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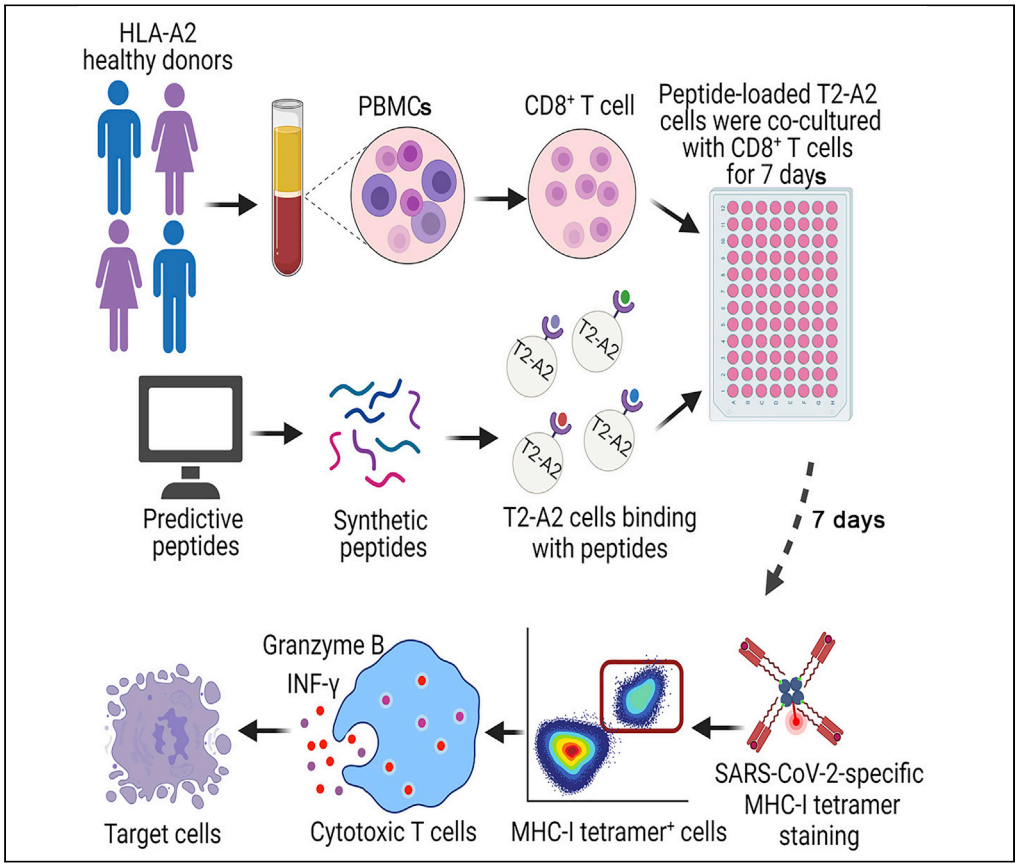
Protocol

Optimization of antigen-specific CD8$^+$ T cell activation conditions for infectious diseases including COVID-19



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.

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Highlights

Protocol for convenience to validate SARS-CoV-2 CD8+ T-cell epitopes

Rapid cell model applicable to produce a large number of specific CD8+ T cells

A cell model applicable to activation of CD8+ T cells with various infectious diseases

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Optimization of antigen-specific CD8$^+$ T cell activation conditions for infectious diseases including COVID-19

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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.

