The explosive spread of the COVID-19 pandemic has made the development of countermeasures an urgent global priority (1–8). However, our understanding of the immunopathogenesis of SARS-CoV-2 is currently very limited. In particular, it is currently not known whether SARS-CoV-2 infection induces natural immunity that protects against re-exposure in humans. Such information is critical for vaccine strategies, epidemiologic modeling, and public health approaches. To explore this question, we developed a rhesus macaque model of SARS-CoV-2 infection, and we assessed virologic, immunologic, and pathologic features of infection as well as protective immunity against rechallenge.

Virology and immunology of SARS-CoV-2 infection in rhesus macaques

We inoculated 9 adult rhesus macaques (6-12 years old) with a total of $1.1 \times 10^6$ PFU (Group 1; N = 3), $1.1 \times 10^5$ PFU (Group 2; N = 3), or $1.1 \times 10^4$ PFU (Group 3; N = 3) SARS-CoV-2, administered as 1 ml by the intranasal (IN) route and 1 ml by the intratracheal (IT) route. Following viral challenge, we assessed viral RNA levels by RT-PCR in multiple anatomic compartments. We observed high levels of viral RNA in bronchoalveolar lavage (BAL) (Fig. 1A) and nasal swabs (NS) (Fig. 1B), with a median peak of 6.56 (range 5.32-8.97) log_{10} RNA copies/ml in BAL and a median peak of 7.00...
(range 5.06-8.55) log_{10} RNA copies/swab in NS. Viral RNA in NS increased in all animals from day 1 to day 2, suggesting viral replication. Viral RNA peaked on day 2 and typically resolved by day 10-14 in BAL and by day 21-28 in NS. Following day 2, viral loads in BAL and NS appeared comparable in all groups regardless of dose. Viral RNA was undetectable in plasma (fig. S1). Animals exhibited modestly decreased appetite and responsiveness suggestive of mild clinical disease (fig. S2) as well as mild transient neutropenia and lymphopenia in the high dose group (fig. S3), but fever, weight loss, respiratory distress, and mortality were not observed.

To help differentiate input challenge virus from newly replicating virus, we developed an RT-PCR assay to assess E gene subgenomic mRNA (sgmRNA). E gene sgmRNA reflects viral replication cellular intermediates that are not packaged into virions and thus represent putative replicating virus in cells (9). Compared with total viral RNA (Fig. 1B), sgmRNA levels were lower in NS on day 1 with a median of 5.11 (range <1.70-5.94) log_{10} sgmRNA copies/swab, but then increased by day 2 to a median of 6.50 (range 4.16-7.81) log_{10} sgmRNA copies/swab (Fig. 1C).

We next evaluated SARS-CoV-2-specific humoral and cellular immune responses in these animals. All 9 macaques developed binding antibody responses to the SARS-CoV-2 Spike (S) protein by ELISA (Fig. 2A) and neutralizing antibody (NAb) responses using both a pseudovirus neutralization assay (Fig. 2C) and a live virus neutralization assay (Fig. 2D). Cellular immune responses of multiple subclasses were observed against the receptor binding domain (RBD), the prefusion S ectodomain (S), and the nucleocapsid (N), and antibodies exhibited diverse effector functions, including antibody-dependent complement deposition (ADCD), antibody-dependent cellular phagocytosis (ADCP), antibody-dependent neutrophil phagocytosis (ADNP), and antibody-dependent NK cell degranulation (NK CD107a) and cytokine secretion (NK MIP1β, NK IFNγ) (I3) (Fig. 2D). Cellular immune responses to pooled S peptides were observed in the majority of animals by IFN-γ ELISPOT assays on day 35, with a trend toward lower responses in the lower dose groups (Fig. 2E). Intracellular cytokine staining assays demonstrated induction of both S-specific CD8+ and CD4+ T cell responses (Fig. 2F).

