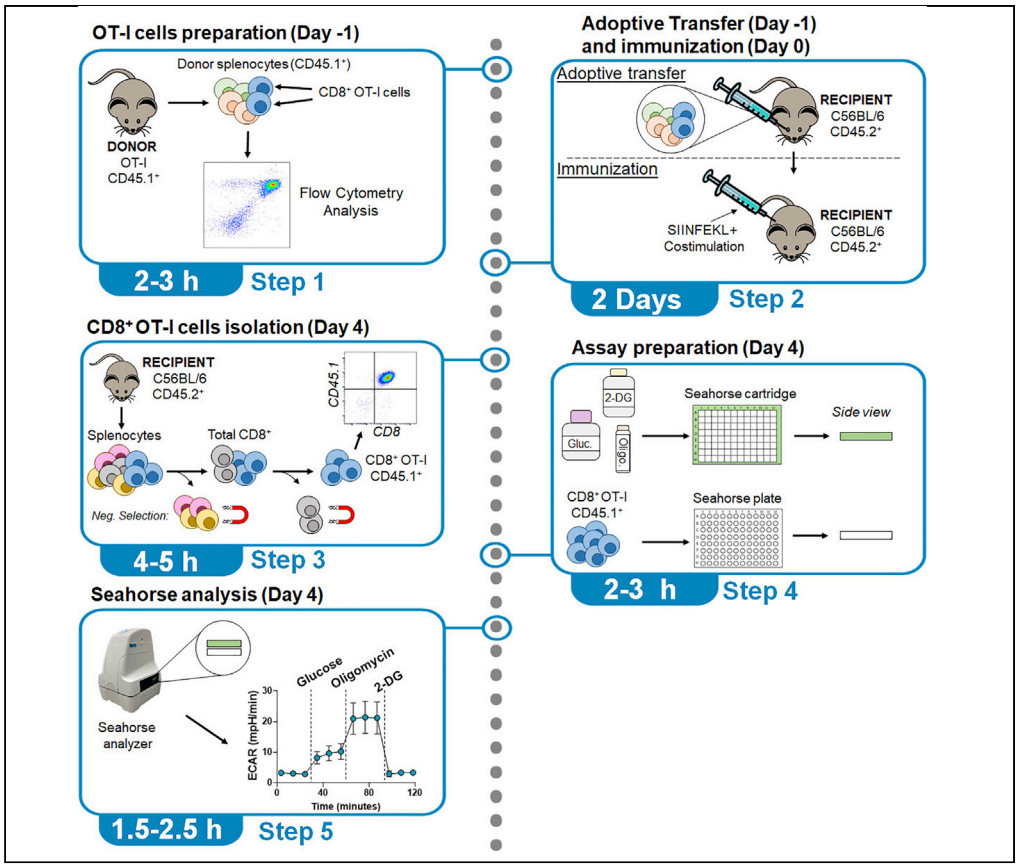
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Evaluating the glycolytic potential of mouse costimulated effector CD8$^+$ T cells *ex vivo*



Studying the metabolic fitness of T cells is fundamental to understand how immune responses are regulated. Here, we describe a step-by-step protocol optimized to efficiently generate and isolate effector antigen-specific CD8$^+$ T cells *ex vivo* using costimulation. We also detail steps to evaluate their metabolic activity using Seahorse technology. This protocol can be used to measure the glycolytic potential of effector murine T cells in response to different manipulations, such as infections, adjuvant studies, gene editing, or metabolite supplementation.

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

Federica Agliano,
Antoine Ménoret,
Anthony T. Vella

agliano@uchc.edu (F.A.)
vella@uchc.edu (A.T.V.)

Highlights

In vivo clonal expansion of mouse OT-I CD8$^+$ T cells

Efficient isolation of effector OT-I CD8$^+$ T cells based on negative selection

Determining glycolytic potential of OT-I CD8$^+$ T cells using Seahorse technology

Agliano et al., STAR Protocols 3, 101441
June 17, 2022
https://doi.org/10.1016/j.xpro.2022.101441

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Evaluating the glycolytic potential of mouse costimulated effector CD8$^+$ T cells *ex vivo*

Federica Agliano,[1,2,*] Antoine Ménoret,[1,2] and Anthony T. Vella[1,3,*]

[1]Department of Immunology, School of Medicine, University of Connecticut, Farmington, CT 06030, USA

[2]Technical contact

[3]Lead contact

*Correspondence: agliano@uchc.edu (F.A.), vella@uchc.edu (A.T.V.)
https://doi.org/10.1016/j.xpro.2022.101441

SUMMARY

Studying the metabolic fitness of T cells is fundamental to understand how immune responses are regulated. Here, we describe a step-by-step protocol optimized to efficiently generate and isolate effector antigen-specific CD8$^+$ T cells *ex vivo* using costimulation. We also detail steps to evaluate their metabolic activity using Seahorse technology. This protocol can be used to measure the glycolytic potential of effector murine T cells in response to different manipulations, such as infections, adjuvant studies, gene editing, or metabolite supplementation.

For complete details on the use and execution of this protocol, please refer to Agliano et al. (2022).

BEFORE YOU BEGIN

The protocol below has been optimized using the widely accepted CD8$^+$ T Cell Receptor (TCR) transgenic OT-I model, where cells from CD45.1$^+$ Rag$^{-/-}$ OT-I donor mice are adoptively transferred to CD45.2$^+$ C57BL/6J recipient mice. Given that the *Rag* coding region is deleted, these mice are unable to initiate V(D)J rearrangement, and fail to produce endogenous mature B or T lymphocytes (Shinkai et al., 1992). Thus, the only expressed lymphocytes carry a transgenic TCR receptor that consists of α-chain variable region 2 (Vα2) and β-chain variable region 5 (Vβ5), specific for a chicken ovalbumin (OVA) peptide fragment (^{257}SIINFEKL^{264}) presented by the MHC class I molecule H2-K^b (Hogquist et al., 1994). OT-I cells are thus adoptively transferred to regular C57BL/6J recipient mice, which are then immunized with the cognate antigen for the transgenic TCR (SIINFEKL), allowing the activation of the OT-I cells only. This way, antigen-specific CD8$^+$ T cells can be studied. Several reports have shown how T cell metabolic fitness is fundamental to T cell function and fate (Makowski et al., 2020; O'Neill et al., 2016). Our protocol describes in detail how to efficiently generate and isolate antigen-specific effector CD8$^+$ T cells and how to successfully measure their glycolytic potential through the detection of the ExtraCellular Acidification Rate (ECAR). We have optimized this protocol to measure the glycolytic potential of effector CD8$^+$ T cells *ex vivo* using the Seahorse glycolysis stress test (# 103020-100, Agilent). Using a Seahorse analyzer, this assay measures the capacity of the glycolytic pathway after glucose starvation. The Seahorse analyzer detects the flux of H$^+$ produced by the conversion of glucose to lactate through glycolysis, and by the TCA cycle activity, inducing the acidification of the assay medium, which is thus measured as extracellular acidification rate (ECAR). More technical information on how a Seahorse analyzer works can be found at: https://www.agilent.com/en/products/cell-analysis/how-seahorse-xf-analyzers-work.

