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Protection from Severe Influenza Virus Infections in Mice Carrying the *Mx1* Influenza Virus Resistance Gene Strongly Depends on Genetic Background

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ABSTRACT

Influenza virus infections represent a serious threat to human health. Both extrinsic and intrinsic factors determine the severity of influenza. The MX dynamin-like GTPase 1 (*Mx1*) gene has been shown to confer strong resistance to influenza A virus infections in mice. Most laboratory mouse strains, including C57BL/6J, carry nonsense or deletion mutations in *Mx1* and thus a nonfunctional allele, whereas wild-derived mouse strains carry a wild-type *Mx1* allele. Congenic C57BL/6J (B6-*Mx1*$^{r/r}$) mice expressing a wild-type allele from the A2G mouse strain are highly resistant to influenza A virus infections, to both mono- and polybasic subtypes. Furthermore, in genetic mapping studies, *Mx1* was identified as the major locus of resistance to influenza virus infections. Here, we investigated whether the *Mx1* protective function is influenced by the genetic background. For this, we generated a congenic mouse strain carrying the A2G wild-type *Mx1* resistance allele on a DBA/2J background (D2-*Mx1*$^{r/r}$). Most remarkably, congenic D2-*Mx1*$^{r/r}$ mice expressing a functional *Mx1* wild-type allele are still highly susceptible to H1N1 virus. However, pretreatment of D2-*Mx1*$^{r/r}$ mice with alpha interferon protected them from lethal infections. Our results showed, for the first time, that the presence of an *Mx1* wild-type allele from A2G as such does not fully protect mice from lethal influenza A virus infections. These observations are also highly relevant for susceptibility to influenza virus infections in humans.

IMPORTANCE

Influenza A virus represents a major health threat to humans. Seasonal influenza epidemics cause high economic loss, morbidity, and deaths each year. Genetic factors of the host strongly influence susceptibility and resistance to virus infections. The *Mx1* (MX dynamin-like GTPase 1) gene has been described as a major resistance gene in mice and humans. Most inbred laboratory mouse strains are deficient in *Mx1*, but congenic B6-*Mx1*$^{r/r}$ mice that carry the wild-type *Mx1* gene from the A2G mouse strain are highly resistant. Here, we show that, very unexpectedly, congenic D2-*Mx1*$^{r/r}$ mice carrying the wild-type *Mx1* gene from the A2G strain are not fully protected against lethal influenza virus infections. These observations demonstrate that the genetic background is very important for the protective function of the *Mx1* resistance gene. Our results are also highly relevant for understanding genetic susceptibility to influenza virus infections in humans.

Influenza A virus represents a major health threat to humans. Seasonal influenza epidemics cause high economic loss, morbidity, and deaths each year (1). Annually, about 500 million people are infected by the influenza A virus worldwide, of whom about 500,000 die (1). In recent history, the emergence of new influenza virus subtypes has caused severe pandemics (2–4), the most severe being the Spanish flu pandemic in 1918, which resulted in 30 to 50 million deaths worldwide (5). Furthermore, a new variant of the H1N1 virus, pH1N1, caused a worldwide pandemic in 2009 (6–12). Seasonal influenza A viruses are transmitted from human to human, but bird influenza A viruses may also directly infect humans who have been in close contact with infected birds. There are presently three virus subtypes, H5N1, H9N2, and H7N9, which are circulating in birds and which have the potential to infect humans. Infection with these subtypes may cause severe disease with lethal outcomes (13–17). There is some evidence from animal models that H7N9 virus may be able to spread by contact and air transmission (18, 19), making it a likely candidate for future pandemics in humans. Therefore, it is important to better understand the biological mechanisms that result in severe outcomes after influenza A virus infection.

The course and outcome of an influenza A virus infection are influenced by viral and host factors. Host risk factors, such as obesity or pregnancy, became evident during the 2009 swine flu pandemic (20, 21). Furthermore, genetic factors in humans associated with a higher susceptibility to influenza virus infections and severe disease outcome have been suspected for the 1918 pandemic, as well as H5N1 virus infections in patients (22–24). However, evidence for genetic predisposition in humans is circumstan-

Received 19 May 2015 **Accepted** 15 July 2015

Accepted manuscript posted online 22 July 2015

Citation Shin D-L, Hatesuer B, Bergmann S, Nedelko T, Schughart K. 2015. Protection from severe influenza virus infections in mice carrying the *Mx1* influenza virus resistance gene strongly depends on genetic background. J Virol 89:9998–10009. doi:10.1128/JVI.01305-15.

Editor: S. Perlman

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doi:10.1128/JVI.01305-15

tial (22–24), and the details of the biological mechanisms for health and genetic factors predisposing to severe influenza in humans remain largely unknown (22–27). Recently, the importance of *IFITM3* as a crucial factor for host susceptibility has been demonstrated in mice and humans (28).

The mouse is one of the most important mammalian model systems for studying host responses to influenza A virus and for assessing, for example, virus virulence, disease severity, genetic predisposition, immune responses, and vaccine efficacy (reference 29 and references therein). The importance of host factors for host susceptibility and resistance has been demonstrated clearly in animal models. We and others have shown in mouse models that susceptibility of the host to influenza A virus infection strongly depends on the genetic background (30–38).

Also in mice, the MX dynamin-like GTPase 1 (*Mx1*) gene has been identified as one of the most important influenza virus resistance genes (reviewed in references 39 to 41). *Mx1* acts as a cell-autonomous restriction factor against many viral pathogens. Expression of *Mx1* is induced by type I or type III interferons (42). Structure analysis of MX1 proteins revealed a globular G domain connected to a stalk region (43). The stalk is able to mediate self-assembly into a ring-like oligomer that is thought to interact directly with viral RNP particles and thereby block replication (43). The amino acid sequence in the L4 loop of the stalk determines specificity against different virus pathogens (44). It has been further suggested that additional cellular host factors may be involved in the antiviral activity of *Mx1* (45, 46).

The protective activity of *Mx1* against myxoviruses has been originally discovered in A2G mice that carry a wild-type allele (47). However, most laboratory mice are deficient for *Mx1* because of deletions or nonsense mutations (48, 49), whereas many wild-derived strains carry a functional *Mx1* allele (49, 50). The A2G allele of *Mx1* has subsequently been demonstrated to be highly protective against lethal influenza virus infections in various mouse models (51–55). Congenic C57BL/6J.A2G-*Mx1*$^{r/r}$ (B6-*Mx1*$^{r/r}$) mice survive infections with mouse-adapted H1N1 virus (56) and are also resistant to lethal infections with highly virulent polybasic H5N1 virus (54). Furthermore, SPRET/Ei mice, which carry another *Mx1* wild-type allele, are strongly protected against influenza virus infections (57). A genetic mapping study in a backcross of (C57BL/6 × SPRET/Ei)F1 × C57BL/6 mice identified *Mx1* as the major resistance locus (57). Furthermore, the founder strains of the Collaborative Cross recombinant inbred population (58) carry five different haplotypes in the *Mx1* genomic region, two of which (PWK/PhJ and NZO/HILtJ) were highly protective against influenza virus infections (49). A/J, C57BL/6J, 129S1/SvImJ, and NOD/ShiLtJ carry a deletion or stop codon in the *Mx1* gene and were highly susceptible (49). A third wild-derived allele was found in CAST/EiJ mice, exhibiting one amino acid difference from the presumed ancestral PWK/PhJ allele. It was expressed after influenza A virus infection but did not protect CAST/EiJ mice from a lethal infection (49). It is yet unclear whether genetic background or the specific *Mx1* allele in CAST/EiJ mice is responsible for the susceptible phenotype. In a mapping study using pre-Collaborative Cross mice, *Mx1* was found as the strongest resistance quantitative trait locus (QTL), explaining 42% of the variation in body weight loss in this population (49).

We showed previously that in the absence of *Mx1*, C57BL/6J (B6-*Mx1*$^{-/-}$) mice survive infections with a less virulent strain of a mouse-adapted H1N1 (PR8M) virus, whereas DBA/2J (D2-*Mx1*$^{-/-}$) mice were highly susceptible (30). On the other hand, *Mx1*-deficient (B6-*Mx1*$^{-/-}$) mice were highly susceptible to the more virulent mouse-adapted H1N1 (PR8) virus (55, 59). However, in the presence of the *Mx1* allele from A2G mice, congenic B6-*Mx1*$^{r/r}$ mice were strongly protected against infections with this virus (55).

