deficient C57BL/6 mice (38). We concluded that the ability to rapidly induce IFN-γ represents an important factor, but not the sole determining factor, responsible for the different sensitivities of C57BL/6 and CAST/EiJ mice.

The participation of IFN-γ in the host response to other poxvirus infections is well known (5, 13, 18, 19, 28, 31, 32), and this cytokine likely has multiple roles, as it promotes a Th1 response in

addition to more direct antiviral activity (8). Previous studies have shown that the susceptibility of inbred mouse strains to ectromelia virus (ECTV) correlates with their ability to produce IFN-γ in the spleen following ECTV infection (38). Thus, BALB/c is a low IFN-γ producer and is highly susceptible to ECTV whereas C57BL/6 is a high IFN-γ producer and is relatively resistant. However, both low- and high-IFN-γ-producer classical inbred strains of mice are resistant to MPXV as shown here and previously (2, 14). Therefore, we did not anticipate that a deficiency in IFN-γ production in the lungs would be a distinguishing feature of the sensitivity of CAST/EiJ mice.

Since IFN-γ is produced mainly by NK and T lymphocytes, the deficiency of this cytokine could be related to a failure to activate or recruit such cells into the lungs. Interestingly, the percentages of NK cells in the peripheral blood of CAST/EiJ and BALB/c are 1.25% and 7.28%, respectively, whereas the percentages of CD4 T cells are similar and those of CD8 T cells are 2-fold higher in BALB/c mice than in CAST/EiJ mice (http://phenome.jax.org). Investigation into the function and trafficking of lymphocytes may help to illuminate the basis for the defect in IFN-γ production in the lungs of CAST/EiJ mice.

To counteract IFN-γ, many poxviruses encode a soluble IFN-γ binding protein with homology to the extracellular part of the IFN-γ receptor (1, 29, 34, 41). However, there is considerable sequence diversity of IFN-γ from different species, e.g., the mouse and human proteins are only 40% identical in amino acid sequence (8). Although the VACV IFN-γ binding protein inhibits IFN-γ from humans, cows, rabbits, rats, and chickens, it has a low affinity for mouse IFN-γ. This can explain the finding that deletion of the B8 gene, encoding the VACV IFN-γ binding protein, did not affect VACV virulence in mice (43), although a reduction had been reported by another group (45). In contrast, the ECTV homolog binds mouse IFN-γ and deletion of the gene reduces virulence for mice (38). MPXV encodes a homolog of the B8 protein with 95% and 91% identities to VACV and ECTV, respectively, but its binding properties have not yet been determined.

In conclusion, intranasal infection of CAST/EiJ mice with MPXV leads to seeding and replication of the virus in the lungs followed by rapid and uncontrolled dissemination to other major organs. To achieve equivalent levels of MPXV replication in the lungs, BALB/c mice were infected with 100 times the lethal dose used for CAST/EiJ mice. However, even then spread of MPXV to other organs was minimal in BALB/c mice. The resistance of BALB/c mice correlated with the induction of IFN-γ in the lungs, which did not occur in CAST/EiJ mice. Moreover, the resistance of CAST/EiJ mice was enhanced by administration of exogenous IFN-γ and the resistance of C57BL/6 mice was reduced by knockout of the gene encoding IFN-γ or the gene encoding the IFN-γ receptor. To follow up these observations, we plan to determine the cellular response to MPXV infection of sensitive and resistant mice and to determine the role of the MPXV IFN-γ binding protein.

