Here, we describe the use of the artificial antigen-presenting cell (aAPC) system for the verification of T-cell epitopes. We purify and activate CD8+ T cells from blood samples from HLA-A2 that are negative for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). CD8+ T cells are combined with peptide-loaded T2-A2 cells, which are then stained with a SARS-CoV-2-specific MHC-1 tetramer to identify specific HLA-A2-restricted T-cell epitopes. The use of aAPC and healthy donors means that only BSL2 lab conditions are needed.
Protocol
Optimization of antigen-specific CD8+ T cell activation conditions for infectious diseases including COVID-19

Chanchan Xiao,1,2,5 Congling Qiu,1,2,3,5 Jieping Deng,1,2 Jiezhou Ye,1,2 Lijuan Gao,1,2 Jun Su,3
Oscar Junhong Luo,2,4 Pengcheng Wang,1,2,* and Guobing Chen1,2,3,6,7,*

1Department of Microbiology and Immunology, Institute of Geriatric Immunology, School of Medicine, Jinan University, Guangzhou, China
2Guangdong-Hong Kong-Macau Great Bay Area Geroscience Joint Laboratory, Guangzhou, China
3Affiliated Huaqiao Hospital, Jinan University, Guangzhou, China
4Department of Systems Biomedical Sciences, School of Medicine, Jinan University, Guangzhou, China
5These authors contributed equally
6Technical contact
7Lead contact
*Correspondence: twangpc@jnu.edu.cn (P.W.), guobingchen@jnu.edu.cn (G.C.)
https://doi.org/10.1016/j.xpro.2021.100789

SUMMARY
Here, we describe the use of the artificial antigen-presenting cell (aAPC) system for the verification of T-cell epitopes. We purify and activate CD8+ T cells from blood samples from HLA-A2 that are negative for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). CD8+ T cells are combined with peptide-loaded T2-A2 cells, which are then stained with a SARS-CoV-2-specific MHC-1 tetramer to identify specific HLA-A2-restricted T-cell epitopes. The use of aAPC and healthy donors means that only BSL2 lab conditions are needed. For details of the use and implementation of this protocol, please refer to Deng et al. (2021).

BEFORE YOU BEGIN
This protocol describes to use of the artificial antigen presenting cell (aAPC) system for the verification of T cell epitopes without the need of a high-level biosafety laboratory. T2 is deficient in antigen polypeptide transporter, but overexpresses HLA-A2; thus the cell line is called T2-A2. We selected T2-A2 cell line for the following reasons. First, the inherent deficiency of endogenous antigen presentation made it more reliable to evaluate the presentation of exogenous epitopes by HLA-A2, without the interference from endogenous epitopes. Second, it showed an excellent capability to activate CD8+ T cells, inducing the generation of a high percentage of antigen-specific CD8+ T cells after the primary immune challenge. Third, it could also be used as a target cell for cytotoxicity assessment of activated CD8+ T cells when loaded with given epitopes. The timeline for the overall experimental procedure is shown in Figure 1.

Recruitment of study subjects
This work was approved by the Institutional Review Board of the Affiliated Huaqiao Hospital of Jinan University. The unexposed donors were healthy people registered in Guangzhou Blood Center and were confirmed negative for SARS-CoV-2 RT-PCR assay. These donors had no known history of any significant systemic diseases, including, but not limited to, hepatitis B or C, HIV, diabetes, kidney or liver diseases, malignant tumors, or autoimmune diseases. All subjects had informed consent that their samples would be used in this study. The HLA-A2+ donors were identified by using flow cytometry.
Synthesis of candidate peptides

The candidate peptides were synthesized in GenScript Biotechnology Co., Ltd (Nanjing, China) and dissolved in DMSO or in water with the stock concentration at 10 mM (Table 1).

⚠️ CRITICAL: For preparing peptide solutions, it is recommended to first dissolve the peptide in a minimal amount of solvent (e.g., 1 mL water for 1 mg peptide if the peptide solubility is 1 mg/mL) and then re-adjust the solution with medium for T2-A2 cells buffer.

⚠️ CRITICAL: Peptides containing methionine (M), cysteine (C), or tryptophan (W) are sensitive to DMSO oxidation, and aqueous-based solvent should be used. It is best to aliquot the peptides into appropriate volume (10 μL) per tube for storage.

⚠️ CRITICAL: It is recommended to resuspend peptides in DMSO immediately before use or stored at −20 °C (or preferably −80 °C).

⚠️ CRITICAL: Please note that distinct dissolution behaviors may happen for small amounts and large amounts of gross peptide in the same solvent. Generally, it takes longer for larger amounts of peptide to dissolve. A brief incubation at warm temperatures (<40 °C) or sonicat-ion can assist in dissolving the peptide.

Table 1. Details of SARS-CoV-2 proteins

| Protein | Number | Start position | End position | Length | Sequence        |
|---------|--------|----------------|--------------|--------|-----------------|
| ORF1ab  | 01     | 1000           | 1008         | 9      | TTIQTIVEV      |
|         | 02     | 1707           | 1716         | 10     | AANFCALILA     |
|         | 03     | 2225           | 2234         | 10     | KLINIWFL       |
|         | 04     | 2230           | 2238         | 9      | IWFLLLSTV     |
|         | 05     | 3673           | 3683         | 11     | SLSFGKLKDCV    |
|         | 06     | 3672           | 3683         | 12     | TSLSGFKLKDCV   |
| Spike   | 07     | 62             | 70           | 9      | VTFWHAIHV     |
|         | 08     | 136            | 145          | 10     | CNDPPLGVYY    |
|         | 09     | 495            | 503          | 9      | YGFPQPTNGV    |
|         | 10     | 567            | 576          | 10     | RDIADTDHAV    |
|         | 11     | 673            | 684          | 12     | SYQQTNSPRRA   |
|         | 12     | 713            | 722          | 10     | AIPTNFTISV    |
|         | 13     | 976            | 984          | 9      | VLDNLSRL      |
|         | 14     | 1114           | 1122         | 9      | ITTWDNTFV     |
| ORF8    | 15     | 73             | 81           | 9      | YIDIGNYTV     |
|         | 16     | 18             | 27           | 10     | QECSSLQCTQ    |
|         | 17     | 50             | 58           | 9      | GARKSAPLI     |
| N       | 18     | 1              | 9            | 9      | MSDNGPQNQ     |
|         | 19     | 228            | 236          | 9      | NQLESKMSG     |
Generation of antigen-specific HLA-A2 tetramer