To further investigate the role of *Mx1* in different genetic backgrounds, we generated a congenic D2(B6).A2G-*Mx1*$^{r/r}$ (D2-*Mx1*$^{r/r}$) mouse line carrying the wild-type *Mx1* allele from A2G and challenged these mice with H1N1 (PR8) virus. Most surprisingly, we found that D2-*Mx1*$^{r/r}$ mice were highly susceptible to PR8 infections even in the presence of the wild-type A2G *Mx1* allele.

(Part of this work has been performed as Ph.D. thesis work [D.-L.S.] at the University of Veterinary Medicine, Hannover, Germany.)

MATERIALS AND METHODS

Ethics statement. All experiments in mice were approved by an external committee according to the national guidelines of the animal welfare law in Germany (BGBl. I S. 1206 and 1313 and BGBl. I S. 1934). The protocol used in these experiments has been reviewed by an ethics committee and approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany (permit numbers 33.9.42502-04-051/09 and 3392 42502-04-13/1234). No approval was necessary for work with 10-day-old embryonated chicken eggs. Laboratory C57BL/6J (B6-*Mx1*$^{-/-}$) and DBA/2J (D2-*Mx1*$^{-/-}$) mice carrying mutant *Mx1* alleles were purchased from Janvier, France. Mice were maintained under specific-pathogen-free conditions at the animal facilities of the Helmholtz Centre for Infection Research (HZI). Embryonated chicken eggs were purchased from Charles River Laboratories, Germany.

Virus. Original stocks of mouse-adapted PR8 virus were obtained from Peter Stäheli, University of Freiburg (PR8, A/PuertoRico/8/34 H1N1, Freiburg variant [59, 60]). Mouse-adapted H3N2 virus (A/Hong Kong/1/68 H3N2) was obtained from Otto Haller, University of Freiburg. All viruses were propagated in the chorioallantoic cavity of 10-day-old pathogen-free embryonated chicken eggs, aliquoted, and stored at −80°C.

Mice. Congenic B6.A2G-*Mx1*$^{r/r}$ (B6-*Mx1*$^{r/r}$) mice carrying a functional A2G *Mx1* allele were provided by Peter Stäheli, University of Freiburg, Germany. Congenic D2(B6).A2G-*Mx1*$^{r/r}$ (D2-*Mx1*$^{r/r}$ mice) carrying a wild-type *Mx1* allele were generated in our laboratory by backcrossing D2-*Mx1*$^{-/-}$ mice for 10 generations onto B6-*Mx1*$^{r/r}$ mice. In each generation, the presence of the *Mx1* wild-type-containing region on chromosome 16 was confirmed by PCR genotyping.

Genotyping of mice. For genotyping, genomic DNA was extracted from mouse tails with the DNeasy blood and tissue kit according to the manufacturer's instructions (Qiagen). DNA concentration was quantified with a spectrophotometer (NanoDrop 1000; Thermo Scientific). A total of 100 ng DNA and 10 pmol primer oligonucleotides were used for PCR with LightCycler 480 Probes Master (Roche) according to the manufacturer's instructions. For the PCR genotyping, polymerase was activated at 95°C for 10 min, followed by 40 cycles of a denaturation step at 94°C for 1 min, primer annealing at 61°C for 1 min, and an elongation reaction at 72°C for 1 min. A three-primer PCR strategy was used for *Mx1* allele genotyping (Peter Stäheli, personal communication). Primers were designed for sequences flanking the *Mx1* locus (exon8 forward, e8fn, 5′-GGAGCTCACCTCCCACATCT-3′; exon8 reverse, e8r, 5′-AGCATGGCTGTGTCACAAGCA-3′; exon12 reverse, e12r, 5′-CGAAGGCAGTTTGGACCATCT-3′). Mice carrying a wild-type *Mx1* gene yielded a 950-bp product, whereas mutant *Mx1* alleles were detected by the presence of a 1,255-bp product. The correct background in congenic mice after backcrossing was verified by the Mouse Universal Genotyping Array (MUGA). Array processing was performed by Neogen. The analysis demonstrated that 99.02% of the single nucleotide polymorphisms (SNPs) in D2-*Mx1*$^{r/r}$

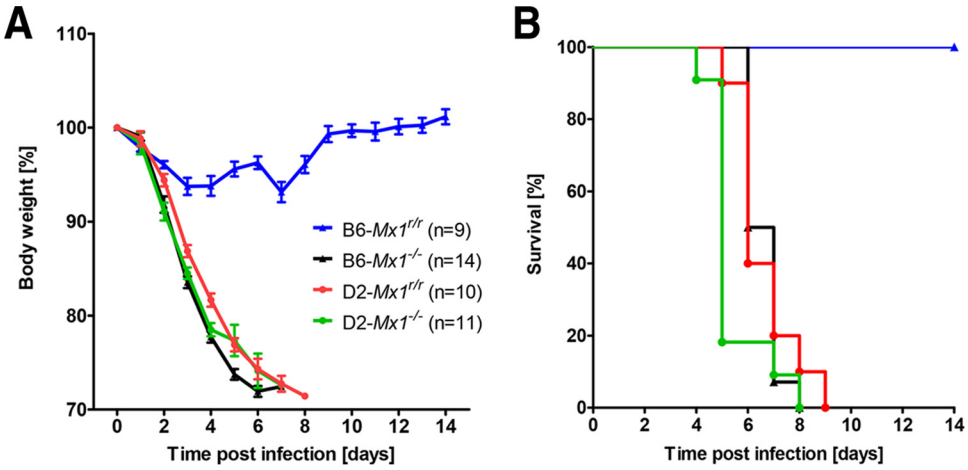

FIG 1 D2-*Mx1*$^{r/r}$ mice were highly susceptible to H1N1 virus (PR8) infections whereas B6-*Mx1*$^{r/r}$ mice were resistant. Eight- to 12-week-old female mice (D2-*Mx1*$^{r/r}$, B6-*Mx1*$^{r/r}$, D2-*Mx1*$^{-/-}$, and B6-*Mx1*$^{-/-}$) were infected intranasally with 2×10^3 FFU of PR8 (H1N1) influenza A virus. Body weight loss (A) and survival rates (B) were monitored over a period of 14 days. Mice that lost 30% or more of the starting body weight were sacrificed and recorded as dead. Data represent mean percentages of body weight change (± SEM) compared to starting body weight (100%). Differences in body weight loss were significant between D2-*Mx1*$^{r/r}$ and B6-*Mx1*$^{r/r}$ mice after day 3 p.i. ($P < 0.0001$, nonparametric Mann-Whitney test). Survival rates were significantly different between D2-*Mx1*$^{r/r}$ and B6-*Mx1*$^{r/r}$ mice ($P < 0.0001$, log rank Mantel-Cox test). *n*, number of mice per group.

mice matched the DBA/2J genotype and carried a 32.73-Mb region from the B6-*Mx1*$^{r/r}$ mice on chromosome 16, which includes 1.5 Mb of the original A2G region.

Infection of mice. Female mice at the age of 8 to 12 weeks were anesthetized by intraperitoneal injection of ketamine-xylazine solution in sterile NaCl (100 mg/ml ketamine [WDT, Garbsen, Germany]; 20 mg/ml Xylavet [CP-Pharma, Burgdorf, Germany]) with a dose adjusted to the individual body weight (200 μl/20 g body weight). Infection was performed by intranasal application of virus solution in 20 μl sterile phosphate-buffered saline (PBS). Subsequently, survival and body weight loss were monitored until day 14 postinfection (p.i.). In addition to mice that were found dead, mice with a weight loss of more than 30% of the starting body weight were euthanized and recorded as dead.

