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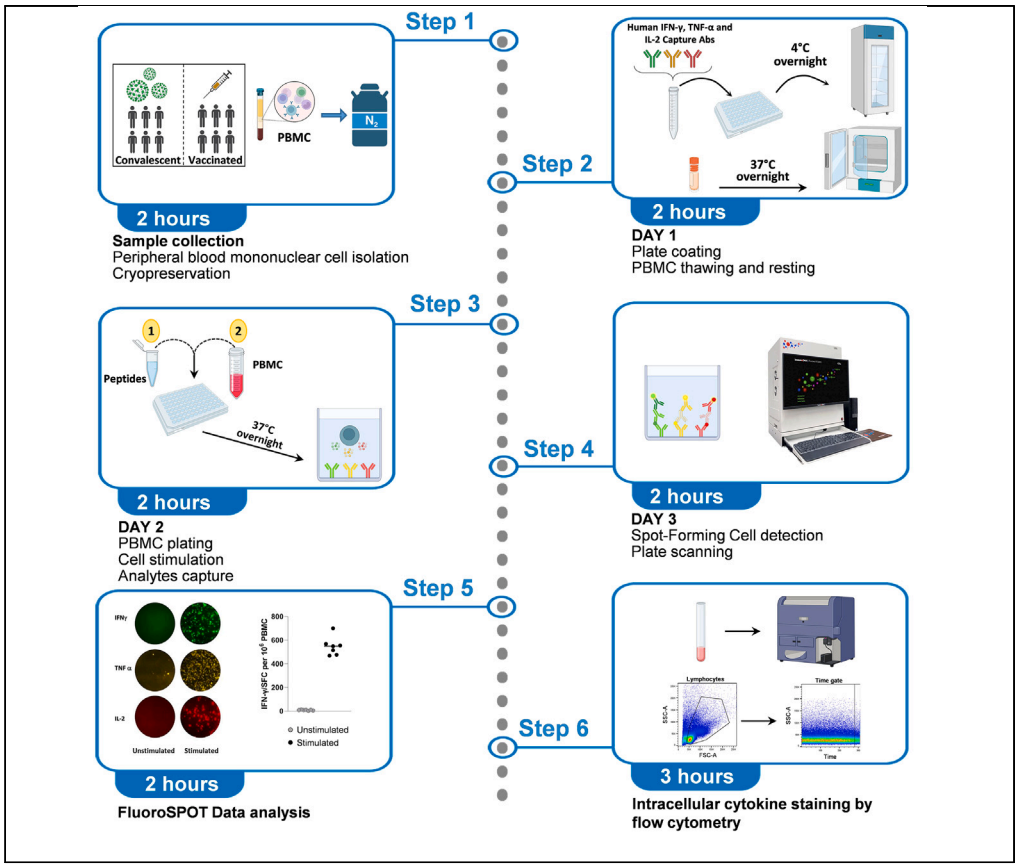
FluoroSpot assay to analyze SARS-CoV-2-specific T cell responses



Monitoring antigen-specific T cell frequency and function is essential to assess the host immune response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Here, we present a FluoroSpot assay for concurrently detecting *ex vivo* antiviral cytokine production by SARS-CoV-2-specific T cells following peptide stimulation. We then detail intracellular cytokine staining by flow cytometry to further validate the FluoroSpot assay results and define the specific T cell subpopulations.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Evaluation of SARS-CoV-2-specific T cell responses following infection or immunization

FluoroSpot assay to assess antiviral cytokine production by SARS-CoV-2-specific T cells

Phenotyping of SARS-CoV-2-specific T cell subpopulations with flow cytometry

Protocol can be performed for detection of other antigen-specific T cells

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FluoroSpot assay to analyze SARS-CoV-2-specific T cell responses

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SUMMARY

Monitoring antigen-specific T cell frequency and function is essential to assess the host immune response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Here, we present a FluoroSpot assay for concurrently detecting *ex vivo* antiviral cytokine production by SARS-CoV-2-specific T cells following peptide stimulation. We then detail intracellular cytokine staining by flow cytometry to further validate the FluoroSpot assay results and define the specific T cell subpopulations.

For complete details on the use and execution of this protocol, please refer to Tiezzi et al. (2023).[1]

BEFORE YOU BEGIN

This protocol provides reagents and steps for quantifying the SARS-CoV-2-specific T cell response from peripheral blood mononuclear cells (PBMCs) of patients after the appropriate peptide stimulation, as described in Tiezzi et al. (2023).[1] We recommend to include positive controls consisted of PBMCs stimulated with CMV, EBV and influenza peptides.