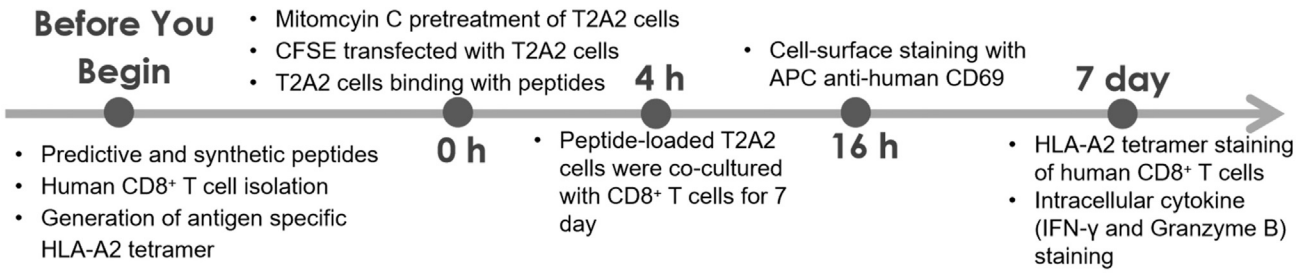

Figure 1. Timeline for the overall experimental procedures

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 aliquote 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 sonication 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	KLINIIIWFL
	04	2230	2238	9	IIWFLLLSV
	05	3673	3683	11	SLSGFKLKDCV
	06	3672	3683	12	TSLSGFKLKDCV
Spike	07	62	70	9	VTWFHAIHV
	08	136	145	10	CNDPFLGVYY
	09	495	503	9	YGFQPTNGV
	10	567	576	10	RDIADTTDAV
	11	673	684	12	SYQTQTNSPRRA
	12	713	722	10	AIPTNFTISV
	13	976	984	9	VLNDILSRL
	14	1114	1122	9	IITTDNTFV
ORF8	15	73	81	9	YIDIGNYTV
	16	18	27	10	QECSLQSCTQ
	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)

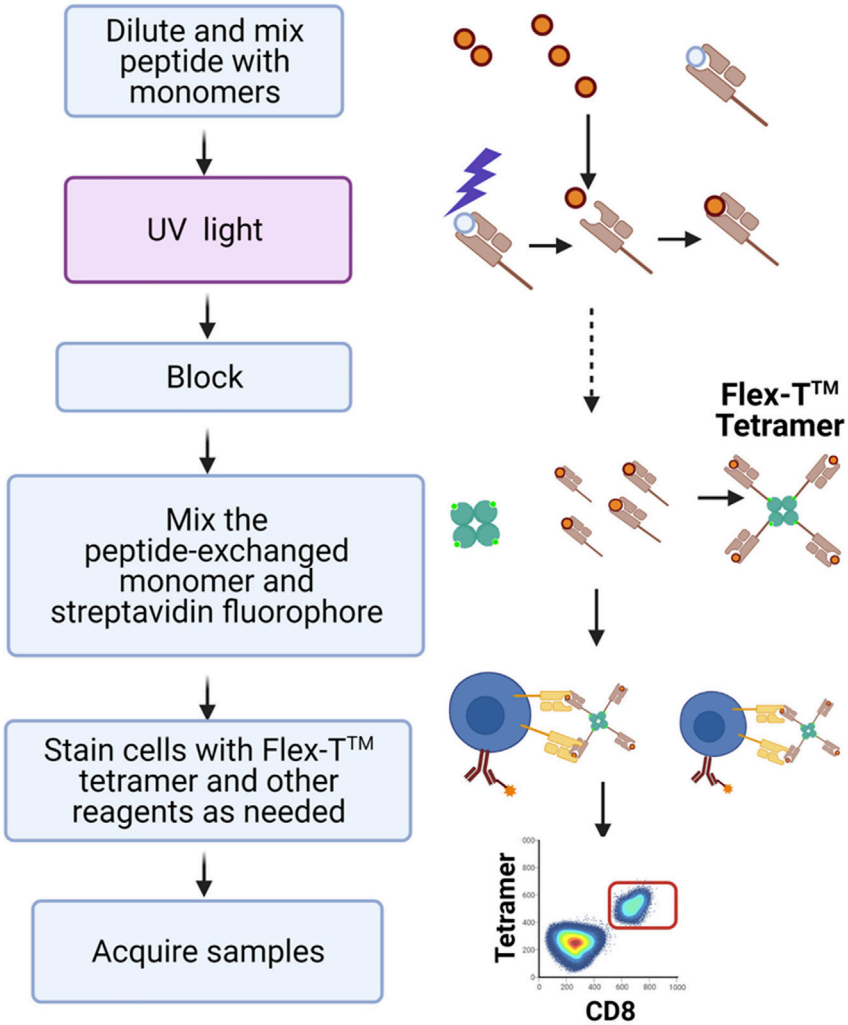

Figure 2. Generation of antigen-specific HLA-A2 tetramer

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 FcX™ (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)	LONSERA	Cat#S711-001S
RPMI 1640 medium	Gibco	Cat# 11875093

