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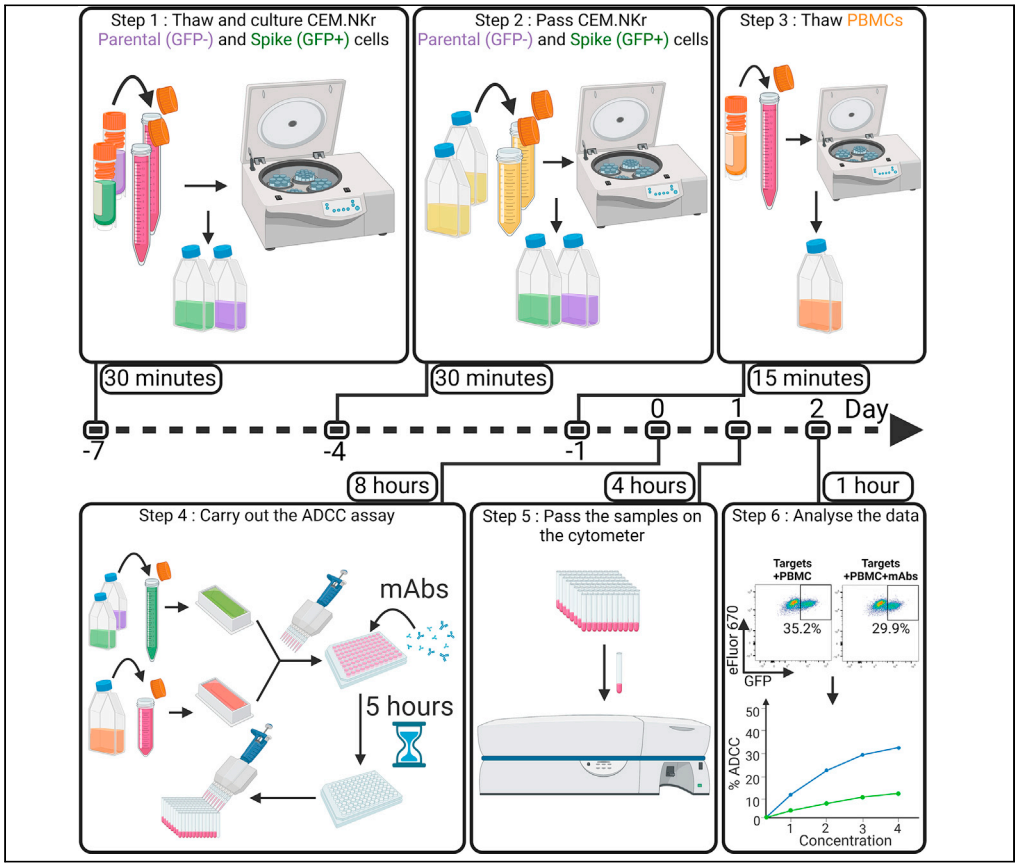
A new flow cytometry assay to measure antibody-dependent cellular cytotoxicity against SARS-CoV-2 Spike-expressing cells



Antibodies can engage specific receptors at the surface of effector cells and mediate several functions beyond viral neutralization. Increasing evidence suggests that Fc-mediated effector functions, such as antibody-dependent cellular cytotoxicity (ADCC), have an important role in protection against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections. We engineered a cell line stably expressing a GFP-tagged SARS-CoV-2 Spike to measure ADCC. This protocol provides an optimized way of measuring ADCC activity mediated by anti-SARS-CoV-2 Spike monoclonal antibodies or plasma from previously infected or vaccinated individuals.

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Highlights

A novel FACS-based assay to measure ADCC against SARS-CoV-2 Spike-expressing cells

A cell line stably expressing a GFP-tagged SARS-CoV-2 Spike was generated

This cell line is susceptible to antibody-mediated ADCC in a dose-dependent manner

This assay can measure ADCC activity of plasma from infected/vaccinated individuals

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A new flow cytometry assay to measure antibody-dependent cellular cytotoxicity against SARS-CoV-2 Spike-expressing cells

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SUMMARY

Antibodies can engage specific receptors at the surface of effector cells and mediate several functions beyond viral neutralization. Increasing evidence suggests that Fc-mediated effector functions, such as antibody-dependent cellular cytotoxicity (ADCC), have an important role in protection against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections. We engineered a cell line stably expressing a GFP-tagged SARS-CoV-2 spike to measure ADCC. This protocol provides an optimized way of measuring ADCC activity mediated by anti-SARS-CoV-2 Spike monoclonal antibodies or plasma from previously infected or vaccinated individuals.

For complete details on the use and execution of this protocol, please refer to Anand et al. (2021b).

BEFORE YOU BEGIN

We recently developed a new method to measure antibody dependent cellular cytotoxicity (ADCC) against SARS-CoV-2 Spike expressing cells. In this flow cytometry-based assay, a human T-lymphoid cell line resistant to direct NK cell-mediated lysis and stably expressing a full length GFP-tagged SARS-CoV-2 Spike (CEM.NKr.Spike cells) is mixed with parental control cells (CEM.NKr cells). These cells are stained with a cellular marker and subsequently used as target cells. Overnight rested peripheral blood mononuclear cells (PBMCs) from healthy individuals stained with another cellular marker are used as effector cells. Stained target and effector cells are then mixed and incubated with anti-Spike monoclonal antibodies. ADCC activity is then calculated by measuring the loss of Spike-expressing GFP^{High} among the target cell population. The following protocol describes the specific steps to measure the ADCC activity of monoclonal antibodies with mutations enhancing or reducing antibody engagement with FcγRIIIa, but can also be used to measure ADCC activity of recombinant proteins (such as ACE2-Fc) and plasmas from SARS-CoV-2 infected, previously infected or vaccinated individuals as previously published (Anand et al., 2021b; Tauzin et al., 2021).

Preparation of the media for target and effector cells

Timing: 10 min

1. Add the following reagents to 445 mL of RPMI 1640 media:

a. 50 mL of Fetal Bovine Serum (FBS).
b. 5 mL of Penicillin-Streptomycin (10 000 U/mL of penicillin and 10 000 μg/mL of streptomycin).

2. Shake the media and wait at least 5 min before using this newly prepared media. See the materials and equipment section for the recipe of the ''Supplemented RPMI 1640 media''.

⚠ CRITICAL: The medium must be prepared in a sterile environment.

Target cell maintenance

Timing: 30 min

To study the ADCC response against SARS-CoV-2, we generated a human T-lymphoid cell line resistant to NK cell lysis and stably expressing a full length GFP-tagged SARS-CoV-2 Spike (CEM.NKr.Spike) (Anand et al., 2021b). As presented in Figure 1, CEM.NKr.Spike is efficiently recognized by anti-Spike monoclonal antibodies (CV3-13 WT and CV3-25 WT) or plasma from COVID-19+ individuals, but not by control monoclonal antibodies (Trastuzumab and A32) or plasma from COVID-19- individuals. The CEM.NKr.Spike cells, as well as the parental CEM.NKr cells are used as target cells in this assay. These cells can be stored for years in the vapor phase of a nitrogen storage tank.

3. Take two 15 mL tubes and label each one for the appropriate cell line.
4. Pipette 10 mL of supplemented RPMI 1640 media in each tube.
5. Thaw CEM.NKr parental and CEM.NKr.Spike cells.
   a. Take the cryogenic vials containing the cells and put them in a hot-water bath at 37°C.
   b. Wait a few moments (1 or 2 min) until the cells are no longer frozen.
6. Quickly pipette the content of both vials in the tubes.
7. Centrifuge the cells at 484 × *g* for 3 min.
8. Discard the supernatant of the cells.
9. Resuspend the cells in supplemented RPMI 1640 media.
10. Count the cells.
11. Centrifuge the cells at 484 × *g* for 3 min.
12. Throw away the supernatant of the cells.
13. Add the appropriate volume of supplemented RPMI 1640 media so that cells are at concentration of 0.25×10^6 cells/mL.
14. Pipette the cells in 2 different cell culture treated flasks.
15. Put the flasks to incubate at 37°C and 5% CO_2.
16. Cells are passaged every 3–4 days (when they reach a concentration of about 1.25×10^6 cells/mL). If the CEM.NKr parental and/or CEM.NKr.Spike cells are in an irregular shape, please refer to the troubleshooting section (problem 1).

⚠ CRITICAL: Do not mix both cell lines in the same flask or tube.

⚠ CRITICAL: The cell lines must have been in culture at least 7 days before starting the assay.

⚠ CRITICAL: When thawing the cells, both cell lines should not be let without supervision in the hot-water bath. As soon as the cells are no longer frozen, you should proceed to the next step to prevent cell mortality.

Isolation and conservation of effector cells

17. Label the cryo-sheets and stick them on the cryotubes for the PBMCs you will isolate.
18. Place 10 freezing containers at 4°C after adding the appropriate volume of isopropyl alcohol.
19. Place 1.5 L of RPMI 1640 at room temperature.
20. Pipette 15 mL of LSM (Lymphocyte separation medium) in 20 tubes of 50 mL.

