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SHORT COMMUNICATION

Age-related rhesus macaque models of COVID-19

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Abstract

Background: Since December 2019, an outbreak of the Corona Virus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus (SARS-CoV-2) in Wuhan, China, has become a public health emergency of international concern. The high fatality of aged cases caused by SARS-CoV-2 was a need to explore the possible age-related phenomena with non-human primate models.

Methods: Three 3-5 years old and two 15 years old rhesus macaques were intratracheally infected with SARS-CoV-2, and then analyzed by clinical signs, viral replication, chest X-ray, histopathological changes and immune response.

Results: Viral replication of nasopharyngeal swabs, anal swabs and lung in old monkeys was more active than that in young monkeys for 14 days after SARS-CoV-2 challenge. Monkeys developed typical interstitial pneumonia characterized by thickened alveolar septum accompanied with inflammation and edema, notably, old monkeys exhibited diffuse severe interstitial pneumonia. Viral antigens were detected mainly in alveolar epithelial cells and macrophages.

Conclusion: SARS-CoV-2 caused more severe interstitial pneumonia in old monkeys than that in young monkeys. Rhesus macaque models infected with SARS-CoV-2

National Team for COVID-19 animal model development.

Pin Yu, Feifei Qi, Yanfeng Xu, Fengdi Li, Peipei Liu, and Jiayi Liu contributed equally to this work.

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provided insight into the pathogenic mechanism and facilitated the development of vaccines and therapeutics against SARS-CoV-2 infection.

KEYWORDS

pathogenicity, pneumonia, rhesus macaque model, SARS-CoV-2

1 | INTRODUCTION

In late December of 2019, a cluster of severe pneumonia cases caused by severe acute respiratory syndrome coronavirus (SARS-CoV-2) were reported from Wuhan, China.[1-3] It is critical to understand the pathogenicity of this virus for the prevention and treatment of Coronavirus disease 2019 (COVID-19). SARS-CoV-2 was isolated and classified as Betacoronavirus, belonging to the lineage B or subgenus Sarbecovirus, which also includes the human SARS coronavirus.[4,5] The full virus genome of SARS-CoV-2 has about an 89% nucleotide identity with bat-SL-CoVZC45, the Spike protein has a 78% nucleotide identity with the human SARS coronavirus, and an 84% nucleotide identity with the bat-SL-CoVZC45 coronavirus.[6,7] The SARS-infected rhesus monkey model was once established,[8] and now the SARS-CoV-2-infected rhesus monkey model can dynamically understand the pathogenicity of COVID-19. A more comprehensive understanding of the pneumonia caused by the SARS-CoV-2 infection is essential for developing more scientific and effective prevention, diagnosis, and treatment measures. Meanwhile, the high fatality of aged cases caused by SARS-CoV-2 was a need to explore the possible age-related phenomena with non-human primate models.[9]

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Rhesus macaques were raised in an animal biosafety level 3 (ABSL3) facility in accomplishment with all the infectious experiments. The Institutional Animal Care and Use Committee of the Institute of Laboratory Animal Science, Peking Union Medical College, reviewed and approved the programs in this study (BLL20001). Briefly, all the samples were collected after anesthetized with 10 mg/kg ketamine hydrochloride.

2.2 | Cells and Viruses

Professor Tan from the China Centers for Disease Control and Prevention (China CDC) warmly afforded the SARS-CoV-2 named strain HB-01. The complete genome for this SARS-CoV-2 had been put in to GISAID (BetaCoV/Wuhan/IVDC-HB-01/2020|EPI_ISL_402119). This strain was stored in the China National Microbiological Data Center (accession number NMDC10013001 and genome accession numbers MDC60013002-01). Vero cells were used for the reproduction of SARS-CoV-2 stocks. Vero cells are maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37°C, 5% CO_2. Titers for SARS-CoV-2 were resolved by a 50% tissue-culture infectious doses ($TCID_{50}$) assay.

2.3 | RNA extraction and RT-PCR

Total RNA was extracted from organs as described in previous report. The RNeasy Mini Kit (Qiagen) and the PrimerScript RT Reagent Kit (TaKaRa) were used following the manufacturer's instructions. RT-PCR reactions were run using the PowerUp SYBR Green Master Mix Kit (Applied Biosystems) following the cycling protocol: 50°C for 30 min, followed by 40 cycles at 95°C for 15 min, 94°C for 15 s, and 60°C for 45 s. The primer sequences used for RT-PCR targeting against the envelope (E) gene of SARS-CoV-2 were as follows: forward primer: 5'- TCGTTTCGGAAGAGACAGGT-3' and reverse primer: 5'-GCGCAGTAAGGATGGCTAGT-3'.