RT-PCR for *Mx1* transcript analysis. Reverse transcription-PCR (RT-PCR) was performed to confirm wild-type *Mx1* expression in D2-*Mx1*$^{r/r}$ mice. Mice were anesthetized and infected intranasally with 2×10^3 focus-forming units (FFU) of PR8 in 20 μl PBS. Lungs were prepared, washed in PBS, and stored in 2 ml RNAlater (Qiagen). Subsequently, lungs were homogenized using a PolyTron 2100 homogenizer. Total RNA was prepared using TRIzol chloroform according to the manufacturer's instructions (Invitrogen). One microgram of total RNA was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen, USA) according to the manufacturer's instructions. Five microliters of cDNA product was amplified with specific primers (e8fn and e12r) to determine expression of the *Mx1* wild-type allele. Only D2-*Mx1*$^{r/r}$ but not D2-*Mx1*$^{-/-}$ mice yielded a product of 467 bp that is generated from expression of the *Mx1* wild-type allele. For quantitative RT-PCR, after alpha interferon (IFN-α) treatment or influenza virus infection, RNA was reverse transcribed using SuperScript III reverse transcriptase. Subsequently, the quantitative PCR was performed in a LightCycler 480 real-time PCR system (Roche) using the SsoFast EvaGreen Supermix kit (Bio-Rad) according to the manufacturer's instructions. Primers for the *Mx1* transcript were designed to cross an intron-exon boundary (Mx1EE-F, 5′-CCTGGAGGAGCAGAGTGACAC-3′; Mx1EE-R, 5′-GGTTAATCGGAG AATTTGGCAA-3′). Primers for the *Ifnb1* transcript were modified from a previous study by Takaki et al. (61): Ifnb1-F (5′-CCAGCTCCAAGAAA GGACGA-3′) and Ifnb1-R (5′-CGCCCTGTAGGTGAGGTTGAT-3′). Primers for β-actin were used as the housekeeping gene control (Bact2-F, 5′-AGGTGACAGCATTGCTTCTG-3′; Bact2-R, 5′-GCTGCCTCAACA CCTCAAC-3′). The specificity of the PCR amplification was assessed by the melting curve at the end of the reaction. Relative expression levels of *Mx1* and *Ifnb1* were calculated by the threshold cycle ($2^{-\Delta\Delta CT}$) method (62) and calculated as fold change induction compared to PBS-treated controls.

Determination of infectious viral particles. For determining viral load in lungs, lungs were prepared and put into 2 ml PBS containing 0.1% bovine serum albumin (BSA). Lung tissue was subsequently homogenized using the PolyTron 2100 homogenizer. Debris was removed by centrifugation, and aliquots were stored at −70°C. Virus titers were determined on MDCK II (Madin-Darby canine kidney II) cells as focus-forming units (FFU) as described previously (59). Briefly, MDCK II cells were seeded in 96-well plates and serial 10-fold dilutions of homogenized lung samples in Dulbecco's modified Eagle's medium (DMEM) containing 5 μg/ml *N*-acetylated trypsin (NAT; Sigma) were added. After incubation for 24 h at 37°C, cells were washed, fixed with 4% formalin, and permeabilized with quencher buffer (0.5% Triton X-100 with 20 mM glycine in PBS), followed by incubation with a primary anti-influenza virus polyclonal antibody (Virostat) and a secondary horseradish peroxidase (HRP) antibody (KPL). Subsequently, a substrate (True Blue; KPL) was used for immunological staining. Foci were counted and calculated as FFU per lung homogenate. The detection limit of the assay was 80 infectious particles/lung. Thus, for samples where no foci were detected, data points were set to 80 FFU/lung.

Cytokine and chemokine analysis in BAL fluid. Female B6-*Mx1*$^{r/r}$ and D2-*Mx1*$^{r/r}$ mice (five in each group and time point) at the age of 10 to 12 weeks were infected with 2×10^3 FFU of PR8. Control mice were mock infected with PBS. After 3 and 5 days p.i., mice were euthanized by isoflurane. A sterile 22-gauge catheter was inserted into the exposed trachea lumen. By instillation of PBS, a volume of 0.5 ml bronchoalveolar lavage (BAL) fluid per mouse was collected. BAL fluid was stored at −70°C until measurement. Chemokine and cytokine levels of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), gamma interferon (IFN-γ), interleukin-1α (IL-1α), IL-6, IL-10, IL-17, IP-10, KC, monocyte chemoattractant protein 1 (MCP-1), MIP-1a, RANTES, tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF) were analyzed using the mouse cytokine/chemokine magnetic bead panel Mcytomag-70K from Millipore according to the instruction manual of the manufacturer. Plates were read in the Luminex 100 apparatus.

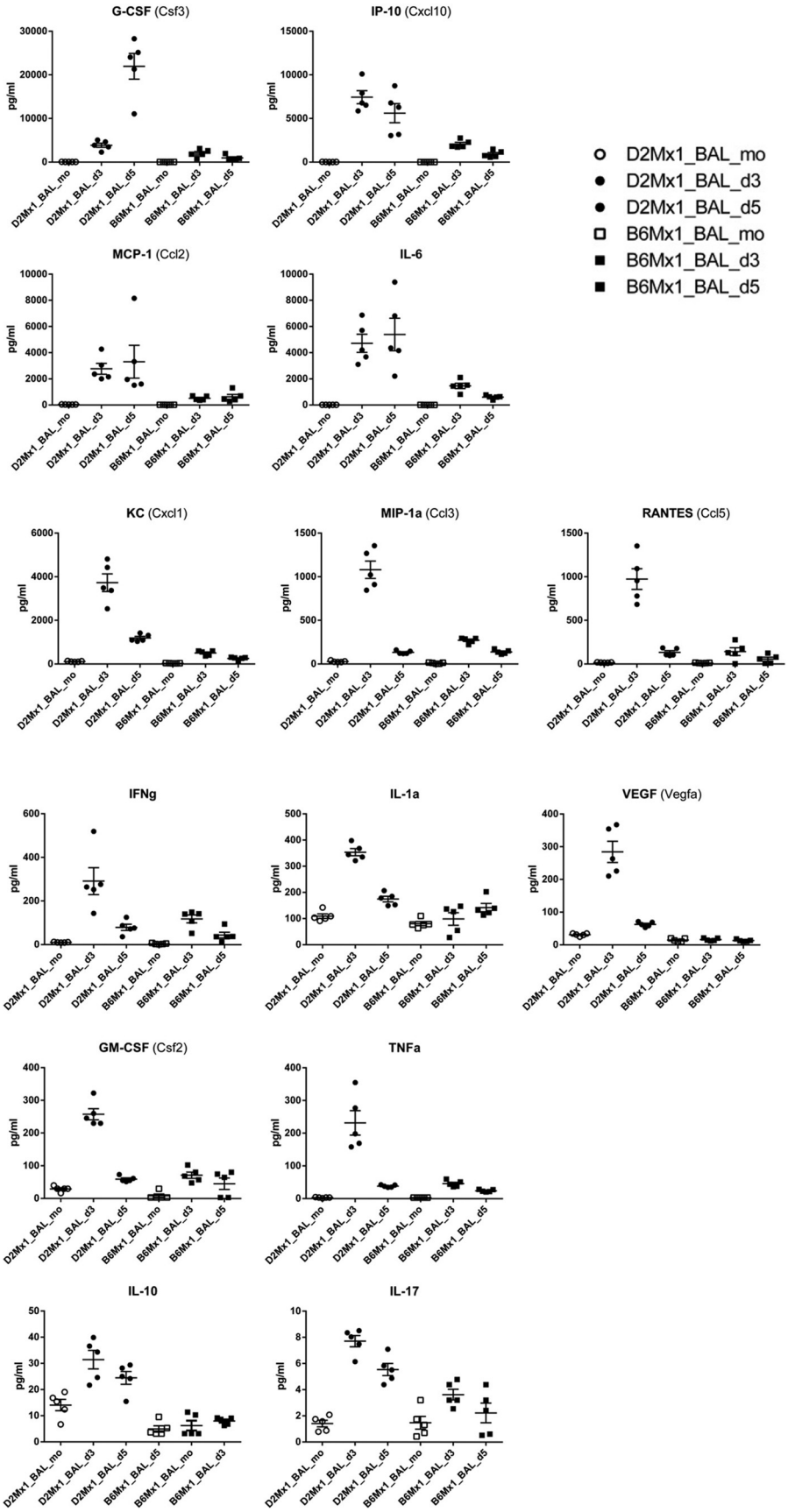

FIG 2 Chemokine and cytokine levels in BAL fluid of D2-*Mx1*$^{r/r}$ mice exhibit stronger inflammatory responses than do those of B6-*Mx1*$^{-/-}$ mice. Female D2-*Mx1*$^{r/r}$ (circles) and B6-*Mx1*$^{r/r}$ (squares) mice were infected with 2×10^3 FFU of PR8F intranasally. Bronchoalveolar lavage (BAL) fluid was collected from mock-infected control mice at day 3 posttreatment and from infected mice at day 3 and day 5 p.i. The concentration of chemokines and cytokines was determined by using the mouse cytokine/chemokine magnetic bead panel (Mcytomag-70K) from Millipore. At each time point, five biological replicates were analyzed.