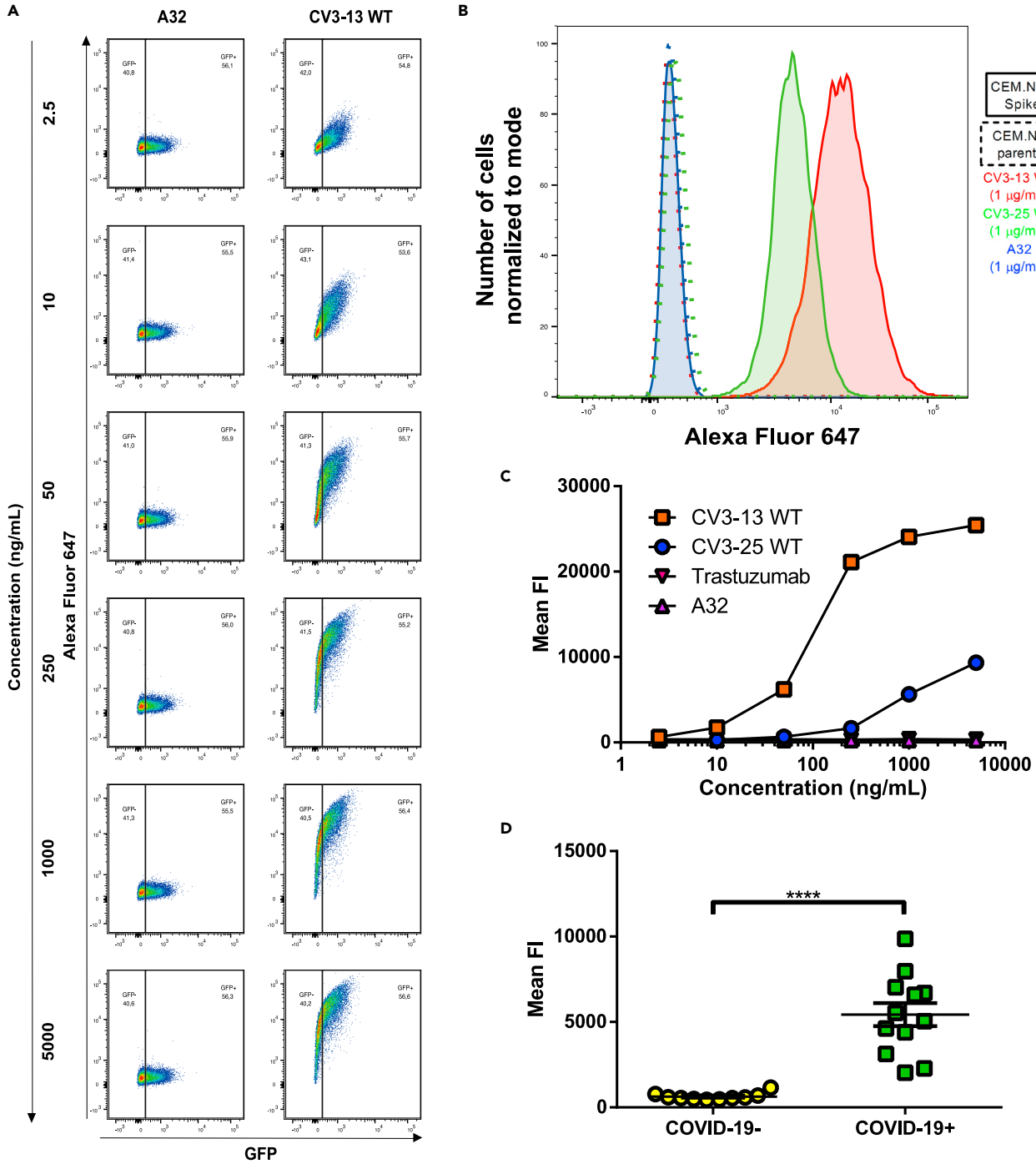

Figure 1. Staining the CEM.NKr.Spike cell line

(A) Example of staining by A32 and CV3-13 WT on the CEM.NKr.spike cell line. Staining of CEM.NKr.Spike cells was done at 2.5 ng/mL, 10 ng/mL, 50 ng/mL, 250 ng/mL, 1000 ng/mL and 5000 ng/mL.

(B) Histogram of the staining of CEM.NKr parental cells and of CEM.NKr.Spike cells by CV3-13 WT, CV3-25 WT and A32 at 1 µg/mL.

(C) Staining of CEM.NKr.Spike cells at 2.5 ng/mL, 10 ng/mL, 50 ng/mL, 250 ng/mL, 1000 ng/mL and 5000 ng/mL by monoclonal antibodies targeting the SARS-CoV-2 Spike (CV3-13 WT and CV3-25 WT) and by monoclonal antibodies not targeting the SARS-CoV-2 Spike (A32 and Trastuzumab).

(D) Staining of CEM.NKr.Spike cells by COVID-19- plasmas and COVID-19+ plasmas at a dilution of 1/500. Mean FI : Mean Fluorescence intensity. Mean values ± Standard error of the Mean (SEM). The *p* value was obtained by the non-parametric Mann-Whitney test. ****, $p < 0.0001$.

21. Pick up the leukapheresis (this protocol is designed for leukapheresis but could also be modified to process other types of blood samples).
22. Disinfect the scissors and the pipe of the plasma bag with 70% ethanol.
23. Cut the pipe with the scissors and empty the blood bag (200 mL) in a T175 cell culture flask which can contain 600 mL. Dilute the blood by adding 400 mL of RPMI 1640 in the T175 cell culture flask (the blood must be at a dilution of at least 1/3).
24. Pipette gently 30 mL of the diluted blood on the LSM cushion.
25. Centrifuge the 20 tubes at 860 × *g* for 21 min. The deceleration needs to be as slow as possible.
26. Prepare 300 mL of Virkon 2% in a 500 mL bottle.
27. Pipette 4 mL of RPMI 1640 in 10 new tubes of 50 mL.
28. Remove 15 mL–20 mL of the surface phase present at the top of the 20 tubes of 50 mL.
29. With a 10 mL pipette, pipette gently the buffy coat that is located on top of the LSM cushion in one 50 mL tube that contains 4 mL of RPMI 1640. Pipette 2 buffy coats per 50 mL tube containing 4 mL of RPMI 1640.
30. Add RPMI 1640 up to a total volume of 50 mL.
31. Centrifuge these 10 tubes at 551 × *g* for 6 min.
32. From the 500 mL bottle containing 300 mL of Virkon 2%, pipette 100 mL into two other 500 mL bottles.
33. Empty the remaining volume of the 20 tubes containing the LSM cushion into these 3 bottles.
34. When the centrifugation is finished, gently pour the supernatant in the waste bottles.
35. Resuspend the PBMCs in 5 mL of RPMI 1640.
36. Pipette the cells of 5 of the 50 mL tubes into the other 5 tubes of 50 mL. You should now have 5 tubes of 50 mL containing 10 mL of RPMI 1640 and PBMCs.
37. Add 40 mL of RPMI 1640 in the 5 tubes of 50 mL.
38. Centrifuge these tubes at 484 × *g* for 5 min.
39. When the centrifugation is finished, gently pour the supernatant in the waste bottles.
40. Resuspend the PBMCs in 5 mL of RPMI 1640.
41. Pipette the cells of 3 of the 50 mL tubes into the other 2 tubes of 50 mL. You should now have 2 tubes of 50 mL containing 12,5 mL of RPMI 1640 and PBMCs.
42. Add 37,5 mL of RPMI 1640 in the two tubes of 50 mL.
43. Centrifuge these tubes at 484 × *g* for 5 min.
44. Gently pour the supernatant in the waste bottles.
45. Add 5 mL of RPMI 1640 in both tubes and pool them together.
46. Add 40 mL of RPMI 1640 in the 50 mL tube and mix the PBMCs.
47. Prepare two 1.5 mL tubes for the cell count:
    a. In the first tube, pipette 380 µL of RBCLB (Red Blood Cell Lysing Buffer Hybri-Max™).
    b. In the second tube, pipette 180 µL of Trypan Blue.
48. Pipette 20 µL of the resuspended PBMCs in the 1.5 mL tube containing 380 µL of RBCLB. Mix the solution well.
49. Wait for 2 min.
50. Pipette 20 µL of the RBCLB solution in the 180 µL of Trypan blue in the second 1.5 mL tube. Mix the solution well. (This corresponds to a final dilution of 1/200)
51. Pipette the PBMCs in a hemocytometer and count the PBMCs.
52. Centrifuge the PBMCs for 5 min at 484 × *g*.
53. Prepare 50 mL of a solution of FBS 20% DMSO.
54. Gently pour the supernatant in the waste bottle.
55. Calculate the volume of FBS 20% DMSO needed to dilute the PBMCs at a concentration of 100 × 10^6 cells/mL in the solution of FBS 20% DMSO.
56. Put the FBS 20% DMSO at 4°C for at least 5 min.
57. During the 5 min, dilute the cell pellet in FBS at a concentration of 100 × 10^6 cells/mL.
58. In a new 50 mL tube, pipette 9 mL of the cell suspension and 9 mL of the FBS 20% DMSO and mix the solution.
59. Pipette 1 mL of the new cell suspension solution in 18 cryogenic tubes. There is now about 50 × 10^6 PBMCs/tube.