2.4 | Animal experiments

To develop a macaque animal infection model with SARS-CoV-2, three rhesus macaques (*Macaca mulatta*) (3-5 years old) and two 15 years old rhesus macaques were subsequently inoculated with SARS-CoV-2 by intratracheal routes containing 10^6 $TCID_{50}$/mL. On 0, 5, 6, 7, 8, 9, 11, 13, and 15-day post-inoculation (dpi), imageological examinations using X-rays were performed. On 3, 5, 7, 9, 11, and 14 dpi, the nasal, throat, and anal swabs were collected in 1 mL DMEM with 50 U/mL penicillin and 50 μg/mL streptomycin. The IPTT-300 temperature probes (BMDS), which were injected interscapularly into the macaques prior to the start of the experiment, were used to monitor the temperature. Blood samples were collected before the beginning of this research and on 0, 3, 4, 6, 7, 11, and 14 dpi for hematological analysis. Lung tissues were collected at 7 dpi. All sera were collected at 3, 7 and 14 dpi to test the IgG antibodies reactive with SARS-CoV-2 antigens. For the OD450 value, more than twice higher than the negative control was regarded as positive result.

2.5 | Preparation of homogenate supernatant

An electric homogenizer was employed to prepare tissue homogenates for 2 minutes 30 seconds in 1 mL of DMEM. The homogenates were centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant was gathered and stored at −80°C for viral titer.

TABLE 1 Clinical signs, viral replication and immune response from the monkeys following SARS-CoV-2 inoculation

Days post inoculation (DPI)	Clinical Signs								Viral Loads						Positive Specific antibody IgG (SARS-CoV-2)	
	Weight loss		Fever		Asthenia		Lethality		Nasal swabs		Throat swabs		Anal swabs			
	YG	EG	YG	EG	YG	EG	YG	EG	YG	EG	YG	EG	YG	EG	YG	EG
3	2/3	2/2	0/3	0/2	2/3	1/2	0/3	0/2	3/3	2/2	3/3	2/2	3/3	2/2	0/3	0/2
5	2/3	2/2	0/3	0/2	2/3	2/2	0/3	0/2	3/3	2/2	3/3	2/2	1/3	2/2	NE	NE
7	2/3	2/2	0/3	0/2	2/3	2/2	0/3	0/2	3/3	2/2	3/3	2/2	2/3	2/2	0/3	0/2
9[a]	1/2	1/1	0/3	0/1	1/2	1/1	0/2	0/1	0/2	1/1	0/2	1/1	0/2	1/1	NE	NE
11	1/2	1/1	0/3	0/1	0/2	1/1	0/2	0/1	0/2	1/1	1/2	1/1	1/2	1/1	NE	NE
14	1/2	1/1	0/3	0/1	0/2	0/1	0/2	0/1	0/2	0/1	0/2	0/1	0/2	0/1	2/2	1/1

Abbreviation: NE, not examined.

[a]One animal per group was euthanized and necropsied on 7 dpi.

