Zoonotic coronaviruses, including the one that caused severe acute respiratory syndrome (SARS), cause significant morbidity and mortality in humans. No specific therapy for any human coronavirus is available, making vaccine development critical for protection against these viruses. We previously showed that recombinant SARS coronavirus (SARS-CoV) (Urbani strain based) lacking envelope (E) protein expression (rU-ΔE) provided good but not perfect protection in young mice against challenge with virulent mouse-adapted SARS-CoV (MA15). To improve vaccine efficacy, we developed a second set of E-deleted vaccine candidates on an MA15 background (rMA15-ΔE). rMA15-ΔE is safe, causing no disease in 6-week-, 12-month-, or 18-month-old BALB/c mice. Immunization with this virus completely protected mice of three ages from lethal disease and effected more-rapid virus clearance. Compared to rU-ΔE, rMA15-ΔE immunization resulted in significantly greater neutralizing antibody and SARS-CoV-specific CD4 and CD8 T cell responses. After challenge, inflammatory cell infiltration, edema, and lung destruction were decreased in the lungs of rMA15-ΔE-immunized mice compared to those in rU-ΔE-immunized 12-month-old mice. Collectively, these results show that immunization with a species-adapted attenuated coronavirus lacking E protein expression is safe and provides optimal immunogenicity and long-term protection against challenge with lethal virus. This approach will be generally useful for development of vaccines protective against human coronaviruses as well as against coronaviruses that cause disease in domestic and companion animals.

Severe acute respiratory syndrome (SARS), caused by a novel coronavirus (SARS-CoV), was contracted by approximately 8,000 individuals during the 2002-2003 epidemic, with a consequent 10% rate of mortality (1, 2). Most strikingly, 50% of patients greater than 60 years of age succumbed to the infection, while no patient less than 24 years old died. SARS-CoV has not reappeared in human populations since 2004, but several species of coronaviruses with similarities to SARS-CoV have been identified in bat populations (3–6). A human coronavirus (Middle East respiratory syndrome coronavirus [MERS-CoV]) related to two of these bat viruses (BtCoV-HKU4 and BtCoV-HKU5) was recently isolated from several patients in the Middle East who developed severe pneumonia and renal disease (7). How these SARS-like CoVs changed host range to infect humans is not known with certainty, but the fact that they did provides the impetus for development of a SARS-CoV vaccine. Most importantly, the development of such a vaccine would provide a guide to rapid engineering and deployment of a vaccine that would be useful against a new, highly pathogenic coronavirus, even if that virus were not SARS-CoV.

Several vaccine candidates have been developed since 2003 (8, 9). Antivirus neutralizing antibodies, which are useful for protecting select populations, such as health care workers during an outbreak, have been isolated and prepared in large quantities. Several protein subunit vaccines, in which one or more SARS-CoV structural proteins are expressed by a heterologous virus or replicon, have also been developed (10). Some of these approaches will be useful in human populations, but the most efficacious vaccines will elicit both antibody and T cell responses directed against the virus. One strategy has been to use nonreplicating Venezuelan equine encephalitis replicon particles (VRP) to induce T and B cell responses (11). However, T cell epitopes are located in both the surface glycoproteins and internal proteins, such as the nucleocapsid protein (12). An unexpected problem was that VRPs containing only the N or the N and S proteins induce an eosinophilic infiltrate in the lung after challenge with virulent virus, especially in older mice, making such vaccines not useful (11, 13).