60. Put these tubes in the freezing container.
61. Place the freezing container at −80°C.
62. Repeat the steps 58–61 until there are no PBMCs left.
63. 24 h after the PBMCs are placed at −80°C, transfer them into liquid nitrogen.

⚠ CRITICAL: For step 25, the deceleration needs to be extremely slow. Do not neglect this step.

Anti-Spike antibodies

In this experiment, we used the anti-Spike antibody CV3-25. This monoclonal antibody targets the S2 subunit of SARS-CoV-2 Spike (Jennewein et al., 2021) and shows ADCC activity against Spike-expressing cells (Figure 6A). We generated Leucine to Alanine (L234A/L235A, LALA) mutant version of CV3-25 to impair interaction with Fc receptors (Saunders, 2019) and reduce its ADCC activity (Figure 6A). To enhance the ADCC activity of CV3-25, we generated a Glycine to Alanine, Serine to Aspartic Acid, Alanine to Leucine and Isoleucine to Glutamic Acid (G236A/S239D/A330L/I332E, GASDALIE) mutant version of CV3-25 (Figure 6A) which strengthen the interaction between the Fc portion of an antibody and Fc receptors (Bournazos et al., 2014; DiLillo and Ravetch, 2015; Lazar et al., 2006; Richards et al., 2008; Smith et al., 2012). Importantly, the differences in ADCC were not the result of differential binding of the different monoclonal antibodies to the CEM.NKr.Spike cells as they all bind the CEM.NKr.Spike cells similarly (Figure 6B). Briefly, for the staining on the CEM.NKr.Spike cell line, approximately 300 000 cells were stained in 100 µL of an antibody solution at a concentration of 2.5 ng/mL, 10 ng/mL, 50 ng/mL, 250 ng/mL, 1000 ng/mL and 5000 ng/mL. These cells were incubated at room temperature (20°C–25°C) for 45 min before being washed twice with PBS. Following this, 100 µL of a solution of goat anti-human IgG 647 secondary antibody (mixed with the viability marker AquaVivid at a dilution of 1:1000) was added to the cells at a concentration of 2 µg/mL for 20 min at room temperature (20°C–25°C). These cells were then washed twice in PBS. Following this, these cells were fixed in a solution of PFA 2% before being passed and analyzed on a cytometer.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CV3-25 WT	Andrés Finzi Laboratory (Jennewein et al., 2021; Ullah et al., 2021)	N/A
CV3-25 L234A-L235A (CV3-25 LALA)	Andrés Finzi Laboratory (Jennewein et al., 2021; Ullah et al., 2021)	N/A
CV3-25 G236A-S239D-A330L-I332E (CV3-25 GASDALIE)	Andrés Finzi Laboratory (Jennewein et al., 2021)	N/A
CV3-13 WT	Andrés Finzi Laboratory (Jennewein et al., 2021)	N/A
A32	Andrés Finzi Laboratory	N/A
Trastuzumab	Michael McLean Laboratory (Anand et al., 2021a)	N/A
Biological samples		
Peripheral blood mononuclear cells (PBMCs)	Andrés Finzi Laboratory	N/A
COVID-19+ plasmas	Andrés Finzi Laboratory (Tauzin et al., 2021)	N/A
COVID-19- plasmas	Andrés Finzi Laboratory (Tauzin et al., 2021)	N/A
Chemicals, peptides, and recombinant proteins		
Formaldehyde, 37% by Weight (With Preservative/Certified ACS), Fisher Chemical™	Fisher Scientific	Cat#F79-500
Dimethyl Sulfoxide, Fisher BioReagents™	Fisher Scientific	Cat#BP231-1
Trypan blue	Wisent Bioproducts	Cat#609-130-EL
Red Blood Cell Lysing Buffer Hybri-Max™	Sigma-Aldrich	Cat#R7757-100ML
ISO-PROPYL ALCOHOL, Reagent Grade	BioShop Canada Inc.	Cat#ISO920.4
LSM (Lymphocyte Separation Medium)	Wisent Bioproducts	Cat#305-010-CL
Pharmacal Research Laboratories VIRKON S 50TABLETS 12BTLS/CS	Fisher Scientific	Cat#NC9549979

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
eBioscience™ Cell Proliferation Dye eFluor™ 670	Thermo Fisher Scientific	Cat#65-0840-85
eBioscience™ Cell Proliferation Dye eFluor™ 450	Thermo Fisher Scientific	Cat#65-0842-85
Experimental models: Cell lines		
CEM.NKr-CCR5 parental cells (referred to as CEM.NKr parental cells)	ATCC Laboratory	N/A
CEM.NKr.SARS-CoV-2.Spike cells (referred to as CEM.NKr.Spike cells)	Andrés Finzi Laboratory (Anand et al., 2021b)	N/A
Software and algorithms		
FlowJo 10.3	BD Biosciences	www.flowjo.com
Other		
RPMI 1640 Medium	Thermo Fisher Scientific	Cat#11875-093
Fetal Bovine Serum (FBS), qualified, Canada	Thermo Fisher Scientific	Cat#12483-020
Penicillin-Streptomycin Solution	Wisent Bioproducts	Cat#450-201-EL
PBS (phosphate buffered saline), 1X	Wisent Bioproducts	Cat#311-010-LL
Falcon® 50 mL High Clarity PP Centrifuge Tube, Conical Bottom, Sterile, 25/Bag, 500/Case	Corning	Cat#352070
Falcon® 15 mL High Clarity PP Centrifuge Tube, Conical Bottom, with Dome Seal Screw Cap, Sterile, 50/Bag, 500/Case	Corning	Cat#352096
Falcon® 75cm^2 Rectangular Straight Neck Cell Culture Flask with Vented Cap	Corning	Cat#353110
Thermo Scientific™ BioLite Cell Culture Treated Flasks	Thermo Fisher Scientific	Cat#12-556-011
CELLSTAR® Filter Cap Cell Culture Flasks, Greiner Bio One (25cm^2)	Avantor	Cat#82051-074
Titertubes® Micro Test Tubes	Bio-Rad	Cat#2239391
Corning® 96-well Clear V-Bottom TC-treated Microplate, Individually Wrapped, with Lid, Sterile	Corning	Cat#3894

MATERIALS AND EQUIPMENT

FBS 20% DMSO		
Reagent	Final concentration	Amount
DMSO	20%	10 mL
FBS	80%	40 mL
Total	**N/A**	**50 mL**
Store at 4°C for up to 6 months.		

Supplemented RPMI 1640 media		
Reagent	Final concentration	Amount
Penicillin (10 000 Units/mL) -Streptomycin (10 000 µg/mL) Solution	Penicillin (100 Units/mL) Streptomycin (100 µg/mL)	5 mL
FBS	10%	50 mL
RPMI 1640 media	N/A	445 mL
Total	**N/A**	**500 mL**
Store at 4°C for up to 6 months.		

Antibody solution 1			
Reagent	Stock concentration	Final concentration	Amount
Antibody (CV3-25 WT, CV3-25 LALA or CV3-25 GASDALIE)	1 mg/mL	100 µg/mL	4 µL
PBS	N/A	N/A	36 µL
Total	**N/A**	**N/A**	**40 µL**
Store at 4°C for up to 1 day (until further use in the experiment).			

Alternatives: The goal is to have an antibody solution at 100 μg/mL. If the stock antibody solution is not at an initial concentration of 1 mg/mL, dilute the antibody stock in order for the ''Antibody solution 1'' to have a final concentration of 100 μg/mL.

Alternatives: If the stock solution of the antibody is already at a concentration of 100 μg/mL you do not have to prepare the ''Antibody solution 1''.

Antibody solution 2

Reagent	Stock concentration	Final concentration	Amount
Antibody (CV3-25 WT, CV3-25 LALA or CV3-25 GASDALIE)	1 mg/mL	1 μg/mL	1 μL
PBS	N/A	N/A	999 μL
Total	**N/A**	**N/A**	**1000 μL**

Store at 4°C for up to 1 day (until further use in the experiment).

Alternatives: The goal is to have an antibody solution at 1 μg/mL. If the stock antibody solution is not at an initial concentration of 1 mg/mL, dilute the antibody stock in order for the ''Antibody solution 2'' to have a final concentration of 1 μg/mL.

Effector cells solution

Reagent	Final concentration	Amount
eBioscience™ Cell Proliferation Dye eFluor™ 450	1:1000	2.5 μL
PBS	N/A	2.5 mL
Total	**N/A**	**2.5 mL**

Store at 4°C for up to 1 day (until further use in the experiment).

Note: As soon as the solution is prepared, protect it from light by covering it with aluminum foil.

Note: The effector cells (PBMCs) are stained at a concentration of 10×10^6 PBMCs/mL. *For an experiment using 25 × 10^6 PBMCs, 2.5 mL of ''Effector cells solution'' in needed.*

Target cells solution

Reagent	Final concentration	Amount
eBioscience™ Cell Proliferation Dye eFluor™ 670	1:1000	3.5 μL
PBS	N/A	3.5 mL
Total	**N/A**	**3.5 mL**

Store at 4°C for up to 1 day (until further use in the experiment).