© Timing: 2 h

To generate MHC/peptide monomers containing candidate peptides, conditional Flex-T™ monomers were mixed with the desired peptides, which could replace the UV-sensitive peptide through the UV-induced peptide exchange. These new MHC/peptide monomers are then polymerized with streptavidin-fluorophore conjugates, which can subsequently be applied to antigen-specific T cell staining and flow cytometry analysis. Chart protocol (Figure 2)

1. Peptide exchange
   a. Put all the reagents on ice
   b. Dilute 10 mM peptide stocking solution to 400 μM working solution with PBS, and keep on ice
   c. Add 20 μL conditional Flex-T™ monomer (200 μg/mL) and 20 μL diluted peptide solution into 96-well U-bottom plates. Mix by pipetting up and down
   d. Seal the plate; centrifuge at 3300 × g at 4 °C for 2 min to spin the liquid down
   e. Carefully remove the seal; place the plate on ice and illuminate with ultraviolet light for 30 min (the distance between UV lamp and sample should be 2–5 cm)

Figure 2. Generation of antigen-specific HLA-A2 tetramer
f. Seal the plate; incubate at 37°C for 30 min in the dark

2. Generation of tetramers
   a. Transfer 30 μL of peptide-exchanged monomer into a new plate
   b. Add 3.3 μL of conjugated streptavidin, and mixed thoroughly
   c. Incubate on ice for 30 min in the dark

Note: It is recommended to use BioLegend streptavidin products coupled with fluorophore. For 30 μL of exchanged Flex-T™ monomer, it is recommended to use 3.3 μL (Total volume 33.3 μL) of APC- streptavidin (Cat#405207) or 1.3 μL (Total volume 31.3 μL) of BV421-streptavidin conjugate (Cat#405225). For the optimal reaction of streptavidin products coupled with other fluorophores, the molar ratio of monomer to streptavidin was ensured to be 6:1.

d. During the incubation, prepare blocking solution by mixing 50 mM D-Biotin (1.6 μL) and PBS (198.4 μL), and vortex. At the end of incubation, add 2.4 μL blocking solution and stop the reaction with pipette up and-down

e. Seal the plate and incubate on ice for 30 min in the dark (or at 2°C–8°C for 12 h in the dark)

Note: It is recommended that Flex-T™ monomers are assembled with two different streptavidin conjugates (e.g., APC and PE) in separate reactions. In this way, the same tetramer allele can be used for two-color staining to ensure the highest specificity.

△ CRITICAL: The peptide could be dissolved in DMSO, but the final concentration was controlled below 10% (v/v) in the exchange reaction. Flex-T™/peptide solution should be kept in the dark and to avoid repeated freezing and thawing. Centrifuge all vials before use (3000 × g at 4°C for one minute). We used a short wavelength (365 nm), since broadband UV lamp is harmful to the MHC complexes.

3. Cell staining and flow cytometric analysis

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| PE anti-human HLA-A2 (clone BB7.2) | BioLegend | Cat#343305;RRID:AB_1877228 |
| FITC anti-human HLA-A2 (clone BB7.2) | BioLegend | Cat#343303;RRID:AB_1659246 |
| APC labeled human CD8⁺ (clone T8) | BioLegend | Cat#301049;RRID:AB_2562054 |
| Anti-human CD28 Antibody (clone CD28.2) | BioLegend | Cat#302901;RRID:AB_314303 |
| Human TruStain FoX™ (Fc Receptor Blocking Solution) | BioLegend | Cat#422302;RRID:AB_2818986 |
| 7-AAD viability staining solution | BioLegend | Cat#420403 |
| APC anti-human CD69 (clone FN50) | BioLegend | Cat#310909;RRID:AB_314844 |
| APC Annexin V | BioLegend | Cat#640919 |
| PerCP anti-human IFN-γ (clone 4S.B3) | BioLegend | Cat#502524;RRID:AB_2616613 |
| FITC anti-human Granzyme B (clone GB11) | BioLegend | Cat#515403;RRID:AB_2114575 |
| Bacterial and virus strains |        |            |
| SARS-CoV-2 Wuhan-Hu-1 strain (NC_045512.2) | N/A | N/A |
| Biological samples |        |            |
| Blood samples from healthy donors | Guangzhou Blood Center | N/A |
| Chemicals, peptides, and recombinant proteins |        |            |
| Lymphocyte separation medium (density: 1.077 g/mL) | GE | Cat#MQ0148 |
| Fetal bovine serum (FBS) | LONSDA | Cat#S711-001S |
| RPMI 1640 medium | Gibco | Cat# 11875093 |