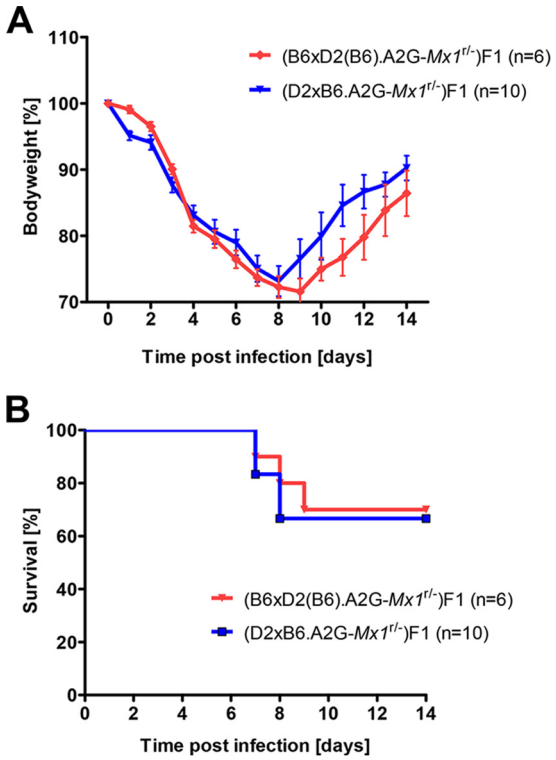

FIG 3 Confirmation of functional wild-type *Mx1* in D2-*Mx1*$^{r/r}$ mice by outcrossing to B6-*Mx1*$^{-/-}$ mice. Congenic D2-*Mx1*$^{r/r}$ mice were outcrossed to B6-*Mx1*$^{-/-}$ mice, and the phenotypes of the resulting F1 mice [(B6 × D2(B6).A2G-*Mx1*$^{r/-}$)F1 or reciprocal crosses] were compared to the phenotype of F1 mice derived from an outcross of B6-*Mx1*$^{r/r}$ to D2-*Mx1*$^{-/-}$ [(D2 × B6.A2G-*Mx1*$^{r/-}$)F1 or reciprocal cross]. F1 mice from both crosses did not show significant differences in body weight loss (A) or survival (B) (Mann-Whitney U test for body weight change analysis and log rank Mantel-Cox test for survival curves). *n*, number of mice per group.

Interferon pretreatment. One day prior to influenza virus infection, mice were anesthetized and treated with 50,000 IU of recombinant human alpha interferon B/D (type I interferon [IFN-α], provided by Peter Stäheli, University of Freiburg) in 20 μl of sterile phosphate-buffered saline by intranasal application. The control group received 20 μl of sterile PBS.

Statistical analysis. Data and statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, California). Results were presented as means ± standard errors of the means (SEM) for body weight change and virus titers. Statistical significance between groups was determined using the Mann-Whitney U test for body weight and virus titers. The log rank test was used to determine significant differences between survival curves. For analysis of BAL fluid proteome data, the Kruskal-Wallis test was used.

RESULTS

D2-*Mx1*$^{r/r}$ mice are not resistant to lethal H1N1 influenza A virus infections. The A2G wild-type allele of *Mx1* was shown to confer high resistance against many influenza virus subtypes in mice with an A2G, C57BL/6J, or BALB/c genetic background. On the other hand, we found that DBA/2J mice lacking *Mx1* were highly susceptible compared to C57BL/6J mice lacking *Mx1*. Therefore, we wanted to investigate if the wild-type *Mx1* allele was also able to protect highly susceptible DBA/2J mice from lethal infection. For this, we generated a congenic DBA/2J(B6).A2G-*Mx1*$^{r/r}$ (D2-*Mx1*$^{r/r}$) mouse strain by backcrossing DBA/2J mice for 10 generations with congenic C57BL/6J.A2G-*Mx1*$^{r/r}$ (B6-*Mx1*$^{r/r}$) mice (received from Peter Stäheli, Freiburg, Germany) that carried the A2G *Mx1* wild-type allele. By SNP genotyping (data not shown), we confirmed that the congenic D2-*Mx1*$^{r/r}$ strain carried a 32.73-Mb region from the B6-*Mx1*$^{r/r}$ strain on chromosome 16 which includes 1.5 Mb of the original A2G region. Furthermore, the presence of the wild-type allele was confirmed by diagnostic PCR (data not shown). Also, congenic D2-*Mx1*$^{r/r}$ mice expressed the *Mx1* wild-type allele after infection with H1N1 (PR8) by RT-PCR (data not shown).

We then infected D2-*Mx1*$^{r/r}$ and B6-*Mx1*$^{r/r}$ mice as well as D2-*Mx1*$^{-/-}$ and B6-*Mx1*$^{-/-}$ mice with A/PuertoRico/8/34 (H1N1) virus (59, 60). As described before, B6-*Mx1*$^{-/-}$ and D2-*Mx1*$^{-/-}$ mice were highly susceptible to these infections. They rapidly lost body weight and died between days 4 and 8 p.i. (Fig. 1). On the other hand, B6-*Mx1*$^{r/r}$ mice exhibited only slight body weight losses and survived the infection, confirming previous observations (Fig. 1). Most surprisingly, infected D2-*Mx1*$^{r/r}$ mice were not protected from lethal infections. They showed severe clinical symptoms and lost body weight similarly to *Mx1*-deficient DBA/2J mice, and all infected D2-*Mx1*$^{r/r}$ mice were dead at day 9 p.i. (Fig. 1). Furthermore, D2-*Mx1*$^{r/r}$ mice produced high levels of chemokines and cytokines in their lungs (Fig. 2), indicating strong inflammatory responses which are associated with high levels of virus replication and a severe course of infection.

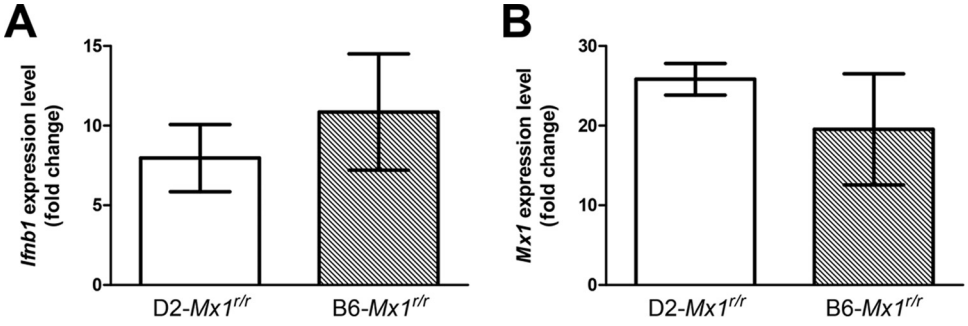

FIG 4 Upregulation of *Ifnb1* and *Mx1* in congenic D2-*Mx1*$^{r/r}$ mice after influenza A virus infection. Eight- to 12-week-old female D2-*Mx1*$^{r/r}$ and B6-*Mx1*$^{r/r}$ mice were inoculated with 2×10^3 FFU of PR8 H1N1 virus or PBS intranasally. On day 3 postinoculation, lung homogenates were prepared and levels of *Ifnb1* (A) and *Mx1* (B) mRNA expression were measured by quantitative RT-PCR and compared to PBS-treated controls. Infection with PR8 H1N1 virus induced comparable fold changes of *Ifnb1* and *Mx1* expression levels in both D2-*Mx1*$^{r/r}$ and B6-*Mx1*$^{r/r}$ mice ($P = 0.5303$ for *Ifnb1* and $P = 0.4346$ for *Mx1*, two-tailed Student's *t* test, $n = 3$).