Note: As soon as the solution is prepared, protect it from light by covering it with aluminum foil.

Note: The target cells (CEM.NKr parental and CEM.NKr.Spike) are stained at a concentration of 1×10^6 cells/mL. *For an experiment using a total of 3.5 × 10^6 target cells, 3.5 mL of ''Target cells solution'' is needed.*

Note: Target and effector cells are stained with two different cellular dye to allow the specific gating of target cells by flow cytometry.

PFA 4%

Reagent	Final concentration	Amount
Formaldehyde, 37% by Weight (With Preservative/Certified ACS), Fisher Chemical™	4%	292 μL
PBS	N/A	2.41 mL
Total	**N/A**	**2.7 mL**

Store at room temperate for up to 1 day (until further use in the experiment).

⚠ CRITICAL: Formaldehyde is toxic (by inhalation, by contact with the skin and if swallowed) and flammable. Make sure to wear the appropriate personal protective equipment (gloves, laboratory coat, etc.), handle carefully under a chemical hood and handle the formaldehyde according to your institution's safety guidelines. Read product description and Material Safety Data Sheet before use.

Note: You will need 100 µL of the PFA 4% solution per condition in the 96 well V-bottom plate. *For this experiment, you will need 2.6 mL of PFA 4% (always prepare a volume superior to what is needed).*

STEP-BY-STEP METHOD DETAILS

Preparation of the experiment and the cells

Timing: 2 h

This section describes how to plan your experiment, how to calculate the appropriate number of cells and on what conditions to thaw the PBMCs.

1. Calculate the number of cells you will need for your experiment. You will need 5×10^4 CEM.NKr parental cells, 5×10^4 CEM.NKr.Spike cells and 1×10^6 PBMCs per condition. An example of a typical template can be found in Figure 2A and can greatly help for the calculations and design of the experiment.
2. One day before the planned experiment, look at your cells. They should be nice and round. If the cells are in nice condition proceed to step 3.
3. Count the target cells to make sure you have enough cells to conduct the experiment. If you do not have enough cells, wait a few more days. If the CEM.NKr parental and/or CEM.NKr.Spike cells doesn't grow, please refer to the troubleshooting section (problem 2).

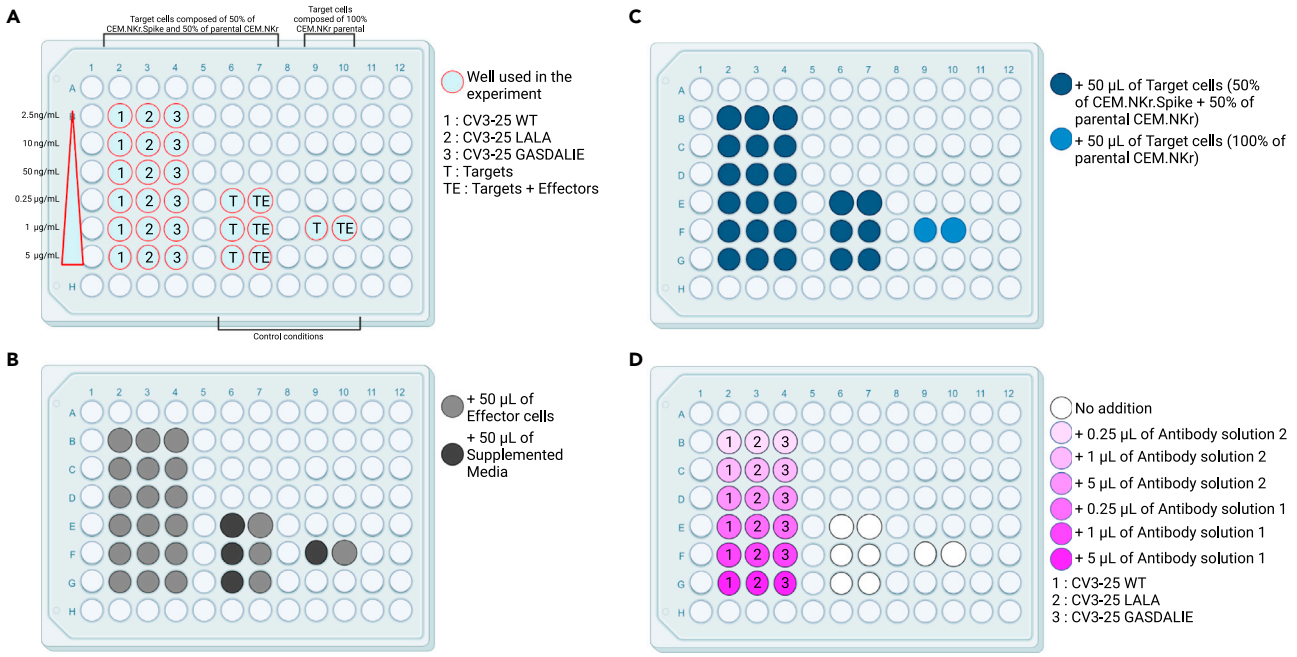

Figure 2. Template of the designed experiment

(A) The template is showing the number of wells required for the experiment and how to identify them.
(B) The template is showing in what wells to add the effector cells (PBMCs) or the supplemented RPMI 1640 media.
(C) The template is showing in what wells to add the appropriate target cells.
(D) The template is showing the volume of each specific antibody to add in each specific well.

4. If you have enough cells, thaw the PBMCs.
5. Take a sterile 15 mL tube and pipette 10 mL of supplemented RPMI 1640 media in the tube.
   a. Take the cryogenic vial containing the PBMCs and put it in a hot-water bath at 37°C.
   b. Wait a few moments (1 or 2 min) until the cells are no longer frozen.
   c. Quickly pour the content of the vial in the tube.
   d. Centrifuge the cells at 484 × *g* for 3 min.
   e. Discard the supernatant of the PBMCs.
   f. Add the appropriate volume of supplemented RPMI 1640 media and mix the PBMCs. The PBMCs should be at a concentration of 5 × 10^6 cells/mL.
   g. Pipette the PBMCs in a 25 cm^2 cell culture treated flask.
   h. Put the flask to incubate at 37°C and 5% CO_2 until the beginning of the experiment the next day.

Preparation of the solutions

Timing: 0.5–1 h

This section describes how to prepare the antibody solutions as well as how to prepare the target cells and the effector cells solutions used to mark the cells.

6. Take out two 1.5 mL tubes per antibody you want to titrate. *Since 3 antibodies are used in this experiment (CV3-25 WT, CV3-25 LALA and CV3-25 GASDALIE), take out six 1.5 mL tubes.*
7. Label these tubes appropriately. One tube will have an antibody solution of 100 μg/mL (Antibody solution 1) and the other will have an antibody solution of 1 μg/mL (Antibody solution 2).
8. In half of these tubes dilute the antibodies at a concentration of 100 μg/mL. See the materials and equipment section for the recipe of the ''Antibody solution 1''.
   a. Add 36 μL of PBS in the tube labeled as ''Antibody solution 1'' for each antibody.
   b. Add 4 μL of the stock antibody solution (if at a concentration of 1 mg/mL) in the tube.
   c. Mix very well the antibody solution.
9. In half of the remaining tubes, dilute the antibodies at a concentration of 1 μg/mL. See the materials and equipment section for the recipe of the ''Antibody solution 2''.
   a. Add 999 μL of PBS in the second tube labeled for each antibody.
   b. Add 1 μL of the stock antibody solution (if at a concentration of 1 mg/mL) in the appropriate tube.
   c. Vortex the antibody solution.
10. Prepare the cell marking solution for the PBMCs that are used as the effector cells. See the materials and equipment section for the recipe of the ''Effector cells solution''.
    a. Take a 15 mL tube and label it.
    b. Add the appropriate amount of PBS in the tube. Stain the PBMCs at a concentration of 10 × 10^6 PBMCs/mL. *For this experiment, add 2.5 mL of PBS in the tube.*
    c. Add 2.5 μL of cell proliferation dye eFluor-450 (eBioscience™ Cell Proliferation Dye eFluor™ 450).
    d. Vortex the solution.
    e. Cover the solution with aluminum foil.
11. Prepare the cell marking solution for the CEM.NKr parental and the CEM.NKr.Spike cells that are used as the target cells. See the materials and equipment section for the recipe of the ''Target cells solution''.
    a. Label a 15 mL tube.
    b. Add the appropriate amount of PBS in the tube. Stain both cell line at a concentration of 1 × 10^6 Target cells/mL. *In the case of this experiment, add 3.5 mL of PBS in the 15 mL tube.*
    c. Add 3.5 μL of cell proliferation dye eFluor-670 (eBioscience™ Cell Proliferation Dye eFluor™ 670).

d. Vortex the solution.
e. Cover the solution with aluminum foil.

Starting the ADCC assay

Timing: 7 h

This section describes how to prepare and organize the cells for the experiment and how to add the antibodies in order to start the ADCC assay.