This protocol includes handling of human samples and all experiments should be performed in appropriate biosafety cabinets.

Institutional permissions

The study was approved by the competent local Ethic Committee and all patients provided written, informed consent. Please acquire all permissions needed for the use of human T cells from relevant authorities of your institution.

Peptide preparation

Timing: 1–3 h

This protocol uses synthetic peptides to stimulate T cells. This enables antigen-induced effector molecules and cytokines production by T cells.

1. Use an appropriate solvent to dissolve the lyophilized peptides and dilute to a desired concentration. The solvent should not promote peptide degradation and be compatible with the experimental application.
   a. The manufacturer's guidelines should be followed for how to dissolve peptides and the condition of storage. The peptides must be completely dissolved without aggregates inside.

⚠ CRITICAL: In general, DMSO is recommended as the initial solvent and a stock peptide concentration of 2–10 mM. DMSO used in peptide stocks should not exceed a maximum final concentration of 0.1% DMSO in cell cultures.

2. Aliquot the dissolved peptides to avoid multiple freeze-thaw cycles and store at −80°C, protected from light.

The assay can be performed using a single peptide or pooled peptides. To ensure successful T cell stimulation, pilot experiments should be performed to identify the optimal peptide concentration and number of peptides to be pooled.

Samples collection

Timing: 2 h

We collected whole blood samples from vaccinated healthy donors recruited 1–2 months after the second dose of Pfizer/BioNTech BNT162b2 vaccination and from SARS-CoV-2 convalescent patients enrolled for this study.

3. Collect about 35–40 mL of peripheral blood from each patient using BD Vacutainer NH (Sodium Heparin) tubes.
4. PBMCs from these cohorts have been isolated by Ficoll-Hypaque density gradient centrifugation and cryopreserved in liquid nitrogen until the day of analysis.
   a. Add 12 mL of density gradient medium to a 50 mL tube.
   b. Gently layer the blood sample on top of the density gradient medium. Blood samples and Ficoll gradient medium should stay as two different layers.
   c. Centrifuge at 1,000 × *g* for 20 min with the brake OFF.
   d. Carefully transfer the mononuclear cells to a clean 15 mL centrifuge tube.
   e. Wash the cells twice with Hank's solution (centrifuge at 600 × *g* for 7 min each time).
   f. Count collected PBMCs and freeze 10^7 cells/vial with 10% DMSO-FBS.

Note: Ensure all reagents are at room temperature (RT, 15°C–25°C).

⚠ CRITICAL: Do not mix the layer of the blood samples and density gradient medium.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Peripheral blood mononuclear cells (PBMCs)	University Hospital of Parma	N/A
Antibodies		
APC-H7 anti-human CD3, dilution 2:100	BD Biosciences, NJ, USA	Cat#560176; RRID: AB_1645475
PE anti-human CD4, dilution 6:100	BD Biosciences, NJ, USA	Cat#555347; RRID: AB_395752