**SARS CoV-2 infection induces acute viral interstitial pneumonia in rhesus macaques**

Only limited pathology data from SARS-CoV-2 infected humans are currently available. To assess the pathologic characteristics of SARS-CoV-2 infection in rhesus macaques, we inoculated 4 animals with 1.1 x 10^6 PFU virus by the IN and IT routes as above and necropsied them on day 2 (N = 2) and day 4 (N = 2) following challenge. Multiple regions of the upper respiratory tract, lower respiratory tract, gastrointestinal tract, lymph nodes, and other organs were harvested for virologic and pathologic analyses. High levels of viral RNA were observed in all nasal mucosa, pharynx, trachea, and lung tissues, and lower levels of virus were found in the gastrointestinal tract, liver, and kidney (fig. S4). Viral RNA was readily detected in paratracheal lymph nodes but was only sporadically found in distal lymph nodes and spleen (fig. S4).

Upper airway mucosae, trachea, and lungs were paraformaldehyde fixed, paraffin embedded, and evaluated by histopathology. On day 2 following challenge, both necropsied animals demonstrated multifocal regions of inflammation and evidence of viral pneumonia, including expansion of alveolar septae with mononuclear cell infiltrates, consolidation, and edema (Fig. 3, A and B). Regions with edema also contained numerous polymorphonuclear cells, predominantly neutrophils. Terminal bronchiolar epithelium was necrotic and sloughed with clumps of epithelial cells detected within airways and distally within alveolar spaces (Fig. 3, C and D) with formation of occasional bronchiolar epithelial syncytial cells (Fig. 3E). Hyaline membranes were occasionally observed within alveolar septa, consistent with damage to type I and type II pneumocytes (Fig. 3F). Diffusely reactive alveolar macrophages filled alveoli, and some were multinucleated and labeled positive for nucleocapsid by immunohistochemistry (Fig. 3G). Alveolar lining cells (pneumocytes) also prominently labeled positive for nucleocapsid (Fig. 3H).

Multifocal clusters of virus infected cells were present throughout the lung parenchyma, as detected by immunohistochemistry and in situ RNA hybridization (RNAscope) (I4, I5) (Fig. 3I). Both positive-sense and negative-sense viral RNA was observed by RNAscope (fig. S5), suggesting viral replication in lung tissue. The dense inflammatory infiltrates included polymorphonuclear cells detected by endogenous myeloperoxidase staining (MPO), CD68 and CD163 positive macrophages, CD4+ and CD8+ T lymphocytes, and diffuse up-regulation of the type 1 interferon gene MX1 (fig. S6). SARS-CoV-2 infection led to a significant increase in polymorphonuclear cell infiltration of lung alveoli compared with uninfected animals (P = 0.0286) as well as extensive MX1 staining in approximately 30% of total lung tissue (P = 0.0286) (fig. S7). Inflammatory infiltrates were also detected in the respiratory epithelial submucosa of larger airways with transmigration of inflammatory cells into bronchiolar lumen (Fig. 3J). Ciliated epithelial cells also stained positive for both SARS CoV-2 RNA (Fig. 3K) and SARS nucleocapsid.
Local immunity was stimulated by a rapid anamnestic immune response, including increased virus-specific ELISA titers (P = 0.0003, two-sided Mann-Whitney test), pseudovirus NAb titers (P = 0.0003), and live virus NAb titers (P = 0.0003) as well as a trend toward increased IFN-γ ELISPOT responses (P = 0.1837) by day 7 after rechallenge (Fig. 6). In particular, NAb titers were markedly higher on day 14 following rechallenge compared with day 14 following primary challenge (P < 0.0001, two-sided Mann-Whitney test) (fig. S11). All animals developed anamnestic antibody responses following rechallenge, regardless of the presence or absence of residual viral RNA or sgRNA in BAL or NS, and thus we speculate that the protective efficacy against rechallenge was mediated by rapid immunologic control.

Discussion

Individuals who recover from certain viral infections typically develop virus-specific antibody responses that provide robust protective immunity against re-exposure, but some viruses do not generate protective natural immunity, such as HIV-1 (17). Human challenge studies for the common cold coronavirus 229E have suggested that there may be partial natural immunity (18). However, there is currently no data whether humans who have recovered from SARS-CoV-2 infection are protected from re-exposure (World Health Organization, Scientific Brief, April 24, 2020; https://www.who.int/news-room/commentaries/detail/immunity-passports-in-the-context-of-covid-19). This is a critical issue with profound implications for vaccine development, public health strategies, antibody-based therapeutics, and epidemiologic modeling of herd immunity. In this study, we demonstrate that SARS-CoV-2 infection in rhesus macaques provided protective efficacy against SARS-CoV-2 rechallenge.