12. Take the cells out of the incubator.
13. Mix the cells in order to homogenize them.
14. Count the cells in order to determine the concentration of the CEM.NKr parental cells, the CEM.NKr.Spike cells and the PBMCs.
15. Label the tubes as shown in Figure 3.
    a. Label the first tube as ''Effector cells''.
    b. Label the second tube as ''Target cells ①''.
    c. Label the third tube as ''Target cells ②''.
    d. Label the fourth tube as ''CEM.NKr parental cells 0.5 × 10^6''.
16. Pipette the appropriate volume to have the needed number of PBMCs in the ''Effector cells'' tube. The ADCC assay requires 1 × 10^6 PBMCs/condition. *For this experiment, pipette 25 × 10^6 PBMCs.* If there are not enough effector cells (PBMCs) to perform the experiment, please refer to the troubleshooting section (problem 3).
17. Pipette the appropriate volume to have the needed number of CEM.NKr.Spike cells in the ''Target cells ①'' tube. The ADCC assay requires 5 × 10^4 CEM.NKr.Spike cells/condition. *For this experiment, pipette 1.5 × 10^6 CEM.NKr.Spike cells.*
18. Pipette the appropriate volume to have the needed number of CEM.NKr parental cells in the ''Target cells ②'' tube. The ADCC assay requires 5 × 10^4 CEM.NKr parental cells/condition. *For this experiment, pipette 1.5 × 10^6 CEM.NKr parental cells.*
19. Pipette the appropriate volume to have 0.5 × 10^6 CEM.NKr parental cells in the ''CEM.NKr parental cells 0.5 × 10^6'' tube. Each time you do an experiment (no matter the size of the experiment), prepare this tube.

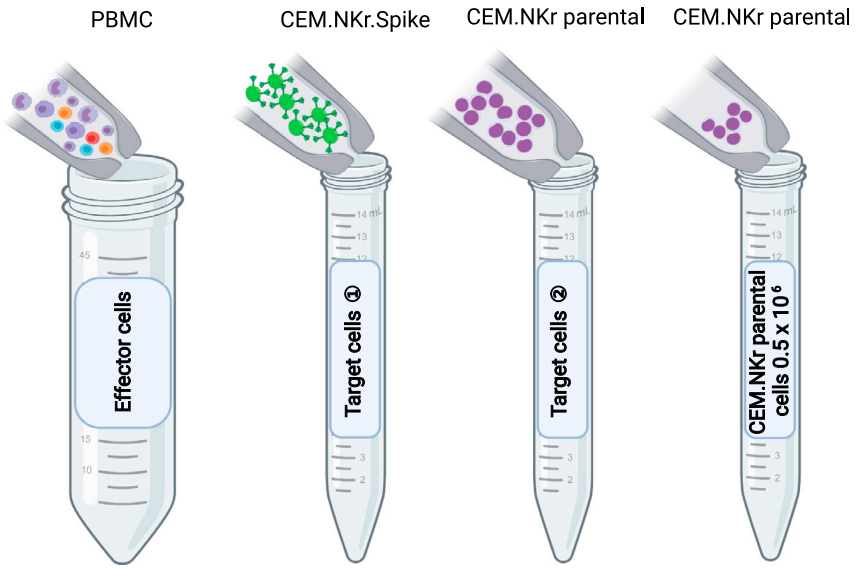

Figure 3. Identification of the different tubes as well as what cells to add in each tube

20. Centrifuge the cells at 484 × *g* for 3 min.
21. Carefully remove the supernatant without touching the cells.
22. Resuspend the PBMCs at a concentration of 10 × 10^6 PBMCs/mL with the ''Effector cells solution'' and mix well. *For this experiment, add 2.5 mL of the ''Effector cells solution''.*
23. Resuspend the CEM.NKr parental cells in the ''Target cells ②'' tube at a concentration of 0.5 × 10^6 cells/mL with the ''Target cells solution'' and mix well. *For this experiment, add 3 mL of the ''Target cells solution''.*
24. Immediately after resuspending the CEM.NKr parental cells in the ''Target cells ②'' tube with the ''Target cells solution'', pipette the whole volume of this solution in the ''Target cells ①'' tube and mix well. The target cells (now composed of 50% of CEM.NKr parental cells and of 50% of CEM.NKr.Spike cells) should now be at a concentration of 1 × 10^6 cells/mL.
25. Use the remaining 500 µL of the ''Target cells solution'' to resuspend the cells in the ''CEM.NKr parental cells 0.5 × 10^6'' tube. These cells should now be at a concentration of 1 × 10^6 cells/mL.
26. Wait 20 min for the cells to be stained with the different cell markers.
27. Add an identical volume of supplemented RPMI 1640 media as there is of ''Effector cells solution''. *For this experiment, add 2.5 mL of supplemented RPMI 1640 media.*
28. Add an identical volume of supplemented RPMI 1640 media as there is of ''Target cells solution'' in each of the 2 tubes containing the target cells. *For this experiment, add 3 mL of supplemented RPMI 1640 media in the ''Target cells ①'' tube and add 0.5 mL of supplemented RPMI 1640 media in the ''CEM.NKr parental cells 0.5 × 10^6'' tube.*
29. Centrifuge the tubes at 484 × *g* for 3 min.
30. Remove carefully the supernatants.
31. Add a volume of supplemented RPMI 1640 media to dilute the PBMCs at a concentration of 10 × 10^6 PBMCs/mL. *For this experiment, add 2.5 mL of supplemented RPMI 1640 media.*
32. Add a volume of supplemented RPMI 1640 media to dilute the target cells at a concentration of 1 × 10^6 cells/mL. *For this experiment, add 3 mL and 0.5 mL of supplemented RPMI 1640 media in the ''Target cells ①'' tube and the ''CEM.NKr parental cells 0.5 × 10^6'' tube, respectively.*
33. Centrifuge the tubes at 484 × *g* for 3 min.
34. Remove carefully the supernatants.
35. Add an appropriate volume of supplemented RPMI 1640 media to dilute the PBMCs at a concentration of 20 × 10^6 PBMCs/mL. Mix the cells very well. *For this experiment, add 1.25 mL of supplemented RPMI 1640 media.*
36. Add an appropriate volume of supplemented RPMI 1640 media to dilute the target cells at a concentration of 2 × 10^6 cells/mL. Mix the cells very well. *For this experiment, add 1.5 mL and 0.25 mL of supplemented RPMI 1640 media in the ''Target cells ①'' and the ''CEM.NKr parental cells 0.5 × 10^6'' tubes, respectively.*
37. Take out a 96 well V-bottom plate.
38. Pipette the PBMCs in a reservoir and mix them.
39. Pipette 50 µL of the PBMC solution in every well that requires it according to your template. *For this experiment, PBMCs should be introduced in 22 wells (18 wells for the experiment with the 3 different antibodies and 4 wells for the control conditions) as shown in Figure 2B.* There should be a total of 1 × 10^6 PBMCs/well.
40. In the 4 control wells that do not require any PBMCs, add 50 µL of supplemented RPMI 1640 media as shown in Figure 2B.
41. Pipette the target cells from the ''Target cells ①'' tube (composed of 50% of CEM.NKr parental cells and of 50% of CEM.NKr.Spike cells) in a reservoir and mix these cells very well.
42. Pipette 50 µL of this solution in every well that requires it according to your template. *For this experiment, target cells should be introduced in 24 wells (18 wells for the experiment with the 3 different antibodies and 6 wells for the control conditions) as shown in Figure 2C.* There should be a total of 1 × 10^5 Target cells/well (composed of 5 × 10^4 CEM.NKr parental cells and of 5 × 10^4 CEM.NKr.Spike cells).

43. Mix the target cells in the ''CEM.NKr parental cells 0.5 × 10^6'' tube (composed of 100% of CEM.NKr parental cells).
44. Pipette 50 μL of the target cells from the previous step in the two remaining control wells as showed in Figure 2C.
45. Mix and vortex every 1.5 mL tube containing the antibodies you will use for the experiment.
46. Add 0.25 μL of the 3 antibodies at a concentration of 1 μg/mL in the 3 appropriate wells according to your template as shown in Figure 2D. These antibodies will be at a final concentration of 2.5 ng/mL.
47. Add 1 μL of the 3 antibodies at a concentration of 1 μg/mL in the 3 appropriate wells according to your template as shown in Figure 2D. These antibodies will be at a final concentration of 10 ng/mL.
48. Add 5 μL of the 3 antibodies at a concentration of 1 μg/mL in the 3 appropriate wells according to your template as shown in Figure 2D. These antibodies will be at a final concentration of 50 ng/mL.
49. Add 0.25 μL of the 3 antibodies at a concentration of 100 μg/mL in the 3 appropriate wells according to your template as shown in Figure 2D. These antibodies will be at a final concentration of 0.25 μg/mL.
50. Add 1 μL of the 3 antibodies at a concentration of 100 μg/mL in the 3 appropriate wells according to your template as shown in Figure 2D. These antibodies will be at a final concentration of 1 μg/mL.
51. Add 5 μL of the 3 antibodies at a concentration of 100 μg/mL in the 3 appropriate wells according to your template as shown in Figure 2D. These antibodies will be at a final concentration of 5 μg/mL.
52. Mix every well of the 96 well V-bottom plate containing cells (even if no antibody was added to the well).
53. Centrifuge the 96 well V-bottom plate at 300 × *g* for 1 min.
54. Put the 96 well plate in the incubator at 37°C and 5% CO_2 for 5 h.
55. During the 5 h of incubation, prepare a PBS-Formaldehyde 4% (PFA 4%) solution. See the materials and equipment section for the recipe of the ''PFA 4%'' solution.
    a. Take a 15 mL tube and label it a PFA 4%.
    b. Pipette the appropriate volume of PBS in the 15 mL tube. You will need 100 μL of the PFA 4% solution per well used in the 96 well V-bottom plate. *For this experiment, pipette 2.41 mL of PBS in the tube.*
    c. Pipette the appropriate volume of formaldehyde 37% in the tube in order to make a PFA 4% solution. *For this experiment, add 292 μL of formaldehyde 37%.*
    d. Vortex the solution.
56. Prepare and label the appropriate number of micro test tubes (Titertubes® Micro Test Tubes) you will need once you stop the experiment. You need 1 micro test tube per well. *For this experiment, prepare a total of 26 micro test tubes.*
57. Once the 5 h of incubation are finished, add 100 μL of the PFA 4% solution to each well of the 96 well V-bottom plate containing cells and mix extremely well.
58. Pipette the content of each well in the corresponding micro test tube.
59. Put the micro test tubes at 4°C and shelter them from light until further use. We do not recommend letting the cells more than 1 week at 4°C before further use.

