1. INTRODUCTION

Coronavirus infections are associated with severe diseases of the lower respiratory and gastrointestinal tract in humans and animals, yet little is known about the underlying molecular mechanisms governing virulence and pathogenesis. Among the human coronaviruses, the etiologic agent of SARS, the SARS coronavirus (SARS-CoV) is an attractive model to study the molecular basis for pathogenesis, given its robust in vitro growth characteristics, the availability of a reverse genetic system, animal models, wealth of clinical data, and several solved replicase and accessory protein structures. SARS-CoV infection afflicted about 8,000 humans and resulted in about 800 deaths, worldwide. Disease severity has been linked to age, with approximate mortality rates of <1% under 24 years of age, 6% for ages 15–44, 15% for ages 45–64, and >50% over 65. Many survivors have suffered long-lasting lung and cardiac complications. The underlying mechanisms governing SARS-CoV pathogenesis are only now being unraveled.

SARS-CoV is a zoonotic pathogen that crossed the species barrier, the most likely host being civet cats and raccoon dogs, although virus has also been isolated or detected from domesticated cats, swine, and rodents. Aggressive public health efforts contained the 2003 epidemic but it is unclear whether the epidemic strains are extinct in the wild. Given the significant health and economic impact of the SARS-CoV outbreak, an effective vaccine strategy for SARS that includes protection against epidemic and zoonotic strains of virus in at risk elderly populations who are most vulnerable to severe disease is essential.

Phylogenetic analyses have suggested that SARS-CoV either represented the prototype group IV coronavirus while other studies have placed the virus as an early split-off of group II. Molecular evolutionary studies on isolates obtained from different stages in the outbreak have implicated changes in ORF1a, the S glycoprotein,
and various accessory ORFs (ORF3a and ORF8) as being associated with increased virulence, transmission and pathogenesis during the epidemic.3,4

The SARS-CoV virion contains a single-stranded positive polarity 29,700 nucleotide RNA genome bound by the nucleocapsid protein, N. The capsid is surrounded by a lipid bilayer containing at least three structural proteins, designated S, M, and E. The 180-kDa spike glycoprotein (S) interacts with its receptor, angiotensin II converting enzyme (ACE2), to mediate entry into cells.11 In addition to the 23-kDa M glycoprotein, the E protein is likely essential for efficient virion maturation and release. The SARS-CoV genome contains 14 principal ORFs. ORF1a and ORF1b encode the viral replicase proteins required for subgenomic and genome length RNA synthesis and virus replication.12,16 ORFs 2–8 are encoded in eight subgenomic mRNAs synthesized as a nested set of 3′ co-terminal molecules in which the leader RNA sequences, encoded at the 5′ end of the genome, are joined to body sequences at distinct transcription regulatory sequences containing a highly conserved consensus sequence. The development of a SARS-CoV molecular clone provides a useful tool for developing novel SARS-CoV isolates for identifying the genetic determinants responsible for increased pathogenesis during the epidemic and developing zoonotic strains for vaccine testing.22

2. METHODS

The 2003 epidemic Urbani strain of SARS-CoV and a molecularly derived recombinant virus (icSARS-CoV) are used throughout these studies. The GDO3T0013 S glycoprotein (GD03) was synthetically reconstructed from published sequences (AY304486),3 inserted into the molecular clone of SARS-CoV, and used to isolate a recombinant virus (icGD03) encoding the icGD03 S glycoprotein.22 The Urbani S glycoprotein or nucleocapsid genes were inserted into Venezuelan equine encephalitis virus replicon particles (VRP) using methods described in the literature.1 VRP vaccine stocks were titered at ~1.0 x 10^9 and used to vaccinate at 1 x 10^6 VRPs. Twenty-eight days later, the animals were boosted by footpad innoculation and challenged with wild-type icSARS-CoV or icGD03 several weeks to months later. Neutralization titers were determined by treating ~100 plaques of icSARS-CoV or icGD03 with varying concentrations of serum from humans or animals and measuring the reduction of infectivity on Vero cell monolayers.

3. RESULTS

3.1. Evolution of the SARS-CoV

Phylogenetic analysis of SARS-CoV isolates from animals and humans makes a compelling argument that the virus originated in animals, most likely in palm civets or raccoon dogs, and was transmitted to human populations via live animal markets.7 However, the actual reservoir for the SARS-CoV has not been clearly determined, although recent unpublished reports from China suggest a possible origin in bats. Using Bayesian methods and sequences from animal and human SARS-CoV isolates, we note that SARS-CoV isolates can be divided into 3 genoclusters including the animal isolates like SZ16 (GI), a cluster of isolates associated with sporadic mild human and animal
infections (GII), and a cluster of highly pathogenic strains associated with the early, middle, or late phase isolates from the 2002–2003 epidemic (GIII) (Figure 1). In general, GII isolates are usually isolated in animals although rare mild cases of infection in humans have been reported and the GI isolates have only been detected in animals. The GD03 S glycoprotein sequence was obtained from a sporadic, mild human case reported on Dec 22, 2003 from a virus that was never successfully cultured \textit{in vitro} and is the most diverse human isolate.