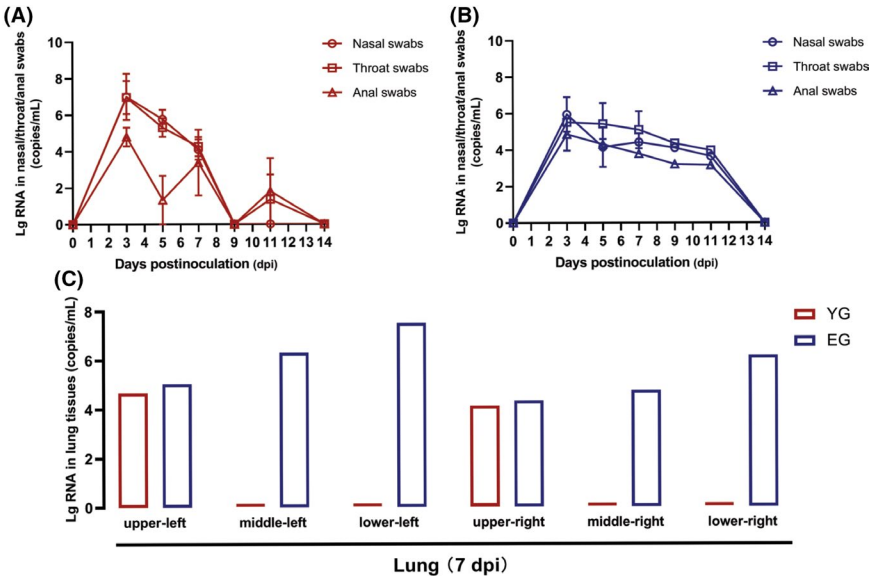

FIGURE 1 Viral load of the SARS-CoV-2-infected rhesus macaque model. A. Average viral loads of swabs from the younger group (YG, n = 3, red line) monkeys. B. Average viral load of swabs from the elder group (EG, n = 2, blue line) monkeys. Viral loads of nasal, throat, and anal swab specimens collected from the inoculated macaques on 0, 3, 5, 7, 9, 11, and 14 dpi. C. Viral loads in varies lobe of lung tissue from YG and EG monkeys at day 7 post-inoculation. RNA was extracted and viral load was determined by qRT-PCR. All data are presented as mean ± SEM

2.6 | ELISA antibody assay

Sera from each animal were collected to detect the SARS-CoV-2 antibody through ELISA at 0, 3, 7, 11, and 14 dpi. The 96-well plates coated with 0.1 μg Spike protein from SARS-CoV-2 (Sino Biological; Product code:40591-V08H) at 4℃ overnight were blocked by 2% BSA/PBST at room temperature for 1 hour. Sera samples were diluted at 1:100, and then were added to different wells and maintained at 37℃ for 30 minutes, followed by the goat anti-monkey antibody labeled with horseradish peroxidase (Abcam, ab112767) incubated at room temperature for 30 minutes. The reaction was built using TMB substrate and determined at 450 nm.

2.7 | Hematoxylin and eosin staining

All the collected organs were preserved in 10% buffered formalin solution fixed, and paraffin sections (3-4 μm in thickness) were prepared according to routine practice. All the tissues sections were stained with hematoxylin and eosin (H&E). The histopathological changes of different tissues were observed under an Olympus microscope.

2.8 | Immunohistochemistry (IHC)

All the collected organs were preserved in 10% buffered formalin solution fixed, and paraffin sections (3-4 μm in thickness) were prepared routinely. An antigen retrieval kit (AR0022; Boster) was prepared the sections for 1 minute at 37°C. Three percent H_2O_2 in methanol were used to quench endogenous peroxidases for 10 minutes. The slices were maintained at 4℃ overnight with a laboratory prepared-7D2 monoclonal antibody[11] after blocking in 1% normal goat serum. HRP-labeled goat anti-mouse IgG secondary antibody (ZDR-5307; Beijing ZSGB Biotechnology) were maintained at 37°C for 60 minutes. The 3,30-diaminobenzidine tetrahydrochloride were treated to make the results visual. The sections were counterstained

