Comparison of SARS-CoV-2 infection in two non-human primate species: rhesus and cynomolgus macaques


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Abstract

SARS-CoV-2 is a coronavirus that sparked the current COVID-19 pandemic. To stop the shattering effect of COVID-19, effective and safe vaccines, and antiviral therapies are urgently needed. To facilitate the preclinical evaluation of intervention approaches, relevant animal models need to be developed and validated. Rhesus macaques (Macaca mulatta) and cynomolgus macaques (Macaca fascicularis) are widely used in biomedical research and serve as models for SARS-CoV-2 infection. However, differences in study design make it difficult to compare and understand potential species-related differences. Here, we directly compared the course of SARS-CoV-2 infection in the two genetically closely-related macaque species.

After inoculation with a low passage SARS-CoV-2 isolate, clinical, virological, and immunological characteristics were monitored. Both species showed slightly elevated body temperatures in the first days after exposure while a decrease in physical activity was only observed in the rhesus macaques and not in cynomolgus macaques. The virus was quantified in tracheal, nasal, and anal swabs, and in blood samples by qRT-PCR, and showed high similarity between the two species. Immunoglobulins were detected by various enzyme-linked immunosorbent assays (ELISAs) and showed seroconversion in all animals by day 10 post-infection. The cytokine responses were highly comparable between species and computed tomography (CT) imaging revealed pulmonary lesions in all animals. Consequently, we concluded that both rhesus and cynomolgus macaques represent valid models for evaluation of COVID-19 vaccine and antiviral candidates in a preclinical setting.

Author summary
SARS-CoV-2 infection can have a wide range of symptoms. It can cause asymptomatic or mild disease, but can also have a severe, potentially deadly outcome. Vaccines and antivirals will therefore be crucial in fighting the current COVID-19 pandemic. For testing these prophylactic and therapeutic treatments, and investigating the progression of infection and disease development, animal models play an essential role. In this study, we compare the course of SARS-CoV-2 infection in rhesus and cynomolgus macaques. Both species showed moderate disease symptoms as shown by pulmonary lesions by CT imaging. Shedding of infectious virus from the respiratory system was also documented. This study provides a detailed description of the pathogenesis of a low-passage SARS-CoV-2 isolate in two macaque models and suggests that both species represent an equally good model in research for both COVID-19 prophylactic and therapeutic treatments.
Introduction

At the end of 2019, the first cases of coronavirus disease 2019 (COVID-19) were described in the Wuhan region, China [1]. Since then, the causative agent of COVID-19, the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has spread rapidly across the globe, leading the World Health Organization to officially declare COVID-19 a pandemic on 11 March 2020. To halt the devastating impact of this disease and the ongoing pandemic, vaccines and antiviral therapies are desperately needed, as well as fundamental knowledge to understand the mode of infection and its associated pathologies. Hence, enormous efforts are initiated to develop a wide range of COVID-19 vaccines.

Between humans, transmission mainly occurs via aerosolized droplets after sneezing or coughing or via direct contact with contaminated surfaces. Similar to the related SARS-CoV, SARS-CoV-2 enters the body via docking with its spike (S) protein to the angiotensin-converting enzyme-2 (ACE2) receptor protein [2]. ACE2 is abundantly expressed on cells of the respiratory system but also on a variety of other organ tissues, including those of the brain and the gastrointestinal tract [3]. This wide-spread distribution of the ACE2 receptor may explain the complex clinical picture of COVID-19. The spectrum of COVID-19 ranges from asymptomatic or mild disease, via flu-like illness to a severe, potentially deadly disease [4].

The most typical hallmark of severe COVID-19 is pneumonia leading to respiratory failure, which occurs after the onset of dyspnea and hypoxemia [5]. However, COVID-19 patients may also suffer from a variety of other symptoms, including amongst other gastrointestinal symptoms, neurological disorders, coagulopathy, and cardiac injury [5-8].