Live attenuated vaccines are considered most effective in their ability to induce a long-lived balanced immune response. The major problems of using live attenuated vaccines relate to the possibility that viruses may revert to virulence and to the risk that even attenuated live vaccines may cause disease in immunocompromised vaccine recipients. Coronaviruses are well known to recombine (14), so any attenuated SARS-CoV should be attenuated at several sites to make the probability of reversion as close to nil as possible. Several approaches have been used to minimize the risk of reversion to virulence, including deletion of a minor structural protein, the envelope (E) protein (15–17). In addition to the N and S proteins, all coronaviruses encode at least two additional structural proteins, the E and transmembrane (M) proteins. The E protein is present in the virion in very small amounts and was initially believed to be
primarily a structural protein. Subsequent work suggested that E protein is involved in virus assembly and virus pathogenesis (14). Deletion of the E protein from SARS-CoV impaired replication but did not prevent release of infectious virus, although titers were lower than they were after infection with E-containing virus (17, 18). Based on these results, we previously developed a recombinant SARS-CoV (human Urbani strain) lacking the E protein (rU−ΔE) and showed that immunization with this virus completely protected hamsters and partially protected mice transgenic (Tg) for the expression of the SARS-CoV receptor human angiotensin-converting enzyme 2 (hACE2) against challenge with SARS-CoV (19, 20). hACE2-Tg mice are very sensitive to infection with SARS-CoV, developing an overwhelming encephalitis (21).

Human isolates of SARS-CoV, including the Urbani strain, cause no or mild disease in young or older wild-type mice, respectively (22, 23). To analyze the effect of vaccination with E protein-deleted virus in the context of a more severe respiratory infection, we also challenged mice with SARS-CoV that had been adapted to growth in mice by serial passage through BALB/c mice (MA15 strain) (24). MA15 causes severe pneumonia in young BALB/c mice and older mice of all strains examined (24–26). Immunization of BALB/c mice with rU−ΔE was partly protective against subsequent challenge with MA15 but induced a weak T cell and antibody response (20). Consequently, we have now engineered another virus on an MA15 background (rMA15−ΔE) with the expectation that it will be more immunogenic than rU−ΔE because it is more fit for growth in the mouse lung. We show here that this is indeed the case, with rMA15−ΔE eliciting more-potent antiviral neutralizing antibody and T cell responses than rU−ΔE but remaining highly attenuated and safe. Further, immunization with rMA15−ΔE fully protected 6- to 10-week-old, 12-month-old, and 18-month-old BALB/c mice from challenge with a lethal dose of MA15 and also induced long-term protection.

MATERIALS AND METHODS

Mice, virus, and cells. Specific-pathogen-free BALB/c mice with ages ranging from 6 weeks to 18 months were purchased from the National Cancer Institute. Mice were maintained in the animal care facility at the University of Iowa. All protocols were approved by the University of Iowa Institutional Animal Care and Use Committee. Mouse-adapted SARS-CoV (MA15) (24), a gift from Kanta Subbarao (National Institutes of Health, Bethesda, MD), was grown in Vero E6 cells.

Development of recombinant virus rMA15−ΔE. Mutations required for mouse adaptation were introduced into the Urbani strain of SARS-CoV using a previously described bacterial artificial chromosome (BAC)-based reverse genetics system (27). Specifically, mutations were introduced into Nsp5 (H133Y, K268N), Nsp9 (T67A), Nsp13 (A4V), S protein (Y436H), and M protein (E11K), resulting in rMA15 (24). All these amino acid substitutions except substitution K268N were previously described (24). Introduction of this additional change did not compromise the virulence of MA15 in BALB/c mice (M. L. DeDiego and L. Enjuanes, unpublished results). Virus deleted in E protein was then generated as previously described (15).

Virus infection and titration. BALB/c mice were lightly anesthetized with isoflurane and immunized intranasally with 6,000 PFU of rMA15−ΔE or phosphate-buffered saline (PBS). Some mice were then challenged with an intranasal inoculation of 10^6 PFU of MA15 (nonrecombinant virus). Mice were monitored daily for morbidity and mortality. All work with SARS-CoV was conducted in the University of Iowa biosafety level 3 (BSL3) laboratory. To obtain SARS-CoV titers, lungs were homogenized in PBS. Virus titers were determined on Vero E6 cells as previously described (15, 20). Viral titers are expressed as PFU/g of tissue for SARS-CoV.

Histology. Animals were anesthetized and transcardially perfused with PBS followed by zinc formalin. Lungs were removed, fixed in zinc formalin, and paraflin embedded. Sections were stained with hematoxylin and cosin.