We developed a rhesus macaque model of SARS-CoV-2 infection that recapitulates many aspects of human SARS-CoV-2 infection, including high levels of viral replication in the upper and lower respiratory tract (Fig. 1) and clear
pathologic evidence of viral pneumonia (Figs. 3 and 4). Histopathology, immunohistochemistry, RNAscope, and CyCIF imaging demonstrated multifocal clusters of virus infected cells in areas of acute inflammation, with evidence for virus infection of alveolar pneumocytes and ciliated bronchial epithelial cells. These data suggest the utility of rhesus macaques as a model for SARS-CoV-2 infection for testing vaccines and therapeutics and for studying immunopathogenesis, and our findings complement and extend recently published data in cynomolgus macaques (I9). However, neither nonhuman primate model led to respiratory failure or mortality, and thus further research will be required to develop a nonhuman primate model of severe COVID-19 disease.

SARS-CoV-2 infection in rhesus macaques led to humoral and cellular immune responses (Fig. 2) and provided protection against rechallenge (Fig. 5). Residual low levels of subgenomic mRNA in nasal swabs in a subset of animals (Fig. 5) and anamnestic immune responses in all animals (Fig. 6) following SARS-CoV-2 rechallenge suggest that protection was mediated by immunologic control and likely was not sterilizing.

Given the near-complete protection in all animals following SARS-CoV-2 rechallenge, we were unable to determine immune correlates of protection in this study. SARS-CoV-2 infection in rhesus monkeys resulted in the induction of neutralizing antibody titers of approximately 100 by both a pseudovirus neutralization assay and a live virus neutralization assay, but the relative importance of neutralizing antibodies, other functional antibodies, cellular immunity, and innate immunity to protective efficacy against SARS-CoV-2 remains to be determined. Moreover, additional research will be required to define the durability of natural immunity.

In summary, SARS-CoV-2 infection in rhesus macaques induced humoral and cellular immune responses and provided protective efficacy against SARS-CoV-2 rechallenge. These data raise the possibility that immunologic approaches to the prevention and treatment of SARS-CoV-2 infection may in fact be possible. However, it is critical to emphasize that there are important differences between SARS-CoV-2 infection in macaques and humans, with many parameters still yet to be defined in both species, and thus our data should be interpreted cautiously. Rigorous clinical studies will be required to determine whether SARS-CoV-2 infection effectively protects against SARS-CoV-2 re-exposure in humans.