3.2. SARS-CoV S Glycoprotein Antigenicity

Detailed mapping studies have indicated that at least three neutralizing epitopes reside within the Urbani S glycoprotein. Monoclonal antibodies that bind site A and B possess significant neutralizing activity against wildtype viruses that completely protect animals from infection. Site B overlaps the receptor binding site known to interact with ACE2.\textsuperscript{10,18,19} Site C represents a 3–4 times less robust neutralizing site, presumably antibodies interfere with virus docking and entry mechanisms dependent upon the function of the heptad repeats encoded in the C-terminus of S. Variation in the SARS-CoV S glycoprotein has been reported, although detailed cross neutralization studies and cross protection studies in animal models comparing the susceptibility of these
heterologous glycoproteins to neutralization have not been evaluated. The most extensive variation has been noted in isolates from group II that were isolated from raccoon dogs and civets. About ~2% amino acid sequence variation in the S glycoprotein has been reported as compared with the Urbani epidemic strain.4,7 The GD03 S glycoprotein contains variation within all 3 neutralizing sites, although it is less clear whether this heterogeneity alters the neutralization kinetics associated with antiserum generated against epidemic strains.

3.3. SARS-CoV Vaccine Development

After the 2002–2003 epidemic, experimental vaccines were developed and tested in animal models, primarily in rodents.6 In the murine model, the principal component of protective immunity was neutralizing antiserum directed against the SARS S glycoprotein and passive transfer of neutralizing antibodies was sufficient to protect against virus replication.18 Not surprisingly, killed and recombinant virus vaccines that elicit neutralizing antibody protect mice from SARS-CoV replication in the lung.6 Importantly, more adverse reactions were noted in ferrets vaccinated with recombinant poxviruses encoding the SARS-CoV S glycoprotein including a lack of protection from
Figure 3. Strategy for resurrection of rare SARS-CoV spikes. Synthetic cDNAs encoding the icGD03 (human/animal isolate, GII), SZ16 (animal isolate, GI), and GZ02 (early isolate, GIII) were obtained and inserted into the Urbani molecular clone, replacing the Urbani S glycoprotein with a variant S gene.

infection and increased hepatic pathology in vaccinated animals. The molecular basis for the increased pathology in vaccinated animals remains unknown.

Important unresolved questions remain regarding the development of efficacious SARS-CoV vaccines. Severe disease and high death rates were noted in elderly human populations, and vaccine efficacy in more vulnerable senescent animal models for SARS-CoV infection have not been evaluated. The ability of vaccines to induce robust immune responses in aged populations, should be evaluated to determine if protection can be elicited in elderly populations with senescent immune systems. Long-term and short-term immunity, waning immunity, and cross protection between strains from different genoclusters has languished due to the lack of heterologous isolates. Finally, the molecular mechanisms governing vaccine mediated immunopathogenesis and enhanced disease in ferrets must be elucidated.

3.4. Synthetic Resurrection of Rare SARS-CoV Isolates

Many animal and early human isolates were sequenced but never successfully cultured in vitro. To address this problem, a systematic approach involving a molecular clone of SARS-CoV and synthetic biology was used to successfully resurrect early human and animal isolates (Figure 3). The details of the resurrection of the icGD03 S glycoprotein gene in the Urbani molecular clone is discussed in more detail in the article by Sheahan et al. (chapter 7.19). The icGD03 virus replicated to high titers in Vero cells and in mice and was more resistant to neutralization with antiserum or monoclonal
Table 1. Properties of icSARS-CoV and icGD03-S recombinant viruses.

| Virus strain | Virus titer | Neutralizing titer (PRNT<sub>50</sub>)<sup>1</sup> |
|--------------|-------------|---------------------------------|
|              | Vero       | BALB/c Mice                     | S monoclonal | Polyclonal sera |
| icSARS-CoV   | 1.0 x 10<sup>7</sup> | 1.0 x 10<sup>7</sup> | >1:1600 | >1:1600 |
| icGD03-S     | 5.0 x 10<sup>6</sup> | 5.0 x 10<sup>6</sup> | 1:100  | 1:100  |

<sup>1</sup>PRNT<sub>50</sub> represents plaque reduction neutralization serum titers that reduce 50% of the SARS-CoV plaques.

antibodies directed against the Urbani strain of SARS-CoV (Table 1,<sup>9</sup>) consistent with the noted sequence variation in domains recognized by neutralizing antibodies.