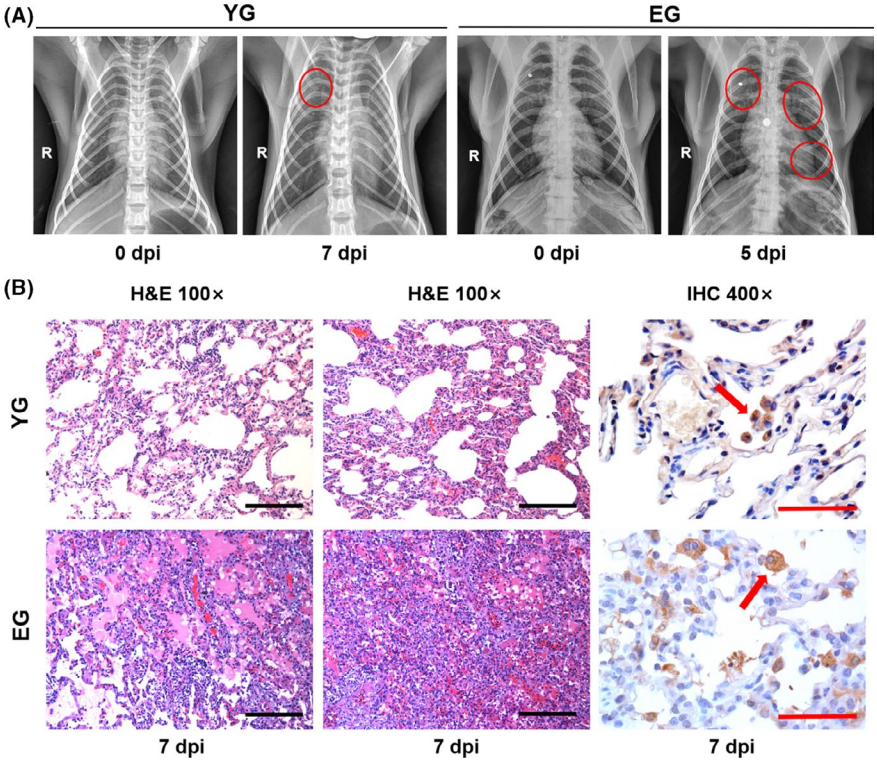

FIGURE 2 The comparison of lesions in the lung between younger group (YG) and elder group (EG) by radiographic alterations, histopathological and immunohistochemical (IHC) observation of the SARS-CoV-2-inoculated-rhesus macaque. A. Anterior-posterior thoracic X-rays from of rhesus macaque imaged prior to SARS-CoV-2 inoculation (day 0) and on 7 dpi of YG and 5 dpi of EG. Areas of interstitial infiltration, indicative of pneumonia, are highlighted (red circle). Positional indicators are included (R = right). B. Histopathological changes in lungs from YG and EG. Lung tissue was collected and stained with hematoxylin and eosin. Black scale bar = 40 μm. IHC staining demonstrated that SARS-CoV-2 antigens were mainly in the epithelial cells and macrophages. SARS-CoV-2 antigens were indicated by red arrows. Red scale bar = 50 μm

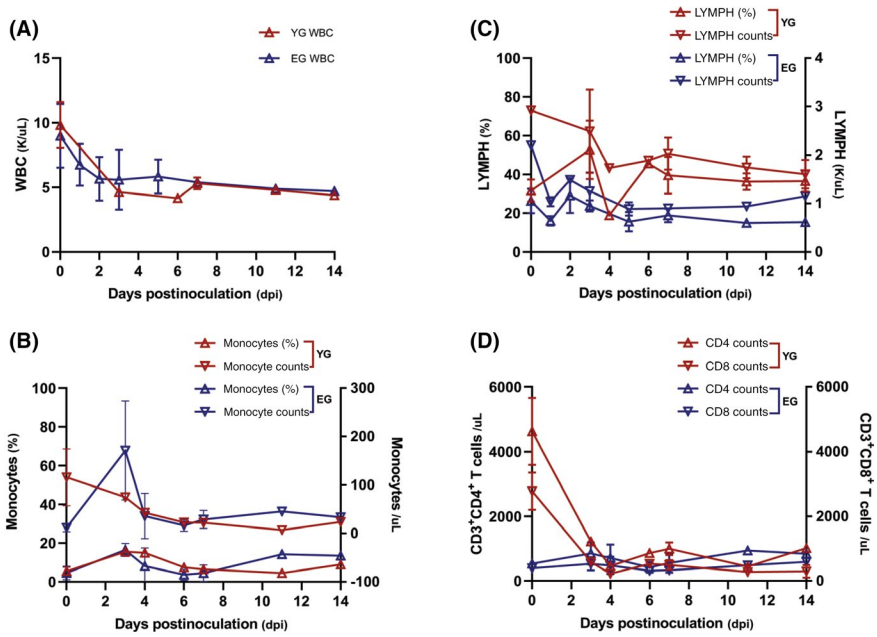

FIGURE 3 Hematological analysis in rhesus macaques inoculated with SARS-CoV-2. A. The counts of white blood cells (WBC) were analysed. B. The percentage and counts of monocytes were determined. C. The percentage and counts of lymphocytes were detected. D. The percentage and counts of $CD3^+$ $CD8^+$ T cells, $CD3^+$ $CD4^+$ T cells were shown. YG (red line) and EG (blue line) were indicated in the upper right corner of each panel. All data are presented as mean ± SEM

with hematoxylin, dehydrated, and mounted on a slide, and observed using an Olympus microscope.