Animal models to investigate the progression of infection and disease development, and to evaluate prophylactic and therapeutic treatment options, are essential to warrant progress in SARS-CoV-2 fundamental and applied research. Different animal species have shown their
value in SARS-CoV-2 research. Originally developed to study SARS-CoV [9], transgenic mice that express the ACE2 receptor elucidated various aspects of COVID-19 [10, 11]. Rodent species, such as the Syrian hamster, turned out to be susceptible to infection with SARS-CoV-2, and hamsters were subsequently utilized to test a live-attenuated YF17D-vectored SARS-CoV2 vaccine candidate [12, 13]. Ferrets represent an established animal model to study the pathology and transmission of respiratory viruses, like influenza viruses [14]. During the course of SARS-CoV infection, viral replication was documented in the upper and lower respiratory tract [15]. Transmission of SARS-CoV-2 among ferrets via either air or direct contact has been shown recently, where virus was shed in nasal washes, saliva, feces, and urine of infected animals [16, 17]. Notwithstanding the great importance of rodent and ferret models, also non-human primates (NHPs) will likely play a pivotal role in COVID-19 research. Like with SARS-CoV, NHPs are susceptible to infection with human SARS-CoV-2. Due to their close phylogenetic relationship, NHPs and humans share many immunological and pathological characteristics [18-20]. This makes NHPs suitable for preclinical evaluation of vaccines and antiviral or immunomodulatory compounds to combat SARS-CoV-2. So far, five species of NHPs; rhesus and cynomolgus macaques, common marmosets (Callithrix jacchus), African green monkeys (Chlorocebus sabaeus), and sacred baboons (Papio hamadryas) have featured in SARS-CoV-2 studies. Common marmosets, a small New World monkey species, are susceptible to infection with SARS and MERS coronaviruses [21, 22], but appear to be relatively resistant to infection with SARS-CoV-2 as only low levels of virus replication could be measured [23, 24]. African green monkeys presented robust virus replication and also showed evidence of respiratory disease [25, 26], in contrast to baboons where variable levels of virus replication were measured [24]. The two most widely-used NHP species in biomedical research are rhesus macaques and cynomolgus macaques. Both macaque species had already
proven their value in research on the related coronaviruses that caused the SARS and MERS epidemics [27, 28], and thus are considered relevant NHP models for preclinical COVID-19 studies. Cynomolgus macaques have been deployed in studies describing aspects of SARS-CoV-2 pathogenesis [23, 29, 30], and have been utilized to evaluate the efficacy of hydroxychloroquine as an antiviral compound [31]. Rhesus macaques have also been applied in COVID-19 pathogenesis studies [22, 24, 32, 33], and to test the efficacy of remdesivir in the treatment of SARS-CoV-2 infection [34]. Additionally, several prototype COVID-19 vaccine candidates have received their first efficacy evaluation in the rhesus macaque model [35-42]. Some research groups [23, 24] shed light on the heterogeneity in SARS-CoV-2 infection and investigated disease progression in different NHP species. Most of these studies were conducted by different research teams, and a controlled comparative approach is lacking thus far.

In other NHP disease models, like those developed for AIDS, TB, and influenza research, the choice of macaque (sub)species can influence the disease outcome considerably [43-47]. The choice of a specific NHP species for research on a new and complex disease, like COVID-19, is therefore not a trivial one and the key question which macaque species is best suited to investigate specific aspects of COVID-19 research needs to be answered. To address this issue, we compared SARS-CoV-2 replication in rhesus and cynomolgus macaque species and monitored signs of COVID-19-like disease symptoms for three weeks after infection. The macaques were infected in parallel with the same virus stock, received completely identical treatment, and the course of infection was followed using the same analyses, including monitoring of lung pathology using computed tomography (CT), and continuous telemetric recording of body temperature and activity of the animals.
Results

Infection of macaques with SARS-CoV-2

After administration of the virus in the upper trachea and nose, levels of viral RNA were detectable in the tracheal and nasal swabs of all monkeys at day 1 pi. Viral RNA remained evident in swab samples for several days. In the tracheal swab sample of rhesus macaque R14002, viral RNA was first time below the detection time at 10 days pi. (Fig 1A, S1 Table). The individual variation of SARS-CoV-2 RNA levels detected in the macaques, regardless of species, was considerable. Peak viral RNA levels in the trachea varied between $1.7 \times 10^4$ copies/ml (R15096; day 1 pi) and $1.8 \times 10^8$ copies/ml (J16017; day 2 pi). The time frame in which viral genetic material could be detected varied from only one day (R15096; day 1 pi) up to day 10 pi. (animal R14002, RNA in the trachea).

Peak viral loads detected in nasal swabs were generally lower than levels observed in the throat samples and did not exceed $9.5 \times 10^4$ copies/ml (R15090; day 1 pi). The high virus loads measured in the first two days post-infection may suggest that some remaining RNA from the original inoculum was still present. However, in all macaques, viral RNA was also isolated from nasal swabs at later time points, showing that SARS-CoV-2 was excreted via the nose, and thus indicative for viral replication. The total viral RNA production over time is shown in Fig 1B.