⚠ CRITICAL: The experiment will only work if there is an equivalent number of PBMCs in each well (~ 1 × 10^6) and, initially, an equivalent number of target cells in each well (~ 0.1 × 10^6). Also, it is crucial that the initial proportion of target cells (1 CEM.NKr parental : 1 CEM.NKr.Spike) in each well be the same. The proportion of effector cells to target cells must also be the same in every well (10:1). To make sure this is respected, mix the cells frequently while pipetting the cells from the reservoirs into the wells of the 96 well V-bottom plate.

⚠ CRITICAL: Do not use the wells in the rows and columns at the extremities of the 96 well V-bottom plate. Higher evaporation and consequently higher variability in the data is observed when using these wells.

Pause point: Between steps 59 and 60 you can wait up to 1 week.

Analyzing the cells on the cytometer

Timing: 1–4 h

This section describes the gating strategy used to calculate the % ADCC mediated by the different antibodies. It also describes in what order to pass and analyze the cells on the cytometer.

60. Prepare the gating strategy as shown in Figure 4.
    a. Prepare the first gate by selecting the target cells based on their side scatter area (SSC-A) and their forward scatter area (FSC-A). To know where to place your gate, pass and select the cells that were in a well of the control conditions without the addition of any PBMCs. The purpose of this gate is to select the target cells.
    b. Prepare the second gate by selecting the cells based on their forward scatter width (FSC-W) and their forward scatter height (FSC-H) in order to exclude the target cells doublets. To know where to place your gate, pass and select the cells that were in a well of the control conditions without the addition of any PBMCs.
    c. Prepare the third gate by selecting the target cells based on their eFluor-670 signal and their eFluor-450 signal. The target cells are eFluor-670^{High} and eFluor-450^{Low}. To know where to place your gate, pass and select the cells that were in a well of the control conditions without the addition of any PBMCs. The purpose of this gate is to select the target cells, while excluding the effector cells.
    d. Prepare the fourth gate by selecting the target cells based of their eFluor-670 signal and their GFP signal. The target cells expressing the SARS-CoV-2 Spike are eFluor-670^{High} and GFP^{High}. To know where to place your gate, pass and exclude the target cells that were in the well of the control conditions without the addition of any PBMCs and where the target cells were composed of 100% of CEM.NKr parental cells (i.e., the cells that are 100% GFP^{Low}). The purpose of this gate is to select the cells that express the SARS-CoV-2 Spike (i.e., the cells that are GFP^{High}).
61. Start passing the samples in the cytometer by beginning with the 2 wells where 100% of the target cells are CEM.NKr parental cells. Following these 2 controls, start passing the other 6 control samples.
62. Pass the other samples in the cytometer. If there are very few cells in the samples, please refer to the troubleshooting section (problem 4).
63. Once you finish passing the samples, export the data.

⚠ CRITICAL: Once you optimized your gating strategy and start passing your samples, never change the parameters mid experiment.

Note: The target cells in the first column of Figure 4 should be gated on by using the 4 control wells where the only cells introduced in the wells were the target cells.

Note: The target cells expressing the SARS-CoV-2.Spike (CEM.NKr.Spike) also express GFP. Use the wells where 100% of the target cells are CEM.NKr parental cells to delineate the GFP^{Low} from the GFP^{High} population as shown in the fourth column of Figure 4.

Note: Pass at least 10 000 target cells per sample in the third gate.

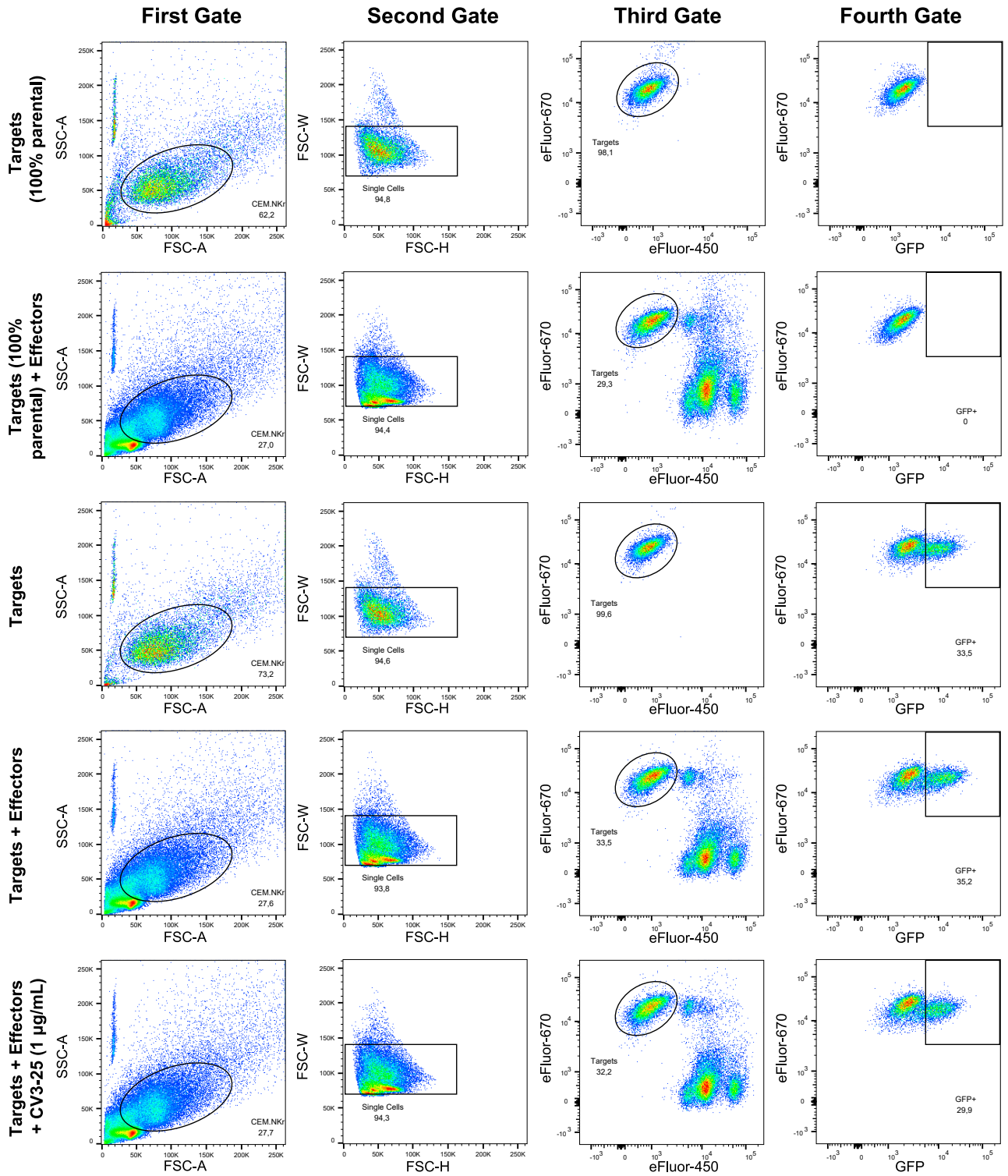

Figure 4. Gating strategy of the ADCC assay when passing the cells in the cytometer and analyzing them

Note: We suggest every lab to prepare compensation tubes (Unstained CEM.NKr.Spike (GFP), PBMCs stained with eFluor-450, CEM.NKr parental cells stained with eFluor-670 and unstained CEM.NKr parental cells) to determine if they require compensation or not.

Analyzing the data

Timing: 1 h

This section describes how to analyze the data obtained from the samples that were used in flow cytometry.