TABLE 1 Survival after H1N1 virus infections is influenced by wild-type *Mx1* copy number and genetic background

Mouse strain	Genetic background	*Mx1* allele	No. of killed mice/no. of infected mice	Survival proportion (%)
B6-*Mx1*$^{r/r}$	B6	*r/r*	0/9	100
B6-*Mx1*$^{r/-}$	B6	*r/−*	3/14	78.57
B6-*Mx1*$^{-/-}$	B6	−/−	14/14	0
D2-*Mx1*$^{r/r}$	D2	*r/r*	10/10	0
D2-*Mx1*$^{r/-}$	D2	*r/−*	7/7	0
D2-*Mx1*$^{-/-}$	D2	−/−	11/11	0
F1(B6 × D2)-*Mx1*$^{r/r}$	B6 × D2	*r/r*	1/14	92.83
F1(B6 × D2)-*Mx1*$^{r/-}$	B6 × D2	*r/−*	5/16	68.75
B6 × F1(B6 × D2)-*Mx1*$^{r/-}$	B6 × F1(B6 × D2)[a]	*r/−*	2/11	81.82
D2 × F1(B6 × D2)-*Mx1*$^{r/-}$	D2 × F1(B6 × D2)[a]	*r/−*	5/8	37.50

[a] Secondary outcross performed by outcrossing F1(B6 × D2)-*Mx1*$^{r/r}$ with B6-*Mx1*$^{-/-}$ or D2-*Mx1*$^{-/-}$.

To confirm that congenic D2-*Mx1*$^{r/r}$ mice carried a functional *Mx1* allele, we outcrossed them to B6-*Mx1*$^{-/-}$ mice and compared the phenotype of the resulting F1 mice with the phenotype of F1 mice derived from an outcross of B6-*Mx1*$^{r/r}$ to D2-*Mx1*$^{-/-}$. Thus, in the first case, the *Mx1* wild-type allele is inherited from the congenic D2-*Mx1*$^{r/r}$ mice, whereas in the second case, the wild-type allele is derived from the original B6-*Mx1*$^{r/r}$ congenic strain. After infection with PR8, F1 mice from both crosses showed increased survival compared to D2-*Mx1*$^{-/-}$ mice (Fig. 3; differences not significant). These observations further demonstrated that the A2G *Mx1* allele in D2-*Mx1*$^{r/r}$ mice is fully functional. D2-*Mx1*$^{r/r}$ mice showed no difference in upregulation of *Mx1* and *Ifnb1* genes after infection compared to B6-*Mx1*$^{r/r}$ mice, demonstrating that the interferon pathway and activation of downstream genes are not affected in D2-*Mx1*$^{r/r}$ mice (Fig. 4).

The protective effect of *Mx1* on survival and virus replication is influenced by copy number and genetic background. We then compared systematically the effect of *Mx1*$^{r/r}$ copy number and combinations of DBA/2J and C57BL/6J background on survival after PR8 infections (Table 1; Table 2 shows pairwise significance comparisons). The presence of only one instead of two copies of *Mx1* increased mortality rates in C57BL/6J mice to 21.5%, and mice died between days 10 and 12 (B6-*Mx1*$^{r/r}$ versus B6-*Mx1*$^{r/-}$, Table 1 and Fig. 5; differences not significant). The increase in mortality was also observed for mice with a hybrid B6 × D2 genetic background [F1(B6 × D2)-*Mx1*$^{r/r}$ versus F1(B6 × D2)-*Mx1*$^{r/-}$, Table 1 and Fig. 5; differences not significant). Mice that were homozygous for the mutant *Mx1* allele were most susceptible and succumbed to the infection, independent of their genetic background (D2-*Mx1*$^{-/-}$ and B6-*Mx1*$^{-/-}$; Table 1 and Fig. 1, differences significant). Furthermore, the hybrid B6 × D2 genetic background decreased survival in the presence of either one or two wild-type *Mx1* alleles compared to a pure C57BL/6J background [F1(B6 × D2)-*Mx1*$^{r/-}$ versus B6-*Mx1*$^{r/-}$, differences not significant, and F1(B6 × D2)-*Mx1*$^{r/r}$ versus B6-*Mx1*$^{r/r}$, differences significant; Table 1 and Fig. 1 and 5]. Mice with a pure DBA/2J background did not survive infection in the presence or absence of *Mx1* (D2-*Mx1*$^{r/r}$ and D2-*Mx1*$^{-/-}$, Table 1 and Fig. 1 and 5, differences not significant).

Next, we compared virus replication in the lungs of D2-*Mx1*$^{r/r}$ mice, B6-*Mx1*$^{r/r}$ mice, F1 mice expressing one copy of the wild-type *Mx1* allele, and D2-*Mx1*$^{-/-}$ and B6-*Mx1*$^{-/-}$ mice carrying a mutant allele. After infection with PR8 virus, DBA/2J mice (with or without a functional *Mx1* allele) exhibited very high levels of viral load in infected lungs at day 1 p.i. (Fig. 6B and D), whereas infected B6-*Mx1*$^{r/r}$ mice rapidly reduced viral titers in lungs at day 3 p.i. (Fig. 6A). Most interestingly, F1(B6 × D2)-*Mx1*$^{r/-}$ mice initially exhibited high viral loads in infected lungs but reduced viral titers in their lungs at day 3 p.i., which further decreased until day 5 p.i. (Fig. 6E). Thus, the *Mx1* restrictive function on viral replication requires a hybrid or pure C57BL/6J background.

D2-*Mx1*$^{r/r}$ mice are partially resistant to low-dose infection with H1N1 and to infections with H3N2 influenza A virus. In addition, we studied pathology in D2-*Mx1*$^{r/r}$ mice after infection with a low dose of H1N1 virus (10 FFU). In this case, D2-*Mx1*$^{r/r}$ showed significantly less body weight loss and almost all infected mice survived compared to D2-*Mx1*$^{-/-}$ infected mice that rapidly lost body weight and all died (Fig. 7A). Also, D2-*Mx1*$^{r/r}$ mice were partially protected against infections with the H3N2 virus subtype, which was evident by a shift in the survival curve in D2-*Mx1*$^{r/r}$ compared to D2-*Mx1*$^{-/-}$ mice (Fig. 7B). However, overall survival rates were much lower than those of B6-*Mx1*$^{r/r}$ mice, which all survived an infection with H3N2 (Fig. 7B). Of note, F1(B6 × D2)-*Mx1*$^{r/-}$ mice with a hybrid C57BL/6J genetic background were also fully protected against mortality from H3N2 infections (Fig. 7B).

TABLE 2 Pairwise comparison of survival rates (log rank test)

Mouse strain	Significance for mouse strain: B6-*Mx1*$^{r/r}$	B6-*Mx1*$^{r/-}$	B6-*Mx1*$^{-/-}$	D2-*Mx1*$^{r/r}$	D2-*Mx1*$^{r/-}$	D2-*Mx1*$^{-/-}$	F1(B6 × D2)-*Mx1*$^{r/r}$	F1(B6 × D2)-*Mx1*$^{r/-}$	B6 × F1(B6 × D2)-*Mx1*$^{r/-}$
B6-*Mx1*$^{r/r}$									
B6-*Mx1*$^{r/-}$	0.1476								
B6-*Mx1*$^{-/-}$	<0.0001	<0.0001							
D2-*Mx1*$^{r/r}$	<0.0001	<0.0001	0.8207						
D2-*Mx1*$^{r/-}$	<0.0001	<0.0001	0.0043	0.0288					
D2-*Mx1*$^{-/-}$	<0.0001	<0.0001	0.0135	0.0357	0.9404				
F1(B6 × D2)-*Mx1*$^{r/r}$	0.4227	0.3207	<0.0001	<0.0001	<0.0001	<0.0001			
F1(B6 × D2)-*Mx1*$^{r/-}$	0.0706	0.4231	<0.0001	<0.0001	<0.0001	<0.0001	0.1147		
B6 × F1(B6 × D2)-*Mx1*$^{r/-}$	0.1900	0.9504	<0.0001	<0.0001	<0.0001	<0.0001	0.4187	0.4735	
D2 × F1(B6 × D2)-*Mx1*$^{r/-}$	0.0056	0.0162	0.0003	0.0040	0.0002	0.0003	0.0060	0.1695	0.0645