Measurement of CD8 and CD4 T cell responses in the lungs. Mice were sacrificed at the indicated times after infection, and single-cell suspensions were prepared using collagenase D (Roche Applied Science, Indianapolis, IN) and 0.1 mg/ml DNase (Roche) to digest the lung (28). Virus-specific CD8 and CD4 T cells were identified by intracellular cytokine staining (ICS) for gamma interferon (IFN-γ) (28, 29). Briefly, cells were incubated for 5 h with brefeldin A (BD Pharmingen, San Diego, CA) in the presence or absence of the SARS-CoV-specific peptides S366 (CD8; HNKKYRYL) and N353 (CD4; VNFENFNGL) (BioSynthesis Inc., Lewisville, TX). A total of 10^6 cells were then labeled at 4°C for cell surface markers using rat anti-mouse CD4 (RM4-5), rat anti-mouse CD8α (53-6.7) (both from BD Biosciences), and rat anti-mouse IFN-γ (XMG1.2) (eBioscience, San Diego). Cells were then fixed/permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and labeled with anti-IFN-γ antibody. All flow cytometry data were acquired on a BD FACSCalibur or FACSVerse (BD Biosciences, San Jose, CA) and were analyzed using FlowJo software (Tree Star, Inc.).

Measurement of ELISA titers. Whole blood was collected, and sera were prepared. Enzyme-linked immunosorbent assay (ELISA) titers were obtained as previously described (20). Briefly, 96-well MaxiSorp Immuno plates (Nunc) were coated with 2 × 10^6 PFU of formaldehyde and UV-inactivated SARS-CoV (BEI Resources, Manassas, VA). After being washed, wells were exposed to 3-fold dilutions of sera from naive or immunized mice for 1.5 h. Wells were washed and developed. The ELISA titer was defined as the highest dilution of serum that gave a 2-fold increase over the background.

Measurement of neutralizing antibody titers. A virus plaque reduction assay was used to determine serum neutralizing antibody titers (15). Sera were diluted at the indicated ratios and incubated with 50 PFU of MA15 for 30 min. The limit of detection was below 1:30.

Statistical analysis. Student’s t test was used to analyze differences in mean values between groups. All results are expressed as means ± standard errors of the means (SEM). P values of <0.05 were considered statistically significant.

RESULTS

rMA15−ΔE is safe in 6-week-old and 12-month-old BALB/c mice. Previously, we showed that rU−ΔE was attenuated in hamsters, young BALB/c mice, and hACE2-Tg mice, which are highly susceptible to SARS-CoV (15, 17, 19, 20). However, this recombinant virus was constructed on the background of a human coronavirus strain that caused no disease and induced weak antivirus T cell and antibody responses in young BALB/c mice (20). Mice, even if older, develop only mild clinical disease after infection with human-adapted strains (22). In contrast, BALB/c mice of all ages infected with the mouse-adapted MA15 strain develop clinical pneumonia, with more-severe disease observed in mice greater than 20 weeks of age (24). To determine whether deletion of the E protein attenuated the MA15 strain, we infected 6-week-old BALB/c mice with 5 × 10^4 PFU of rMA15−ΔE. Mice developed no signs of clinical disease or weight loss, while mice infected with the same dosage of MA15 succumbed to the infection (Fig. 1A and B). Mice that are 20 weeks of age and older develop more-severe disease after infection with MA15 (25, 26). To evaluate the safety of rMA15−ΔE in older mice, we infected 12-month-old BALB/c mice with the same dosage of rMA15−ΔE. These mice also re-
mained asymptomatic and lost no weight following immunization (Fig. 1C and D).

rMA15–ΔE immunization protects 6-week-old BALB/c mice from MA15-mediated pulmonary disease. rU–ΔE immunization provides imperfect protection against challenge with MA15 (20), so we assessed the efficacy of rMA15–ΔE immunization against challenge with a lethal dose of MA15, comparing it to that of control (PBS-treated) mice. In preliminary results, we found that intranasal immunization of 6-week-old BALB/c mice with 3,000 to 12,000 PFU of rMA15–ΔE resulted in optimal CD4 and CD8 T cell responses at day 7 after immunization; we used 6,000 PFU in all subsequent experiments. Intranasal infection of 6-week-old BALB/c mice with 6,000 PFU of rMA15–ΔE resulted in a small amount of peribronchial/perivascular inflammatory cell infiltration over the first few days following immunization, as assessed on histological examination (data not shown). Infectious virus was detected at days 2, 4, and 6 after immunization in the lungs of 6-week-old mice immunized with 6,000 PFU of rMA15–ΔE but was no longer detectable by day 8 (Fig. 2A).