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
BV786 anti-human CD8, dilution 2:100	BD Biosciences, NJ, USA	Cat#563823; RRID: AB_2687487
APC-R700 anti-human IFN-γ, dilution 2:100	BD Biosciences, NJ, USA	Cat#564981; RRID: AB_2739031
BV650 anti-human TNF-α, dilution 2:100	Miltenyi	Cat#563418; RRID: AB_2738194
Chemicals, peptides, and recombinant proteins		
10-mer synthesized peptides (Tiezzi et al.)[1]	Mimotopes Pty Ltd, Australia	N/A
15-mer SARS-CoV-2 overlapping spike (Tiezzi et al.)[1]	GenScript	N/A
GolgiPlug protein transport inhibitor (containing brefeldin A)	BD Biosciences, NJ, USA	Cat#555029
GolgiStop protein transport inhibitor (containing monensin)	BD Biosciences, NJ, USA	Cat#554724
Lympholyte cell separation media	Euroclone	Cat#DVCL5020
Phosphate-buffered saline, pH 7.4 (PBS) 10×	Euroclone	Cat#ECM4004XL
Hank's balanced salts solution (HBSS)	Euroclone	Cat#ECB4006L
RPMI 1640	Euroclone	Cat#ECM2001L
Fetal bovine serum (FBS)	Euroclone	Cat#ECS5000L
Tween 20	Sigma-Aldrich	Cat#P7949-100mL
Human serum	Sigma-Aldrich	Cat#H6914-100mL
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#D5879
2-mercaptoethanol (2-ME)	Merck	Cat#M6250
Non-essential amino acids (NEAA) (MEM)	Euroclone	Cat#ECB3054D
Gentamicin sulfate 80 mg/2 mL	LFM	N/A
Amphotericin-B 250 μg/mL	Euroclone	Cat#ECM0009D
Ethanol (EtOH)	Carlo Erba	Cat#414601
L-glutamine 100×	Euroclone	Cat#ECB3000D
Penicillin-Streptomycin 100×	Euroclone	Cat#ECB3001D
Critical commercial assays		
Human IFN-γ/TNF-α/IL-2 Three-Color FluoroSpot Kit	Cellular Technology Limited (CTL), DE	Cat#HT3001F-1M/5
LIVE/DEAD Fixable Viability Stain 575V Kit	BD Biosciences, NJ, USA	Cat#565694
FIX&PERM Cell Fixation and Permeabilization Kit	Nordic-MUbio	Cat#GAS-002
Software and algorithms		
FlowJo 10.8.1	BD Biosciences	https://www.flowjo.com/solutions/flowjo/downloads/previous-versions
ImmunoSpot 7 Software	Cellular Technology Limited (CTL), DE	https://immunospot.com/immunospot-software.html
GraphPad Prism 7	GraphPad	https://www.graphpad.com/features
Other		
LSR Fortessa	BD Biosciences	N/A
S6 Ultra M2 analyzer	CTL Analyzers, LLC	N/A

MATERIALS AND EQUIPMENT

Complete RPMI medium		
Reagent	Final concentration	Amount
RPMI 1640	N/A	489 mL
2-ME	0.05 mM	25 μL
MEM	1×	5 mL
Gentamicin sulfate	40 μg/mL	500 μL
Amphotericin-B	2.5 μg/mL	5 mL
Total	N/A	500 mL

Note: Complete RPMI Medium can be stored at 4°C for up to 1 month and should be warmed to 37°C before use.

ICS culture media		
Reagent	Final concentration	Amount
Complete RPMI Medium	N/A	46 mL
Human Serum	8%	4 mL
Total	N/A	50 mL

Note: ICS Culture Media can be stored at 4°C for up to 1 month and should be warmed to 37°C before use.

FluoroSpot CTL-Resting medium		
Reagent	Final concentration	Amount
CTL-Test Medium	N/A	49 mL
L-glutamine	1%	0.5 mL
Penicillin-Streptomycin	1%	0.5 mL
Total	N/A	50 mL

Note: FluoroSpot CTL-Resting Medium can be stored at 4°C for up to 1 week and should be warmed to 37°C before use.

FluoroSpot CTL-Test medium		
Reagent	Final concentration	Amount
CTL-Test Medium	N/A	49.5 mL
L-glutamine	1%	0.5 mL
Total	N/A	50 mL

Note: FluoroSpot CTL-Test Medium can be stored at 4°C for up to 1 week and should be warmed to 37°C before use.

70% ethanol		
Reagent	Final concentration	Amount
Ethanol Absolute	70%	10 mL
Distilled water	N/A	4.3 mL
Total	N/A	14.3 mL

Note: 70% Ethanol should be prepared right before using.

PBS 1×		
PBS 1×	1×	50 mL
Distilled water	N/A	450 mL
Total	N/A	500 mL

Note: PBS 1× can be stored at 4°C for up to 3 months and should be warmed to 37°C before use.

0.05% Tween-PBS		
Reagent	Final concentration	Amount
PBS 1×	N/A	500 mL
Tween 20	0.05%	0.25 mL
Total	N/A	500.25 mL

Note: 0.05% Tween-PBS can be stored at 4°C for up to 3 months and should be warmed to 37°C before use.

8% human serum RPMI 1640		
Reagent	Final concentration	Amount
Complete RPMI Medium	N/A	46 mL
Human Serum	8%	4 mL
Total	N/A	50 mL

Note: 8% human serum RPMI 1640 can be stored at 4°C for up to 1 month and should be warmed to 37°C before use.