64. Export the data to the computer for the analysis.
65. Drag the data from the computer into the FlowJo™ program to analyze the data.
66. Prepare the gating as shown in Figure 4.
67. Once the gating is completed, add the % GFP^{High} cells from the gating in the fourth column as shown in Figure 4.
68. Export the % GFP^{High} cells for each condition in a file on your computer.
69. Using the % GFP^{High} cells in each wells, calculate the % ADCC using this formula:

$$\%\ ADCC = \frac{(Average\ of\ (Targets + Effectors)) - (Targets + Effectors + Antibody)}{Average\ of\ Targets\ alone}\ x\ 100$$

70. If the calculated % ADCC differs significantly from an experiment to another, please refer to the troubleshooting section (problem 5).

Note: For further explanations on the quantification of ADCC, please refer to the quantification and statistical analysis section.

EXPECTED OUTCOMES

As the CV3-25 antibody specifically recognized the CEM.NKr.Spike cells and not the parental cell line, incubation with this monoclonal antibody led to a dose-dependent ADCC-mediated elimination of the GFP^{High} CEM.NKr.Spike cells. Introduction of mutations known to impair (L234A/L235A also known as LALA (Saunders, 2019)) Fc effector functions, significantly reduced the ADCC-mediated killing of Spike-expressing cells. In contrast, introduction of mutations (G236A/S239D/A330L/I332E also known as GASDALIE (DiLillo and Ravetch, 2015; Lazar et al., 2006; Richards et al., 2008; Smith et al., 2012; Bournazos et al., 2014)) increasing the affinity between the Fc portion of an antibody and the Fc receptor, significantly enhanced the ADCC activity of CV3-25. Raw data are presented in Figure 5. Once the % ADCC is calculated from the % GFP^{High} cells, data can be presented with more clarity as shown in Figure 6A.

As previously reported, this assay can also be used to measure the ADCC mediating capacity of plasma from infected, vaccinated or convalescent individuals (Brunet-Ratnasingham et al., 2021; Anand et al., 2021b; Tauzin et al., 2021; Group et al., 2021). Figure 7 shows examples of ADCC mediating plasmas from non-infected, vaccinated, previously infected and previously infected and vaccinated individuals. Figure 7 also shows that COVID-19 negative plasmas, as expected, do not kill SARS-CoV-2 Spike expressing cells. This shows that the assay is specific towards SARS-CoV-2 Spike expressing cells. We recently used this assay to evaluate the Fc-mediated effector functions of antibodies present in plasma from convalescent donors used in the CONCOR-1 convalescent plasma transfer clinical trial (Group et al., 2021).

QUANTIFICATION AND STATISTICAL ANALYSIS

In this assay, the cells expressing the SARS-CoV-2 Spike are GFP^{High}. A loss of the GFP^{High} population indicates that the cells expressing the Spike were specifically killed. The more the cells are killed, the

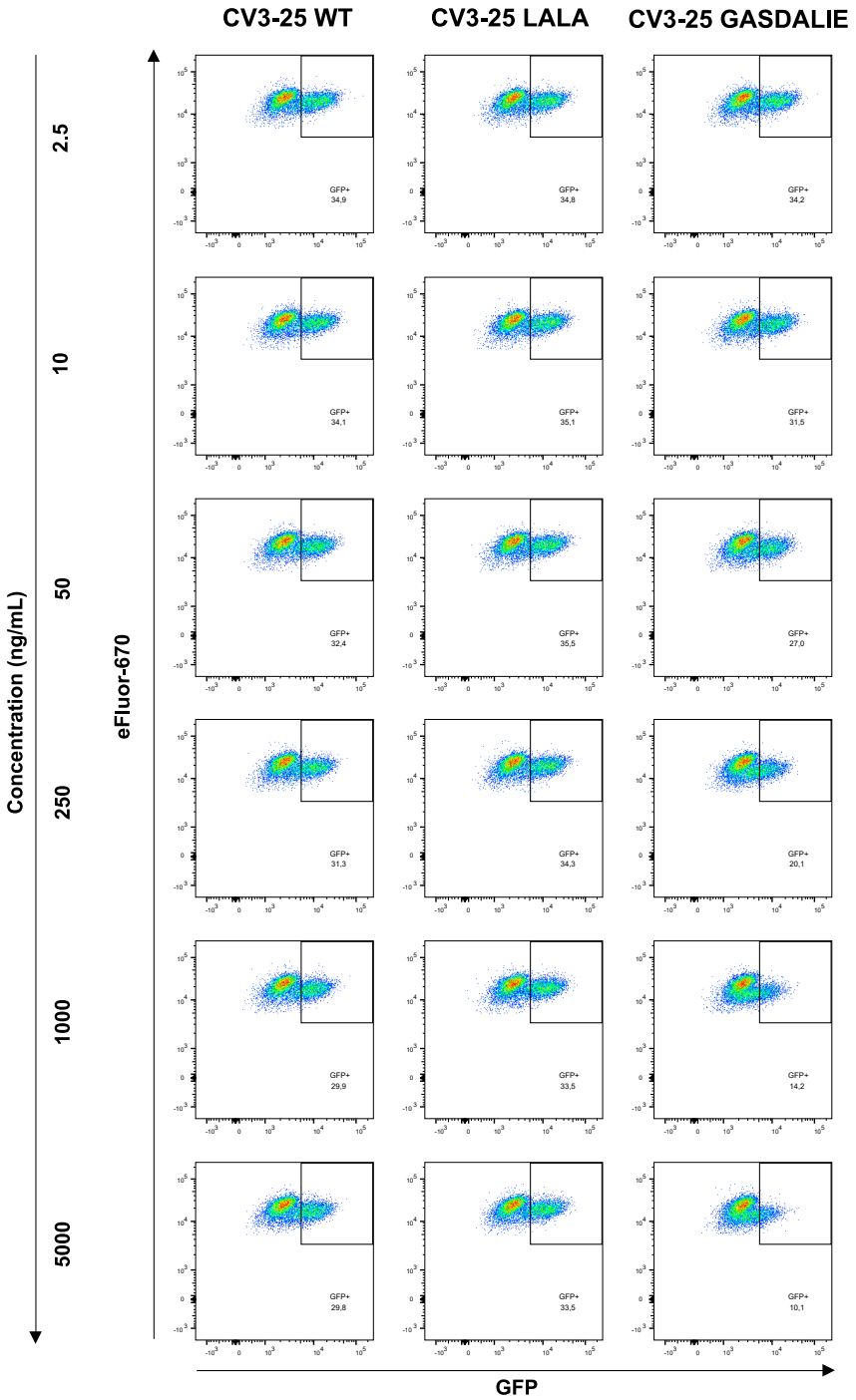

Figure 5. Gating strategy used on the target cells for each of the antibodies (CV3-25 WT, CV3-25 LALA, and CV3-25 GASDALIE) at every concentration of antibody tested (2.5 ng/mL, 10 ng/mL, 50 ng/mL, 250 ng/mL, 1000 ng/mL and 5000 ng/mL)

more there will be a loss in the GFP^{High} population. In order to calculate the % ADCC mediated by CV3-25 WT, LALA and GASDALIE (or any other antibody or plasma), the following formula was used:

$$\%\ ADCC = \frac{(Average\ of\ (Targets + Effectors)) - (Targets + Effectors + Antibody)}{Average\ of\ Targets\ alone} \times 100$$

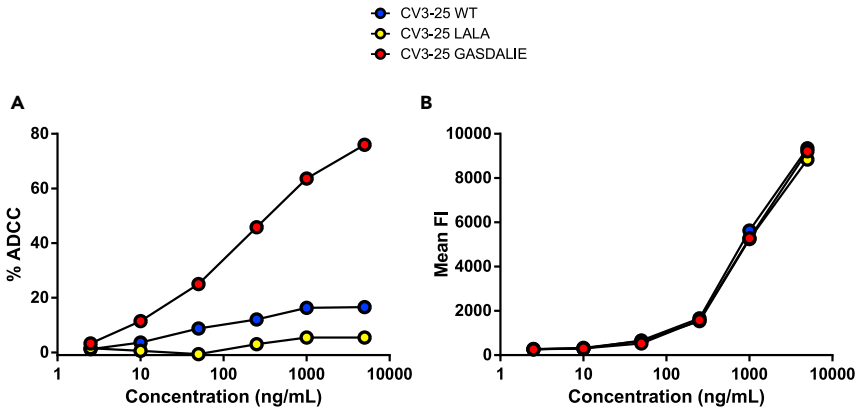

Figure 6. Characterization of the CV3-25 WT, CV3-25 LALA, and CV3-25 GASDALIE antibodies

(A) Percentage of ADCC calculated from the % GFP^{High} cells (obtained in Figure 5) for the CV3-25 WT, CV3-25 LALA and CV3-25 GASDALIE antibodies.

(B) Staining of CV3-25 WT, CV3-25 LALA and CV3-25 GASDALIE on CEM.NKr.Spike cells. The antibodies were tested at 2.5 ng/mL, 10 ng/mL, 50 ng/mL, 250 ng/mL, 1000 ng/mL and 5000 ng/mL. Mean FI : Mean Fluorescence intensity.