The patterns of viral RNA detection in swabs also varied between individuals. The most outstanding observation was made for cynomolgus macaque J16017 that was positive in the nose at day 1 pi, then had no detectable viral RNA for a period of three days, but later the animal became again positive in the nose swabs for three consecutive days. Other animals (R15096, J16004, J16012, and Ji40805) also became PCR-positive again after a period of one or more days characterized by undetectable levels. In the anal swabs, viral genetic material was rarely detected. Only at a few time points, three macaques tested positive, with a
maximum viral RNA load of $3 \times 10^3$ copies/ml at day 1 pi., namely in cynomolgus macaque J16017. One animal tested positive for viral RNA in blood at a single time point; R15080 at day 5 pi. (S1 Table). Notably, no significant differences in viral RNA loads were calculated between the macaque species (Fig 1B).

**Body temperature, activity, clinical symptoms and blood parameters after SARS-CoV-2 infection**

Body temperature and activity of each animal was continuously monitored using a Physiotel Digital telemetric device during the entire study. Upon infection, elevated body temperatures were measured in both macaque species, which could be correlated to the episodes of viral replication in the nose and trachea as was evidenced by qRT-PCR. In Fig 2, we show the body temperature alterations from the baseline during the study. In both groups of animals, the body temperature was significantly higher during the first two weeks after infection as compared to later time points (Fig 2). The temperature curves for the individual animals are depicted in the supplementary data (S1 Fig). The group of cynomolgus macaques showed elevated body temperature in the first 8 to 10 days following infection. This is in contrast with the measurements of the rhesus macaques where no substantial rise in temperature was measured, except for two animals (R14002 and R15090) that showed a sudden peak in body temperature of 0.7°C at day 8 pi.

The activity curves measured in the 3-weeks observation period for all individuals are documented in the S2A Fig. The cumulative activity scores in the first 2 weeks pi. were compared with activity scores in the last week of the study period (S2B Fig). The paired t-test illustrated a significantly lower activity in all four rhesus macaques during the first period after
infection (p=0.0072), while this difference in cynomolgus macaques was less obvious and only found in 2 out of 4 macaques.

We applied a clinical scoring list to enumerate clinical symptoms that may be caused by the SARS-CoV-2 infection (S2 Table). The cumulative clinical scores per week did not exceed 50 (of a maximum 490 score per week; data not shown), confirming the absence of serious COVID-19-related symptoms. However, in the second week of infection, cynomolgus macaques showed more, but still mild, clinical symptoms than rhesus macaques. This was less evident during the first and third weeks, probably due to outlier clinical scores of individual animals (Fig 3).

Blood samples were analyzed for changes in cell subsets and in biochemical parameters. These data were related to a set of normal (standard) values derived from uninfected, healthy rhesus, and cynomolgus macaques from the same breeding colony. No significant deviations from the normal values were seen in blood cell subsets of the infected monkeys. C-reactive protein levels, which are increased in COVID-19 patients with pneumonia [48], were not found higher in infected macaques. In humans, acute kidney injury has been related to SARS-CoV-2 infection [49, 50], and elevated levels of serum creatinine and blood urea were detected in 10-15% of a cohort of COVID-19 patients [51]. Hence, we measured creatinine and urea levels in macaque blood samples at days 0, 5, 10, 14, and 22 pi., but did not find evidence of kidney malfunction in the infected, but otherwise seemingly healthy monkeys. Equally, depending on the severity of the disease, blood coagulation disorders, like highly elevated D-dimer levels, have been reported for patients [52, 53], but no elevated D-dimer levels were measured in either macaque species. Elevated levels of glucose and alanine transferase were measured in the first week pi. in the blood of most animals, and amylase was increased in one rhesus macaque, R15080 (S3 Fig).
Detection of lung lesions in macaques after SARS-CoV-2 infection.

Chest CTs of the macaques after infection revealed several manifestations of COVID-19 with a variable time course and lung involvement (Table 1). The most common lesion types that were found in both rhesus and cynomolgus macaques were ground glass opacities (GGO), consolidations, and crazy paving patterns (CCP) (Fig 4).