2% FBS		
Reagent	Final concentration	Amount
FBS	2%	2 mL
PBS 1×	N/A	98 mL
Total	N/A	100 mL

Note: 2% FBS can be stored at 4°C for up to 3 months.

Freezing solution		
Reagent	Final concentration	Amount
FBS	90%	90 mL
DMSO	10%	10 mL
Total	N/A	100 mL

Note: Freezing solution can be stored at 4°C for up to 3 months.

LIVE/DEAD fixable viability stain 575V 1×		
Reagent	Final concentration	Amount
LIVE/DEAD Fixable Viability Stain 575V 1000×	1×	1 μL
PBS 1×	N/A	999 μL
Total	N/A	1 mL

Note: LIVE/DEAD Fixable Viability Stain 575 1× should be prepared right before using.

FluoroSpot antibody	
Antibodies	Final concentration
Capture solution	
Human IFN-γ Capture Ab	4 μL in 1 mL of diluent A
Human TNF-α Capture Ab	8 μL in 1 mL of diluent A
Human IL-2 Capture Ab	10 μL in 1 mL of diluent A
Detection solution	
Anti-Human IFN-γ (FITC)	2 μL in 1 mL of diluent B
Anti-Human TNF-α (Hapten1)	3 μL in 1 mL of diluent B
Anti-Human IL-2 (Hapten2)	2 μL in 1 mL of diluent B
Tertiary solution	
Anti-FITC Alexa Fluor 488	2.5 μL in 1 mL of diluent C
Anti-Hapten1 CTL-Yellow	2.5 μL in 1 mL of diluent C
Anti-Hapten2 CTL-Red	2.5 μL in 1 mL of diluent C

Note: FluoroSpot antibody dilutions should be prepared the same day of the experiment and stored at 4°C until use.

Note: For this study, we chose this kit to investigate IFN-γ, TNF-α and IL-2 production, but several sets with different associations of metabolites are commercially available.

Flow cytometry staining antibody	
Antibodies	Final concentration
APC-H7 anti-human CD3	2 μL per 10^6 cells
PE anti-human CD4	6 μL per 10^6 cells
BV786 anti-human CD8	2 μL per 10^6 cells
APC-R700 anti-human IFN-γ	2 μL per 10^6 cells
BV650 anti-human TNF-α	2 μL per 10^6 cells
Brilliant Stain Buffer	50 μL per tube
LIVE/DEAD Fixable Viability Stain 575V 1×	100 μL per tube

Note: Staining antibodies should be prepared right before use.

STEP-BY-STEP METHOD DETAILS

Day 1 – Plate Coating and PBMCs resting

Timing: 2 h

This section describes how to prepare plates for FluoroSpot test setup. The plate pretreatment permits to obtain high-quality spots.

1. Plate coating.
   a. Prepare Human *IFN-γ/TNF-α/IL-2 Capture Solution* and prepare 70% ethanol.
   b. Remove plate underdrain, pipette 15 μL of 70% ethanol into each well quickly.
   c. Add 150 μL of PBS, decant, and wash with 150 μL of PBS 1× two more times.
   d. Replace underdrain and immediately (before plate dries) pipette 80 μL/well Human *IFN-γ/TNF-α/IL-2 Capture Solution.*
   e. Seal plate with parafilm and incubate at 4°C for 24 h.

Note: Activation of the membrane with ethanol is instantaneous and can be seen visually as a graying of the membrane. Wash ethanol off as quickly as possible following activation.

2. Cryopreserved PBMCs thawing and resting.
   a. Thaw frozen PBMCs in a 15 mL centrifuge tube adding *CTL-Resting Medium* at 37°C up to 15 mL.
   b. Centrifuge samples at 600 × *g* for 5 min at RT, then discard the supernatant.
   c. Resuspend the thawed PBMCs in 1–2 mL of *CTL-Resting Medium*.
   d. Count PBMCs using a viability stain and calculate the total number of live cells.
   e. Adjust the total volume of *CTL-Resting Medium* to achieve a final concentration of 2×10^6 PBMC/mL.
   f. Incubate PBMCs at 37°C for 18 h resting.

Note: since a 10%–15% loss in cell number is expected after resting, it is ideally advisable to thaw a higher number of PBMCs to achieve the desired number of cells.

Day 2 - PBMCs plating and peptide stimulation

Timing: 2 h

This section describes the steps to stimulate PBMCs for 24 h with SARS-CoV-2 spike peptide. Antigen-specific T cells will produce IFN-γ, TNF-α and IL-2 and will be detected as fluorescent spot forming units.