The following tables (Tables 1 and 2) show the % GFP^{High} cells that was used to calculate the % ADCC of the three monoclonal antibodies tested in this experiment. We encourage the reader to calculate for themselves the % ADCC of the 3 monoclonal antibodies with the provided Tables 1 and 2.

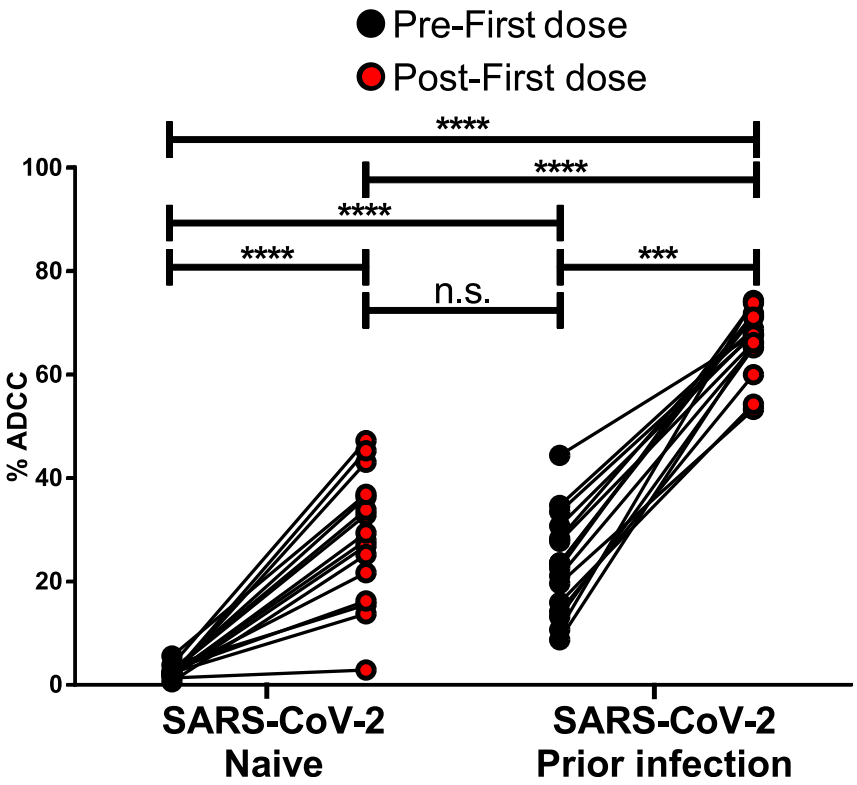

Figure 7. Percentage of ADCC mediated by plasma from SARS-CoV-2 naïve or infected individuals, before or after a first dose of vaccine

The plasmas were tested at a dilution of 1/500. The *p* values were obtained by the parametric t-test or paired t-test or the non-parametric Mann-Whitney test or Wilcoxon signed rank test depending on the normality of the dataset. ***, $p < 0.001$; ****, $p < 0.0001$.

Table 1. Percentage of GFP^{High} cells for the control conditions

-	Targets alone			Targets + effectors		
Well number	1	2	3	1	2	3
% GFP^{High} cells	33.5	32.9	33.1	35.3	35.4	35.2
Average (%)	33.17			35.3		

LIMITATIONS

To test donor to donor variability in mediating ADCC, we compared 5 donors against the recently described antibody CV3-13 WT (Jennewein et al., 2021) (Figure 8). This antibody targets the N-terminal domain (NTD) of the Spike and is known to mediate good Fc effector functions. As it is possible to see in Figure 8, PBMCs from different individuals are not uniform as to their capacity to mediate Fc effector functions (Figure 8). A differential proportion of Fc-gamma receptor-bearing effector cells among the PBMCs population between donors could explain this variation. For this reason, when comparing ADCC activity between monoclonal antibodies or plasma, PBMCs from the same donor must be used.

It is relevant to note that for large experiments it is important to limit to a minimum the time between when you pipette the antibody/plasma in the first and last wells. The incubation time should be the same in each condition. Because of this, you should not spend more than 45 min pipetting the antibodies or plasmas in the different wells.

TROUBLESHOOTING

Problem 1

The CEM.NKr parental and/or CEM.NKr.Spike cells are in an irregular shape.

Potential solution

CEM.NKr parental and CEM.NKr.Spike cells are normally round. These cells might be in an irregular shape if they were recently thawed. If this is the case, wait a few more days and if the cells are still in an irregular shape they might be contaminated. In that case, throw them away and thaw new cells. CEM.NKr parental and CEM.NKr.Spike cells can also be in an irregular shape when they are too concentrated. If this seems to be the case, dilute them at a concentration of 0.25×10^6 cells/mL.

Problem 2

The CEM.NKr parental and/or CEM.NKr.Spike cells don't grow.

Potential solution

If the cells do not multiply, they might be contaminated. If this is the case, throw them and thaw new cells. If you diluted your cells in a concentration less than 0.15×10^6 cells/mL, they might take more time to grow and give the impression that they are not multiplying. If this is the case, count them again after 2–3 more days.

Problem 3

There are not enough effector cells (PBMCs) to perform the experiment.

Table 2. Percentage of GFP^{High} cells and corresponding % ADCC for the experiment

	CV3-25 WT		CV3-25 LALA		CV3-25 GASDALIE	
Concentration (ng/mL)	% GFP^{High}	% ADCC	% GFP^{High}	% ADCC	% GFP^{High}	% ADCC
2.5	34.9	1.21	34.8	1.51	34.2	3.32
10	34.1	3.62	35.1	0.603	31.5	11.5
50	32.4	8.74	35.5	-0.603	27.0	25.0
250	31.3	12.1	34.3	3.01	20.1	45.8
1000	29.9	16.3	33.5	5.43	14.2	63.6
5000	29.8	16.6	33.5	5.43	10.1	76.0

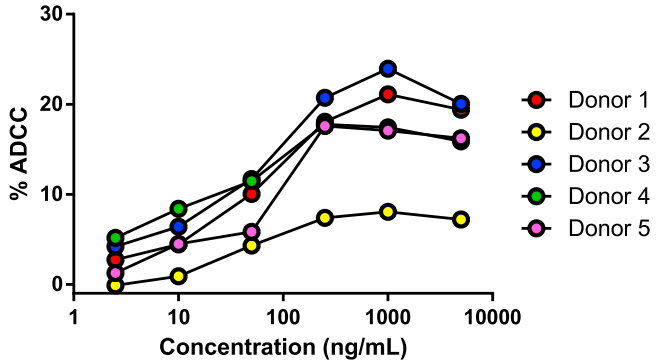

Figure 8. Percentage of ADCC of the CV3-13 WT antibody calculated from a single experiment using five different healthy donors of PBMCs at concentrations of 2.5 ng/mL, 10 ng/mL, 50 ng/mL, 250 ng/mL, 1000 ng/mL, and 5000 ng/mL

Potential solution

PBMCs will not multiply and you can only use them the day after you thaw them. In order to not waste them, perform the experiment with less conditions than you initially prepared. To make sure this does not happen, you can count the PBMCs immediately after thawing them. If you do not have enough, thaw one or more vials of PBMCs.

Problem 4

There are very few cells in the samples.

Potential solution

When passing the samples on the cytometer, you might notice that for some of your samples there are very few cells. This might be because you did not pipette well your cells in the micro test tubes. In order to pipette most of the cells in your wells, do multiple up-and-downs in each well. This will put the cells in suspension and make them easier to pipette in your micro test tubes.

Problem 5

Variation in the calculated percentage of ADCC across different experiments.

Potential solution

Having some variation between experiments is normal. However, having variation above 30% is not. Use PBMCs from the same donor as not all PBMCs are good at mediating ADCC as shown in Figure 8. Also important to note, measure the concentrations of your different antibody stocks to confirm that the solutions are at the concentrations you think they are. Other sources of variation can be that the proportion of target to effector cells (1:10) is not the same between experiments or that there was some variation in the incubation time. We suggest to include one or two positive control antibodies (at a single concentration, for example 1 μg/mL) for each experiment that you can use to normalize your data. Such antibodies can include any anti-Spike monoclonal antibodies such as CV3-25 WT or CV3-13 WT described here.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Andrés Finzi (andres.finzi@umontreal.ca).

Materials availability

CEM.NKr parental cells can be directly obtained from ATCC. CEM.NKr.Spike cells could be obtained after establishing a standard Materials Transfer Agreement (MTA).

Data and code availability

The published article includes all datasets for the CV3-25 WT, CV3-25 LALA and CV3-25 GASDALIE antibodies generated and analyzed during this study.

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AUTHOR CONTRIBUTIONS

G.B.-B., J.R., and J.P. designed the ADCC assay. G.B.-B. performed and optimized the assay. G.B.-B., J.R., and A.F. wrote the manuscript with input from all authors. J.R. and G.B.-B. created the CEM.NKr.Spike cell line. J.P. designed the primers and performed the mutagenesis to obtain the LALA and GASDALIE mutants of CV3-25. G.G. produced CV3-25 WT, CV3-25 LALA, and CV3-25 GASDALIE.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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