Table 1. CT scores of lung lesions in SARS-CoV-2-infected macaques

<table>
<thead>
<tr>
<th>Animals</th>
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Lung lesions (max. CT score 2/35) were already seen in CTs early after infection on day 2 in 5 out of 8 monkeys, three rhesus, and two cynomolgus macaques. Thereafter, lung involvement was seen in most animals and CT scores increased. Around days 8 and 10 pi., lung lesions were manifest in all animals, and in several macaques the coverage had increased (Table 1). Again, individual variations in lung pathology were considerable; for instance, rhesus macaque R15090 showed low CT scores of 2 only at days 2 and 8 pi, while rhesus macaque R15096 had positive CT scores at all time points after experimental infection, varying from 1 to 4. Cynomolgus macaque J16012 suffered from serious lung damage as early as day 4 pi (score 5) and had a positive chest CT at 8 out of 9 time points. In Fig 5, the cumulative CT scores over
the monitoring period are depicted. The increase in cumulative CT score was 0.692 points per
day (95% CI 0.657 to 0.726) and no difference was observed between rhesus and cynomolgus
macaques (P = 0.2708).

Immune response to SARS-CoV-2 infection

Humoral immune responses developed relatively fast after infection (Fig 6 and S4 Fig). Total
immunoglobulin (Ig) in serum was detected by the DR-ELISA. The antibody response directed
to the nucleoprotein (N) of SARS-CoV-2 became evident between day 10 and 12 pi., and
continued to rise thereafter, but with individual variations in total Ig levels and patterns. While
Ig levels in several animals, both rhesus and cynomolgus macaques, continued to rise until day
21 pi., the Ig levels in others already showed first signs of a decline at day 17 pi. The total Ig
pattern seen in the sera of all macaques was reflected by the measurement of IgG directed to
the N protein in the same sera. Notably, the development of IgM titers was barely detected
in the longitudinal serum samples. In sera from only one animal, cynomolgus macaque
J106012, IgM was detectable, beginning at day 12 pi. but titers already started to decline to
baseline levels at the end of the study at day 22 pi.

Cytokine and chemokine measurements

Serum levels of 13 cytokines and chemokines were measured to examine the nature of the
inflammatory response triggered by the infection with SARS-CoV-2. We investigated if any
species differences could be distinguished in the inflammatory response to infection. All
metadata obtained from the individual animals are depicted in S5 Fig. Directly upon infection,
IP-10 and MCP-1 levels peaked in sera of all cynomolgus macaques. The IP-10 peak at day 2
pi. quickly returned to baseline values. In contrast, in rhesus macaques, only a minor rise in
IP-10 levels was observed at the same time interval. In the same time span, but in both macaque species, the levels of chemokine MCP-1 (CCL2) increased. Levels of other chemokines, like eotaxin (CCL11), MIP-1α (CCL3), MIP-1β (CCL4), but also the cytokines IL-6 and IFN-γ dropped in the first week after infection and started rising around two weeks pi. This decline of particular serum cytokine and chemokine levels coincided with the time period that viral RNA was detectable in nasal and tracheal swabs, indicative for virus production. A different pattern was seen for RANTES (CCL5) and TNFα proteins; serum levels were high in the first 2 weeks after infection and then started decreasing to undetectable levels. RANTES serum concentrations also showed an initial drop very early after infection. This was most prominent in the cynomolgus macaques where RANTES levels dropped to zero on day 1 pi. and started to increase at day 6 pi. Post-infection levels of I-TAC (CXCL11), MIG (CXCL9), and IL-8 (CXCL8) were below detection or were not influenced by the SARS-CoV-2 infection.