3. Stimulate PBMCs with peptide pools and incubate for ex-vivo FluoroSpot.
   a. Prepare peptide pool solutions at two times final concentration (2 μM) in *CTL-Test Medium*.
   b. Decant plate containing *Capture Solution* from the day before and wash one time with 150 μL of PBS.
   c. Plate peptide pool solutions and provided stimulatory reagent Anti-CD28 (0.1 μg/mL final concentration for each well), 50 μL/well.
   d. Count PBMCs using a viability stain and calculate the total number of live cells.
   e. Adjust PBMCs to desired concentration in *CTL-Test Medium*, e.g.,: 6 million/mL corresponding to 300,000 cells/50 μL for each well (cell numbers can be adjusted according to expected spot counts since 300,000–500,000 cells/well will provide linear results). Keep cells at 37°C in humidified incubator, 5% CO_2 while processing PBMCs and until plating.
   f. Plate PBMCs, 50 μL/well using large orifice tips. Once completed, gently tap the sides of the plate and immediately place into a 37°C humidified incubator, 5% CO_2.
   g. Incubate for 24 h. Do not stack plates. Avoid shaking plates by carefully opening and closing incubator door. Do not touch plates during incubation.

Note: ideally PBMCs per well should be seeded in duplicate or in triplicate. We recommend to include at least 3 unstimulated wells.

Note: Ensure the pH and temperature are ideal for cells by placing the plate containing antigens into a 37°C, 5% CO_2 incubator if it will be more than 10–20 min before plating cells.

Note: each washing step is done manually adding the correct volume of mentioned washing solution, allowing wells to soak for about 1 min.

Day 3 - Spot-forming cell detection and plate scanning

Timing: 2 h

This section describes the Spot Detection by fluorophore-conjugated reagents and the plate count by S6 Ultra M2 Analyzer.

4. Detect spots.
   a. Prepare *0.05% Tween-PBS Solution*.
   b. Prepare Anti-human IFN-γ/TNF-α/IL-2 *Detection Solution*.
   c. Wash plate two times with PBS and then two times with 0.05% Tween-PBS, 200 μL/well each time.
   d. Add 80 μL/well Anti-human IFN-γ/TNF-α/IL-2 *Detection Solution*. Incubate at RT, 2 h.
   e. Prepare *Tertiary Solution*.
   f. Wash plate three times with 0.05% Tween-PBS, 200 μL/well.
   g. Add 80 μL/well of *Tertiary Solution*. Incubate at RT, 1 h.
   h. Decant and wash plate three times with distilled water, 200 μL/well.
   i. Remove protective underdrain from the plate and rinse back of plate with sterile water.
   j. Air-dry plate for 2 h in running laminar flow hood.
5. Scan and count plate according to the manufacturer's instructions.
   a. example of FluoroSpot representation is shown in Figure 1.

   Note: each washing step done manually adding the correct volume of mentioned washing solution, allowing wells to soak for about 1 min.

Intracellular cytokine staining (ICS)

Timing: 3 h

This section describes steps to better characterize SARS-CoV-2 T cells upon peptide stimulation and to quantify their cytokine production with flow cytometry.

6. Peptide stimulation.
   a. Resuspend at least 10^6 PBMCs with 200 μL of *8% human serum RPMI 1640* in a 5 mL round-bottom polystyrene tube.
   b. Stimulate PBMCs with or without SARS-CoV-2 peptide pools (1 μM) in the presence of Brefeldin A (GolgiPlug 1 μg/mL) and Monensin (GolgiStop 0.5 μg/mL).
   c. Incubate at 37°C for 18 h.
7. Stain the cells with Abs.
   a. Wash the tube with at least 2 mL of *2% FBS-PBS* (centrifuge samples at 670 × *g* for 4 min).
   b. Add 100 μL of *LIVE/DEAD fixable dead cell cell stain 1*×.
   c. Incubate at RT for 15 min.
   d. Add 60 μL of human anti-CD3, anti-CD4, anti-CD8 and brilliant stain buffer cocktail and mix. See Flow cytometry Staining antibody table.
   e. Incubate 30 min in the dark at 4°C.
   f. Wash the tube with at least 2 mL of 2% FBS-PBS (centrifuge samples at 670 × *g* for 4 min).
   g. Add 100 μL of Fix reagent, as manufacturer's instructions.
   h. Incubate 15 min in the dark at 4°C.
   i. Wash the tube with at least 2 mL of 2% FBS-PBS (centrifuge samples at 670 × *g* for 4 min).
   j. Add 100 μL of Perm reagent, as manufacturer's instructions, and 4 μL of human anti-IFN-γ and anti-TNF-α cocktail and mix. See Flow cytometry Staining antibody table.
   k. Incubate 30 min in the dark at 4°C.
   l. Wash the tube with at least 4 mL of 2% FBS-PBS (centrifuge samples at 670 × *g* for 4 min).
   m. Acquire samples on a BD LSR Fortessa and analyze with the FlowJo software.
   n. Gating strategy is shown in Figure 2.