(Continued on next page)
**Continued**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HEPES               | Gibco  | Cat#15630080 |
| 2-Mercaptoethanol   | Gibco  | Cat# 21985023 |
| Sodium pyruvate     | Sigma-Aldrich | Cat#S8636 |
| Non-essential amino acids in MEM | Gibco  | Cat# 1140050 |
| MEM Vitamins Solution | Gibco  | Cat#11120052 |
| Bovine serum albumin (BSA) | Absin  | Cat#absa9157 |
| Phosphate buffered saline (pH 7.4) | Gibco  | Cat# 10010049 |
| Iscove’s Modified Dulbecco’s Medium (IMDM) | HyClone | Cat#SH30228.01 |
| Penicillin-Streptomycin solution, 100× | Solarbio | Cat# P1400 |
| Fixation buffer     | BioLegend | Cat#420801 |
| Cell staining buffer | BioLegend | Cat#420201 |
| 10× Permeabilization wash buffer | BioLegend | Cat#421002 |
| DNase I Solution    | STEMCELLL | Cat#07900 |
| EasySep™ Buffer     | STEMCELLL | Cat#20144 |
| Dimethyl sulfoxide  | Sigma-Aldrich | Cat#02650 |
| MHC monomer         | BioLegend | Cat#280003 |
| PE streptavidin     | BioLegend | Cat#405203 |
| Mitomycin C (MC)    | GLPBIO | Cat#GC12353; CAS Number:50-07-7 |
| Carboxyfluorescein succinimidyl ester (CFSE) | TargetMol | Cat#T6802 |
| IL-2                | SL Pharm | N/A |
| Leukocyte Activation Cocktail, GolgiPlug™ | BD Biosciences | Cat#550583 |
| 50 mM Biotin        | Invitrogen | Cat#2110450 |
| Influenza A M P1-38,44 GILGFVFTL peptide | GenScript | Customized |
| EBV EBNA4 A256-243 VTDFSVIK peptide | GenScript | Customized |
| SARS-CoV-2 ORF1b 1290-1300 ITTQITIVEV peptide | GenScript | Customized |
| SARS-CoV-2 ORF1b 1265-1281 AANFCALLA peptide | GenScript | Customized |
| SARS-CoV-2 ORF1b 1275-1291 KLINIIWFL peptide | GenScript | Customized |
| SARS-CoV-2 ORF1b 1230-1248 IWFLLLTV peptide | GenScript | Customized |
| SARS-CoV-2 ORF1b 1233-1248 LSLGFKKDCV peptide | GenScript | Customized |
| SARS-CoV-2 ORF1b 1243-1258 TSLGFKKDCV peptide | GenScript | Customized |
| SARS-CoV-2 S 525-537 VTVFHAIHV peptide | GenScript | Customized |
| SARS-CoV-2 S 527-542 CNDPFLGVYY peptide | GenScript | Customized |
| SARS-CoV-2 S 541-556 YGFOPTGNY peptide | GenScript | Customized |
| SARS-CoV-2 S 623-637 RDIADTDADV peptide | GenScript | Customized |
| SARS-CoV-2 S 630-648 SYQQTQNSPRRA peptide | GenScript | Customized |
| SARS-CoV-2 S 713-729 AIPNTNTISV peptide | GenScript | Customized |
| SARS-CoV-2 S 857-864 VNDILSRIL peptide | GenScript | Customized |
| SARS-CoV-2 S 1124-1132 ITTNTNYPV peptide | GenScript | Customized |
| SARS-CoV-2 ORF8 77-87 YIDIGNYTV peptide | GenScript | Customized |
| SARS-CoV-2 ORF8 87-98 QECSDLSTCOQ peptide | GenScript | Customized |
| SARS-CoV-2 ORF8 99-108 GARKAPLI peptide | GenScript | Customized |
| SARS-CoV-2 N 29-39 MSDNGPONQ peptide | GenScript | Customized |
| SARS-CoV-2 N 39-58 NQLESKMSG peptide | GenScript | Customized |

**Critical commercial assays**

| EasySep™ Human CD8+ T Cell Isolation Kit (Negative Selection) | STEMCELLL | Cat #17953 |

**Experimental models: cell lines**

| T2-A2 | Dr. Anna Gil | N/A |

**Software and algorithms**

| FlowJo software version 10.7 | FlowJo LLC | https://www.flowjo.com/ |
| Prism version 8 | GraphPad | https://www.graphpad.com/ |

**Other**

| Each epitope corresponds to a tetramer | Home-made | N/A |
| Microcentrifuge tube (1.5 mL) | Axygen | Cat#MCT-150-C |
| Falcon tube (50 mL) | Corning | Cat# CLS430290-500EA |

(Continued on next page)
MATERIALS AND EQUIPMENT

Preparation of buffers and solutions

| Solution                        | Contents                                                                 | Final volume | Storage               |
|---------------------------------|--------------------------------------------------------------------------|--------------|-----------------------|
| Medium for T2-A2 cells          | IMDM medium with 20% (v/v) FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin | 50 mL        | 4°C, 4 weeks          |
| Medium for primary T cells      | RPMI1640 medium with 20% (v/v) FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin, 1% 1M HEPES, 0.2% 55 mM 2-Mercaptoethanol, 1% 100 mM sodium pyruvate, 1% 100 mM non-essential amino acids in MEM, 0.4% 100 mM MEM-Vitamins solution | 100 mL       | 4°C, 4 weeks          |
| Cell freezing medium            | RPMI1640 medium with 40% (v/v) FBS, 10% (v/v) DMSO                       | 100 mL       | 4°C, make fresh       |
| 5 μM CFSE                       | Prepare CFSE stock solution in DMSO (5 mM), and dilute to 5 μM in sterile PBS | 1 mL         | 4°C, make fresh       |
| 20 μg/mL Mitomycin C lysate buffer | 100 mg/mL mitomycin C stock in DMSO is diluted to a final concentration of 20 μg/mL in IMDM complete medium | 100 mL       | –20°C, 2 months       |
| Red blood cell (RBC) lysis buffer | 155 mM ammonium chloride (4.145 g), 10 mM potassium bicarbonate (0.5 g), 0.1mM EDTA (100 μL), and 500 mL sterile nuclease-free ddH2O, adjust pH to 7.3 | 500 mL       | 4°C, 2 weeks          |
| 1X Permeabilization wash buffer | Prepare 10 x permeabilization wash buffer stock solution, and dilute to 1X with sterile nuclease-free ddH2O | 100 mL       | 4°C, make fresh       |
| BSM buffer                      | Sodium chloride (8.0 g), potassium chloride (0.4 g), dibasic sodium phosphate (0.12 g), potassium dihydrogen phosphate (0.06 g), glucose (1.0 g), BSA (2.0 g), 100X antibiotics (10 mL), 100X HEPES (10 mL), and 980 mL sterile nuclease-free ddH2O, adjust pH to 7.3–7.6 | 1 L          | 4°C, 2 weeks          |
| EasySep™ Buffer                 | D-PBS with 2% (v/v) FBS, and 1 mM EDTA                                  | 1 L          | 4°C, 2 weeks          |

The pH of all buffers was measured at 25°C.