When performing this protocol, access to a Flow Cytometer with at least 8 channels, a Seahorse XFe96 analyzer and a 37°C non-CO_2 incubator is necessary.

Institutional permission

This protocol requires the use of mice. Users who follow this protocol must acquire permissions from their institutions and perform experiments in accordance with relevant institutional and national guidelines and regulations. All animal procedures described in this protocol were approved by the UConn Health Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health Animal Care and Use Guidelines.

Seahorse equipment preparation

Timing: 1–1.5 h

Prepare the Seahorse equipment the day before the Seahorse assay. In order to form a monolayer and obtain consistent measurements, suspension cells must be immobilized to the bottom of the wells. For this purpose, we used a cell and tissue adhesive, Cell-Tak (Cat# 354240, Corning). Furthermore, the sensor cartridge must be hydrated.

⚠ CRITICAL: The Seahorse analyzer must be switched on the day before the experiment to allow the machine to pre-warm.

1. Coat the Seahorse 96-well plate with Cell-Tak:
   a. Prepare 50 mL of 0.1 M $NaHCO_3$, pH 8.

   ⚠ CRITICAL: Ensure that $NaHCO_3$ maintains a pH of 8, as acidification will affect cell adhesion after cell plating. If necessary, pH can be adjusted by adding 1 N NaOH.

   b. Prepare a diluted Cell-Tak solution such that each well will receive 0.5 μL (0.8 μg) of undiluted Cell-Tak and 24.5 μL of 0.1 M $NaHCO_3$, pH 8.

   ⚠ CRITICAL: Always use Seahorse 96-well plates. The surface of Seahorse 96-well plates is 40% the area of a typical 96-well plate. These plates have been developed specifically to maximize the sensitivity of the sensor cartridge measurements.

   c. Dispense 25 μL/well.
   d. Incubate for 25 min at 21°C–24°C.
   e. Flick the plate and wash with sterile 200 μL of distilled H_2O twice.
   f. Air dry the plate for 30 min under the hood and store at 4°C for 18–20 h or for a maximum of a week.

   Alternatives: Poly-D-Lysine (PDL) can also be used for cell adhesion. In addition, Agilent provides ready-to-use PDL-coated XFp Cell Culture Microplates (for 96 well plates: Cat# 103730-100, Agilent).

2. Hydrate the Seahorse sensor cartridge:
   a. Aliquot 20 mL of calibrant (Cat# 102601-100, Agilent) in a 50 mL tube and place it in a 37°C non-CO_2 incubator for 18–20 h.
   b. Fill utility plate with 200 μL of sterile tissue culture grade H_2O.
   c. Lower the sensor cartridge into the utility plate and place in a 37°C non-CO_2 incubator for 18–20 h.

   Note: Sensor cartridge can be hydrated for a minimum of 4 h to a maximum of 72 h before the experiment. However, for best results we recommend hydration for 18–20 h. For hydration >24 h, it is recommended to wrap the cartridge and utility plate in parafilm to prevent evaporation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat monoclonal α-CD134 (OX40), clone OX-86 (1:200)	BioXCell	Cat# BE0031; RRID: AB_1107592
Rat monoclonal α-CD137 (4-1BB), clone 3H3 (1:200)	BioXCell	Cat# BE0239; RRID: AB_2687721
Rat monoclonal α-CD8a, clone 53-6.7 (1:200)	BD Biosciences	Cat# 558106; RRID: AB_397029
Rat monoclonal α-TCR Vα2, clone B20.1 (1:200)	Thermo Fisher Scientific	Cat# 46-5812-80; RRID: AB_11039487
Mouse monoclonal α-TCR Vβ5, clone MR9-4 (1:200)	BD Biosciences	Cat# 553190; RRID: AB_394698
Mouse monoclonal α-CD45.1, clone A20 (1:200)	Thermo Fisher Scientific	Cat# 17-0453-82; RRID: AB_469398
Mouse monoclonal α-CD45.2, clone 104 (1:50)	Thermo Fisher Scientific	Cat# 13-0454-82; RRID: AB_46645
Rat monoclonal α-CD44, clone IM7 (1:200)	Thermo Fisher Scientific	Cat# 56-0441-82; RRID: AB_494011
Rat monoclonal α-CD4, clone RM4-5 (1:200)	BD Biosciences	Cat# 552775; RRID: AB_394461
Rat monoclonal α-B220/CD45R, clone RA3-6B2 (1:200)	BD Biosciences	Cat# 553093; RRID: AB_394622
Purified anti-mouse CD16/CD32, clone 2.4G2 (1:20)	BioXCell	Cat# BE0307, RRID: AB_2736987
LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit	Invitrogen	Cat# L34962
Chemicals, peptides, and recombinant proteins		
OVA peptide SIINFEKL	Invivogen	Cat# vac-sin, CAS# 138831-86-4
Cell-Tak™	Corning	Cat# 354240
L-glutamine 200 mM	Thermo Fisher Scientific	Cat# 25030081
Critical commercial assays		
Dynabeads™ Untouched™ Mouse CD8 Cells Kits from ThermoFisher Scientific	Thermo Fisher Scientific	Cat# 11417D
Dynabeads™ Biotin binder	Thermo Fisher Scientific	Cat# 11047
Seahorse Glycolysis stress test kit	Agilent Technologies	Cat# 103020-100
Seahorse XFe96 FluxPaks	Agilent Technologies	Cat# 102601-100
Seahorse XF DMEM Medium	Agilent Technologies	Cat# 103575-100
Experimental models: Organisms/strains		
C57BL/6J CD45.2, wild type mice; age: 6–12 weeks; sex: males and females	The Jackson Laboratory	JAX: 000664
C57BL/6J Rag$^{-/-}$ OT-I CD45.1^{+} mice; age: 6–12 weeks; sex: males and females	University of Washington	N/A
Software and algorithms		
FlowJo v10.6.1	FlowJo	RRID: SCR_008520 https://www.flowjo.com/solutions/flowjo/downloads
GraphPad Prism v9	GraphPad	RRID: SCR_00279 https://www.graphpad.com/
Seahorse Wave	Agilent Technologies	RRID: SCR_014526 https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-wave-desktop-software-740897
Other		
LSR II Flow Cytometer	BD Biosciences	N/A
Seahorse analyzer XFe96	Agilent Technologies	N/A
DynaMag™-50 Magnet	Thermo Fisher Scientific	Cat# 12302D
DynaMag™-15 Magnet	Thermo Fisher Scientific	Cat# 12301D
100 μm Cells Strainers	Corning	Cat# 431752
37°C non-CO_2 incubator	Fisher Scientific	N/A