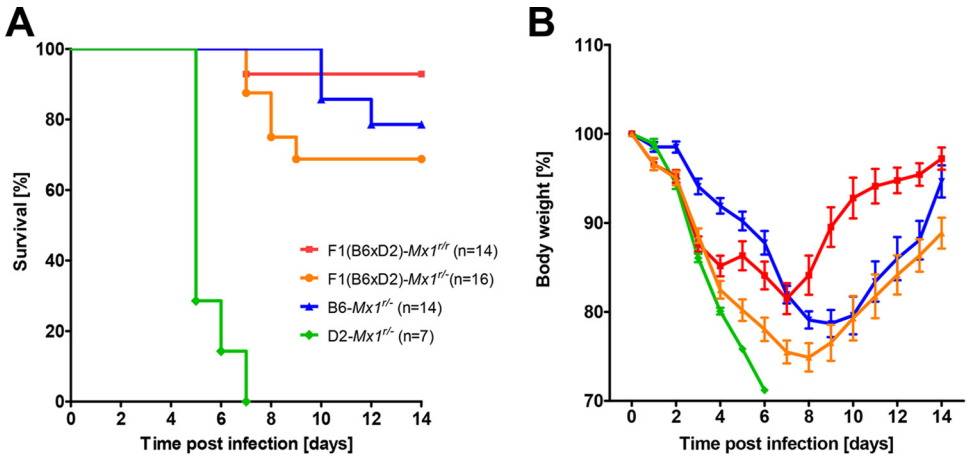

FIG 5 Resistance to lethal H1N1 virus infections is controlled by *Mx1* copy number and genetic background. F1 mice of different *Mx1* allele combinations and different C57BL/6J and DBA/2J background combinations were tested for susceptibility to PR8 H1N1 virus. Groups of 8- to 12-week-old female mice [F1(B6 × D2)-*Mx1*$^{r/r}$, F1(B6 × D2)-*Mx1*$^{r/-}$, B6-*Mx1*$^{r/-}$, and D2-*Mx1*$^{r/-}$] were infected intranasally with 2×10^3 FFU of PR8 virus, and survival was monitored until day 14 p.i. Mice that lost 30% or more of the starting body weight were sacrificed and recorded as dead. For the F1(B6 × D2)-*Mx1*$^{r/-}$ group, data from reciprocal crosses [(B6 × D2(B6).A2G-*Mx1*$^{r/-}$)F1 ($n = 6$) and (D2 × B6.A2G-*Mx1*$^{r/-}$)F1 ($n = 10$)] were combined. Two copies of the wild-type *Mx1* locus increased resistance compared to that with one copy. Introduction of the DBA/2J background in hybrid F1(B6 × D2)-*Mx1*$^{r/r}$ mice increased susceptibility, and the pure DBA/2J background in *Mx1*$^{r/r}$ mice increased susceptibility further.

D2-*Mx1*$^{r/r}$ mice are resistant to H1N1 influenza A virus after interferon pretreatment. *Mx1* is one of the main interferon response genes and can be induced by exogenous treatment with interferon (55). Therefore, we investigated the pathology in D2-*Mx1*$^{r/r}$ mice after pretreatment with IFN-α 1 day before infection. We first confirmed that IFN-α pretreatment induces *Mx1* expression in both D2-*Mx1*$^{r/r}$ and B6-*Mx1*$^{r/r}$ mice (Fig. 8). Most remarkable, all pretreated D2-*Mx1*$^{r/r}$ mice survived an infection with PR8 virus, whereas all PBS mock-treated mice lost body weight and died (Fig. 9A). Furthermore, D2-*Mx1*$^{r/r}$ mice pretreated with IFN-α exhibited lower viral loads than did mock-treated animals at day 1 p.i. (Fig. 9B). In immunohistochemical staining, we did not observe viral antigen at the day 3 p.i. point in IFN-α-pretreated mice compared to a wide spread of virus in D2-*Mx1*$^{r/r}$ mice that were

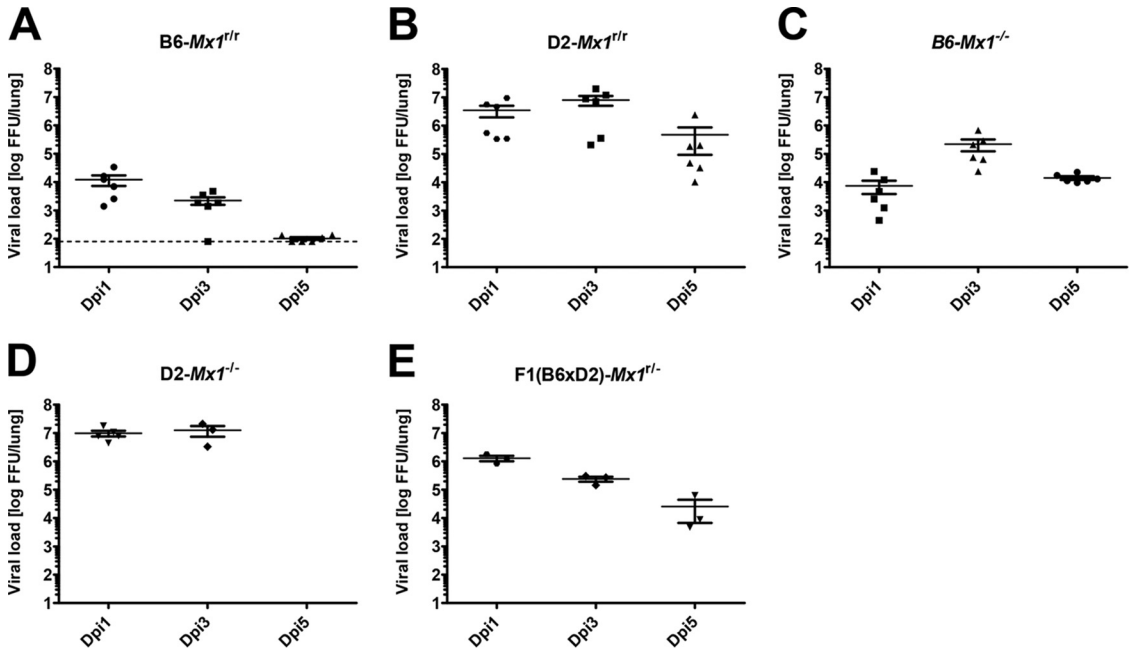

FIG 6 Restriction of virus replication is determined by the presence of a functional *Mx1* allele and genetic background. Eight- to 12-week-old female B6-*Mx1*$^{r/r}$ (A), D2-*Mx1*$^{r/r}$ (B), B6-*Mx1*$^{-/-}$ (C), D2-*Mx1*$^{-/-}$ (D), and F1(B6 × D2)-*Mx1*$^{r/-}$ (E) mice were infected intranasally with 2×10^3 FFU of PR8 virus. Infectious virus particles in lung homogenates were determined by focus-forming assay at days 1, 3, and 5 p.i. Viral loads on day 1 p.i. were significantly different between infected mice that carried a DBA/2J genetic background and those that carried a C57BL/6J genetic background (B6-*Mx1*$^{r/r}$ compared to D2-*Mx1*$^{r/r}$ using Mann-Whitney test, $P = 0.0022$), and only mice carrying both a functional *Mx1* allele and a C57BL/6J genetic background reduced viral loads from day 1 to day 3 p.i. D2-*Mx1*$^{r/r}$, B6-*Mx1*$^{r/r}$, and B6-*Mx1*$^{-/-}$ mice, $n = 6$ per time point; D2-*Mx1*$^{-/-}$ mice, $n = 5$ per time point; F1(B6 × D2)-*Mx1*$^{r/-}$ mice, $n = 3$ per time point.