Additional materials

- Buckets containing chlorine disinfectant to rinse tips and tubes before being discarded into waste bins
- A sharp container to discard glass pipettes for separating peripheral blood mononuclear cells (PBMCs)
- Empty waste bottle for liquid waste
- Alcohol sprayer for glove and hand disinfection
- Aliquots of the media and buffers required
- Phosphate buffer saline
- Cell culture medium
- CFSE
- Mitomycin C
- fixation buffer (on ice)
cell staining buffer (on ice)
permeabilization wash buffer (on ice)
RBC lysis buffer
BSM buffer (on ice)

STEP-BY-STEP METHOD DETAILS

Note: If the same samples are used for both red blood cell (RBC) lysis and PBMCs isolation, the samples should be divided into 2 aliquots. The volumes depend on the amount of the starting material and ought to be adjusted to meet the requirement for next experiments.

Red blood cell lysis

Timing: 25 min

In this section, procedures for RBC lysis are described. RBC lysis is performed in whole blood samples from healthy volunteers prior to identification of HLA-A2 by using flow cytometry.

△ CRITICAL: RBC lysis and buffer—Sterilized by filtration through 0.22 μm filter.

1. Prepare the RBC lysis solution
2. RBC lysis
   a. Manipulate the blood tube in the biological hood, and gently transfer 1 mL blood (About 1 × 10^6 PMBCs) to a 15 mL microcentrifuge tube
   b. Add 10 mL RBC lysis buffer carefully by using a serological pipette
   c. Vortex the microcentrifuge tube briefly and put it still for 10 min at 25°C
   d. Centrifuge the cell suspension at 300 × g for 5 min at 4°C
   e. Discard the supernatant and add 15 mL BSM buffer, repeat centrifugation as in step d
   f. Discard the supernatant and resuspend cells in 100 μL BSM buffer
   g. Count viable cells and adjust to 5–10 × 10^6 cells/mL with BSM buffer, and distribute cell suspension into 1.5 mL microcentrifuge tubes with 100 μL/tube (5–10 × 10^5 cells/tube, at least 2 tubes)

Cell staining and flow cytometric analysis

Timing: 1 h

3. Block Fc-receptors
   Human TruStain includes specialized human IgG that binds to Fc receptors through the Fc terminal of the antibodies. Human TruStain is compatible with flow cytometry staining with anti-human CD64 (clone 10.1), CD32 (clone FUN-2), and CD16 (clone 3G8) antibodies. The reagents blocking the Fc receptors may help to reduce non-specific immunofluorescent staining.
   a. Add 5 μL Human TruStain FcX™ per million cells in 100 μL staining volume, mix and incubate for 10 min at 25°C

   Note: There is no need to wash cells between these blocking and immunostaining steps.

4. Cell-Surface staining with PE anti-human HLA-A2 antibody
   HLA-A2 is the most dominant MHC class I subtype in population in North America and Northern Asia. MHC class I heavy chain and β2-microglobulin are expressed on all human nucleated cells.
   a. For flow cytometric staining, it is suggested to use 5 μL reagent per million cells in a 100 μL staining volume, and incubate on ice for 20 min in the dark
   b. Wash twice with 1.5 mL cell staining buffer by centrifugation at 300 × g for 5 min at 4°C
c. Resuspend cell pellet in 200 μL cell staining buffer and add 5 μL/million cells of 7-AAD viability staining solution to exclude dead cells
d. Incubate on ice for 3–5 min in the dark
e. Perform flow cytometric analysis

**Pause point:** If you cannot read your samples immediately on a flow cytometer, keep them foil wrapped and in a refrigerator at 4°C–8°C for less than 4 hours. The samples should be re-suspended in cell staining buffer. It is worth noting that the samples should not remain in a fixation buffer for a long time, which will affect fluorophore conformation and fluorescence.

**Isolation of human PBMCs (density gradient centrifugation)**

- **Timing:** 1 h

In this section, the procedures from PBMCs isolation from whole blood of healthy volunteers are described. These healthy volunteers enrolled in Guangzhou Blood Center and confirmed with a negative report for SARS-CoV-2 RNA real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay. These donors had no known history of any significant systemic diseases, including, but not limited to, hepatitis B or C, HIV, diabetes, kidney or liver diseases, malignant tumors, or autoimmune diseases. The average yield of whole blood per mL was 1–2 × 10^6 cells PBMCs. Lymphocyte Separation Medium (LSM) is designed for the rapid, simple isolation of lymphocytes from diluted defibrinated whole blood layered on a solution of Ficoll® and centrifuged at low speed (805 × g) for 20 min. Blood cells migrate through the solution during centrifugation to form density-specific layers. Lymphocytes and other
mononuclear cells form a unique band between LSM fractions and the plasma (Figure 3A). Lymphocytes are retrieved by aspirating the plasma layer and then removing the cells. Excess plasma, platelets, and LSM can then be removed by cell washing. To achieve the best results, use blood drawn within two hours. Blood drawn more than 24 h before should not be used.