Discussion

In humans, COVID-19 was initially regarded as a respiratory disease, but now it is clear that individuals that succumb to this disease can display a complex array of pathologies that cover a broad spectrum of symptoms. To sort out the factors leading to the different COVID-19 manifestations, animal models are essential. Due to their similarity to humans, specifically, non-human primates can play a pivotal role in this type of preclinical research. To best appreciate the potential of the various macaque species as SARS-CoV-2 infection models, a thorough characterization of the course of infection is needed. The comparative study reported in this communication contributes to the knowledge, but more importantly validates the macaque models that are currently in use in SARS-CoV-2 research.
Unlike in some human patients, we found no evidence for renal involvement or coagulation disorders in our monkeys. Equally, increasing C-reactive protein (CRP) levels are a marker in the early diagnosis of pneumonia [48], but different from humans; these levels were not subject to change in the infected macaques during the 3-weeks monitoring period. A direct comparison with humans is hampered by the fact that the macaques were infected, but seemingly healthy, while most published findings in humans were obtained from COVID-19 patients in various stages of disease. Unbiased measurement of the body temperature and activity of each animal was done using a telemetric device implanted in the abdomen. Monitoring by telemetry is an important asset as in both macaque species a notable difference in body temperature was recorded in the first 2 weeks (period of active virus replication) as compared to the third week. Significant differences in animal activity indicated that SARS-CoV-2 infection also influenced the well-being of the animals without causing obvious clinical symptoms. Notably, the elevation in body temperature on the first day of infection, as was reported by Munster et al., was not seen in our study, possibly pointing to a potential difference in the sampling method. While we used 24/7 monitoring, in the study described by Munster et al., temperature was measured only on selected days on anesthetized animals [54]. However, variation can also be due to a difference between the challenge viruses used, the methods used for virus inoculation, or the origin and adaptation of the animals used. Antibody responses were detectable in all animals after infection. Interestingly, no IgM was detected in 7 out of 8 animals. This result was confirmed by a second, in-house developed IgM-ELISA, but instead, the SARS-CoV-2 S protein was used as coating antigen. This approach excludes a technical flaw in the serological assay used. We cannot explain the lack of IgM response to infection, but similar observations were made in an infection study using African Green monkeys. Hartman et al. did detect IgM, but the titers were very low, not exceeding
Log_{10} above the assay limit of detection [26]. In contrast, the development of IgG accurately followed the course of virus infection, as it became first detectable within one week after the virus had become undetectable in sera by RT-PCR. This was in line with findings of others [25, 26, 29].

In a subset of COVID-19 patients, the acute respiratory syndrome coincides with a ‘cytokine storm’ or hypercytokinemia, which eventually can result in multi-organ failure [55]. Patients with severe COVID-19 symptoms on intensive care units had significantly elevated plasma levels of proinflammatory cytokines, like IL-2, IL-7, IL-10, GSCF, IP-10, MCP-1, MIP-1α, and TNF-α [56]. In this study, macaques did not show overt disease symptoms of COVID-19, but certain cytokines, like IL-6, IFN-γ, MIP-1α, and MIP-1β increased in the plasma of both macaque species, indicating the involvement of the chemokine system during SARS-CoV-2 infection.

The cytokine profiles after SARS-CoV-2 were highly comparable between species, except for IP-10 and MCP-1, suggesting differential involvement of monocyte activation between the two species. The similarity in cytokine response after SARS-CoV-2 infection contrasts with observations made after infection of macaques with another respiratory virus, pandemic H1N1 influenza [47]. In that study, macaque species-specific cytokine responses (IL-6, MCP-1, IL-15, IL-1Ra, MIP-1α, and IL-8) were induced upon infection with pH1N1, highlighting the virus type-specific reaction of the chemokine system.

Unlike most published studies, we decided not to conduct a necropsy on animals early, 4-5 days, post-infection. At that time point after infection, evidence was found for acute viral interstitial pneumonia [30, 32, 34, 54]. Instead, we performed CT imaging to visualize lung pathology induced by SARS-CoV-2. In humans, the sensitivity of CT scanning for lung pathology is high (positive predictive value of 92%), but the type of lesions found are not COVID-19-specific, and can also be observed in a number of other infectious and non-infectious diseases.
In this study, we used purpose-bred NHPs with a well-documented health status and we could compare the scans with a CT obtained just before infection. Therefore, CT imaging provides a valuable tool to specifically monitor the progression of COVID-19-related lung pathology during the entire course of the study. Based on the criteria set to determine clinical severity [59], the macaques in our panel featured moderate disease levels as all eight individuals show levels of pneumonia. In another study using only cynomolgus macaques and using CT imaging as well, lesions were found as early as 2 days post-infection in infected animals [29]. Type-wise, the lung lesions described in that report were comparable to the ones in this communication, but they tend to be located deeper in the lungs. An explanation for this difference may be that the method of instillation of the virus is the underlying cause. Finch et al. administered the virus into each bronchus by direct bilateral primary post-carinal intrabronchial instillation, whereas we applied the virus relatively high in the trachea, just below the vocal cords. For similar reasons as reported by the same research team [29], we did not collect bronchoalveolar lavage (BAL) samples from our animals to avoid unwanted interventions in the natural infection process caused by SARS-CoV-2. Studies performed at our institute with respiratory viruses have shown that lung lavages can indeed significantly influence the infection process (manuscript in preparation). Instead of BAL samples, we collected tracheal swabs for virological analysis. The viral RNA loads, but also the temporal pattern of detection in swabs samples were like those observed in BAL samples from other studies [22, 24, 34, 38]. This demonstrates that tracheal swabs are a good alternative for BAL sampling. In addition, the collection of tracheal swabs is a less invasive technique that causes relatively minor discomfort to the animals.

