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Protocol

Isolation and enrichment of mouse splenic T cells for ex vivo and in vivo T cell receptor stimulation assays

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SUMMARY

Specific antigen recognition by T cell receptor (TCR) activates TCR signaling pathway, leading to T cell proliferation and differentiation into effector and memory cells. Herein, we describe protocols for TCR stimulation assays, including procedures for the isolation and enrichment of mouse splenic T cells for ex vivo TCR stimulation with anti-CD3/CD28 antibodies, and the use of ovalbumin-OT-II mouse model for in vivo TCR stimulation. We applied this protocol to show that MYC protein is essential for T cell proliferation and differentiation. For complete details on the use and execution of this protocol, please refer to Nozais et al. (2021).

BEFORE YOU BEGIN

In vivo assays are carried out with mouse models, thus those assays require qualified staff to perform experimental procedures on mice and to be validated by an ethical committee. The protocol below describes the specific steps for TCR stimulation of wild-type and MYC-deficient T cells with anti-CD3 and anti-CD28 antibodies or with ovalbumin. The procedure can be performed with cells expressing various types of transgenic TCR to investigate response to other kinds of antigens such as LACK (Wang et al., 2001) or Der p 1 (Dullaers et al., 2017).

Mouse models

Besides wild-type C57BL/6 mice, mouse models used in this protocol are the following. Myc\textsuperscript{flx/flx} mice which allow conditional inactivation of Myc gene (Trumpp et al., 2001). Transgenic CD4-cre mice in which Cre recombinase is activated at the CD4^CD8^ (DP) stage of thymocyte differentiation (Lee et al., 2001). ROSA26-LSL-eYFP reporter mice, in those mice Cre-expressing cells express the enhanced yellow fluorescent protein (eYFP) (Srinivas et al., 2001). OT-II mice that harbor OT-II transgene encoding for a V\textsubscript{z2/V\textsubscript{b5.1} TCR. OT-II TCR recognizes the chicken ovalbumin antigen in the context of MHC-II molecules (Barnden et al., 1998). These mice were crossed in different combination to obtain: 1) ‘Control’ that corresponds to CD4-Cre X ROSA26-LSL-eYFP mouse; 2) ‘Control OT-II’ corresponds to ‘Control’ X OT-II mouse; 3) ‘Myc\textsuperscript{del}’ corresponds to CD4-Cre X ROSA26-LSL-eYFP X Myc\textsuperscript{flx/flx} mouse; and 4) ‘Myc\textsuperscript{del} OT-II’ corresponds to Myc\textsuperscript{del} X OT-II mouse. Both female and male mice were used, they were aged between 6 and 12 weeks.