5. Preparation of the necessary materials
   a. Prewarm the LSM at 25°C and thoroughly mix it by gently flipping the bottle. For each sample (4 mL heparinized blood, 1–2 × 10^6 cells/mL), fill one 15 mL Falcon tube with 4 mL LSM and another one with 4 mL PBS
   b. Properly label the tubes, including sample ID, PBS, LSM etc.

6. Density-gradient centrifugation
   a. Mix 4 mL heparinized blood with 4 mL PBS (equal volume of heparinized blood and PBS)
   b. Carefully LAYER the diluted blood over the 4 mL LSM in the 15 mL centrifuge tube, creating a distinct blood-LSM interface
   c. DO NOT MIX! The quality of separation depends on the distinct interface between the diluted blood and density gradient
   d. Centrifuge the tube at 805 × g for 20 min at 25°C

   Note: For PBMCs separation, the speeds of acceleration and brake are set 0.

   e. After centrifugation, open the centrifugation bucket in the hood to get the Falcon; PBMCs are carefully retrieved into the 15 mL Falcon pre-filled with PBS by using a 2 mL serological pipette

   Note: The volume of PBS should be twice as the expected PBMCs fraction, and adjust the final volume accordingly.

   △ CRITICAL: Avoid mixing of density phases to prevent the contamination from red blood cells and granulocytes.
   f. Wash cells twice with 10 mL PBS by centrifugation at 300 × g for 10 min at 4°C

   Note: Washing the cells removes LSM and decreases the amount of platelets.

   g. Discard supernatant and resuspend the PBMCs (1 × 10^6 cells/mL) in the desired volume of primary T cell medium

Cryopreservation of PBMCs samples

© Timing: 15 min

In the present section, the procedures for long-term storage of PBMCs for T cell activation later are described. Cells are resuspended in cell freezing medium containing DMSO and stored in liquid nitrogen.

7. Prepare required media and equipment
   a. Cell freezing medium
   b. Medium for primary T cells
   c. Chilled cell freezing container
   d. Chilled cryovials

8. Cryopreservation protocol
   a. Label cryovials properly, and put them on ice in the biological safety cabinet
   b. Centrifuge the isolated PBMCs at 300 × g for 10 min at 4°C
   c. Resuspend the cells in a suitable volume of cell freezing medium to make a cell concentration of 2–10 × 10^6 cells/mL (keep the cells on ice)
d. Mix the cell suspension gently 

e. Transfer 1 mL cell suspension into the cryovials on ice 

f. Transfer the cryovials into the pre-chilled cell freezing container 

g. Place the container in a –80°C freezer for a minimum of 12 h 

h. Transfer the cryovials to liquid nitrogen for long-term storage 

Human CD8+ T-cell isolation

© Timing: 8 min

In this section, high purity CD8+ T cells are isolated from fresh or frozen human PBMCs by immuno-magnetic negative selection. Non-CD8+ T cells are recognized by antibodies specific to their cell surface markers, and labeled with magnetic beads, which help to remove these cells by using an EasySep™ magnet. Starting with a single cell suspension of human PBMCs, the CD8+ T cell content of the isolated fraction is typically 98.6% (Figure 3B). The wanted CD8+ T cells are then transferred into a new tube, and can be used immediately for downstream applications such as culture and flow cytometry.

Note: Yield of CD8+ T cell isolation can greatly vary depending if they are isolated from fresh blood or frozen PBMCs. If derived from fresh blood, CD8+ T cells (about 15%–30% of PBMCs) are more than 95% viability. If derived from frozen PBMCs, CD8+ T cells (about 10%–25% of PBMCs) are more than 90% viability.

9. Preparation of required media and equipment

a. PBMCs suspension

Note: Incubate the frozen PBMCs with 100 μg/mL Deoxyribonuclease (DNase) I solution for at least 15 min at 25°C before labeling and separation. Filter the cell suspension with 37 μm cell filters to obtain optimal results.

Note: DNase I solution is useful to reduce or prevent the clumping of concentrated and/or cryopreserved cell suspensions following thawing.

b. 5 mL polystyrene round-bottom

c. EasySep™ Magnet

10. CD8+ T cells isolation protocol

a. Prepare cell suspension at 5 x 10^7 cells/mL in optimum EasySep™ buffer

△ CRITICAL: EasySep™ Buffer—This buffer should be free of Ca++ and Mg++.

b. Add samples to 5 mL polystyrene round-bottom tubes

c. Add isolation cocktail to sample, 50 μL/mL of sample volume

d. Mix and incubate at 25°C for 5 min

e. Vortex RapidSpheres™, put at 25°C for 30 s

Note: Beads should evenly dispersed.

f. Add RapidSpheres™ to sample and mix, 50 μL/mL of sample volume

g. Add appropriate amount of EasySep™ buffer to top up to 2.5 mL. Mix by gently pipetting up and down for 2–3 times

h. Place the tube (without lid) into the magnet and incubate at 25°C for 3 min

i. Pick up the magnet, and in one continuous movement invert the magnet and tube, pouring the enriched cell suspension into a new tube
△ CRITICAL: Keep the magnet and tube inverted for 2–3 seconds, then return upright. Don’t shake or wipe off any drops that may remain hanging from the tube orifice.

j. Centrifuge the purified CD8+ T cells at 400 × g for 5 min at 4°C
k. Remove supernatant and resuspend the CD8+ T cells in the desired volume of primary T cell medium

Activation of CD8+ T cells

Timing: ~7 days

Once the human CD8+ T cells have been isolated in step 10, they will be stimulated in a primary culture in order to induce the production of specific CD8+ T cells and cytokines.

11. Preparation of required media and equipment
   a. T2-A2 cells
   b. Medium for T2-A2 cells
   c. Candidate peptides (Table 1)
   d. Mitomycin C
   e. CFSE
   f. IL-2
   g. Anti-human CD28 antibody
   h. Clear 6-well TC-treated microplates
   i. 96-well TC-treated microplates

12. Mitomycin C (20 μg/mL) pretreatment of T2-A2 cells
   Mitomycin C induces cross-linking of the double-stranded DNA, thereby inhibiting DNA synthesis, triggering DNA repair events, and inducing apoptosis. The aminoquinone group in mitomycin C contributes to the inhibition of DNA synthesis.