In most SARS-CoV-2 studies in non-human primates, the animals are euthanized shortly after infection in the first week, or after a period of 3 weeks. The animals from this study were not...
euthanized to be able to perform re-infection studies or to monitor them for late clinical signs, or co-morbidities related to COVID-19.

We conclude that the course of SARS-CoV-2 infection of both macaque species is highly similar, indicating that they are equally suitable models to test vaccines and antivirals in a preclinical setting for safety and efficacy. The macaque model for SARS-CoV-2 infection in humans manifests important virological aspects of this disease in humans. Given their immunological and physiological resemblance to humans, NHPs likely will continue to play a pivotal role in research for both COVID-19 prophylactic and therapeutic treatments.

Materials and Methods

Ethics and Biosafety Statement

All housing and animal care procedures took place at the Biomedical Primate Research Centre (BPRC) in Rijswijk, the Netherlands. The BPRC is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) International and is compliant with European directive 2010/63/EU as well as the “Standard for Humane Care and Use of Laboratory Animals by Foreign Institutions” provided by the Department of Health and Human Services of the US National Institutes of Health (NIH, identification number A5539-01). Upon positive advice by the independent ethics committee (DEC-BPRC) the competent authorities (CCD, Central Committee for Animal Experiments) issued a project license (license AVD50200209404). Approval to start was obtained after further assessment of the detailed study protocol by the institutional animal welfare body (AWB) (in Dutch: Instantie voor Dierenwelzijn, IvD). All animal handlings were performed within the Department of Animal Science (ASD) according to Dutch law. ASD is regularly inspected by the responsible national authority (Nederlandse Voedsel- en Warenautoriteit, NVWA), and the AWB.
**Animals**

Four Indian-origin rhesus macaques and four cynomolgus macaques were used in this study (S3 Table). All macaques were mature, outbred animals, purpose-bred, and housed at the BPRC. The animals were in good physical health with normal baseline biochemical and hematological values. All were pair-housed with a socially compatible cage-mate in cages of at least 4 m³ with bedding to allow foraging and were kept on a 12-hour light/dark cycle. The monkeys were offered a daily diet consisting of monkey food pellets (Ssniff, Soest, Germany) supplemented with vegetables and fruit. Enrichment was provided daily in the form of pieces of wood, mirrors, food puzzles, and a variety of other homemade or commercially available enrichment products. Drinking water was available *ad libitum* via an automatic watering system. Animal Care staff provided daily visual health checks before infection, and twice-daily after infection. The animals were monitored for appetite, general behavior, and stool consistency. All possible precautions were taken to ensure the welfare and to avoid any discomfort to the animals. All experimental interventions (intratracheal and intranasal infection, swabs, blood samplings, and CT scans) were performed under anesthesia.

**Virus stock**

The animals were infected with the SARS-CoV-2 strain BetaCoV/BavPat1/2020. This strain was isolated from a patient who traveled from China to Germany, and an aliquot of a Vero E6 cell culture was made available through the European Virus Archive-Global (EVAg). The viral stock for the infection study was propagated on Vero E6 cells. For this study, a fifth passage virus stock was prepared with a titer of $3.2 \times 10^6$ TCID₅₀ per ml. The integrity of the virus stock was confirmed by sequence analysis.
Experimental infections and post-exposure study follow-up

Three weeks before the experimental infection, a Physiotel Digital device (DSI Implantable Telemetry, Data Sciences International, Harvard Bioscience, UK) was implanted in the abdominal cavity of each animal. This device allowed the continuous real-time measurement of the body temperature and the animals’ activity remotely using telemetry throughout the study.