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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#abs9157
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#D2650
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 $MP_{58–66}$ GILGFVFTL peptide	GenScript	Customized
EBV $EBNA4_{416–424}$ IVTDFSVIK peptide	GenScript	Customized
SARS-CoV-2 $ORF1ab_{1000–1008}$TTIQTIVEV peptide	GenScript	Customized
SARS-CoV-2 $ORF1ab_{1007–1016}$AANFCALILA peptide	GenScript	Customized
SARS-CoV-2 $ORF1ab_{2225–2234}$KLINIIIWFL peptide	GenScript	Customized
SARS-CoV-2 $ORF1ab_{2230–2238}$IIWFLLLSV peptide	GenScript	Customized
SARS-CoV-2 $ORF1ab_{3673–3683}$SLSGFKLKDCV peptide	GenScript	Customized
SARS-CoV-2 $ORF1ab_{3672–3683}$TSLSGFKLKDCV peptide	GenScript	Customized
SARS-CoV-2 $S_{62–70}$VTWFHAIHV peptide	GenScript	Customized
SARS-CoV-2 $S_{136–145}$ CNDPFLGVYY peptide	GenScript	Customized
SARS-CoV-2 $S_{495–503}$ YGFQPTNGV peptide	GenScript	Customized
SARS-CoV-2 $S_{567–576}$ RDIADTTDAV peptide	GenScript	Customized
SARS-CoV-2 $S_{673–684}$ SYQTQTNSPRRA peptide	GenScript	Customized
SARS-CoV-2 $S_{713–722}$ AIPTNFTISV peptide	GenScript	Customized
SARS-CoV-2 S $S_{976–984}$ VLNDILSRL peptide	GenScript	Customized
SARS-CoV-2 $S_{1114–1122}$ IITTDNTFV peptide	GenScript	Customized
SARS-CoV-2 ORF8 $_{73–81}$ YIDIGNYTV peptide	GenScript	Customized
SARS-CoV-2 $ORF8_{18–27}$ QECSLQSCTQ peptide	GenScript	Customized
SARS-CoV-2 $ORF8_{50–58}$ GARKSAPLI peptide	GenScript	Customized
SARS-CoV-2 $N_{1–9}$ MSDNGPQNQ peptide	GenScript	Customized
SARS-CoV-2 $N_{228–236}$ 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

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Falcon tube (15 mL)	Corning	Cat# CLS430053-500EA
Serological pipettes (2, 5, 10, 25 mL)	KIRGEN	Cat#KG1421, KG1431, KG1441, KG1451
T-75 cell culture flasks	Corning	Cat#CLS430720-100EA
5 mL Polystyrene round-bottom tube	STEMCELLL	Cat #38007
6-Well clear TC-treated microplates	Corning	Cat#3516
96-Well TC-treated microplates	Corning	Cat#CLS3997
Cryovials	NEST	Cat#607101
Improved neubauer hemocytometer	Brand	Cat#717810
37 μm Cell strainer	STEMCELLL	Cat #27250
Triple-purpose UV analyzer ZF-1(365 nm)	Qilin Bell	Cat#1903274
Magnet EasySep™	STEMCELLL	Cat #18000
Eppendorf centrifuge 5810R	Eppendorf	Cat #5810000394
BD Flow Cytometer FACS Canto	BD Biosciences	Cat #BD FACSCanto II

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× 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	100 mg/mL mitomycin C stocks 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
1× Permeabilization wash buffer	Prepare 10 × permeabilization wash buffer stock solution, and dilute to 1× 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), 100× antibiotics (10 mL), 100× 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 ssuggested 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