A

VAX 019

	1	2	3	4	5	6	7
A		pool 3	pool 11	pool 19	pool 27	pool L7	pool S6
B		pool 4	pool 12	pool 20	pool 28	pool L8	pool S7
C		pool 5	pool 13	pool 21	pool L1	pool L9	Unstimulated
D		pool 6	pool 14	pool 22	pool L2	pool S1	Unstimulated
E		pool 7	pool 15	pool 23	pool L3	pool S2	
F		pool 8	pool 16	pool 24	pool L4	pool S3	
G	pool 1	pool 9	pool 17	pool 25	pool L5	pool S4	
H	pool 2	pool 10	pool 18	pool 26	pool L6	pool S5	

IFN-γ

	1	2	3	4	5	6	7
A		22	4	7	20	60	587
B		6	16	14	11	6	83
C		10	11	10	30	8	26
D		6	8	20	4	608	33
E		9	10	11	47	101	
F		6	10	11	11	186	
G	6	21	10	9	3	66	
H	8	17	9	12	24	177	

TNF-α

	1	2	3	4	5	6	7
A		983	774	680	721	71	1021
B		591	639	1020	873	78	883
C		684	6041	647	61	72	297
D		838	627	723	44	1002	220
E		722	620	644	106	927	
F		719	740	743	83	907	
G	172	756	826	790	86	937	
H	977	891	876	788	84	919	

IL-2

	1	2	3	4	5	6	7
A		47	39	56	60	56	449
B		47	57	50	41	26	180
C		38	47	33	50	32	112
D		36	27	54	21	461	113
E		39	39	30	71	212	
F		40	47	40	36	229	
G	39	44	49	50	26	163	
H	59	47	53	46	40	209	

B

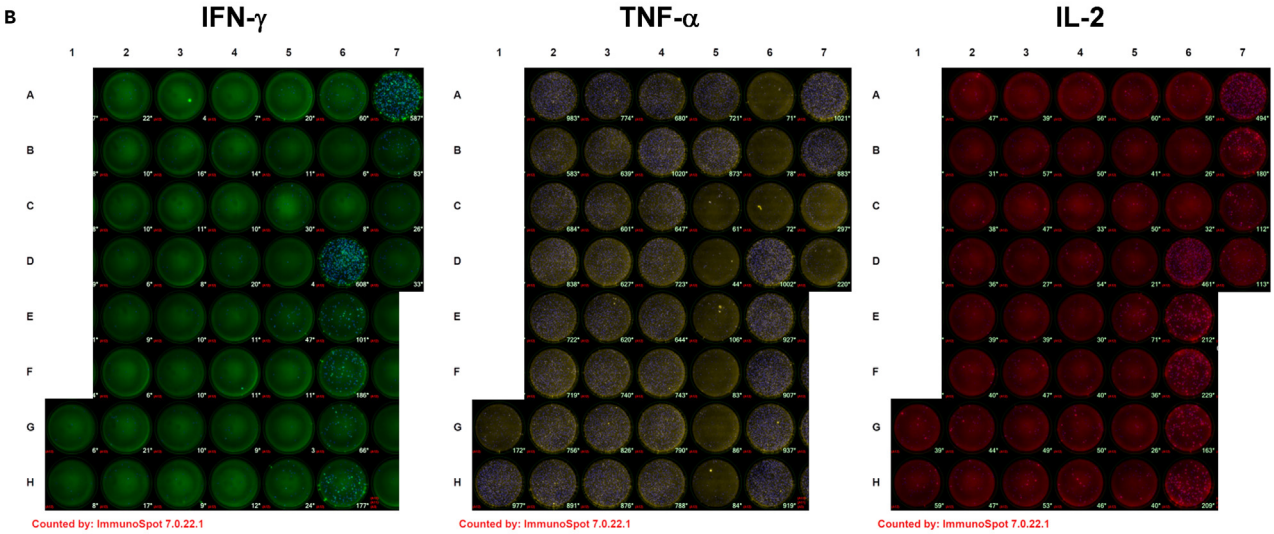

Figure 1. Results of FluoroSpot assay from a representative vaccinated subject

(A) Example of raw data derived from the plate reader. Red numbers indicate positive spots.