Reagents preparation

© Timing: 1 h
Prepare the buffers and antibodies cocktails as described in the ‘materials and equipment’ section.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| **Antibodies**       |        |            |
| CD3 APC Cy7 (1:400)  | BD Pharmingen | Cat#: 560590, RRID: AB_1727461 |
| CD4 V450 (1:500)     | BD Pharmingen | Cat#: 560468, RRID: AB_1645271 |
| CD4 APC (1:500)      | BD Pharmingen | Cat#: 553051, RRID: AB_398528 |
| CD4 PerCP Cy 5.5 (1:400) | BD Pharmingen | Cat#: 561115, RRID: AB_10563934 |
| CD8a PerCP Cy5.5 (1:400) | BD Pharmingen | Cat#: 561109, RRID: AB_10563417 |
| CD8a PE Cy7 (1:400)  | BD Pharmingen | Cat#: 552877, RRID: AB_394506 |
| CD25 PE (1:800)      | BD Pharmingen | Cat#: 561065, RRID: AB_10563211 |
| CD44 APC Cy7 (1:400) | BD Pharmingen | Cat#: 560568, RRID: AB_1727481 |
| CD62L APC (1:500)    | BD Pharmingen | Cat#: 561919, RRID: AB_1645257 |
| CD69 PerCP Cy5.5 (1:400) | BD Pharmingen | Cat#: 561931, RRID: AB_10892815 |
| TCR Vb5 PE (1:400)   | BD Pharmingen | Cat#: 553190, RRID: AB_394698 |
| TCR Va2 APC (1:400)  | eBioscience | Cat#: 17-5812-80, RRID: AB_469460 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Dynabeads® Mouse T-Activator CD3/CD28 | Life Technologies | Cat#: 11456D |
| Annexin V-APC       | BD Pharmingen | Cat#: 550474 |
| Complete Freund’s adjuvant | Sigma-Aldrich | Cat#: F5881 |
| EndoFit Ovalbumin   | Invivogen | Cat#: vac-pova |
| RPMI 1640           | Life Technologies | Cat#: 10716-016 |
| Dulbecco’s Phosphate Buffered Saline 10× | Life Technologies | Cat#: 14200-067 |
| RBC lysis buffer    | Life Technologies | Cat#: 00-4333-57 |
| Fetal Bovine Serum | Life Technologies | Cat#: 10270-106 |
| Annexin V Binding Buffer | BD Pharmingen | Cat#: 51-66121E |
| β-mercaptoethanol   | Life Technologies | Cat#: 31350-010 |
| GlutMax             | Life Technologies | Cat#: 20300-024 |
| Sodium pyruvate     | Life Technologies | Cat#: 11360-039 |
| Penicillin-Streptomycin | Life Technologies | Cat#: 15140122 |
| Ethylenediaminetetraacetic acid | Life technologies | Cat#: 15576-028 |
| Trypan Blue solution | Sigma-Aldrich | Cat#: 78154 |
| Ionomycin           | Life technologies | Cat#: 124222 |
| Phorbol myristate acetate | Sigma-Aldrich | Cat#: P8139 |
| **Critical commercial assays** | | |
| EasySepTM Mouse T cell isolation kit | Life Technologies | Cat#: 19851 |
| CellTrace Violet    | Invitrogen | Cat#: C34557 |
| **Experimental models: Organisms/strains** | | |
| Mycflx/flox mice    | Andreas Trump (DKFZ) | Trumpp A. Nature. 414, 768-73 (2001). |
| CD4-Cre mice        | European Mouse Mutant Archive | EM: 01139 |
| OT-II mice          | Barnden MJ. Immunol Cell Biol. 76, 34–40 (1998) | MGI: 3046083 |
| ROSA26-LSL-eYFP reporter mice | The Jackson Laboratory | MGI: 2449038 |
| CS7BL/6 mice        | The Jackson Laboratory | MGI: 2159769 |
| **Software and algorithms** | | |
| FlowJo version 10   | FlowJo | https://www.flowjo.com/ |
| Diva version 8.0.1  | BD Biosciences | https://wwwbdbiosciences.com/ |
| **Other**           |       |            |
| 6-well plate        | Falcon | Cat#: 353046 |
| 48-well plate       | Falcon | Cat#: 353078 |
| 5mL polystyrene tubes | Falcon | Cat#: 352054 |
| 15mL tubes          | Sarstedt | Cat#: 62.554.502 |
| 50mL tubes          | Sarstedt | Cat#: 62.547.254 |
| 70 μm cell strainer | Sarstedt | Cat#: 83.3945.070 |

(Continued on next page)
## MATERIALS AND EQUIPMENT

### PBS1 x

| Reagent                                      | Final concentration | Amount       |
|----------------------------------------------|---------------------|--------------|
| Dulbecco’s Phosphate Buffered Saline (DPBS) 10 x | 1 X                 | 100 mL       |
| dH2O                                         | n/a                 | Up to 1 L    |
| **Total**                                   | n/a                 | 1 L          |

Store at 4°C for up to one month.

### PBS1 x/2%FBS

| Reagent         | Final concentration | Amount |
|-----------------|---------------------|--------|
| Fetal Bovine Serum | 2%                  | 8 mL   |
| PBS1 x          | 1 X                 | Up to 400 mL |
| **Total**       | n/a                 | 400 mL |

Store at 4°C for up to one month.

### Complete RPMI medium

| Reagent                          | Final concentration | Amount       |
|----------------------------------|---------------------|--------------|
| β-mercaptoethanol                 | 50 µM               | 500 µL       |
| Fetal Bovine Serum               | 10%                 | 50 mL        |
| GlutaMax                         | 1 X                 | 5 mL         |
| Sodium pyruvate                  | 1 X                 | 5 mL         |
| Penicillin-Streptomycin          | 1 X                 | 5 mL         |
| RPMI 1640                        | n/a                 | Up to 500 mL |
| **Total**                        | n/a                 | 500 mL       |

Store at 4°C for up to one month.

