Dynamic changes in host immune system and gut microbiota are associated with the production of SARS-CoV-2 antibodies

Maozhen Han[1,†], Yixuan Huang[3,†], Hongya Gui[2,†], Yixuan Xiao[2,†], Maozhang He[2], Jiling Liu[2], Xiujing Cao[4], Meijuan Zheng[5], Min Lu[5], Weihua Jia[1], Hui Li[6], Xiaoyan Wang[7], Na Zhang[1], Shu-an Kong[3], Xiaohui Liu[8], Yonggui Wu[9,*], Fengchang Wu[8,*], Shenghai Huang[1,2*]

[1] School of Life Sciences, Anhui Medical University, Hefei, Anhui 230032, China

[2] Department of Microbiology, The Key Laboratory of Microbiology and Parasitology of Anhui Province, The Key Laboratory of Zoonoses of High Institutions in Anhui, School of Basic Medical Sciences, Anhui Medical University, Hefei, Anhui 230032, China

[3] Department of Clinical Medicine, the First School of Clinical Medicine, Anhui Medical University, Hefei, Anhui 230032, China

[4] Department of Epidemiology and Biostatistics, School of Public Health, Anhui Medical University, Hefei, Anhui 230032, China

[5] Department of Clinical Laboratory, the First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230032 China

[6] Hefei City Maternal and Child Health & Family Planning Service Center, Hefei, Anhui 230001, China

[7] Department of Pediatrics, the First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230032, China

[8] State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences, Beijing 100012, China

[9] Department of Nephropathy, the First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230032, China

[†]These authors contributed equally to this work.

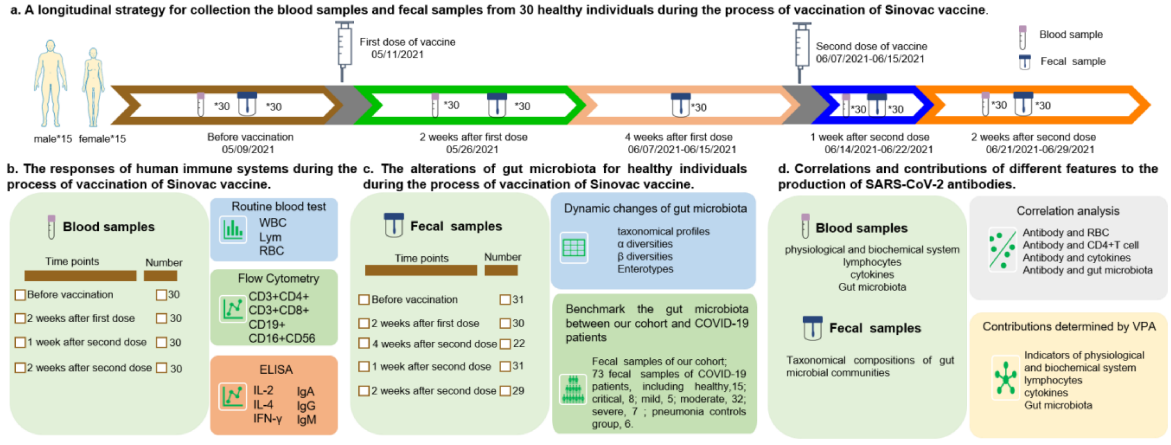
* Corresponding author:

Shenghai Huang, huangshh68@aliyun.com; Fengchang Wu, wufengchang@vip.skleg.cn; Yonggui Wu, wuyonggui@medmail.com.cn

Materials and methods

Study design and sample collection

In our present study, to highlight the dynamic changes in the gut microbiota, host response, and production of SARS-CoV-2 antibodies and their interactions during the process of SARS-CoV-2 vaccination, which involves various doses, a total of 30 healthy volunteers (undergraduates of School of Life Sciences, Anhui Medical University) whose age ranged from 20 to 23 years were recruited from May to July 2021. Their height and weight were measured to calculate the BMI. Blood samples were collected four times, and fecal samples were collected five times. The procedure is detailed in **Supplementary Figure S1**. Specifically, blood and fecal samples were collected before vaccination with the Sinovac vaccine, 2 weeks after vaccination with the first dose of the vaccine, and 1 and 2 weeks after vaccination with the second dose of the vaccine, and fecal samples were also obtained on the day at which the second dose was administered, which resulted in a total of four blood samples and five fecal samples **(Figure 1A, Supplementary Figure S1**). Each blood sample was divided into three parts: one part was used for routine blood test, a second part was utilized for flow cytometry, and the remaining serum was maintained at -80 °C for cytokine and antibody detection. The first blood sample was used as a control. Immediately after collection, the fecal samples were stored at -80 °C for the extraction of DNA and sequencing. Subsequently, 30 blood samples and 31, 30, 22, 31, and 29 fecal samples were collected at each of the four blood and five fecal sampling time points, respectively, to explore the dynamic patterns of the host response, gut microbiota, production of SARS-CoV-2 antibodies, and the interaction among the gut microbiota, host response, and production of SARS-CoV-2 antibodies and assess their interactions during the SARS-CoV-2 vaccination process (**Figure 1A, Supplementary Figure S1**). The study protocols were all approved by the Biomedical Ethics Committee of Anhui Medical University (No 2021H021).