(B) Image of IFN-γ, TNF-α and IL-2 production exported from the ImmunoSpot 7 Software.

Note: Brefeldin A and Monensin are used to study protein transport and secretion in immune cells. They both prevent protein secretion, allowing the accumulation of proteins in the endoplasmic reticulum or the Golgi complex.

EXPECTED OUTCOMES

Following the steps of the FluroSpot assay, specific SARS-CoV-2 T cell responses induced by mRNA vaccination or natural infection can be assessed. This protocol combines the sensitivity of ELISpot with the capacity to examine additional functional and qualitative parameters of T cell-mediated immune responses such as different cytokines and effector molecules secreted by activated T cells. These SARS-CoV-2-specific T cells can be further characterized by flow cytometry to verify FluoroSpot assay results and to better define the specific T cell sub-populations targeted by SARS-CoV-2 spike peptides.

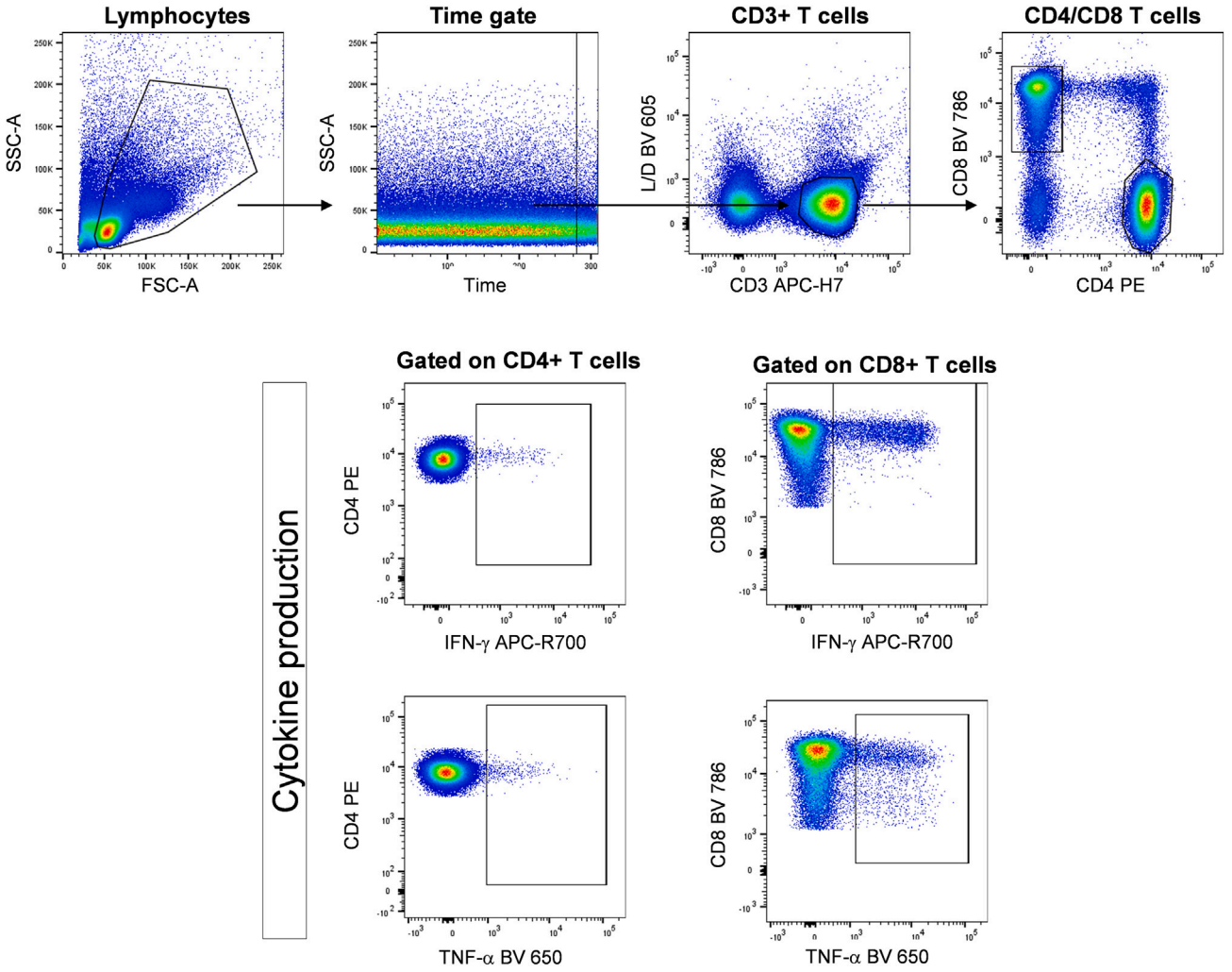

Figure 2. Gating strategy for the intracellular cytokine staining performed with PBMC stimulated by peptide pools

QUANTIFICATION AND STATISTICAL ANALYSIS

FluoroSpot is considered positive if the number of spots in the stimulated wells is at least 3 standard deviations above background and the difference between the number of spots in the stimulated and unstimulated wells is above 10. Cytokine-secreting cells are expressed as spot forming cells (SFC) per 10^6 cells after subtraction of the background.

LIMITATIONS

T cell functional analysis by FluoroSpot assay is limited to three parameters.

These protocols have been validated using peptides to quantify the antigen-specific T-cell response. So, if complex antigens (e.g., whole virus proteins) are used, further improvements will be necessary to investigate the endogenously synthesized antigen processing.

The FluoroSpot assay does not provide information on the phenotype of responding T cells.

TROUBLESHOOTING

Problem 1

Higher background appearing in the control wells.

Potential solution

This problem can be resolved by:

- Wash at least 4 times the plate between each step,
- Use CTL Serum-free Media for freezing, washing and testing PBMC. Even brief exposure to a mitogenic serum can cause high background.

Note: Plates may be washed manually or with a suitable automated plate washer with adjusted pin length and flow rate, so membranes and spots are not damaged