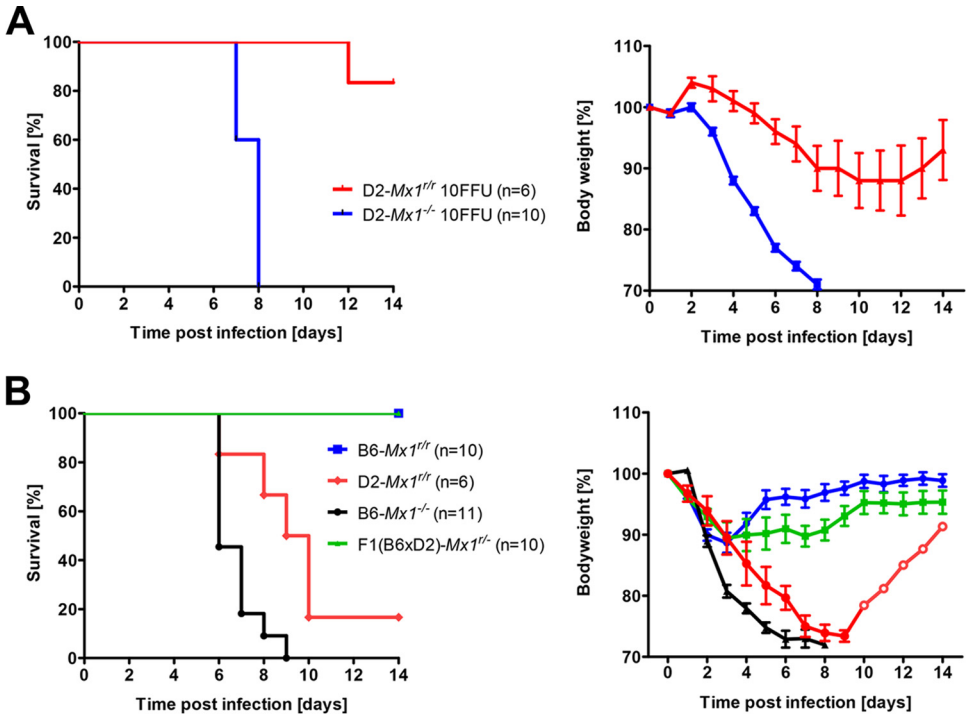

FIG 7 D2-*Mx1*$^{r/r}$ mice were protected against infections with low-dose H1N1 (PR8) virus and partially protected against infections with H3N2 virus. Eight- to 12-week-old female mice [B6-*Mx1*$^{r/r}$, D2-*Mx1*$^{r/r}$, F1(B6 × D2)-*Mx1*$^{r/-}$, B6-*Mx1*$^{-/-}$, and D2-*Mx1*$^{-/-}$] were infected intranasally with 10 FFU of PR8 H1N1 virus (A) or with 2×10^3 FFU of H3N2 virus (B). Survival rates were monitored over a period of 14 days p.i. Mice that lost 30% or more of the starting body weight were sacrificed and recorded as dead. Almost all infected D2-*Mx1*$^{r/r}$ mice survived infection with low-dose PR8 virus. D2-*Mx1*$^{r/r}$ mice were partially protected against H3N2 infections compared to B6-*Mx1*$^{-/-}$ (log rank Mantel-Cox test, $P = 0.0065$). Also, all F1(B6 × D2)-*Mx1*$^{r/-}$ mice with a hybrid C57BL/6J genetic background survived the H3N2 infections.

pretreated with PBS (data not shown). When D2-*Mx1*$^{-/-}$ or B6-*Mx1*$^{-/-}$ mice were pretreated with IFN-α prior to infection, all infected mice died and no difference from mock-treated mice was observed, demonstrating that survival depends on the presence of a functional *Mx1* allele (data not shown).

DISCUSSION

Most laboratory mouse strains, including C57BL/6J, are deficient in *Mx1* and susceptible to H1N1 (mouse-adapted PR8) infections. However, the presence of a wild-type *Mx1* allele makes B6-*Mx1*$^{r/r}$ resistant to H1N1 infections (55). Furthermore, many studies have shown that *Mx1* is a strong genetic resistance factor controlling influenza virus replication and protecting the host from severe pathology and mortality (51–55). These studies combined suggested that the *Mx1* allele from A2G mice is able to protect from lethal infections independently of the genetic background.

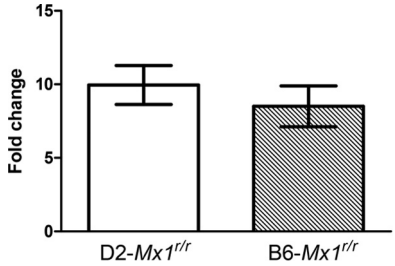

FIG 8 Upregulation of *Mx1* in congenic D2-*Mx1*$^{r/r}$ mouse strains after IFN-α treatment. D2-*Mx1*$^{r/r}$ and B6-*Mx1*$^{r/r}$ mice were treated with 50,000 IU of recombinant IFN-α intranasally. Levels of *Mx1* mRNA expression were measured by quantitative RT-PCR and compared to those in PBS-treated controls. Treatment with IFN-α induces comparable fold increases of *Mx1* expression in both D2-*Mx1*$^{r/r}$ and B6-*Mx1*$^{r/r}$ mice. No difference was detected between B6-*Mx1*$^{r/r}$ and D2-*Mx1*$^{r/r}$ mice ($P = 0.4918$, two-tailed Student's *t* test, $n = 3$).

Most surprisingly, we found that congenic D2-*Mx1*$^{r/r}$ mice that carry the *Mx1* wild-type allele still exhibited an equally highly susceptible phenotype as did D2-*Mx1*$^{-/-}$ mice after infection with H1N1 virus (PR8). All infected D2-*Mx1*$^{r/r}$ mice rapidly lost body weight and died. We confirmed in D2-*Mx1*$^{r/r}$ infected mice that the wild-type allele in D2-*Mx1*$^{r/r}$ was expressed after infection. Furthermore, (B6 × D2(B6).A2G-*Mx1*$^{r/-}$)F1 mice which received the *Mx1* allele from the congenic D2-*Mx1*$^{r/r}$ strain were as resistant to PR8 infections as F1 mice that were generated by crossing B6-*Mx1*$^{r/r}$ to D2-*Mx1*$^{-/-}$ mice. These experiments confirmed that D2-*Mx1*$^{r/r}$ mice carried a fully functional protective *Mx1* allele.

In agreement with previously published data (55), we also observed that B6-*Mx1*$^{r/r}$ mice were resistant to mouse-adapted PR8 (H1N1) virus infections whereas *Mx1*-deficient B6-*Mx1*$^{-/-}$ mice succumbed to the infection. Previously, the 50% lethal dose (LD_{50}) after PR8 infection had been determined for B6-*Mx1*$^{r/r}$ mice at 6.7×10^6 infectious particles (56), for B6-*Mx1*$^{-/-}$ mice at 20 to 32 infectious particles (38), and for D2-*Mx1*$^{-/-}$ at 2 to 3 infectious particles (38). In our studies, D2-*Mx1*$^{r/r}$ mice survived an infection with a low dose (10 FFU) of PR8 virus. However, they succumbed to an infection dose of 2×10^3 infectious particles. We