3.5. SARS-CoV Vaccines and Heterotypic Cross-Protection

To evaluate vaccine efficacy against homologous and heterologous strains, the Urbani S glycoprotein and nucleocapsid genes were cloned and inserted in Venezuelan equine encephalitis virus replicon particles (VRP-S or VRP-N) using standard approaches reported from our laboratory previously.<sup>1</sup> In addition, the influenza A HA glycoprotein (VRP-HA) was used as a control vector. BALB/c mice were vaccinated with 1 x 10<sup>6</sup> infectious units of VRPs-HA or the combination of VRP S+VRP-N, boosted 4 weeks later, and then challenged with icSARS-CoV or icGD03-S about 8 or 40 weeks post-boost (Table 2). Ages of senescent mice exceeded 1 year at the time of challenge. Importantly, VRP vaccines provided complete short-term protection against homologous and heterologous isolates, as all VRP S+VRP-N vaccinated animals were protected against challenge. The VRP vaccines also provided long-term protection against homologous challenge, protecting all senescent mice from icSARS-CoV replication. Although aged mice vaccinated with VRP-HA demonstrated pathologic lesions in the lung similar to that reported in the literature,<sup>15</sup> VRP S+VRP-N vaccinated mice displayed little if any pathologic lesions in the lung (data not shown). In contrast, VRP-S+VRP-N vaccinated mice provided little long-term protection against icGD03-S infection although virus titers were reduced about 1-log as compared with VRP-HA controls. The icGD03 challenge virus also produced pathologic lesions in both the VRP-HA and SARS vaccinated animals and was virtually indistinguishable from icSARS-CoV infection (data not shown). At this time, it is likely that rapid waning immunity against heterologous challenge viruses resulted in vaccine failure in aged animals.

Table 2. Virus replication in the lungs of vaccinated BALB/c mice.

| Virus strains | Young BALB/C mice<sup>1</sup> | Senescent BALB/C mice<sup>1</sup> |
|--------------|-----------------------------|---------------------------------|
|              | VRP-HA | VRP-S+VRP-N | VRP-HA | VRP S+VRP-N |
| icSARS-CoV   | 4/4    | 0/4         | 4/4    | 0/4        |
| icGD03-S     | 4/4    | 0/4         | 4/4    | 4/4        |

<sup>1</sup>Ratio represents infected mice over total mice.
SARS-CoV is a zoonotic pathogen, and several cases of likely animal to human transmission have been reported in China. In most cases, these infections were mild and did not spread beyond the index case suggesting that animal viruses require additional adaptation prior to evolving efficient usage of humans as hosts. After adapting to the human host, most severe disease manifestations occur within elderly populations likely compromised by waning innate immune and acquired immune responses to pathogen insult. Importantly, several zoonotic strains in genocluster II are closely related, display heterogeneity in neutralizing epitope sites, and encode determinants in the S glycoprotein gene that are consistent with rapid human adaptation and spread. These data suggest that current vaccine formulations should be tested not only against epidemic strains, but also to evaluate protective therapeutic potential against zoonotic reintroduction. To address this need, we resurrected live SARS-CoV encoding the GD03 S glycoprotein and demonstrated that this virus replicated efficiently in vitro and in vivo and produced pathologic lesions in aged mice. As shown with poliovirus and 1918 influenza virus genes, our data provide additional support for the use of synthetic DNA and reverse genetics as a means of rescuing “extinct” viruses and viral genes for the improvement of vaccines and enhancing the overall public health.

The icSARS-CoV GD03-S recombinant virus demonstrated gaps in vaccine design for controlling future SARS-CoV epidemics. Our results are consistent with earlier reports suggesting that zoonotic viruses are highly resistant to neutralization with antiserum directed against epidemic strains like Urbani. Importantly, VRP vaccines elicited high levels of neutralizing antiserum against the homologous isolate, but less efficient neutralizing responses against the icGD03-S recombinant virus. These high neutralizing responses likely translated to efficient protection from homologous infection, both in young and aged mice, but also provided short-term protection against homologous protection in younger animals. In aged animals, responses had waned or elderly immune systems had deteriorated sufficiently to allow for robust icGD03 replication and pathogenesis. It is likely that vaccine approaches that induce less robust neutralizing responses like DNA and killed vaccines, might completely fail in protecting against icGD03 challenge. To rectify this problem, booster vaccines should be considered in senescent populations or new vaccine formulations be assembled that include S glycoprotein determinants that protect against epidemic and zoonotic forms of SARS-CoV. Future studies will evaluate single VRP regiments that include either the VRP-S or VRP-N candidate vaccines separately, as this approach may enhance overall protection in VRP-S vaccinated animals. Clearly, the availability of SARS-CoV strains harboring zoonotic S glycoproteins will provide important future reference inoculums for evaluating the robustness of new vaccine candidates in animals.

The growing recognition that human coronaviruses can produce significant pulmonary diseases in humans places the SARS-CoV in an excellent position to serve as a premiere model system to elucidate the molecular mechanisms governing human coronavirus pathogenesis in the lung and to identify the components of protective immunity that prevent severe lower respiratory tract infections in humans and animals. Current animal models for SARS-CoV usually display little clinical disease and rarely cause death, hampering measurements of vaccine efficacy against severe infection and disease. Animal models that mirror the immunopathological and pathophysiological changes noted in humans are needed for future vaccine testing.
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