Problem 2

Too low or too high spot frequency and intensity due to clumping of cells, inadequate incubation time, not appropriate cell concentration.

Potential solution

FluoroSpot conditions should be optimized as follows:

- gain a homogeneous cell suspension in order to avoid clumping of cells.
- determine the optimal T cell (pre)incubation time.
- optimize cell concentration.

Problem 3

High proportion of cell death.

Potential solution

This problem can be resolved by:

- use cells with a viability more than 90%.
- incubate thawed cells for 18 h to promote discarding of dying cells, allowing only live cells to be plated as described in day 1 – Plate Coating and PBMCs Resting.

Problem 4

Unspecific response in the FluoroSpot results. Even brief exposure to a mitogenic serum can cause high background while other sera can have suppressive effects.

Potential solution

This problem can be resolved by using free serum medium during all cell processing steps.

Problem 5

Insufficient number of PBMCs to test all desired conditions.

Potential solution

It is frequently not easy to obtain sufficient PBMCs to test all conditions or individual peptides. For these scenarios, we advise to stimulate the PBMCs with peptides in a pool, which can be made following the instructions in the peptide preparation section.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Carolina Boni (cboni@ao.pr.it).

Materials availability

This study did not generate any new reagents.

Data and code availability

This study did not generate any new data or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Methodology, C.T., M.R., A.V., S.D., A.P., P.F., I.M., C.C.B., V.R., and A.M.; conceptualization, G.M., C.F., and C.B.; investigation, C.F. and C.B.; data curation and writing, C.T., M.R., and C.B.; funding acquisition, C.F.; supervision, G.M., C.F., and C.B.

DECLARATION OF INTERESTS

C.F. has received grants from Gilead and AbbVie and is a consultant at Gilead, AbbVie, Vir Biotechnology Inc, Arrowhead, Transgene, and BMS.

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1. Tiezzi, C., Vecchi, A., Rossi, M., Cavazzini, D., Bolchi, A., Laccabue, D., Doselli, S., Penna, A., Sacchelli, L., Brillo, F., et al. (2023). Natural heteroclitic-like peptides are generated by SARS-CoV-2 mutations. iScience *26*, 106940. https://doi.org/10.1016/j.isci.2023.106940.