To evaluate the effect of rMA15–ΔE immunization on clinical disease, we immunized 6-week-old mice with rMA15–ΔE, rU–ΔE, or PBS and then challenged them with 10⁴ PFU of MA15 at day 21 after immunization. We previously showed that immunization with rU–ΔE resulted in a 90% decrease in virus titer compared to immunization with PBS by day 5 postchallenge (20). rMA15–ΔE immunization was at least as effective, with virus undetectable at day 4 after challenge (Fig. 2B). All mice in the rMA15–ΔE-treated groups survived and showed no weight loss, while 100% of control mice died (Fig. 2C and D). In agreement with our previous report, immunization with rU–ΔE protected mice against death, although mice showed 10 to 15% weight loss at early times after infection (20).

Immunization with rU–ΔE induced an anti-SARS-CoV T cell response in some but not all mice at day 7 and very low (<1:10) neutralizing antibody titers at day 21 (20). To determine whether the enhanced protection afforded by rMA15–ΔE correlated with greater immunogenicity, we measured antiviral T cell and antibody responses at days 7 and 21, respectively, after immunization. Anti-SARS-CoV neutralizing antibody titers were detectable in 7 of 9 rMA15–ΔE-immunized mice, with an average titer of 1:95 ± 21 (Fig. 2E). In agreement with our previous report (20), anti-SARS-CoV neutralizing titers were below the limit of detection (1:30) in mice immunized with rU–ΔE.

Lung-specific CD4 and CD8 T cell responses were assessed at day 7 after immunization by measuring IFN-γ expression after stimulation with peptides N353 and S366, respectively. As shown in Fig. 2F, virus-specific T cell responses were barely detectable in mice inoculated with rU–ΔE. In contrast, approximately 0.4% ± 0.1% and 0.8% ± 0.1% of the CD4 and CD8 T cells, respectively, in the lungs of BALB/c mice immunized with rMA15–ΔE were virus specific.

rMA15–ΔE immunization protects 12-month-old and 18-month-old BALB/c mice after MA15 challenge. As mice age, they become progressively more susceptible to infection with SARS-CoV, so that 12- to 14-month-old BALB/c mice develop mild clinical disease and weight loss even after infection with the Urbani strain of SARS-CoV (22). We did not examine the efficacy of rU–ΔE in older mice in our previous studies. Immunization with 6,000 PFU of rMA15–ΔE caused no clinical disease in 12-month-old mice (data not shown), and histological examination of immunized lungs revealed a small amount of inflammatory cell infiltration at days 4 and 6 after inoculation (Fig. 3A to C). Almost no changes were detected in the lungs after immunization with rU–ΔE (Fig. 3D to F). The kinetics of rMA15–ΔE clearance from the lungs of 12-month-old mice was
delayed compared to that of rMA15-ΔE-immunized 6-week-old mice or rU-ΔE-immunized 12-month-old mice (compare Fig. 2A and 4A), with virus completely cleared by 8 days. This clearance is not due to enhanced replication of rMA15-ΔE in extrapulmonary tissues, since neither rMA15-ΔE nor rU-ΔE was detected in the liver, brain, or spleen of 12-month-old mice at day 2 postinfection (p.i.) (data not shown).