Supplementary Figure S1. A longitudinal sampling strategy for the collection of blood and fecal samples from

30 healthy individuals at different points during the process of SARS-CoV-2 vaccination was used to explore the dynamic patterns of immune systems, the gut microbiota, and the production of antibodies and their interactions. a: Longitudinal strategy for collecting blood and fecal samples from 30 healthy individuals during the process of vaccination with the Sinovac vaccine. **b:** Responses of human immune systems during the process of vaccination with the Sinovac vaccine. **c:** Alterations in the gut microbiota of healthy individuals during the process of vaccination with the Sinovac vaccine. **d:** Correlations and contributions of different features to the production of SARS-CoV-2 antibodies.

Measurement of cytokines and SARS-CoV-2 antibodies in serum by ELISA

To monitor the response of the host's body based on cytokines and SARS-CoV-2 antibodies, the levels of inflammatory factors, including IL-2, IL-4, IFN-γ, and SARS-CoV-2 antibodies, including anti-(N+S) IgA, anti-(N+S) IgG, and anti-(N+S) IgM, were measured by ELISA. Specifically, the levels of cytokines and antibodies in whole-blood serum obtained from all the blood samples (n =30 at each sampling point) were measured. We selected IL-2 and IFN-γ as representatives of Th1 cells and IL-4 as representative of Th2 cells. IL-2, IL-4, IFN-γ, anti-(N+S) IgA, anti-(N+S) IgG and anti-(N+S) IgM were measured using a high-sensitivity enzyme linked assay quantitative kit (the kits for cytokines were procured from Bio-Techne USA Co., Ltd., Minnesota, USA, and the kits for antibodies were obtained from Wuhan Fine Biotechnology Co., Ltd., Wuhan, China) in accordance with the manufacturer's instructions. Specifically, taking the detection of SARS-CoV-2 antibodies as an example, the kit can detect the concentration of SARS-CoV-2 antibodies based on indirect enzyme-linked immunosorbent assay technology. Ninety-six-well plates were percolated with recombinant 2019-nCoV nucleocapsid and spike protein (antigen), and HRP-conjugated antibody was used as the detection antibody. Subsequently, the standards, test samples and HRP-conjugated detection antibody were added to the wells, and the plates were washed with wash buffer. 3,3',5,5'-Tetramethylbenzidine (TMB) substrates were used to visualize the HRP enzymatic reaction, which is catalyzed by HRP to produce a blue product that changes to a yellow color after addition of an acidic stop solution. The density of the yellow color is proportional to the target amount of sample captured in the plate. The O.D. absorbance at 450 nm is read using a microplate reader, and the concentration of the target was then calculated.

Characteristics of peripheral lymphocyte subset alterations measured by flow cytometry

To quantify the variation in lymphocytes during the process of SARA-CoV-2 vaccination, the levels of different types of lymphocytes, including CD3+ T cells, CD3+CD4+ T cells, CD3+CD8+ T cells, CD3+CD4+CD8+ T cells,

CD4+/CD8+ ratio, CD16+CD56+ cells, and CD19+ cells, were measured by flow cytometry. Specifically, a 50-μl peripheral blood sample with EDTA-K2 anticoagulant was first added to a prepared pipe in a biological safety cabinet. Second, the blood sample was incubated with 5 μl of antibodies (BD Multitest™ IMK kit (BD Biosciences, USA, Catalog No.340503) and BD Multitest™ 6-color TBNK reagent (BD Biosciences, USA, Catalog No.644611)) for 15 min in a dark room. Third, 450 μl of BD FACS lysing solution (10X) (BD Biosciences, USA, Catalog No.349202) and sterile water were injected to lyse the erythrocytes for 10 min. The processed samples were then examined using a flow cytometer (BD FACSCanto™, BD Biosciences) and FACSDiva v. 6.1 software, and the results were analyzed using FlowJo software (Version 7.6.1; Tree Star, Inc., Ashland, OR, USA) at the First Affiliated Hospital of Anhui Medical University.

Measurement of indicators using routine blood tests

Various indicators obtained from routine blood tests, such as eosinophils, neutrophils and hemoglobin, were measured via routine blood tests conducted at Hefei City Maternal and Child Health & Family Planning Service Center (Anhui Province). Specifically, a 2-ml blood sample was collected in an EDTA-K2 anticoagulant tube. Immediately after collection, the tube was mixed by being gently reversed several times to ensure adequate anticoagulation of the blood specimen. The blood samples were then sent to Hefei City Maternal and Child Health & Family Planning Service Center, and the indicators obtained from routine blood tests were directly measured using a three-classification blood cell analyzer (BC-5390 CRP, Marry).