△ CRITICAL: Mitomycin C—To get higher solubility, please warm the tube at 37°C and shake it in an ultrasonic bath for a while.

   a. Harvest T2-A2 cells from T-75 cell culture flasks by agitation and aspiration using a 10 mL serological pipette, or equivalent. Cells are then transferred to a fresh 15 mL Falcon.
   b. Centrifuge at 400 × g for 5 min at 4°C
   c. Discard the supernatant and resuspend cells in complete IMDM to a final concentration of approximately 1–2 × 10^6 cells/mL
   d. Transfer the T2-A2 cells in 2 mL/well into the 6-well plate, add mitomycin C (20 μg/mL) per well, and incubate at 37°C with 5% CO₂ for 30 min
   e. Wash twice with PBS and centrifuge at 400 × g for 5 min at 4°C

Note: The solubility of mitomycin C in DMSO is >16.7 mg/mL. To get higher solubility, warm the tube at 37°C and shake it in an ultrasonic bath for a while. Stock solution can be stored foil wrapped below –20°C for up to 6 months.

13. Carboxyfluorescein succinimidyl ester (CFSE) transfected with T2-A2 cells
   CFSE is a fluorescent dye with cellular permeability. It is covalently coupled with intracellular molecules, especially lysine residues and other amine sources, through its succinimide group. The binding of CFSE to cells is stable. It has no toxicity and will not alter the viability of labeled cells. CFSE persists in cells for 24 h after labeling, thus is suitable for long term detection of labeled cells (Xiao et al., 2005). In the process of cell division and proliferation, its fluorescence intensity decreases gradually with the cell division. The labeled fluorescence can be
equally distributed to the two daughter cells, so their fluorescence intensity is half of the parental cells.

**△ CRITICAL: CFSE—Freshly prepared or make small aliquots and store at −80°C for up to 2 years.**

**Note:** The concentration mentioned here is safe (≤5 μM). However, high concentration of CFSE (FITC) might be toxic to T cells.

- The CFSE stock solution (5 mM) in DMSO is diluted to a final concentration of 5 μM in PBS with a total volume of 1 mL
- 2–4 × 10^6 T2-A2 cells are resuspended with 1 mL PBS, mixed with 1 μL 5 mM CFSE (final concentration is 5 μM), and incubated at 37°C, 5% CO₂ for 30 min with agitation
- The labeling reaction is stopped for 10 min by adding 9 mL complete IMDM
- The CFSE labeled cells are washed twice with PBS and recounted, and the cell concentration is adjusted to 5 × 10^6 cells/mL in serum-free IMDM

**Note:** To get higher solubility, warm the tube at 37°C and shake it in an ultrasonic bath for a while.

14. Peptide screening in T2A2 cells
To validate the predicted epitopes, first check if they can be presented by HLA-A2 on the antigen presenting cells. T2A2 is an antigen-presenting cell lacking antigen peptide transporter and expressing HLA-A2 on the cell surface. The peptide-MHC complex would be more stabilized if the epitopes bind with HLA-A2 suitably.

- The candidate peptides (Table 1) are synthesized in GenScript Biotechnology and dissolved in DMSO at the concentration of 10 mM.
- T2A2 cells are seeded into 96-well plates with 10^5 cells in 200 μL per well, and then incubated with peptides at a final concentration of 20 μM at 37°C for 4 h, respectively.

15. Activation of CD8⁺ T cells
- Harvest peptide loaded T2-A2 cells from step 14 and mix all cells together
- Centrifuge cells at 400 × g for 5 min at 4°C
- Discard the supernatant and resuspend cells in primary T cell medium to a final concentration of approximately 2.5 × 10^6 cells/mL
- 0.5 × 10^6 CD8⁺ T cells isolated from healthy donors and 0.5 × 10^6 peptide loaded T2-A2 cells are mixed and seeded into 96-well plates with 200 μL/well. Add 1 μg/mL anti-CD28 antibody and 50 IU/mL IL-2 into each well, and incubate at 37°C, 5% CO₂
- Every two days, 50 μL old medium is carefully taken along the 96-well plate wall and discarded, and 50 μL fresh T cell culture medium containing 50 IU/mL IL-2 and 20 μM mixed peptide is added

**Note:** When transfer the 96-well plate from the incubator, avoid shaking and carefully move it out of the incubator to prevent the cells from floating on the surface of the medium.

- The T cell activation marker CD69 and tetramer specific CD8⁺ T cells are evaluated after 16 h and 7 days, respectively

16. Cell-Surface staining with APC anti-human CD69
CD69 is transiently expressed on activated leukocytes including T, B, and NK cells. CD69 is involved in early events of lymphocyte, platelet, and monocyte activation, and plays a role in redirected lysis mediated by activated NK cells.