At day 0, all animals were exposed to a dose of $1 \times 10^6$ TCID$_{50}$ of SARS-CoV-2, diluted in 5 ml phosphate buffered saline (PBS). The virus was inoculated via a combination of the intratracheal route (4.5 ml) and intranasal route (0.25 ml per nostril). Virus infection was monitored for 22 days, during which period the animals were checked twice-daily by the animal caretakers and scored for clinical symptoms according to a previously published, adapted scoring system [60] (S2 Table). A numeric score of 35 or more per observation point was predetermined to serve as an endpoint and justification for euthanasia. Every time an animal was sedated, the body weight was measured. Blood was collected using standard aseptic methods from the femoral vein at regular time points post-infection (pi). In parallel, tracheal, nasal, and anal swabs were collected using Copan FLOQSwabs (MLS, Menen, Belgium). Swabs were placed in 1 ml DMEM, supplemented with 0.5% bovine serum albumin (BSA), fungizone (2.5 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) and directly transported to the BSL3 lab.

Biochemistry and hematology analysis

Clinical biochemistry was performed using a Vetscan VS2 Chemical analyzer (Zoetis Benelux, Capelle aan de IJssel, The Netherlands) with the use of the Comprehensive Diagnostic profile.
This profile allows testing for alanine aminotransferase, albumin, alkaline phosphatase, amylase, calcium, creatinine, globulin, glucose, phosphorus, potassium, sodium, total bilirubin, total protein, and blood urea nitrogen. Hematology was performed using a Vetscan HM5 Hematology analyser (Zoetis Benelux, Capelle aan de IJssel, The Netherlands). C-reactive protein and D-dimer levels were measured using Cobas Integra 400 plus analyzer (Roche Diagnostics Nederland B.V.).

**Viral RNA detection**

Viral RNA was isolated from plasma and swab sample supernatants using a QIAamp Viral RNA Mini kit (Qiagen Benelux BV, Venlo, The Netherlands) following the manufacturer’s instructions. Viral RNA was reverse-transcribed to cDNA using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics BV, Almere, The Netherlands). Viral RNA was quantified by real-time quantitative RT-PCR specific for the RdRp gene of SARS-CoV-2, as described by Corman et al. [61]. The lower detection limit of the qRT-PCR was 3.6 viral RNA copies per reaction.

**Computed tomography**

Computed tomography (CT) data were acquired on several time points until day 22 (D0, 2, 4, 6, 8, 10, 12, 14, 16, 22 post-infection) using a MultiScan Large Field of View Extreme Resolution Research Imager (LFER) 150 PET-CT (Mediso Medical Imaging Systems Ltd., Budapest, Hungary). Animals were sedated with ketamine (10 mg/kg ketamine hydrochloride (Alfasan Nederland BV, Woerden, The Netherlands) combined with medetomidine hydrochloride, 0.05 mg/kg (Sedastart; AST Farma B.V., Oudewater, The Netherlands) to induce sedation and muscle relaxation, both applied intramuscularly (IM). The monkeys were positioned head first
supine (HFS) with the arms up and fixated in a vacuum pillow. A single CT of the thorax takes
35 seconds by which respiratory motion is inevitable, therefore, to mitigate the impact of
respiratory motion and improve the image quality, respiratory gating was applied. The
respiratory amplitude was detected with a gating pad placed next to the belly button. At the
end of the procedure, when the macaques returned to their home cage,
atipamezole
(Sedastop; AST Farma B.V., Oudewater, The Netherlands) was given IM (0.25 mg/kg).
For the final reconstruction, the expiration phases were exclusively used and manually
selected. A semi-quantitative scoring system for chest CT evaluation was used to estimate
SARS-CoV-2-induced lung disease [29, 62]. Quantification of the CTs was performed
independently by two persons based on the sum of the lobar scores. The degree of
involvement in each zone was scored as: 0 for no involvement, 1 for <5%, 2 for 5-24%, 3 for
25-49%, 4 for 50-74% and 5 for >=75% involvement. An additional increase or decrease of 0.5
was used to indicate alterations in CT density of the lesions. By using this scoring system, a
maximum score of 35 could be reached for the combined lobes per time point.

Assessment of cytokine and chemokine protein levels in serum
Cytokine and chemokine concentrations in sera of infected macaques, including IL-1β, IL-6,
CCL11 (Eotaxin), CXCL10 (IP-10), CXCL11 (I-TAC), CCL2 (MCP-1), CXCL9 (MIG), CCL3 (MIP-1α),
CCL4 (MIP-1β), CCL5 (RANTES), CXCL8 (IL-8), TNFα, and IFNγ, were determined using
LEGENDplex™ NHP Chemokine/Cytokine Panel (13-plex) (Biolegend, San Diego, CA, USA)
according to manufacturer’s instruction. Samples were measured on an LSRII FACS machine
and analyzed by using company software.