MATERIALS AND EQUIPMENT

(Buffered Saline Solution) BSS		
Reagent	Final concentration	Amount
Glucose	1 g/L	4 g
KH_2PO_4	0.06 g/L	0.24 g
Na_2HPO_4	0.19 g/L	0.76 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
$CaCl_2 \times 2H_2O$	0.19 g/L	0.76 g
KCl	0.4 g/L	1.6 g
NaCl	8 g/L	32 g
$MgCl_2 \times 6H_2O$	0.2 g/L	0.8 g
$MgSO_4$	0.1 g/L	0.4 g
Phenol Red 5 g/L	0.01 g/L	8 mL
ddH_2O	n/a	4 L
Total	**n/a**	**4 L**
Store at 4°C for up to 1 year.		

Note: Filter through a 0.2 μm filter cup.

Alternatives: Other commercially available BSS, such as Hanks' Balanced Salt Solution (HBSS) (#SH30031-03, Avantor) can be used.

Ammonium chloride (NH_4Cl)		
Reagent	Final concentration	Amount
NH_4Cl	8.3 g/L	4.15 g
KCO_3	1 g/L	0.5 g
Phenol Red 5 g/L	0.0035 g/L	0.350 mL
ddH_2O	n/a	Up to 500 mL
Total	**n/a**	**500 mL**
Store at 4°C for up to 1 year.		

Note: pH should be 7.2. Adjust by gassing with 10% CO_2.

Filter through a 0.2 μm filter cup.

Alternatives: Other commercially available Red Blood Cell (RBC) lysis buffer such as Ammonium-Chloride-Potassium (ACK) Lysing Buffer (#A10492-01, Gibco) can be used.

Fluorescence activated cell sorting (FACS) buffer		
Reagent	Final concentration	Amount
HBSS with no phenol red	n/a	500 mL
Sodium azide (NaN_3)	0.1%	0.5 g
Heat inactivated fetal bovine serum (FBS) (56°C, 30 min)	0.3%	15 mL
Total	**n/a**	**500 mL**
Store at 4°C for up to 1 year.		

Fc block stock		
Reagent	Final concentration	Amount
BSS	n/a	16.8 mL
Purified anti-mouse CD16/CD32	n/a	1 mg
Heat inactivated mouse serum (56°C, 30 min)	30%	7.2 mL
Sodium azide (NaN_3)	0.1%	24 mg
Total	**n/a**	**24 mL**
Make 4 mL aliquots. Store at −20°C or 4°C for up to 1 year.		

Alternatives: Other commercially available Fc block (e.g., Cat# 553141, BD) can be used.

Isolation buffer		
Reagent	Final concentration	Amount
Phosphate-Buffered Saline (PBS)	n/a	498 mL
EDTA 0.5 M	2 mM	2 mL
BSA	1%	5 g
Total	**n/a**	**500 mL**
Store at 4°C for up to 6 months.		

Note: Filter through a 0.2 μm filter cup.

STEP-BY-STEP METHOD DETAILS

Preparation of OT-I donor-derived cells

Timing: 2–3 h (Day -1)

1. Euthanize OT-I mice according to your approved animal protocol.

   Note: Always follow your institution's IACUC guidelines for all animal procedures.

2. Place mice on a clean dissection board.
3. Spray mice with 75% ethanol to reduce bacterial contamination of cells.
4. Collect spleen (troubleshooting 1):
   a. Make a sub-cutaneous incision using sterile scissors.
   b. Using sterile forceps remove the spleen from the abdomen, excising any excess of fat.
   c. Place the spleen in a 12 well-plate filled with 2 mL of ice-cold BSS (see recipe in materials and equipment).

   Note: BSS can be stored at 4°C for up to 1 year.

   Optional: To increase the number of OT-I transgenic $CD8^+$ T cells, lymph nodes (e.g., cervical, axillary, brachial and inguinal) can also be harvested.

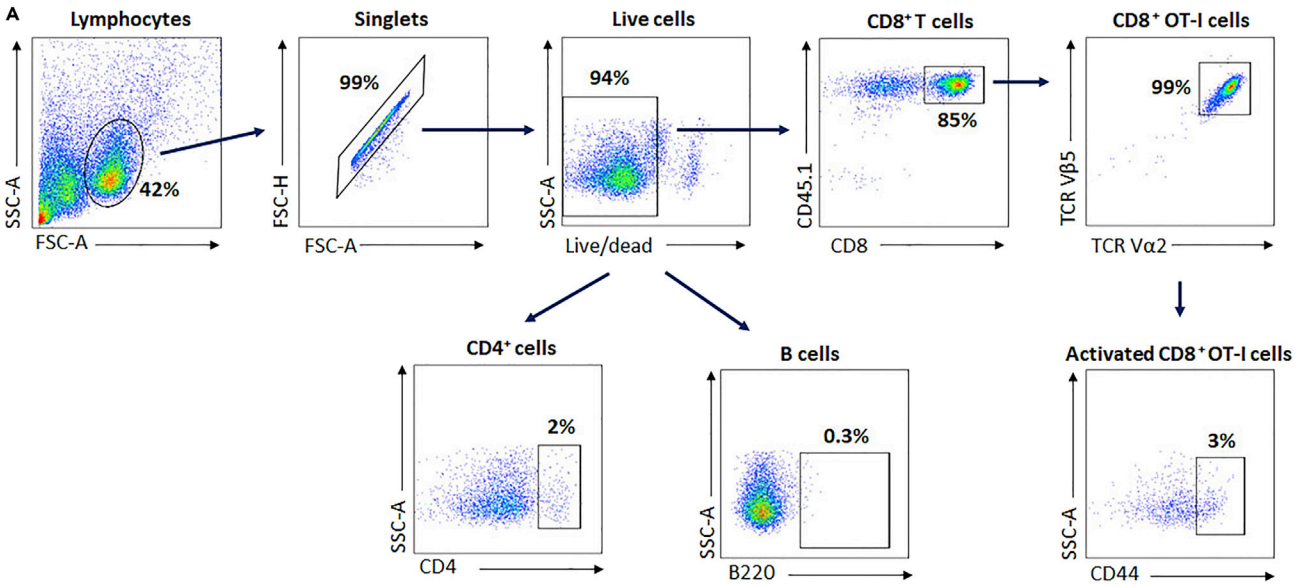
   Note: The subsequent steps must be performed on ice to prevent cell death.