**Note:** The T cell activation marker CD69 was evaluated after 16 hours.
a. Transfer the 96-well plates from the incubator to the hood. Mix the cells in each well and transfer 30 μL cell suspension into 1.5 mL microcentrifuge tubes
b. Add 5 μL Human TruStain FcX per million cells in 100 μL staining volume, mix and incubate at 25°C for 10 min

Note: There is no need to wash cells between these blocking and immunostaining steps.

c. For flow cytometric staining, it is suggested to use 5 μL anti-CD69 antibody per 1 million cells in 100 μL staining volume, and incubate on ice in the dark for 20 min
d. Wash twice with at least 1.5 mL cell staining buffer by centrifugation at 300 x g for 5 min at 4°C
e. Resuspend cell pellet in 200 μL cell staining buffer and add 5 μL 7-AAD viability staining solution to exclude dead cells
f. Incubate on ice for 3–5 min in the dark
g. Perform flow cytometric analysis

17. MHC tetramer staining of human CD8+ T cells
MHC Tetramer is a tetramized peptide-MHC complex for enhancing the binding of the pMHC to T cell receptors. It can be used to detect the antigen-specific T cells. So, SARS-CoV-2 antigen-specific T cells can be directly detected with corresponding tetramers, unlike the indirect detection methods such as cytokine assay. Moreover, it can be applied for cell sorting. It enables detailed phenotypical and functional analysis of T cells.
a. Transfer cells from each well into 1.5 mL microcentrifuge tubes and centrifuge at 300 x g for 5 min at 4°C
b. Discard the supernatant and resuspend each pellet with 100 μL cold cell staining buffer by gentle swirling
c. It is recommended to add 5 μL Human TruStain FcX per million cells in 100 μL staining volume, mix and incubate at 25°C for 10 min

Note: It is not necessary to wash cells between these blocking and immunostaining steps.
d. Add 2 μL per sample of corresponding Flex-T complex prepared in "before you begin" steps 1–2, mix and incubate in the dark for 1 h at 25°C
e. Add 2 μL APC labeled human CD8+ (clone T8), mix and incubate in the dark for 30 min at 4°C
f. Wash once with at least 1.5 mL cell staining buffer by centrifugation at 300 x g for 5 min at 4°C
g. Resuspend cell pellet in 200 μL cell staining buffer and add 5 μL/million cells of 7-AAD viability staining solution to exclude dead cells
h. Incubate on ice for 3–5 min in the dark
i. Perform flow cytometric analysis within 2 h, representative data and a gating strategy are shown in (Figure 4A)

△ CRITICAL: When perform MHC tetramer staining with anti-CD8 antibody in flow cytometry, you need to choose correct CD8 clones to gain the best results. A wrong CD8 clone may lead to undesired reactions on MHC tetramer binding to the TCR, either enhancing or reducing tetramer reaction (Campanelli et al., 2002). We strongly recommend clones T8, Hit8a or MCD8 for human MHC tetramer staining. Also make sure the correct protocol is followed.

Intracellular cytokine (IFN-γ and Granzyme B) staining

△ Timing: ~8 h

Activated cell populations can be generated from in vitro-stimulated cultures or from in vivo-stimulated tissues (e.g., antigen-specific activation). The Leukocyte Activation Cocktail, with BD
GolgiPlug™ is a ready-to-use polyclonal cell activation mixture including the phorbol ester, a calcium ionophore (Ionomycin), the protein transport inhibitor BD GolgiPlug™ (Brefeldin A) and Phorbol 12-Myristate 13-Acetate (PMA). Primary cytokine responses by T cells can be induced with this mixture. Since Brefeldin A blocks the intracellular protein transport processes, the produced cytokines will be localized in the rough endoplasmic reticulum of the cytokine producing cells. Thus, these cells are readily detected by immunofluorescent staining and flow cytometric analysis due to the accumulation of the increased expression of cytokines.

18. Cell-Surface staining with APC anti-human CD8
   a. On day 7, quickly thaw the cocktail in a water bath at 37°C and add 2 μL Leukocyte Activation Cocktail to 1 mL cell culture (e.g., ~10⁶ cells/mL) and mix thoroughly
   b. Culture in CO₂ incubator at 37°C for 6 h

△ CRITICAL: It is suggested not to keep Leukocyte Activation Cocktail with BD GolgiPlug in a cell culture for more than 12 hours.

Note: Store product at -80°C before use or for long-term storage.

   c. Harvest cells from each well into 1.5 mL microcentrifuge tubes and centrifuged at 300 x g for 5 min at 4°C
   d. Discard the supernatant and resuspend each pellet in 100 μL cold cell staining buffer by gentle swirling
   e. It is recommended to add 5 μL of Human TruStain FcX™ per million cells in 100 μL staining volume, mix and incubate at 25°C for 10 min

Figure 4. Visualization of representative flow cytometry data and gating strategy
(A) MHC tetramer staining of human CD8+ T cells.
(B) Intracellular cytokine (IFN-γ and Granzyme B) staining.
f. Add 2 μL APC labeled human CD8+, mix and incubate in the dark for 30 min at 4°C
g. Wash once with at least 1.5 mL cell staining buffer by centrifugation at 300 × g for 5 min at 4°C

**Note:** Some antibodies to surface markers of natural cells may not recognize fixed/denatured antigens. Therefore, it is suggested to stain cell surface antigens with live and unfixed cells before cell fixation/permeabilization and intracellular staining.

19. Fixation

Stain the cell surface antigens as described in step 18, then fix cells in 0.5 mL/tube Fixation Buffer in the dark for 20 min at 25°C

Tip: For mild fixation (especially with tandem fluorophore), FluoroFix™ Buffer (BioLegend Cat# 422101) can be used.

a. Centrifuge at 300 × g for 5 min at 4°C, and discard supernatant

***Pause point:*** To hold the experiment at this point for future staining and analysis, wash cells with Cell Staining Buffer. Re-suspend the cells in Cell Staining Buffer, and store at 4°C for a short time, and stored at –80°C in a cell cryopreservation solution for a long time (fixed cells without surface antigen staining). Alternatively, cells can be kept in Cyto-Last™ Buffer for the storage of cytokine-producing cells for up to 2 weeks. The frequencies of cytokine-producing cells present in activated human PBMCs cultures may be very different due to the donor variability. For this reason, cryopreserved cells from a single donor can be used for longitudinal studies.