Antibody response
The total antibody response in macaque sera was analyzed in a double recognition enzyme-linked immunosorbent assay (DR-ELISA) that detects total immunoglobulins in serum and targeted to the SARS-CoV-2 nucleoprotein (N) protein as described by Hoste et al. [63] (INgezim COVID19 DR; Eurofins-INGENASA, Madrid, Spain). Additionally, two in-house indirect ELISAs were used to detect monkey immunoglobulin G (IgG) and immunoglobulin M (IgM) directed to SARS-CoV-2 N protein. Briefly, Corning® 96-Well High-Binding Flat-Bottom Microplates were coated with N protein and incubated overnight at 4°C in carbonate buffer, pH 9.6. After washing the wells with PBS pH 7.4/0.05% Tween 20 (PBST), a blocking step was performed with StabilZyme® SELECT Stabilizer (SurModics, Inc., Eden Prairie, MN, USA) for 1h at room temperature (RT). The plate was then incubated with serum samples diluted 1:100 in PBST with 2.5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1h at RT. Two positive, cut-off, and negative controls were added to each plate. For macaque-IgG detection, the wells were washed as described above and incubated with Peroxidase-conjugated AffiniPure Goat Anti-Human IgG, Fcy Fragment Specific (Jackson ImmunoResearch Laboratories, Inc., PA, USA) diluted 1:25 000 in StabilZyme® HRP Conjugate Stabilizer (SurModics), supplemented with 0.5M NaCl for 1h at RT. Finally, after a washing step, the plate was incubated for 15 min with the substrate (TMB-MAX, Neogen Corporation, Lexington, KY, USA) and the reaction was stopped by addition of 0.5 M sulfuric acid. The absorbance was measured at 450 nm using a SpectraMax M5 plate reader (Molecular Devices, LLC., San Jose, CA, USA). To detect macaque-IgM, the same protocol was followed, but the secondary antibody used was an anti-Monkey IgM (μ-chain specific)-Peroxidase antibody produced in goat (Sigma-Aldrich, Merck KGaA, MI, USA) at 1:5000 dilution.

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References:


Fig 1. Virus load in swab samples. (A) Viral RNA quantification in tracheal and nasal swabs of rhesus and cynomolgus macaques by qRT-PCR. The limit of quantification (154 RNA copies/mL) is indicated by the dotted line.
copies/ml) is indicated by the dotted horizontal line. (B) Total virus loads in throat and nose samples of macaques throughout the study. Horizontal bars represent geometric means. The sum of the viral copies was calculated rather than area under the curve (AUC), as AUC interpolates for time points when virus loads were not determined.

The different colors used for each animal as shown in the legend of 1A are used to denote the same individual in all figures of this manuscript. The group of rhesus macaques is indicated by yellow to red colors; cynomolgus macaques by green to blue. In each graph, rhesus macaques are depicted left and cynomolgus macaques right.

Fig 2. Body temperature during the study. The body temperature was measured by telemetry throughout the study. The daily average body temperature of rhesus and cynomolgus macaques was calculated and the deviations from baseline body temperature (in °C) are depicted. Measurements done during biotechnical handlings were omitted from the calculations.
Fig 3. Cumulative clinical scores. The cumulative clinical scores were calculated per week and per individual animal (day 1-7, 8-14 and 15-21). Horizontal bars represent medians.

Fig 4. Types of lung lesions detected via CT scans in SARS-CoV-2-infected macaques. (A) Ground glass opacities (GGO), (B) consolidations, and (C) crazy paving patterns (CCP).
Fig 5. **Cumulative CT scores.** Cumulative CT scores for each animal were calculated based on the CT scores depicted in Table 1.
Fig 6. Development of SARS-CoV-2 antibody response in rhesus and cynomolgus macaques. The humoral immune response was determined using (A) DR-ELISA measuring the total antibody response, (B) an IgG-specific IgG assay, and (C) an IgM-specific IgM serological test. Results are shown as S/P: sample to positive control ratio:

\[ S/P = \frac{\text{test sample} - \text{mean negative control}}{\text{mean positive control} - \text{mean negative control}} \quad \text{and} \quad \text{Units} = \frac{\text{test sample at } x \text{ dpi}}{\text{test sample at } 0 \text{ dpi}}. \]