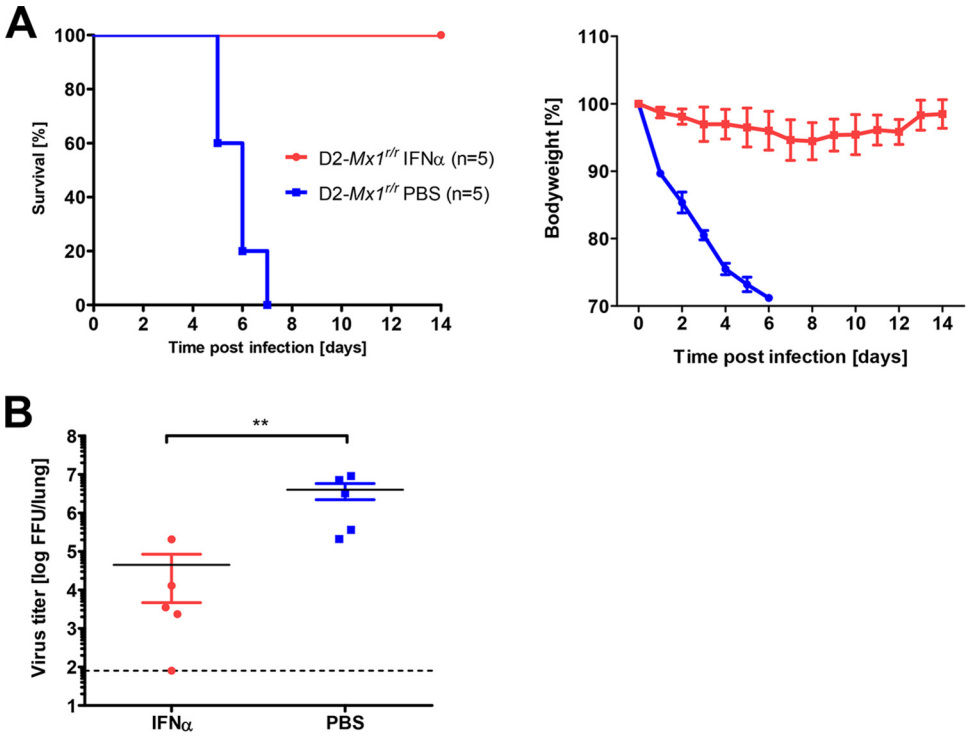

FIG 9 Alpha interferon pretreatment rescues D2-*Mx1*$^{r/r}$ mice. Eight- to 11-week-old female D2-*Mx1*$^{r/r}$ mice were pretreated with 1 μg recombinant human alpha interferon B/D (IFN-α) intranasally 1 day prior to infection, and PBS was given as a mock control. All mice were subsequently infected intranasally with 2×10^3 FFU of PR8 virus. (A) Survival rate was monitored for 14 days p.i. Mice that lost 30% or more of the starting body weight were sacrificed and recorded as dead. IFN-α-pretreated mice showed higher survival rates than did PBS-treated controls (log rank survival, $P = 0.0019$, $n = 5$ mice per group). (B) Virus particles in lung homogenates from IFN-α-pretreated and PBS-treated mice were determined in a focus-forming assay on day 1 p.i. Virus titers were significantly different between IFN-α- and PBS-pretreated groups (**, $P = 0.01$, Mann-Whitney U test). $n = 5$ mice per group.

decided to not further determine the exact LD_{50} for D2-*Mx1*$^{r/r}$ mice with respect to the 3Rs (reduction, refinement, and replacement) of animal ethics, because it would not add any more highly relevant information. Thus, in conclusion, the LD_{50} for B6-*Mx1*$^{-/-}$ mice is about 5 orders of magnitude lower than that for B6-*Mx1*$^{r/r}$ mice whereas the LD_{50} for D2-*Mx1*$^{-/-}$ mice is only about 1 to 2 orders of magnitude lower than that for D2-*Mx1*$^{r/r}$ mice. D2-*Mx1*$^{r/r}$ mice are 3 to 5 orders of magnitude more susceptible than B6-*Mx1*$^{r/r}$ mice.

Thus, our results show for the first time that the presence of the A2G *Mx1* allele, which is able to rescue A2G and congenic C57BL/6J mice from lethal influenza A virus infections, does not exert its protective function in a DBA/2J genetic background. These observations suggest that additional genetic factors are required for the protective *Mx1* functions or that the DBA/2J background is highly permissive to infections and that expression of *Mx1* comes too late.

Recently, the wild-derived mouse strain CAST/EiJ was found to be highly susceptible to H1N1 virus infections, although these mice express a full-length *Mx1* allele with only one amino acid difference from the ancestral PWK/PhJ allele (49). However, it is yet unclear if the high susceptibility in CAST/EiJ mice is caused by the genetic background or the polymorphism in the *Mx1* allele.

Furthermore, we systematically tested different background combinations and *Mx1* allele combinations to determine how resistance and susceptibility were influenced by wild-type *Mx1* and combinations of C57BL/6J and DBA/2J backgrounds. We observed that changing the genetic background from a pure DBA/2J to a hybrid B6 × D2 and then to a pure C57BL/6J background incrementally increased survival. Thus, for protective nonlethal outcome of an H1N1 virus infection, at least one C57BL/6J genome had to be present. In the presence of a C57BL/6J genome, an increase from one to two copies of the wild-type *Mx1* allele also increased survival. These observations suggest that the *Mx1* gene and presumed resistance factors from C57BL/6J act in an additive fashion. Furthermore, we found that the presence of a DBA/2J background in pure DBA/2J or in hybrid B6 × D2 mice resulted in high viral loads in the lung at day 1 p.i., regardless of whether the wild-type *Mx1* allele was present or not. However, in mice with a C57BL/6J or hybrid B6 × D2 background, the presence of the *Mx1* wild-type allele always resulted in a reduction of virus lung titers on day 3 p.i. The absence of the wild-type *Mx1* allele resulted in very high titers in a DBA/2J background, which were not reduced at day 3, and lower initial virus titers in mice with a C57BL/6J background, which increased until day 3 p.i.

These observations suggest that a pure DBA/2J background renders the host highly permissive to an early rapid viral replication whereas the presence of at least one copy of the wild-type *Mx1* gene results in reduction of virus replication on day 3 p.i. The most likely explanation for the high susceptibility of D2-*Mx1*$^{r/r}$ mice is therefore that *Mx1* is induced too late after infection with H1N1 virus (PR8) to exert its protective functions.

Mx1 is upregulated after infection in both DBA/2J and C57BL/6J mice with higher levels in DBA/2J mice (our unpublished data). Further-

more, it has been shown previously that pretreatment of B6-*Mx1*$^{r/r}$ mice with IFN-α can rescue mice from an otherwise lethal infection with a very highly virulent H1N1 virus (hvH1N1) (55). We therefore investigated whether IFN-α pretreatment may have a beneficial effect in D2-*Mx1*$^{r/r}$ mice. Indeed, pretreated D2-*Mx1*$^{r/r}$ mice survived lethal infections with PR8 virus. Thus, our results showed that wild-type *Mx1* is able to protect DBA/2J mice when it is already present at the time of infection. These results further support the hypothesis that in nontreated DBA/2J mice viral replication during the first 2 days is very rapid and expression of *Mx1* is too late to restrict the massive viral replication and prevent severe tissue damage and subsequent death.

Mx1 expression in D2-*Mx1*$^{r/r}$ mice was investigated only at the RNA level. We can thus not exclude the possibility that protein half-lives may be different in different genetic backgrounds. However, our experiments with IFN pretreatment suggest that this possibility is very unlikely, because in this case, D2-*Mx1*$^{r/r}$ mice would not be protected.

D2-*Mx1*$^{r/r}$ mice showed a lower mortality rate when infected with 2×10^3 FFU of H3N2 virus. In this case, we speculate that H3N2 virus may exhibit a lower replication rate very early after infection or that H3N2 virus does not suppress induction of *Mx1* as efficiently as PR8 and that induction of Mx1 protein is early or strong enough to partially rescue infected D2-*Mx1*$^{r/r}$ mice.

Humans, as a species, carry genes that are functional orthologs of *Mx1*, named *MX1* and *MX2* (39). Thus, the differences in susceptibility and resistance to influenza A virus in humans that are attributed to genetic factors (24, 63) are most likely not caused by the presence or absence of *MX1*. Therefore, our observations which demonstrate that the genetic background may render an individual highly susceptible, even in the presence of a functional *MX1* resistance gene, are also highly relevant for understanding genetic susceptibility to influenza virus infections in humans.

In summary, our results show that, in contrast to studies that were performed previously, the wild-type influenza virus resistance *Mx1* gene (derived from A2G) does not necessarily result in high resistance to lethal influenza A virus infections. Rather, the protective effect of *Mx1* depends strongly on the genetic background, the virulence of the virus, and the kinetics of *Mx1* induction.

ACKNOWLEDGMENTS

We thank the animal caretakers at the Central Animal Facilities at the HZI for maintaining the mice for this study and Christin Fricke and Karin Lammert for excellent technical assistance. We thank Claudia Pommerenke for her support in analyzing the genotyping data for congenic D2-*Mx1*$^{r/r}$ mice. Original stocks of influenza A viruses were obtained from Peter Stäheli (University of Freiburg) and Otto Haller (University of Freiburg). Congenic B6-*Mx1*$^{r/r}$ mice, the protocol for genotyping *Mx1* congenic mice, and recombinant human alpha interferon B/D were obtained from Peter Stäheli. We thank Blair Prochnow for critical reading of the manuscript.

This work was supported by intramural grants from the Helmholtz Association (Program Infection and Immunity) and a research grant, FluResearchNet (no. 01KI07137), from the German Ministry of Education and Research to K.S. D.-L.S. received partial financial support from a Studying Abroad Scholarship from the Ministry of Education, Taiwan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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