5. Obtain OT-I donor-derived splenocytes:
   a. Place a 100 μm strainer on a 50 mL tube and crush spleen using a syringe plunger.
   b. Rinse the strainer with ice-cold BSS and fill the 50 mL tube.
   c. Centrifuge at 400 g for 5 min at 4°C.
   d. Discard the supernatant and resuspend the pellet with 5 mL of NH_4Cl for RBC lysis (see recipe in materials and equipment) for 1 min (perform this step at RT).

   Note: NH_4Cl can be stored at 4°C for up to 1 year.

   e. Fill the 50 mL tube with BSS and gently mix.
   f. Centrifuge at 400 g for 5 min at 4°C.

   Alternatives: Other commercially available RBC lysis buffers can be used.



B

No. of CD8$^+$ OT-I = 10.6 x 10^6 x (0.42) x (0.99) x (0.94) x (0.85) x (0.99) = 3.5 x 10^6

Figure 1. Gating strategy to phenotype OT-I cells

(A) Flow cytometry analysis of surface stained splenocytes from CD54.1$^+$ Rag $^{-/-}$ OT-I mice. This gating strategy allows to phonotype OT-I mice and calculates the percentage of single, live CD8 OT-I cells (top panel). A representative plot is shown.

(B) Example of how to calculate the percentage of single live CD8$^+$ OT-I cell based on flow cytometry analysis shown in Figure 1A.

Note: At this step, all red blood cells should be lysed. If a red pellet is still present, repeat steps d-g.

g. Resuspend pellet in 200–500 μL of BSS.

6. Count splenocytes.

Note: A hemocytometer or any automated cell counter can be used.

7. Flow cytometry analysis (Figure 1A):
   a. Transfer a small aliquot (at least 10^4 cells/well) to a flat-bottom 96 well plate.
   b. Spin the plate at 400 g × 3 min at 4°C, then flick buffer off while the cell pellet remains.
   c. Wash by adding 200 μL of FACS buffer, spin, then flick.

Note: FACS buffer can be stored at 4°C for up to 1 year.

d. Add 10 μL of Fc block 42 μg/mL (see materials and equipment) and incubate for 1 min at 21°C–25°C.

Note: Fc block is necessary to prevent non-specific binding of primary antibodies to Fc receptors. Several aliquots can be made and stored at −20°C for up to 1 year. Once thawed, aliquots can be stored at 4°C for up to 1 year.

e. Add 50 μL of the surface staining mix (Table 1) and incubate 30 min at 4°C.
f. Wash twice by adding approximately 200 μL of FACS buffer.

Table 1. Surface staining mix

Antigen	Fluorophore	Dilution v/v	μg/mL final
Rat α-CD8a	Pacific Blue	1:200	1
Mouse α-CD45.1	APC	1:200	1
Rat α-TCR Vα2	PerCP-ef710	1:200	1
Mouse α-TCR Vβ5	PE	1:200	1
CD44	AF-700	1:200	1
CD4	PE-Cy7	1:200	1
B220/CD45R	PerCP	1:200	1
Live/dead Fixable Blue Dead Cell Stain Kit	UV-blue	1:200	n/a

g. Resuspend in 200 μL of FACS buffer.
h. Acquire cells on a Flow Cytometer (we used an LSR II, BD).
i. Analyze using FlowJo.

⚠ CRITICAL: Always check that:

i. All $CD8^+$ T cells are $CD45.1^+$ and TCR $V\alpha2^+$ TCR $V\beta5^+$ (transgenic TCR) to ensure that the cells express the congenic marker (CD45.1) necessary to distinguish them from the recipient mice cells (CD45.2) and that the TCR transgene is conserved. This analysis will work as a rigorous quality control for the OT-I colony;
ii. Only a minor percent of $CD4^+$ cells are present (<5%); this analysis will also work as quality control. If a higher percentage of $CD4^+$ cells is observed, either mice are not in the $Rag^{-/-}$ background or other cells expressing CD4 (e.g., some dendritic cell sub-populations) might have unexpectedly expanded. If this is the case we strongly suggest not to use those cells.
iii. We find undetectable B cell levels ($B220/CD45R^+$);
iv. None or very little cell activation ($CD44^+$) (<10%) is observed;
v. Less that 20%–25% of cell death is observed. If higher cell death is observed, mistakes during the cell preparation step have been made. We strongly encourage to discard the cells if this is the case.

j. Calculate the number of single live $CD8^+$ OT-I cells using the following formula (Figure 1B):
No. of $CD8^+$ OT-I = total cells × (% lymphocytes) × (% single cells) × (% live) × (% $CD8^+$) × (% $V\alpha2^+$ $V\beta5^+$).

Adoptive transfer and immunization

Timing: adoptive transfer 10–20 min (Day -1)

Timing: immunization 10–20 min (Day 0)

Immediately after the flow cytometry analysis, splenocytes from $CD45.1^+$ $Rag^{-/-}$ OT-I mice are adoptively transferred to $CD45.2^+$ C57BL/6J recipient mice. One day after, the ovalbumin (OVA) peptide specific for the transgenic $CD8^+$ T cells (SIINFEKL) is i.p. injected, leading to the activation of antigen-specific $CD8^+$ T cells. At the same time, the costimulatory agonists α-CD134 (OX-40)/CD137 (4-1BB) mAbs are injected to induce antigen-specific $CD8^+$ T cell effector differentiation followed by their survival (Lee et al., 2004, 2007).

8. Prepare the splenocyte suspension from OT-I mice such that each recipient mouse will receive 0.5×10^6 $CD8^+$ OT-I cells in 50–100 μL.
9. Prepare a 1 mL syringe with the splenocyte suspension and a 27 G × 1/2″ needle (Figure 2A).

⚠ CRITICAL: Longer or thicker needles must be avoided to prevent eye damage.