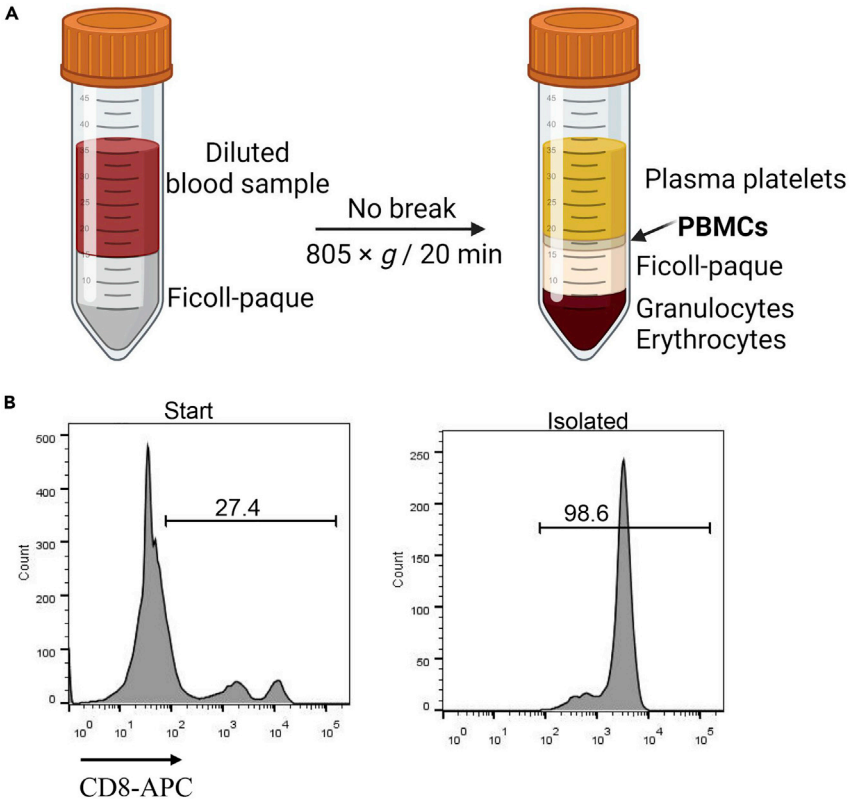

Figure 3. Isolation of human PBMCs and EasySep™ human CD8+ T cells selection
(A) Isolation of human PBMCs.
(B) EasySep™ human $CD8^+$ T cells selection.

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 immunomagnetic 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 5×10^7 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 propriate 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. Mitomcyin 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 mitomcyin 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% CO_2 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 toxic to T cells.

a. 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
b. 2–4 × 10^8 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% CO_2 for 30 min with agitation
c. The labeling reaction is stopped for 10 min by adding 9 mL complete IMDM
d. 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.
a. The candidate peptides (Table 1) are synthesized in GenScript Biotechnology and dissolved in DMSO at the concentration of 10 mM.
b. 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
a. Harvest peptide loaded T2-A2 cells from step 14 and mix all cells together
b. Centrifuge cells at 400 × *g* for 5 min at 4°C
c. Discard the supernatant and resuspend cells in primary T cell medium to a final concentration of approximately 2.5 × 10^6 cells/mL
d. 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% CO_2
e. 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.

f. 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 (1 × 10^6 cells) 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 × *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 per million cells 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 × *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 × *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