### FACS buffer

| Reagent                          | Final concentration | Amount       |
|----------------------------------|---------------------|--------------|
| Fetal Bovine Serum               | 1%                  | 1 mL         |
| Ethylenediaminetetraacetic acid | 2 mM                | 200 µL       |
| PBS1 x                           | 1 X                 | Up to 50 mL  |
| **Total**                        | n/a                 | 50 mL        |

Store at 4°C for up to one month.
### Annexin V Binding Buffer

| Reagent                  | Final concentration | Amount    |
|-------------------------|---------------------|-----------|
| Annexin V Binding Buffer| 1 X                 | 1 mL      |
| dH₂O                    | n/a                 | Up to 10 mL |
| **Total**               | n/a                 | 10 mL     |

Store at 4°C for up to one week.

### CellTrace Violet stock

| Reagent                  | Final concentration | Amount    |
|-------------------------|---------------------|-----------|
| CellTrace Violet kit (CTV) | 5 mM              | 1 vial    |
| DMSO                    | n/a                 | 20 μL     |
| **Total**               | n/a                 | 20 μL     |

Make aliquots, store at −20°C up to one month and protect from light.

### Antibodies cocktail n°1 - immunophenotyping - 20× concentrated

| Reagent                  | Amount      | Dilution in antibodies cocktail | Working dilution (in the final cell suspension) |
|-------------------------|-------------|---------------------------------|-----------------------------------------------|
| CD4 V450                | 1 μL        | 1:25                            | 1:500                                         |
| CD8 PerCP Cy5.5         | 1.25 μL     | 1:20                            | 1:400                                         |
| CD3 APC Cy7             | 1.25 μL     | 1:20                            | 1:400                                         |
| Vα2 APC                 | 1.25 μL     | 1:20                            | 1:400                                         |
| Vβ5 PE                  | 1.25 μL     | 1:20                            | 1:400                                         |
| FACS Buffer             | Up to 25 μL | n/a                            | n/a                                           |
| **Total**               | 25 μL       |                                 |                                               |

Store at 4°C until use and protect from light.

### Antibodies cocktail n°2 - after enrichment – 20× concentrated

| Reagent                  | Amount      | Dilution in antibodies cocktail | Working dilution (in the final cell suspension) |
|-------------------------|-------------|---------------------------------|-----------------------------------------------|
| CD4 APC                 | 1 μL        | 1:25                            | 1:500                                         |
| CD8 PerCP Cy5.5         | 1.25 μL     | 1:20                            | 1:400                                         |
| CD3 APC Cy7             | 1.25 μL     | 1:20                            | 1:400                                         |
| Vβ5 PE                  | 1.25 μL     | 1:20                            | 1:400                                         |
| FACS Buffer             | Up to 25 μL | n/a                            | n/a                                           |
| **Total**               | 25 μL       |                                 |                                               |

Store at 4°C until use and protect from light.

### Antibodies cocktail n°3 – stimulation - 20× concentrated

| Reagent                  | Amount      | Dilution in antibodies cocktail | Working dilution (in the final cell suspension) |
|-------------------------|-------------|---------------------------------|-----------------------------------------------|
| CD25 PE                 | 0.6 μL      | 1:40                            | 1:800                                         |
| CD69 PerCP Cy5.5        | 1.25 μL     | 1:20                            | 1:400                                         |
| CD44 APC Cy7            | 1.25 μL     | 1:20                            | 1:400                                         |
| FACS Buffer             | Up to 25 μL | n/a                            | n/a                                           |
| **Total**               | 25 μL       |                                 |                                               |

Store at 4°C until use and protect from light.
**Note:** The amount of antibody mentioned in the above tables are for 5 samples containing each up to $1 \times 10^6$ cells. Antibodies cocktails are kept at 4°C in the dark and used on the day of preparation, avoid storing for more than 2 days.

△ **CRITICAL:** Isoflurane gaz, xylazine and ketamine are hazardous reagents, so personal protective equipment (PPE) are needed to prevent eyes and skin damages.

### STEP-BY-STEP METHOD DETAILS

#### Spleen harvesting

© Timing: 50 min

This section describes how to obtain splenic cells (Figure 1 depicts the main steps).

1. Euthanize mice using carbon dioxide and place them on a dissection board. Herein we used Myc$^{del}$ mice (for ex vivo assay), Myc$^{del}$ OT-II (for in vivo assay) mice and their Control counterparts. Sterilize the skin using 70% ethanol. Using sterile scissors, cut through the skin and the muscle layer. Visualize the spleen next to the stomach on the left side of the mouse.