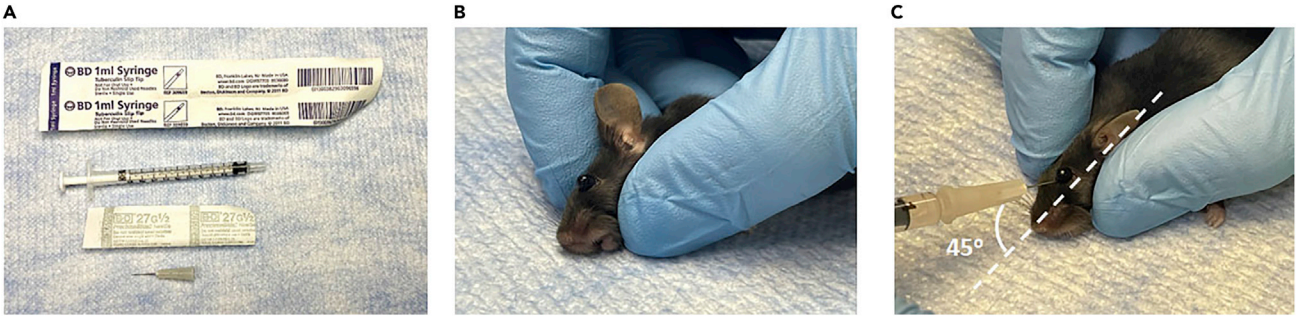


Figure 2. Retro orbital injection

(A) Prepare a 1 mL syringe and a 27 G 1/2″ needle.

(B) After anesthesia, the index and the thumb of the non-dominant hand are used to draw back the skin above the eye to allow the eye to slightly protrude.

(C) Carefully insert the needle (bevel up) at an angle of about 45 to the conjunctival membrane.

10. Anesthetize C57BL/6J mice using isoflurane or other appropriate anesthetic, such as ketamine.

Note: Always follow your institution's IACUC guidelines for anesthesia procedures.

11. Carefully inject about 50 μL of the splenocyte suspension i.v. by retro-orbital injection or other preferred i.v. injection routes (e.g., tail vein injection):
    a. Put the mouse on its side.
    b. With the index and the thumb of the non-dominant hand, draw back the skin above the eye to allow the eye to protrude (Figure 2B).
    c. Insert the needle (bevel up) at an angle of about 45 to the conjunctival membrane (Figure 2C).
    d. Inject into the retro-bulbar sinus.
    e. Pause a second or so to avoid back pressure reflux.
    f. Gently remove the needle to prevent bleeding or any eye injury.
12. Wait for the mice to recover form anesthesia.
13. After 18–20 h, immunize the recipient mice with SIINFEKL and costimulatory antibodies (α-CD134/CD137):
    a. Prepare the injection solution in sterile PBS such that each recipient mouse will receive 50 μg of SIINFEKL, 20 μg of α-CD134 and 10 μg of α-CD137.

Alternatives: to maintain antigen-specific $CD8^+$ T cell differentiation and survival, other adjuvants, such as TLR agonists, can be used (Myers et al., 2006).

    b. Inject 100 μL of this solution i.p.

Alternatives: The immunization step can be done the same day of the adoptive transfer. In this case we recommend to wait for at least 2 h after the adoptive transfer.

⚠ CRITICAL: Perform the immunization step within 24 h after the adoptive transfer, as unstimulated $CD8^+$ OT-I cells do not survive long *in vivo*.

$CD8^+$ OT-I cell isolation

Timing: 4–5 h (Day 4)

We suggest to start the isolation step on day 4 post-immunization, since at this time, effector $CD8^+$ T cells are highly glycolytic (Agliano et al., 2022; Tsurutani et al., 2016), whereas at longer time points

Figure 3. Cell number expected outcome

(A) Number of splenocytes from non-immunized C57BL/6 mice and immunized C57BL/6 mice that previously received naïve CD45.1$^+$ OT-I cells.

(B) Number of CD8$^+$ OT-I cells isolated from immunized C57BL/6 mice that previously received naive CD45.1$^+$ OT-I cells. An average of three independent experiments is shown. Each dot represents an individual mouse. Data are represented as mean ± SEM. ***p<0.001 by two-tailed unpaired t test.

their glycolytic potential decreases (Tsurutani et al., 2016). Moreover, on day 4, antigen-specific CD8$^+$ T cells are greatly expanded, as confirmed by total splenocyte number of immunized mice compared to non-immunized mice (Figure 3A). Isolation of total CD8$^+$ T cells has been performed using a bead-based negative selection (Dynabeads untouched mouse CD8 cells kit; Cat# 11417D, Thermo Fisher). A cocktail of rat antibodies against CD4$^+$ T cells, B cells, NK cells, monocytes/macrophages, dendritic cells, erythrocytes, and granulocytes (non-CD8$^+$ cells) is added to the sample. Next, magnetic beads coated with anti-rat antibodies are added to bind the antibody-labeled non-CD8$^+$ cells. After a short incubation, the bead-bound cells are then separated from unbound cells in 1–2 min using a magnet. A second negative selection step, using a biotinylated α-CD45.2 mAb is necessary to obtain highly enriched CD45.1$^+$ CD8$^+$ OT-I cells (Figure 4A). The use of negative selection steps will avoid bouts of CD8$^+$ OT-I activation due to antibody binding. If preferred, CD45.1$^+$ CD8$^+$ OT-I cells can be isolated by sorting via flow cytometry.

Note: The following steps are for isolation of 5 × 10^7 cells. Scale all volumes depending on cell number.

14. Splenocyte isolation.
    a. Isolate splenocytes as described in steps 4 and 5.

Note: At this step, the spleen of recipient mice should appear highly enlarged due to the clonal expansion of antigen-specific CD8$^+$ T cells.

15. Resuspend splenocytes in 500 μL of isolation buffer (see recipe in materials and equipment) in a 50 mL tube.

Note: Store isolation buffer at 4°C for up to 6 months.

16. Count cells and save an aliquot for flow cytometry analysis (see step 7) (troubleshooting 2).
17. For the remaining cell suspension add 100 μL of heat-inactivated FBS.

Alternatives: Heat-inactivated (fetal calf serum) FCS can also be used.

18. Add 100 μL of antibody mix (cocktail containing rat IgGs that bind mouse CD4$^+$ T cells, B cells, NK cells, monocytes/macrophages, dendritic cells, erythrocytes, and granulocytes).
19. Mix and incubate for 30 min at 4°C with rotation.
20. Wash the cells by adding 10 mL of isolation buffer and mix well.
21. Centrifuge at 350 g for 8 min at 4°C.