20. Permeabilization

a. Dilute 10 × permeabilization wash buffer stock solution to 1 × in sterile nuclease-free ddH2O
b. Resuspend fixed cells in Intracellular Staining Perm Wash Buffer and centrifuge at 300 × g for 5–10 min
c. Repeat step b twice

21. Intracellular staining (IFN-γ and Granzyme B)

Interferon-γ (IFN-γ) and Granzyme B are potent multifunctional cytokines, mainly secreted by activated NK cells and T cells. Originally characterized based on anti-viral activities, IFN-γ also play a role in proinflammatory, immunoregulatory, and anti-proliferative activities. Granzyme B exerts crucial functions to induce rapid apoptosis and cell death.

a. Resuspend fixed/permeabilized cells in residual Intracellular Staining Perm Wash Buffer and add a predetermined optimum concentration of fluorophore-conjugated antibody of interest (PerCP-IFN-γ and FITC-Granzyme B, 5 μL/tests ), incubate for 20 min at 25°C in the dark
b. Wash twice with 1.5 mL of Intracellular Staining Perm Wash Buffer and centrifuge at 300 × g for 5 min

**Note:** It is required to carry out fluorophore conjugated streptavidin incubation if primary intracellular antibody is biotinylated, and subsequent washes in Intracellular Staining Perm Wash Buffer.

c. Resuspend the fixed and intracellular labeled cells with 0.2 mL cell staining buffer and perform flow cytometric analysis, representative data and a gating strategy are shown in (Figure 4B)

**Note:** Set photomultiplier tube voltage and compensation using cell surface staining controls. Set quadrant markers based on isotype controls, unstained cells or blocking controls. To perform appropriate flow cytometric analysis, the cells stained with this method should be
examined by optical microscope and/or flow light scattering mode to confirm that they are well dispersed. Bivariate dot plots or probability contour plots can be generated by data analysis to show the frequencies and patterns by which individual cells demonstrate co-expression of a certain level of cell surface antigen and intracellular cytokine proteins.

EXPECTED OUTCOMES
The function of the immune responses against SARS-CoV-2 is urgently needed for understanding the pathogenesis of the disease and its vaccine development. CD8+ T cells are critical for virus clearance and induce long lasting protection in the host. Here we identified specific HLA-A2 restricted T cell epitopes in the protein of SARS-CoV-2. Epitope peptides were confirmed to bind with HLA-A2 and presented by antigen presenting cells to induce host immune responses. In addition, these epitopes could activate and generate epitope-specific T cells in vitro, and those activated T cells showed cytotoxicity to target cells (T2-A2 cells). Our data strongly indicated that mutant epitopes in SARS-CoV-2 variant caused deficiency in antigen presentation and CD8+ T cell immune responses. It is required to rebuild a new CD8+ T cell immune response for variant SARS-CoV-2.

LIMITATIONS
One of the limitations is the usage of sorted CD8+ T cells rather than total PBMCs. Since the proportion of T cells in PBMCs is relatively low, the proportion of activated specific CD8+ T cells is even less. Furthermore, our study focused on HLA-A*02 restricted epitopes currently, and other HLA class I allotype restricted CD8+ T cells specific epitopes should be included for a broader understanding of SARS-CoV-2 induced immune responses, which shall be addressed in further studies.

TROUBLESHOOTING
Problem 1
Low percentage of specific CD8+T cells generation after stimulation (steps 15–17).

Potential solution
In this work, 50 µL fresh T cell culture medium containing 50 IU/mL IL-2 and 20 µM mixed peptide (Figure 5) (Positive control (Influenza A M1 peptide M58-66 GILGFVFTL) and negative control (EBV virus E416-424 IVTDFSVIK) peptides) was added every two days. Another solution is to extend the stimulation time appropriately 1–3 days.

![Figure 5. Establishment of T2A2 binding assay](image)
Problem 2
A wrong anti-CD8 antibody clone may lead to undesired reactions on MHC tetramer binding to the TCR, either enhancing or reducing tetramer reaction (steps 17).

Potential solution
When perform MHC tetramer staining with anti-CD8 antibody in flow cytometry, you need to choose correct anti-CD8 antibody clone to gain the best results. We strongly recommend clones T8, Hit8a or MCD8 for human MHC tetramer staining. Also make sure the correct protocol is followed.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Guobing Chen (guobingchen@jnu.edu.cn)

Materials availability
The corresponding tetramers may be obtained from the research group of Guobing Chen, Jinan University, China.

Data and code availability
No datasets were generated using this protocol.

ACKNOWLEDGMENTS
This work was supported by grants from the National Key Research and Development Program of China (2018YFC2002003), Natural Science Foundation of China (U1801285, 81971301), Guangzhou Planned Project of Science and Technology (201904010111, 202002020039), Zhuhai Planned Project of Science and Technology (ZH22036302200067PWC), and the Initial Supporting Foundation of Jinan University.

AUTHOR CONTRIBUTIONS
G.C. and P.W. designed the project. C.X., C.Q., and J.D. performed the experiments. J.Y., L.G., and J.S. assisted with experiments; O.J.L. assisted with data analysis. C.X., P.W., and G.C. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
Campanelli, R., Palermo, B., Garbelli, S., Mantovani, S., Lucchi, P., Necker, A., Lantelme, E., and Giachino, C. (2002). Human CD8 co-receptor is strictly involved in MHC-peptide tetramer-TCR binding and T cell activation. Int. Immunol. 1, 39–44.

Deng, J., Pan, J., Qiu, M., Mao, L., Wang, Z., Zhu, G., Gao, L., Su, J., Hu, Y., Luo, O.J., et al. (2021). Identification of HLA-A2 restricted CD8+ T cell epitopes in SARS-CoV-2 structural proteins. J. Leukoc. Biol. https://doi.org/10.1002/JLB.4MA0621-020R.

Xiao, Q., Xiu, M., Li, H., Yan, Q., and Tan, Y. (2005). carboxyfluorescein diacetate succinimidyl ester fluorescent dye for cell labeling. Acta Biochim. Biophys. Sin. 06, 379–385.