Immunization with 6,000 PFU of rMA15-ΔE, but not PBS or rU-ΔE immunization, effected rapid MA15 clearance after challenge, with virus largely cleared by day 2 (Fig. 4B). Immunization with rU-ΔE provided modest protection against lethal disease when 12-month-old mice were challenged 21 days later (Fig. 4C and D). In contrast, however, the same dose of rMA15-ΔE completely protected 12-month-old mice from challenge with 10⁵ PFU of MA15. Histological examination of lungs of rU-ΔE-immunized mice or PBS-immunized mice challenged with MA15 revealed interstitial and peribronchial/perivascular inflammatory cell infiltration with edema and proteinaceous deposition in airways and alveoli (Fig. 3J to L), while the lungs of infected rMA15-ΔE-immunized mice showed minimal evidence of lung damage or cellular infiltration (Fig. 3H and I). In previous vaccine studies, inclusion of the N protein in the immunogen resulted in an eosinophilic infiltration in the lungs on subsequent challenge with SARS-CoV, suggestive of immunopathological disease (11, 13). This was especially evident in older mice. In contrast, after vaccination with rMA15-ΔE, we did not observe significant eosinophil infiltration into the lungs of 12-month-old mice after MA15 challenge.

Finally, protection correlated with higher antivirus neutralizing and ELISA antibody titers and CD4 and CD8 T cell responses in rMA15-ΔE-immunized mice than in rU-ΔE-immunized animals (Fig. 4F). Neutralizing antibody titers were 1:198 ± 51 in rMA15-ΔE-immunized mice, compared to low titers in two mice (1:61) or levels below the limit of detection in six rU-ΔE-immunized animals at day 21 after immunization (Fig. 4E). ELISA titers were low in all mice but significantly higher in rMA15-ΔE-immunized mice than in those immunized with rU-ΔE (Fig. 4F). Similarly, N363-specific CD4 and
S366-specific CD8 T cell responses were significantly higher in rMA15-/H9004 E-immunized mice than in rU-immunized mice at day 7 (S366, 4.3% ± 1.1% versus 1.0% ± 0.1%, respectively; N363, 3.7% ± 0.3% versus 0.03% ± 0.03%, respectively) (Fig. 4G and H).

To determine whether immunization with rMA15-ΔE was safe and protective even in older mice, we immunized 18-month-old BALB/c mice with 6,000 PFU of rMA15-ΔE. Mice showed no signs of clinical disease and minimal weight loss after immunization (Fig. 5A). Histological examination of the lungs of rMA15-ΔE-immunized mice showed a minor amount of peribronchial and perivascular infiltration (data not shown). Next, we challenged PBS- and rMA15-ΔE-immunized 18-month-old mice with 10^5 PFU of MA15 at 21 days after immunization. Immunization with rMA15-ΔE conferred complete protection from lethal disease, with no weight loss observed, while PBS-treated mice developed rapidly fatal disease (Fig. 5B and C).

Prolonged protection against virulent challenge with MA15 after immunization with rMA15-ΔE. Vaccination must result in long-lived protection against challenge with virulent virus to be useful. To begin to address the long-term efficacy of rMA15-ΔE immunization against MA15 challenge, we immunized 6-week- and 12-month-old mice with rMA15-ΔE, rU-ΔE, or PBS and then challenged them with 10^5 MA15 at 66 days after immunization. Virus was rapidly cleared from the lungs of 6-week- and 12-month-old rMA15-ΔE-immunized mice but not from the lungs of rU-ΔE-immunized or control mice after challenge at day 66 (Fig. 6A and B). Further, all rMA15-ΔE-immunized, but not rU-ΔE- or PBS-treated, mice survived subsequent infection with MA15, had no signs of clinical illness, and exhibited virtually no weight loss (Fig. 6C to F). Anti-SARS-CoV antibody titers, whether measured in an ELISA or in a plaque reduction neutralization assay, were substantially lower in rU-ΔE-immunized mice than in rMA15-ΔE-immunized mice at day 66 after immunization in both 6-week- and 12-month-old mice (Fig. 6G to J). Notably, neutralizing antibody titers were at or below the limit of detection in both age groups after immunization with rU-ΔE, while titers measured by ELISA were present at low levels in mice immunized at 6 weeks but were not detected in those immunized at 12 months.