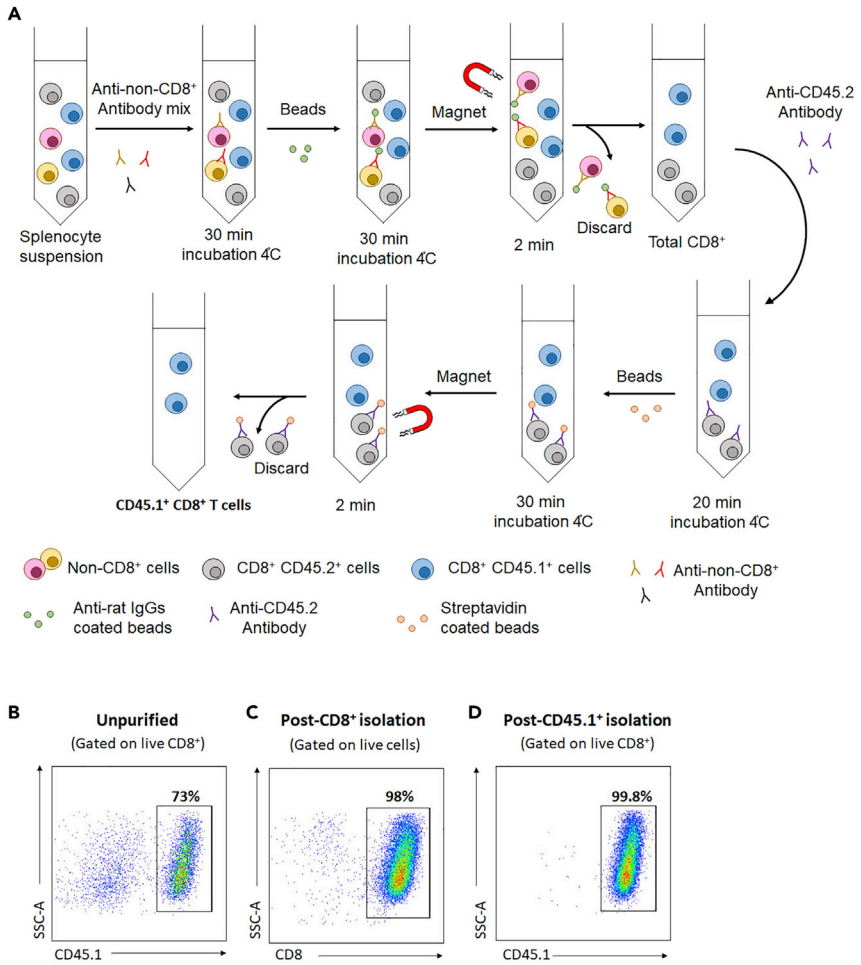


Figure 4. CD8⁺ OT-I cell isolation timeline and expected outcome

(A) Schematic timeline showing how to isolate CD8⁺ OT-I cells from a mouse splenocyte suspension, using two negative selection bead-based steps.

(B) Percentage of OT-I cells out of total CD8 T cells at day 4 post-immunization with SIINFEKL.

(C) Purity yield after CD8 T cell isolation with beads.

(D) Purity yield after CD45.1 isolation with beads. Data show representative plots.

22. Wash depletion beads (coated with an anti-rat IgG antibody):
    a. Resuspend beads by vortexing for >30 s.
    b. Transfer 1 mL of beads to a 15 mL tube.
    c. Add 1 mLof isolation buffer and mix (If cells are > 5 × 10^7 scale up volume. If cells are <5 × 10^7 still use 1 mL).
    d. Place the tube in the magnet (DynaMag-15, Cat# 12301D, Thermo Fisher Scientific) for 1 min.
    e. Discard the supernatant.
    f. Resuspend the washed beads in the same volume used in step b.
23. Resuspend the cells in 4 mL of isolation buffer.
24. Add 1 mL of pre-washed beads.
25. Incubate for 30 min at 4°C with rotation.
26. Add 5 mL of isolation buffer.
27. Resuspend by gently pipetting 5 times. Avoid foaming.
28. Place the 50 mL tube in the magnet (DynaMag-50, Cat# 12302D, Thermo Fisher Scientific) for 2 min.

29. Pour the supernatant containing the untouched $CD8^+$ T cells into a new sterile 50 mL tube.
30. Centrifuge at 400 g for 5 min at 4°C.
31. Resuspend pellet in 1 mL of isolation buffer and count.
32. Save an aliquot for flow cytometry analysis to test for purity.

Note: The following steps are for isolation of 1×10^7 cells. Scale all volumes depending on cell number.

33. Transfer the cell suspension in a 15 mL tube and add 10 μg (adjust antibody amount if cells are > or < 1×10^7) of the biotinylated α-CD45.2 mAb (#13-0454-85, Thermo Fisher).
34. Incubate for 20 min at 4°C with rotation.
35. Wash cells by adding 2 mL of isolation buffer.
36. Centrifuge at 350 g for 8 min at 4°C.
37. Resuspend the cells in isolation buffer to 1×10^7/mL.
38. Wash biotin binder beads (coated with recombinant streptavidin) (#11047, Thermo Fisher):
    a. Resuspend beads by vortexing for >30 s.
    b. Transfer 100 μL of beads to a 1.5 mL tube.
    c. Add 1 mL of isolation buffer and mix (If cells are > 1×10^7 scale up volume. If cells are <1×10^7 still use 1 mL).
    d. Place the tube in the magnet (DynaMag-15) for 1 min.
    e. Discard the supernatant.
    f. Resuspend the washed beads in the same volume used in step b.
39. Incubate for 30 min 4°C with rotation.
40. Add 1 mL of isolation buffer to limit trapping of unbound cells.
41. Place the 15 mL tube in the magnet (DynaMag-15) for 2 min.
42. Pour the supernatant containing the untouched $CD45.1^+$ $CD8^+$ T cells into a new tube.
43. Count cells.
44. Save an aliquot for flow cytometry analysis (troubleshooting 3).
45. Check cell viability (troubleshooting 4).

Measurement of glycolytic potential

Timing: 3–4 h (Day 4)

46. Set up the Seahorse Analyzer:
    a. In the Wave software, open the glycolysis stress test template.
    b. Add your experimental details. Instructions on how to fill the template can be found on the Agilent website: https://www.agilent.com/en/product/cell-analysis/how-to-run-an-assay.
47. Medium preparation.
    a. Supplement the Seahorse XF DMEM medium pH 7.4 with no phenol red (# 103575 100, Agilent) with 2 mM glutamine.
48. Remove the sensor cartridge from the 37°C non-CO_2 incubator, aspirate the dH_2O from the utility plate and add 200 μL/well of warm calibrant. Leave in the incubator for 45–60 min.