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REFERENCES

1. **Alcami A, Smith GL.** 1995. Vaccinia, cowpox, and camelpox viruses encode soluble gamma interferon receptors with novel broad species specificity. J. Virol. **69**:4633–4639.
2. **Americo JL, Moss B, Earl PL.** 2010. Identification of wild-derived inbred mouse strains highly susceptible to monkeypox virus infection for use as small animal models. J. Virol. **84**:8172–8180.
3. **Banus HA, et al.** 2006. Host genetics of Bordetella pertussis infection in mice: significance of Toll-like receptor 4 in genetic susceptibility and pathobiology. Infect. Immun. **74**:2596–2605.
4. **Breman JG, et al.** 1980. Human monkeypox, 1970–79. Bull. World Health Organ. **58**:165–182.
5. **Chaudhri G, et al.** 2004. Polarized type 1 cytokine response and cell-mediated immunity determine genetic resistance to mousepox. Proc. Natl. Acad. Sci. U. S. A. **101**:9057–9062.
6. **Chen N, et al.** 2005. Virulence differences between monkeypox virus isolates from West Africa and the Congo basin. Virology **340**:46–63.
7. **Earl PL, Cooper N, Wyatt LS, Moss B, Carroll MW.** 1998. Preparation of cell cultures and vaccinia virus stocks., p 16.16.11–16.16.13. *In* Ausubel FM, et al. (ed), Current protocols in molecular biology, **vol 2**. Greene Publishing Associates & Wiley-Interscience, New York, NY.
8. **Farrar MA, Schreiber RD.** 1993. The molecular cell biology of interferon-gamma and its receptor. Annu. Rev. Immunol. **11**:571–611.
9. **Fierer J, Walls L, Eckmann L, Yamamoto T, Kirkland TN.** 1998. Importance of interleukin-10 in genetic susceptibility of mice to Coccidioides immitis. Infect. Immun. **66**:4397–4402.
10. **Fuller T, et al.** 2011. Using remote sensing to map the risk of human monkeypox virus in the congo basin. EcoHealth **8**:14–25.
11. **Giavedoni LD, et al.** 1992. Vaccinia virus recombinants expressing chimeric proteins of human immunodeficiency virus and gamma interferon are attenuated for nude mice. Proc. Natl. Acad. Sci. U. S. A. **89**:3409–3413.
12. **Guarner J, et al.** 2004. Monkeypox transmission and pathogenesis in prairie dogs. Emerg. Infect. Dis. **10**:426–431.
13. **Huang S, et al.** 1993. Immune response in mice that lack the interferon-gamma receptor. Science **259**:1742–1745.
14. **Hutson CL, et al.** 2010. Comparison of west African and Congo basin monkeypox viruses in BALB/c and C57BL/6 mice. PLoS One **5**:e8912. doi:10.1371/journal.pone.0008912.
15. **Hutson CL, et al.** 2009. A prairie dog animal model of systemic orthopoxvirus disease using West African and Congo basin strains of monkeypox virus. J. Gen. Virol. **90**:323–333.
16. **Johnson RF, et al.** 2011. Comparative analysis of monkeypox virus infection of cynomolgus macaques by the intravenous or intrabronchial inoculation route. J. Virol. **85**:2112–2125.
17. **Karem KL, et al.** 2007. Monkeypox-induced immunity and failure of childhood smallpox vaccination to provide complete protection. Clin. Vaccine Immunol. **14**:1318–1327.
18. **Karupiah G, Blanden RV, Ramshaw IA.** 1990. Interferon gamma is involved in the recovery of athymic nude mice from recombinant vaccinia virus/interleukin 2 infection. J. Exp. Med. **172**:1495–1503.
19. **Karupiah G, Fredrickson TN, Holmes KL, Khairallah LH, Buller RM.** 1993. Importance of interferons in recovery from mousepox. J. Virol. **67**:4214–4226.
20. **Keckler MS, et al.** 2011. Establishment of the black-tailed prairie dog (Cynomys ludovicianus) as a novel animal model for comparing smallpox vaccines administered preexposure in both high- and low-dose monkeypox virus challenges. J. Virol. **85**:7683–7698.
21. **Khodakevich L, Jezek Z, Kinzanzka K.** 1986. Isolation of monkeypox virus from wild squirrel infected in nature. Lancet **i**:98–99.
22. **Kohonen-Corish MR, King NJ, Woodhams CE, Ramshaw IA.** 1990. Immunodeficient mice recover from infection with vaccinia virus expressing interferon-gamma. Eur. J. Immunol. **20**:157–161.
23. **Langohr IM, Stevenson GW, Thacker HL, Regnery RL.** 2004. Extensive lesions of monkeypox in a prairie dog (Cynomys sp). Vet. Pathol. **41**:702–707.
24. **Learned LA, et al.** 2005. Extended interhuman transmission of monkeypox in a hospital community in the Republic of the Congo, 2003. Am. J. Trop. Med. Hyg. **73**:428–434.
25. **Likos AM, et al.** 2005. A tale of two clades: monkeypox viruses. J. Gen. Virol. **86**:2661–2672.
26. **Liu G, et al.** 2004. Prevention of lethal respiratory vaccinia infections in