Histological examination of the lungs of 12-month-old mice challenged at day 66 paralleled findings observed in mice infected

FIG 3 Histological changes observed after immunization with rMA-ΔE or rU-ΔE and challenge with MA15. Twelve-month-old BALB/c mice were immunized with 6,000 PFU of rMA15-ΔE (A to C), rU-ΔE (D to F), or PBS (G) and sacrificed at day 2, 4, or 6 postimmunization. Additional groups of mice were challenged with 10^5 PFU of MA15 at days 21 (d21) (H to L) and 66 (d66) (M to Q) after immunization. Lungs were harvested and processed for histological examination as described in Materials and Methods. Representative images are shown. X, edema; ○, cellular debris, ↓, denuded epithelium. Original magnification, ×40.
21 days after immunization: we detected few changes in the lungs of rMA15-ΔE-immunized mice, with modest amounts of perivascular and peribronchial cellular infiltration and little alveolar or airway edema observed at day 2 or day 4 (Fig. 3M and N). In contrast, after challenge, PBS- and rU-ΔE-immunized mice showed airway necrosis and alveolar edema compared to rMA15-ΔE-immunized mice (Fig. 3O to Q). Few eosinophils were detected in the inflammatory infiltrate at any time point in any of the mice.

**DISCUSSION**

We showed that immunization of 6-week-old BALB/c mice with a recombinant version of a human strain of SARS-CoV...
detection of infectious rMA15-E of the E-deleted virus augmented virus replication, resulting in mouse adaptation and that long-term protection was induced in 6-week-old mice. Our results show that mouse adaptation of the immunizing agent enhances immunogenicity and protection without compromising safety since mouse adaptation of the immunizing agent enhances immunogenicity. We show that this in fact occurs when examined by electron microscopy (15, 17). In addition to its involvement in virion morphogenesis, the E protein also has ion channel activity, although the role of this activity in the virus life cycle is not established (39–41). Recent work has shown that the E protein reduces the stress response in infected cells as well as when introduced exogenously into cells stressed chemically or by infection with a noncoronavirus (42). The E protein also interacts with several host cell proteins and modifies the expression of genes involved in signal transduction, inflammation, apoptosis, and the cell cycle, in addition to those related to stress (42). Thus, its deletion has multiple advantages of being located at a site distant from that of the E protein, making it less likely that recombination with circulating viruses would result in the generation of a virulent virus. Mutations in another nonstructural protein, Nsp14 (a 3′ to 5′ exonuclease), result in decreased fidelity and virus attenuation in vivo (36). Incorporation of mutations in Nsp14 into rMA15-ΔE would minimize the risk of reversion.

We developed an E protein-deleted virus because previous work suggested that this protein was critical for optimal virus development but was not absolutely required (15, 18). The E protein is a structural protein that is present in the virion in very small amounts (37) and has an important role in virus assembly. Somewhat surprisingly, the requirement for E for the production of infectious virus is not consistent across all coronaviruses. Thus, no infectious virus is released in the absence of E from cells infected with transmissible gastroenteritis coronavirus (TGEV) (38), while virus with an abnormal morphology is released from cells infected with MHV, a betacoronavirus, like SARS-CoV (18). In the case of SARS-CoV, the virus shows normal morphology when examined by electron microscopy (15, 17). In addition to its involvement in virion morphogenesis, the E protein also has ion channel activity, although the role of this activity in the virus life cycle is not established (39–41). Recent work has shown that the E protein reduces the stress response in infected cells as well as when introduced exogenously into cells stressed chemically or by infection with a noncoronavirus (42). The E protein also interacts with several host cell proteins and modifies the expression of genes involved in signal transduction, inflammation, apoptosis, and the cell cycle, in addition to those related to stress (42). Thus, its deletion has multiple advantages of being located at a site distant from that of the E protein, making it less likely that recombination with circulating viruses would result in the generation of a virulent virus. Mutations in another nonstructural protein, Nsp14 (a 3′ to 5′ exonuclease), result in decreased fidelity and virus attenuation in vivo (36). Incorporation of mutations in Nsp14 into rMA15-ΔE would minimize the risk of reversion.

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effects on the infected cell, potentially attenuating the infection by a
variety of mechanisms.

Currently, we are identifying domains of E protein responsible
for its various roles in assembly, morphogenesis, and virulence.
Our future efforts at vaccine development will be to maintain
some E protein function in order to enhance immunogenicity
while introducing one or more of the changes listed above in order
to decrease the likelihood of recombination or reversion to wild-
type virus.

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