   **Note:** For euthanasia with CO$_2$, mice are placed in a hermetically sealed box, then we use an automatic CO$_2$ euthanasia machine (TemSega) which allows a sequence of 3 phases according to a strict and secure protocol 1) 'induction' phase which lasts 1 min and corresponds to a progressive saturation in CO$_2$; 2) 'Euthanasia' phase which lasts 2 min (100% CO$_2$); 3) 'Emptying' phase which lasts 2 min and corresponds to CO$_2$ absorption.

2. Remove the spleen, trimming away any non-specific tissue (like fat) and place the spleen in a six-well plate containing a 3 mL of PBS1 x/2%FBS in each well.

3. Perform the following steps at room temperature (RT; 20°C–25°C) under a cell culture hood.

4. Put a 70 μm cell strainer on top of a 50 mL conical tube.

5. Place the organ on the cell strainer, which was previously moistened with PBS1 x/2%FBS, and dilacerate the spleen with the piston of a syringe. (Add PBS1 x/2%FBS to facilitate the dilaceration).

6. Adjust the volume to 20 mL for each tube with PBS1 x/2%FBS.

7. Centrifuge (450 × g, 7 min, at RT). Discard supernatant.

   **Note:** For all centrifugations performed in this protocol, we used shortest acceleration time/ braking time. For our centrifuge (Eppendorf 5810R) this corresponds to level ACC 9/BRK 9.

8. Add 2 mL of Red Blood Cells lysis buffer on the cell pellet. Resuspend cells by pipetting and incubate 10 min at RT.

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### Antibodies cocktail n°4 – post-graft - 20× concentrated

| Reagent          | Amount   | Dilution in antibodies cocktail | Working dilution (in the final cell suspension) |
|------------------|----------|---------------------------------|-----------------------------------------------|
| CD4 PerCP Cy5.5  | 1.25 μL  | 1:20                            | 1:400                                         |
| CD8 PE Cy7       | 1.25 μL  | 1:20                            | 1:400                                         |
| CD44 APC Cy7     | 1.25 μL  | 1:20                            | 1:400                                         |
| CD62L APC        | 1 μL     | 1:25                            | 1:500                                         |
| Vβ5 PE           | 1.25 μL  | 1:20                            | 1:400                                         |
| FACS Buffer      | Up to 25 μL | n/a                             | n/a                                           |
| **Total**        | 25 μL    |                                 |                                               |

*Store at 4°C until use and protect from light.*
Note: We advise to resuspend cells by gentle pipetting in order to prevent cell death, also do not exceed 10 min incubation, as it might alter cells of interest.

9. Add 10 mL of PBS1 x/2%FBS and centrifuge (450 x g, 7 min, RT).
10. Remove supernatant and resuspend cell pellet with 10 mL of PBS1 x/2%FBS.
11. Take 5 μL of cells and mix them with 45 μL of trypan blue (previously diluted at 1:1 ratio with PBS1 x).
12. Load cell mixture into a hemocytometer. Using a microscope, count viable cells which correspond to bright cells that are not stained in blue (Figure 1G).

Alternatives: Cells can be counted with an automated cell counter.

13. Centrifuge conical tubes containing 10 mL of cell suspension at 450 x g (7 min, RT).
14. Resuspend the pellet in PBS1 x/2%FBS in order to be at 1 x 10^8 cells/mL.
**Immunophenotyping**

- **Timing:** 50 min

After splenic cells harvesting, T cells are characterized by flow cytometry using specific markers to determine the percentage of CD8+, CD4+, DP or OT-II+ cells.

15. Transfer $10^6$ cells into a 5 mL polystyrene tube (FACS tube).
16. Add for each sample, 5 µL of antibodies cocktail n°1 and FACS buffer up to 100 µL.
17. Incubate for 30 min at 4°C, in the dark.
18. Wash cells with 2 mL of PBS1×. Centrifuge cells (650 × g, 3 min, 4°C). Discard supernatant.
19. Resuspend the cell pellet in 100 µL of FACS Buffer.
20. Acquire cells on a flow cytometer (we used a BD Canto).

**Note:** Cells are not fixed so they are kept at 4°C until their acquisition which is performed as soon as possible (within 3 h maximum).

21. Analyze files using an adapted software such as FlowJo.

**Note:** Antibodies cocktail is defined according to surface markers of interest.

**Splenic T cells enrichment**

- **Timing:** 30 min

This section describes the purification of T cells from a splenic suspension. We use mouse T cell Isolation kit (StemCellTM) which is designed to isolate T cells by negative selection and we follow the manufacturer’s instructions 1000003744-PIS_01.pdf (stemcell.com). Below (steps 22–30) is the procedure to process $5 \times 10^7$ cells (from Myc<sup>del</sup>, Myc<sup>del</sup> OT-II or Control mice).