mice with interferon-alpha and interferon-gamma. FEMS Immunol. Med. Microbiol. **40**:201–206.
27. **Marennikova SS, Seluhina EM.** 1976. Susceptibility of some rodent species to monkeypox virus, and course of the infection. Bull. World Health Organ. **53**:13–20.
28. **Mossman K, et al.** 1996. Myxoma virus M-T7, a secreted homolog of the interferon-gamma receptor, is a critical virulence factor for the development of myxomatosis in European rabbits. Virology **215**:17–30.
29. **Mossman K, Upton C, Buller RM, McFadden G.** 1995. Species specificity of ectromelia virus and vaccinia virus interferon-gamma binding proteins. Virology **208**:762–769.
30. **Osorio JE, Iams KP, Meteyer CU, Rocke TE.** 2009. Comparison of monkeypox viruses pathogenesis in mice by in vivo imaging. PLoS One **4**:e6592. doi:10.1371/journal.pone.0006592.
31. **Panchanathan V, Chaudhri G, Karupiah G.** 2005. Interferon function is not required for recovery from a secondary poxvirus infection. Proc. Natl. Acad. Sci. U. S. A. **102**:12921–12926.
32. **Panchanathan V, Chaudhri G, Karupiah G.** 2006. Protective immunity against secondary poxvirus infection is dependent on antibody but not on CD4 or CD8 T-cell function. J. Virol. **80**:6333–6338.
33. **Parker S, Nuara A, Buller RM, Schultz DA.** 2007. Human monkeypox: an emerging zoonotic disease. Future Microbiol. **2**:17–34.
34. **Puehler F, Weining KC, Symons JA, Smith GL, Staeheli P.** 1998. Vaccinia virus-encoded cytokine receptor binds and neutralizes chicken interferon-gamma. Virology **248**:231–240.
35. **Reed KD, et al.** 2004. The detection of monkeypox in humans in the Western Hemisphere. N. Engl. J. Med. **350**:342–350.
36. **Reynolds MG, et al.** 2010. A silent enzootic of an orthopoxvirus in Ghana, West Africa: evidence for multi-species involvement in the absence of widespread human disease. Am. J. Trop. Med. Hyg. **82**:746–754.
37. **Rimoin AW, et al.** 2010. Major increase in human monkeypox incidence 30 years after smallpox vaccination campaigns cease in the Democratic Republic of Congo. Proc. Natl. Acad. Sci. U. S. A. **107**:16262–16267.
38. **Sakala IG, et al.** 2007. Poxvirus-encoded gamma interferon binding protein dampens the host immune response to infection. J. Virol. **81**:3346–3353.
39. **Sbrana E, Xiao SY, Newman PC, Tesh RB.** 2007. Comparative pathology of North American and central African strains of monkeypox virus in a ground squirrel model of the disease. Am. J. Trop. Med. Hyg. **76**:155–164.
40. **Schultz DA, Sagartz JE, Huso DL, Buller RM.** 2009. Experimental infection of an African dormouse (Graphiurus kelleni) with monkeypox virus. Virology **383**:86–92.
41. **Smith VP, Alcami A.** 2002. Inhibition of interferons by ectromelia virus. J. Virol. **76**:1124–1134.
42. **Stabenow J, et al.** 2010. A mouse model of lethal infection for evaluating prophylactics and therapeutics against monkeypox virus. J. Virol. **84**:3909–3920.
43. **Symons JA, Tscharke DC, Price N, Smith GL.** 2002. A study of the vaccinia virus interferon-gamma receptor and its contribution to virus virulence. J. Gen. Virol. **83**:1953–1964.
44. **Tesh RB, et al.** 2004. Experimental infection of ground squirrels (Spermophilus tridecemlineatus) with monkeypox virus. Emerg. Infect. Dis. **10**:1563–1567.
45. **Verardi PH, Jones LA, Aziz FH, Ahmad S, Yilma TD.** 2001. Vaccinia virus vectors with an inactivated gamma interferon receptor homolog gene (B8R) are attenuated In vivo without a concomitant reduction in immunogenicity. J. Virol. **75**:11–18.
46. **von Magnus P, Andersen E, Petersen K-AA.** 1959. A pox-like disease in cynomolgous monkeys. Acta Pathol. Microbiol. Scand. **46**:156–176.
47. **Weaver JR, Isaacs SN.** 2008. Monkeypox virus and insights into its immunomodulatory proteins. Immunol. Rev. **225**:96–113.
48. **Xiao SY, et al.** 2005. Experimental infection of prairie dogs with monkeypox virus. Emerg. Infect. Dis. **11**:539–545.
49. **Yonekawa H, et al.** 1986. A hybrid origin of Japanese mice "Mus musculus molossinus." Curr. Top. Microbiol. Immunol. **127**:62–67.
50. **Zaucha GM, Jahrling PB, Geisbert TW, Swearengen JR, Hensley L.** 2001. The pathology of experimental aerosolized monkeypox virus infection in cynomolgus monkeys (Macaca fascicularis). Lab. Invest. **81**:1581–1600.