DNA extraction and metagenomic sequencing

In this study, we collected fecal samples from 30 volunteers at five time points during the SARS-CoV-2 vaccination process to explore the dynamic changes in the human gut microbiota. A total of 143 fecal samples were collected, and metagenomic DNA was extracted using the Magen HiPure Stool DNA Kit (Magen, Guangzhou, China) according to the manufacturer's instructions. All extracted DNA was dissolved in TE buffer and stored at -20 °C until further use. The metagenomic DNA of the samples was then fragmented randomly to the desired size using a Covaris S/E210 and electrophoresed to obtain DNA fragments of the required lengths. Subsequently, the DNA fragments from each sample were ligated with adapters and evaluated for cluster preparation. All sequencing reactions of these samples were performed using the Illumina X Ten platform with a paired-end sequencing strategy (GENEWIZ, Inc., South Plainfield, NJ, USA). All sequencing data from the 143 fecal samples were deposited into NCBI's Sequence Read Archive (SRA) database with the BioProject number PRJNA778267.

Processing of metagenome datasets

To profile the taxonomic composition of the gut microbial community, 143 fecal samples were collected for metagenome sequencing. The generated sequencing reads were first quality-filtered against the human genome (hg38) using bowtie2 to identify the reads belonging to humans and filter the contaminants. To further obtain the high-quality reads, the 143 metagenome datasets were subjected to quality control using Trimmomatic (v 0.32) with the following parameters: TruSeq3-PE.fa, 2:30:10; leading, 3; trailing, 3; sliding window, 5:20; and min length, 25. Subsequently, an average of 16.7 Gb of high-quality sequences were obtained from each sample (fastq document), which resulted in the generation of 2.4 TB of sequence data in total (fastq document).

Taxonomical and functional compositions based on shotgun metagenomic datasets

We selected two popular tools currently used in metagenome analysis, namely, MetaPhlan2 and HuMAnN2, to accurately identify the taxonomic and functional compositions of the gut microbial communities with the default settings. The taxonomic compositions of the gut microbial communities were identified, and the relative abundance of each taxon was summarized at the phylum, class, order, family, genus, and species levels. The enterotypes of all fecal samples were classified using the 'Biotype' package on the R platform. In addition, the dominant species were selected based on the average abundances (higher than 0.01%), and the interactions between individual species were calculated using the Kendall correlation coefficient with the 'cor' package on the R platform.

Analysis of public datasets related to patients with COVID-19

To track the dynamic changes and patterns of the gut microbial communities of our cohort and patients with COVID-19, we downloaded the gut metagenome datasets of a cohort of COVID-19 patients from SRA databases (BioProject number: PRJNA624223). This cohort consisted of 73 fecal samples, which were divided into healthy Chinese individuals (15 samples), a critical group (8 samples), a mild group (5 samples), a moderate group (32 samples), a severe group (7 samples), and a pneumonia control group (6 samples), and provided evidence of the gut microbial alterations in COVID-19 patients. These 73 gut microbiome datasets were analyzed using the same strategy as that used for the datasets from our cohort, and the similarities among the microbial communities were estimated through linear discriminate analysis (LDA).

Statistical analysis

In the present study, the alpha diversities of the gut microbial communities, including Shannon and Simpson indices, were calculated with the 'vegan' package and compared among different time points during the SARS-CoV-2 vaccination process. ANOSIM and PERMANOVA were performed based on the Bray-Curtis distance matrix to estimate whether the taxonomic compositions of the gut microbial communities differed among different time points during the process of SARS-CoV-2 vaccination. In addition, LDA, which is a supervised learning approach, was applied to distinguish the gut microbial communities based on the taxonomic compositions. To evaluate the effects of body features and the gut microbiota and assess their contributions to the production of antibodies against SARS-CoV-2, a variation partitioning analysis (VPA) was performed using the R platform. Specifically, we utilized the interactive forward selection function in Canoco software (version: 5.0) to eliminate the collinearity between factors based on the contribution, *p* value and adjusted *p* value of each feature. Afterward, the features mean corpuscular volume (MCV), CD3+CD8+ T cells, CD3+CD4+CD8+ T cells, CD16+CD56+ cells, lymphocyte (LYM), and red blood cell volume distribution width-SD (RDW-SD) were selected and defined as body features, whereas a total of 25 taxa (**Supplementary Table S5**), including *Veillonella dispar*, *Lachnospiraceae bacterium*_3_1_46FAA, *Lachnospiraceae bacterium*_2_1_58FAA, *Lachnospiraceae bacterium*_8_1_57FAA, *Akkermansia muciniphila*, *Klebsiella_* sp_MS_92_3, and *Lactobacillus ruminis*, were selected as representative species of the gut microbiota. Moreover, the contributions of body features and the gut microbiota to the production of SARS-CoV-2 antibodies were estimated using the 'varpart' function of the 'vegan' package on the R platform. The analyses were performed using SPSS version 22.0. The differences between groups were assessed by two-way ANOVA, and two-sided exact P-values are reported. A P-value < 0.05 was considered to indicate statistical significance. In the linear correlation analysis, a P-value < 0.05 was considered to indicate statistical significance.