⚠ CRITICAL: the 37°C non-CO_2 incubator is absolutely necessary to prevent a pH alteration of the Seahorse reagents.

49. Count the isolated $CD8^+$ OT-I cells.
50. Centrifuge cells at 400 g for 5 min at 4°C and resuspend in Seahorse medium at a concentration of 6×10^6 cells/mL.
51. Dispense 50 μL of the cell suspension to each well. We suggest to plate 0.3×10^6 cells/well and to use three technical replicates per sample.

Table 2. Glycolysis stress test injections optimized for effector CD8$^+$ T cells

Port	Compound	Concentration injected	Final concentration	Volume from stock	Volume of seahorse medium	Volume per well
A	Glucose	100 Mm	10 mM	3,000 µL	–	20 µL
B	Oligomycin	40 µM	4 µM	720 µL	1,080 µL	22 µL
C	2-DG	500 Mm	50 mM	3000 µL	–	25 µL

⚠ CRITICAL: Dispense 50 µL of medium with no cells in the background wells (four corners of the plate). Plating less than 0.3 × 10^6 CD8$^+$ T cells/well may result in weak ECAR detection.

52. Centrifuge plate at 200 g for 1 min without brake.
53. Place the plate in a 37°C non-CO_2 incubator for 15–25 min.
54. Take the sensor cartridge out from the incubator and add the compounds (for optimized compound dilutions, see Table 2). Instructions on how to correctly load the sensor cartridge can be found at: https://www.agilent.com/cs/library/usermanuals/public/DAY%20OF%20LOADING%20CARTRIDGE%20XFe96-XF96.pdf.

⚠ CRITICAL: When loading the compounds to the injection ports ensure to not touch the bottom of the port with the pipette tips and avoid air bubbles.

55. Remove the plate from the incubator and check with a microscope that the cells are attached to the bottom of the wells (troubleshooting 5).
56. Slowly add 125 µL of Seahorse medium without disturbing the cells.

Note: At this step, inhibitors, drugs or metabolites can be added to the cells as pre-treatment.

57. Return the plate to the 37°C non-CO_2 incubator for 15–25 min.
58. Insert the sensor cartridge with the utility plate in the Seahorse analyzer and start calibration (duration ~ 30 min).
59. Once calibration is over, discard the utility plate and insert the cell plate into the machine.
60. Start the assay (duration ~ 2 h).
61. Remove the plate from the machine.
62. Analyze data. We recommend using GraphPad Prism (troubleshooting 6 and troubleshooting 7).
63. If users suspect that wells do not contain the same number of cells or if a treatment or manipulation that affects cell proliferation is used, normalization must be performed (e.g., using protein quantification or DNA content Hoechst staining). For more details on how to perform normalization please refer to: https://www.agilent.com/cs/library/technicaloverviews/public/Methods_and_Strategies_for_Normalizing_Tech_Overview_022118.pdf.

Note: Oligomycin should be titrated to select the minimum concentration needed to achieve a maximal increase in ECAR (glycolytic reserve capacity).

EXPECTED OUTCOMES

Immunizing recipient mice after OT-I splenocyte transfer with SIINFEKL plus α-CD134/137 agonist mAbs and harvesting T cells after 4 days will lead to substantial antigen-specific CD8$^+$ T cell clonal expansion that is reflected in an increased splenocytes number (Figure 3A). The splenocytes number from an immunized mouse at day 4 is usually ~ 150–200 × 10^6. The number of CD8$^+$ OT-I after purification is ~10–30 × 10^6/mouse (Figure 3B), being ~70%–89% of total CD8$^+$ T cells (Figure 4B). After total CD8$^+$ T cells are isolated, the purity yield is usually ~95%–98% (Figure 4C). After CD45.2$^+$ host cell depletion, the purity yield of the isolated CD45.1$^+$ CD8$^+$ T cells is usually ~95%–99% (Figure 4D).

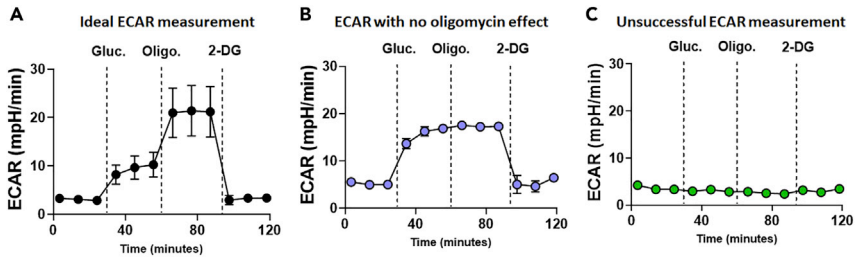


Figure 5. Glycolysis stress test expected outcome

(A) Ideal ECAR measurement in effector OT-I cells: ECAR is increased by Glucose injection (glycolysis); ECAR reaches its maximum after oligomycin injection (maximum glycolytic capacity); ECAR returns to basal level after 2-DG injection.

(B) ECAR measurement where oligomycin did not have an effect.

(C) Unchanged ECAR measurement due to insufficient cell number or incorrect compound loading into the injection ports. Data show representative plots. Data are presented as mean ± SD of technical replicates. Showed outcomes are consistent with data published in Agliano et al. (2022).

During the Glycolysis stress test assay, cells are expected to increase their ECAR after Glucose injection and to reach maximal glycolytic activity after oligomycin injection (Figure 5A. However, if cells are extremely glycolytic, oligomycin may have less of an impact on ECAR (Figure 5B). After 2-DG injection, ECAR is expected to return to levels equal to pre-glucose measurements. If the assay is not successful, a flat line is obtained (Figure 5C).

QUANTIFICATION AND STATISTICAL ANALYSIS

Based on our experience for most studies, experiments should be repeated at least three times to ensure reproducibility. Each plot is automatically generated using the Wave software (free available at: https://www.agilent.com/en/products/cell-analysis/software-download-for-wave-desktop) and can be easily exported to Excel or GraphPad Prism. Statistically significant differences between curves can be evaluated using a one-way ANOVA analysis within each segment of the assay to analyze distinct metabolic states. Alternatively, an area under the curve (AUC) analysis or a two-way ANOVA analysis between curves can be performed.

LIMITATIONS

This protocol is optimized to isolate antigen-specific $CD8^+$ T cell *ex vivo* at 3–4 days post immunization. If considering to isolate antigen-specific $CD8^+$ T at earlier time points (<30 h), this protocol is not recommended as is, since T cells will be retained within secondary lymphoid tissues (Maxwell et al., 2004). In this case, a pre-treatment with collagenase D for 30 min is necessary. This protocol only allows the analysis of OVA peptide-specific $CD8^+$ T cells.