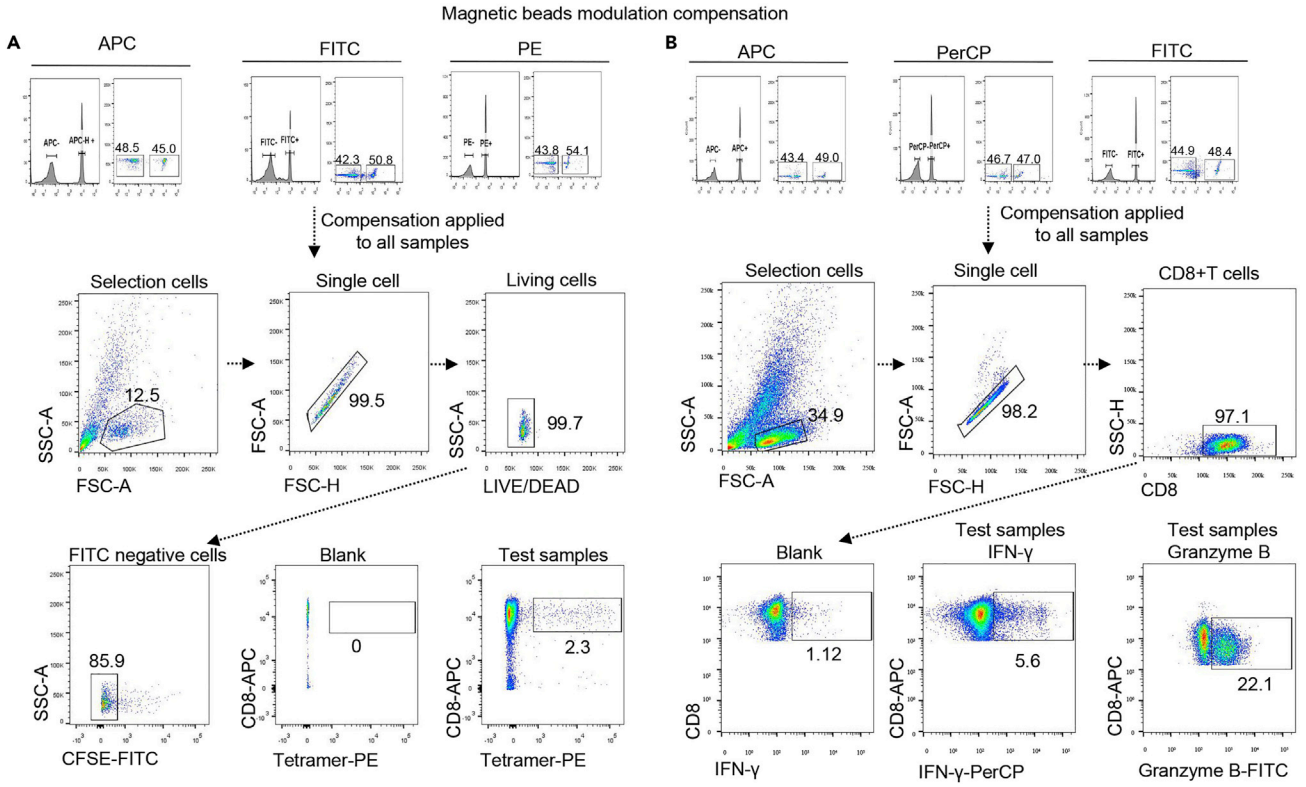

Figure 4. Visualization of representative flow cytometry data and gating strategy

(A) MHC tetramer staining of human $CD8^+$ T cells.

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

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., ~10^6 cells/mL) and mix thoroughly
    b. Culture in CO_2 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 × *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

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.

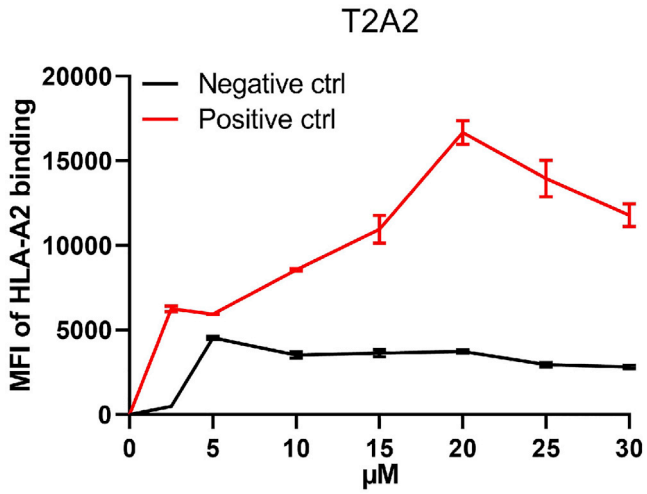

Figure 5. Establishment of T2A2 binding assay

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.

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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.