3 | RESULTS AND DISCUSSION

We report nonhuman primate models for COVID-19 infected with SARS-CoV-2 (HB-01). Three rhesus macaques (divided into younger group, YG) between 3 and 5 years old and two rhesus macaques (divided into elder group, EG) about 15 years old were inoculated with 1×10^6 $TCID_{50}$ of SARS-CoV-2 through intratracheal route. Clinical signs including weight loss, fever, asthenia and lethality were recorded in Table 1, the weight was decreased obviously from 3 dpi in all the monkeys except one young monkey but without significant change of body temperature during the entire observation. Clinical signs were transient and lasted for a few days. Nasal, throat, and anal swabs were obtained from animals on 0, 3, 5, 7, 9, 11, and 14 days post-inoculation (dpi). Viral RNA was found in all animals from nose, pharynx, and crissum. Virus load in the respiratory tract peaked at 3 dpi in both of YG and EG (Figure 1A,B). For young monkeys, the virus load in anal swabs was

lower than that in the nasal and throat specimens (Figure 1A). Moreover, viral load from the anal swabs in EG was persistently detected from 3 dpi to 11 dpi, which is higher that in YG (Figure 1B). One monkey from each group was euthanized and necropsied at 7 dpi. Different lobes of lung were collected for detecting the viral loads. Approximate 104 Viral RNA copies/mL from the upper lung was detected in YG. As shown in Figure 1C, viral replication in EG monkeys was detected in all the lobes of lung with higher viral RNA copies (range from 104 to 107.5 copies/mL). The anterior-posterior thoracic x-rays from the young macaque showed ground-glass opacity and obscure lung markings in the upper lobe of the right lung at 7 dpi (Figure 2A). By comparison, old macaque developed obviously increased radiographic changes at 5 dpi, demonstrating prominent ground-glass opacities in the bilateral lobes and left lower lobe of the lung, and patchy lesions in the bilateral upper lobe of the lung (Figure 2A).Consistent with the thoracic x-rays results, microscopically, lesions were mainly in the lungs where interstitial pneumonia was remarkable. Pneumonia from young macaque was characterized by moderate thickening of alveolar septum, increased numbers of monocytes in the alveolar cavities, degeneration of epithelia and macrophage, and inflammatory cells infiltration including lymphocytes and monocytes (Figure 2B). Old macaque exhibited diffuse, severe pneumonia along with extremely thickened alveolar septum, serious infiltration of inflammatory cells in the alveolar interstitium. The alveolar cavities were filled with abundant exudation and fragments, accompanying with serious edema. Furthermore, viral antigen was detected from the lung of infected monkeys, and more viral antigens was found in elder animal than that from younger animal by immunohistochemistry (IHC), which was predominantly present in alveolar epithelia, macrophages in the alveolar cavities at 7 dpi (Figure 2C). White blood cell counts (Figure 3A), the counts and percentage of monocytes (Figure 3B) and lymphocytes (Figure 3B) were analyzed in blood samples collected along the indicated timeline. Comparisons between YG and EG revealed no changes in the counts or percentages white blood cells (Figure 3A) and monocytes (Figure 3B), a notably higher average counts or percentage of lymphocytes was observed in YG than that in EG during the observation, but the decreased counts of lymphocytes was found both in YG and EG monkeys (Figure 3C). Furthermore, regarding the counts of $CD3^+$ $CD8^+$ T cells and $CD3^+$ $CD4^+$ T cells, the obvious decline in YG but not in EG was observed in Figure 3D. Importantly, the decreased counts of lymphocytes were similar to the clinical manifestations of the SARS-CoV-2-infected-patients.[10] The specific IgG antibody against SARS-CoV-2 from the two groups were detectable at 14 dpi (Table 1).

Collectively, SARS-CoV-2 caused more severe interstitial pneumonia in old monkeys than that in young monkeys, as demonstrated by clinical signs, viral replication, chest X-ray, histopathological changes and immune response. The age-related animal models of SARS-CoV-2 infection are meaningful for further investigation on pathogenicity and evaluation of vaccines and therapeutics.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

CQ and WJT designed the project. PY, FFQ, YFX, FDL, PPL, JYL, LLB, WD, HG, ZGX, CX, QL, SRG, JNL, ZQS, YJQ, JX, QW, MYL, GPW, SYW, HSY, XL, BYH, WLW, LZ, HJW, FY, WMZ, WZ, JH, GZW, QJ, and JWW performed the majority of experiments. JNL, ZQS, JX, LLB, and QW wrote the manuscript. All the authors analyzed the data. CQ, JNL, ZQS, JX, LLB, and QW reviewed the manuscript.

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