The glycolysis stress test assay is optimized for *ex vivo* $CD8^+$ T cells. Adjustments in the protocol may be necessary when working with other lymphocytes or when $CD8^+$ T cells are activated *in vitro*. Moreover, the Glycolysis stress test also accounts for acidification due to mitochondrial oxidative reactions. If the extracellular acidification by glycolysis alone needs to be measured, the Glycolytic Rate Assay (Cat# 103344-100, Agilent) may be more appropriate.

TROUBLESHOOTING

Problem 1

The spleen of OT-I donor mice appears enlarged at step 4.

Potential solution

$Rag^{-/-}$ OT-I mice exhibit tiny spleens, with no more than 10–20 × 10^6 splenocytes per spleen. If enlarged spleens are observed, because these mice are immunodeficient, infection or other immune system-related issues may have occurred. We strongly recommend not to use those mice as donors.

Problem 2

At day 4, no expansion of antigen-specific $CD8^+$ T cell occurred (step 16).

Potential solution

Ensure that the adoptive transfer by retro-orbital injection is done correctly. Depending upon experience, an alternate route is i.v. *via* the tail vein. The number of transferred $CD8^+$ OT-I cells could be insufficient: we recommend transferring 0.3–0.6 × 10^6 $CD8^+$ OT-I cells per recipient mouse. A poor expansion can also be due to the injection of an incorrect antigen amount. Always check the antigen stock and dilution calculations and be certain that the transferred cells have the appropriate OT-I phenotypic markers and are viable as measured by flow cytometry.

Problem 3

Low purity of OT-I $CD8^+$ T cells is observed in step 44.

Potential solution

Increase antibodies and/or beads incubation times. Increasing the number of washes may help reduce contamination from other cell types. Performing a flow cytometry analysis after each isolation step will help identify possible mistake(s).

Problem 4

High cell death was observed in step 45.

Potential solution

It is critical that all steps are carried at 4°C.

When harvesting spleen and/or lymph nodes, certain amounts of fat can be carried over and pass through the strainer. This can affect cell viability. Remove the fat that may be attached to the organs.

Problem 5

Cells did not attach to the bottom of the wells after Cell-Tak coating (step 55).

Potential solution

One possible explanation is that the pH of $NaHCO_3$ was below 8 and Cell-Tak did not work properly. Furthermore, always check Cell-Tak concentration and expiration date to get maximum adhesion of cells.

Problem 6

Different ECAR values are observed between technical replicates (step 62).

Potential solution

This could be due to inaccurate plating of cells. Different number of cells will show different ECAR. Check cell density with a microscope. This issue can be also overcome by performing a proper normalization.

Problem 7

Cells do not respond to one or more injections (step 62).

Potential solution

ECAR detection is highly dependent on cell number. When running an experiment for the first time, plate cells at different cell densities to determine the optimal seeding concentration. Another reason could be due to an incorrect compound loading, which results in inconsistent compound injection. Lastly, ensure that in all wells, each series of ports contains the same injection volume.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anthony T. Vella (vella@uchc.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets or codes.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants P01AI056172 and R21AI139891, institutional support, and Boehringer Ingelheim endowed Chair in Immunology.

AUTHOR CONTRIBUTIONS

F.A. and A.M. designed and optimized the protocol. F.A. wrote the manuscript. A.M. and A.T.V. edited the manuscript. A.T.V. supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Agliano, F., Karginov, T.A., Menoret, A., Provatas, A., and Vella, A.T. (2022). Nicotinamide breaks effector CD8 T cell responses by targeting mTOR signaling. iScience *25*, 103932. https://doi.org/10.1016/j.isci.2022.103932.

Hogquist, K.A., Jameson, S.C., Heath, W.R., Howard, J.L., Bevan, M.J., and Carbone, F.R. (1994). T cell receptor antagonist peptides induce positive selection. Cell *76*, 17–27. https://doi.org/10.1016/0092-8674(94)90169-4.

Lee, S.J., Myers, L., Muralimohan, G., Dai, J., Qiao, Y., Li, Z., Mittler, R.S., and Vella, A.T. (2004). 4-1BB and OX40 dual costimulation synergistically stimulate primary specific CD8 T cells for robust effector function. J. Immunol. *173*, 3002–3012. https://doi.org/10.4049/jimmunol.173.5.3002.

Lee, S.J., Rossi, R.J., Lee, S.K., Croft, M., Kwon, B.S., Mittler, R.S., and Vella, A.T. (2007). CD134 costimulation couples the CD137 pathway to induce production of supereffector CD8 T cells that become IL-7 dependent. J. Immunol. *179*, 2203–2214. https://doi.org/10.4049/jimmunol.179.4.2203.

Makowski, L., Chaib, M., and Rathmell, J.C. (2020). Immunometabolism: from basic mechanisms to translation. Immunol. Rev. *295*, 5–14. https://doi.org/10.1111/imr.12858.

Maxwell, J.R., Rossi, R.J., McSorley, S.J., and Vella, A.T. (2004). T cell clonal conditioning: a phase occurring early after antigen presentation but before clonal expansion is impacted by Toll-like receptor stimulation. J. Immunol. *172*, 248–259. https://doi.org/10.4049/jimmunol.172.1.248.

Myers, L., Lee, S.W., Rossi, R.J., Lefrancois, L., Kwon, B.S., Mittler, R.S., Croft, M., and Vella, A.T. (2006). Combined CD137 (4-1BB) and adjuvant therapy generates a developing pool of peptide-specific CD8 memory T cells. Int. Immunol. *18*, 325–333. https://doi.org/10.1093/intimm/dxh371.

O'Neill, L.A., Kishton, R.J., and Rathmell, J. (2016). A guide to immunometabolism for immunologists. Nat. Rev. Immunol. *16*, 553–565. https://doi.org/10.1038/nri.2016.70.

Shinkai, Y., Rathbun, G., Lam, K.P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M., et al. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, 855–867. https://doi.org/10.1016/0092-8674(92)90029-c.

Tsurutani, N., Mittal, P., St Rose, M.C., Ngoi, S.M., Svedova, J., Menoret, A., Treadway, F.B., Laubenbacher, R., Suarez-Ramirez, J.E., Cauley, L.S., et al. (2016). Costimulation endows immunotherapeutic CD8 T cells with IL-36 responsiveness during aerobic glycolysis. J. Immunol. *196*, 124–134. https://doi.org/10.4049/jimmunol.1501217.