REFERENCES AND NOTES


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**SUPPLEMENTARY MATERIALS**

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Materials and Methods

Table S1

Figs. S1 to S11

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Fig. 1. Viral loads in SARS-CoV-2 challenged rhesus macaques. Rhesus macaques were inoculated by the intranasal and intratracheal route with $1.1 \times 10^6$ PFU (Group 1; $N = 3$), $1.1 \times 10^5$ PFU (Group 2; $N = 3$), or $1.1 \times 10^4$ PFU (Group 3; $N = 3$) SARS-CoV-2. (A) Log$_{10}$ viral RNA copies/ml (limit 50 copies/ml) were assessed in bronchoalveolar lavage (BAL) at multiple timepoints following challenge. (B and C) Log$_{10}$ viral RNA copies/swab (B) and Log$_{10}$ sgRNA copies/swab (limit 50 copies/swab) (C) were assessed in nasal swabs (NS) at multiple timepoints following challenge. Red horizontal bars reflect median viral loads.
Fig. 2. Immune responses in SARS-CoV-2 challenged rhesus macaques. (A to D) Humoral immune responses were assessed following challenge by binding antibody ELISA (A), pseudovirus neutralization assays (B), live virus neutralization assays (C), and systems serology profiles (D) including antibody subclasses and effector functions to receptor binding domain (RBD), soluble spike (S) ectodomain, and nucleocapsid (N) proteins on day 35. Antibody-dependent complement deposition (ADCD), antibody-dependent cellular phagocytosis (ADCP), antibody-dependent neutrophil phagocytosis (ADNP), and antibody-dependent NK cell degranulation (NK CD107a) and cytokine secretion (NK MIP1β, NK IFNγ) are shown. (E and F) Cellular immune responses were also assessed following challenge by IFN-γ ELISPOT assays (E) and multiparameter intracellular cytokine staining assays (F) in response to pooled S peptides. Red and horizontal bars reflect mean responses.
Fig. 3. SARS-CoV-2 induces acute viral interstitial pneumonia. (A to F) H&E sections of fixed lung tissue from SARS-CoV-2 infected rhesus macaques 2 days following challenge showing interstitial edema and regional lung consolidation (A), intra-alveolar edema and infiltrates of neutrophils (B), bronchiolar epithelial sloughing and necrosis [(C) and (D)], bronchiolar epithelial syncytial cell formation (E), and hyaline membranes within alveolar septa (F). (G and H) IHC for SARS nucleocapsid showing virus infected cells within interstitial spaces including a viral syncytial cell within the lumen (G) and virus infected alveolar lining cells (H). (I) Inflammatory infiltrate showing multiple cells containing SARS-CoV-2 RNA by RNAscope in situ hybridization. (J to L) bronchial respiratory epithelium showing inflammation within the submucosa and transmigration of inflammatory cells into the ciliated columnar respiratory epithelium of a bronchus (J). SARS-CoV-2 RNA (K), and SARS nucleocapsid (L). Scale bars (A) = 200 microns; (C, I, K-L) = 100 micron; (G) = 50 micron; (B, D-F, J) = 20 microns, and (H) = 10 microns. H&E = hematoxylin and eosin; IHC = immunohistochemistry; RNAscope = SARS-CoV-2 RNA staining.
Fig. 4. SARS-CoV-2 infects alveolar epithelial cells in rhesus macaques. Cyclic immunofluorescence (CyCIF) staining of fixed lung tissue from SARS-CoV-2 infected rhesus macaques 2 days following challenge. (A) Whole slide image of a lung stained with Hoechst 33342 to visualize cell nuclei (greyscale); regions of nuclear consolidation (arrows), and foci of viral replication (box) are highlighted. (B) Higher magnification image of inset box in (A) showing staining for SARS nucleocapsid protein (SARS-N; green) and cell nuclei (grey scale). (C) Higher magnification image of inset box in (B) showing SARS-N (green) and cell nuclei (blue). (D) Bright-field IHC for SARS-N from corresponding lung region depicted in (C). (E to K) CyCIF staining for DNA (blue; all panels) and SARS-N [(E), (F), and (H-K); green], CD206 [(E) and (K); magenta], pan-cytokeratin (pan-CK) [(G) and (H); red], CD68 (I; yellow), or Iba-1 (J; greyscale) showing virus infected epithelial cells and macrophages near an infected epithelial cell. Scale bars (F-K) = 50 microns.
Fig. 5. Viral loads following SARS-CoV-2 rechallenge in rhesus macaques. On day 35 following initial infection (Fig. 1), rhesus macaques were rechallenged with SARS-CoV-2 by the intranasal and intratracheal route with $1.1 \times 10^6$ PFU (Group 1; N = 3), $1.1 \times 10^5$ PFU (Group 2; N = 3), or $1.1 \times 10^4$ PFU (Group 3; N = 3). Three naïve animals were included as a positive control in the rechallenge experiment. (A) Log$_{10}$ viral RNA copies/ml (limit 50 copies/ml) were assessed in bronchoalveolar lavage (BAL) at multiple timepoints following rechallenge. One of the naïve animals could not be lavaged. (B) Comparison of viral RNA in BAL following primary challenge and rechallenge. (C and E) Log$_{10}$ viral RNA copies/ml (C) and log$_{10}$ sgmRNA copies/swab (limit 50 copies/ml) (E) were assessed in nasal swabs (NS) at multiple timepoints following rechallenge. (D and F) Comparison of viral RNA (D) and sgmRNA (F) in NS following primary challenge and rechallenge. Red horizontal bars reflect median viral loads. P-values reflect two-sided Mann-Whitney tests.
Fig. 6. Anamnestic immune responses following SARS-CoV-2 rechallenge in rhesus macaques. Binding antibody ELISAs, pseudovirus neutralization assays, live virus neutralization assays, and IFN-γ ELISPOT assays are depicted prior to and 7 days following SARS-CoV-2 rechallenge. Red lines reflect mean responses. P-values reflect two-sided Mann-Whitney tests.