22. Transfer $5 \times 10^7$ cells into a FACS tube.
23. Add 25 µL of Normal Rat Serum.
24. Add 25 µL of EasySep<sup>TM</sup> Mouse T cell isolation cocktail.
25. Incubate 10 min at RT.
26. Vortex EasySep<sup>TM</sup> Streptavidin RapidSpheres<sup>TM</sup> for 30 s and transfer 37.5 µL into cell suspension.
27. Incubate 2.5 min at RT.
28. Add 2 mL PBS1×/2%FBS and pipette mix (avoid vortexing).
29. Put the FACS tube (without cap) on a magnet and wait 2.5 min at RT.
30. Transfer cell suspension into a new 15 mL conical collection tube by inverting magnet/FACS tube in a single continuous motion.

**CRITICAL:** It’s very important to make this movement properly and without shaking to prevent beads from coming loose.

- a. Count cells as described in steps 11 and 12.
- b. To check the quality of the purification, take an aliquot (around 50 µL of cell suspension) and stain cells with 5 µL of antibodies cocktail n°2. Then, proceed to steps 17–21 described above. An example of FACS analysis of T cells purification is shown in Figure 2.

31. Centrifuge cells (450 × g, 7 min, RT). Aspirate supernatant.
32. Resuspend cell pellet at $2 \times 10^7$ cells/mL in PBS1×.
FACS analysis step is important to assess the efficiency and the quality of T cell enrichment.

**Cell trace violet staining**

- **Timing:** 25 min

This step describes cell staining with a carboxyfluorescein succinimidyl ester (CFSE)-based reagent in order to follow cell proliferation. Here we use CellTrace™ Violet (CTV), yet other types of CFSE-based reagents are commercially available.

1. Dilute CTV stock solution (5 mM) at 1:1000 with PBS
2. In a microcentrifuge tube, add 20 μL diluted CTV (5 μM) to 3 × 10^6 cells in 1 mL PBS
3. Mix well by gentle pipetting.
4. Incubate at 37°C for 15 min (in the dark).
5. Wash cells twice with 500 μL of cold PBS. Spin cells (700 × g, 5 min, 4°C).
6. Resuspend cell pellet in complete RPMI (for *ex vivo* assays, step 39) or PBS (for *in vivo* assays, step 50) and store at 4°C in the dark until use.

**Note:** The samples can be stored at 4°C until use, but usually cells are used within 3 h in order to prevent cell death.

**Ex vivo T cell receptor stimulation**

- **Timing:** 3 days

In the following section T cells are stimulated by anti-CD3 and anti-CD28 antibodies and analyzed by flow cytometry 24H and 72H post-stimulation.

1. Resuspend cells in complete RPMI at a cell concentration of 3 × 10^6 cells/mL
2. In a 48-well plate, dispatch 10^6 cells of each sample in 2 wells:
   - Well #1: ‘Not stimulated’.
   - Well #2: ‘Stimulated CD3/CD28’: add 10 μL Dynabeads Mouse T-activator CD3/CD28.

**Optional:** Addition of a control well. In a well #3 ‘PMA/ionomycin’: add Phorbol myristate acetate (PMA) and ionomycin at a final concentration of 0.1 μg/mL and 2 μg/mL respectively.
Alternatives: Instead of using Dynabeads for stimulation, it is possible to pre-coat the plate with anti-CD3 antibodies and then add cell suspension together with anti-CD28 antibodies.

41. Incubate at 37°C in a 5% CO₂ incubator.
42. At 24H, resuspend cells by gentle pipetting and take 100 μL cells in FACS tube. Add 1 mL PBS1× in each sample.
43. For stimulated cells, put the tube on a magnet to remove Dynabeads. Wait 2 min and collect the supernatant into another FACS tube.
44. For all samples, centrifuge cells (700 × g, 5 min, 4°C). Discard supernatant.
45. Add 5 μL of antibodies cocktail n°3 and FACS Buffer up to 100 μL for each sample. Incubate for 30 min at 4°C, in the dark.
46. Wash cells by adding 2 mL Annexin-V binding buffer 1×. Centrifuge cells (650 × g, 3 min, 4°C) and discard the supernatant.
47. Resuspend cell pellet in 100 μL of Annexin-V binding buffer 1× and add 1 μL of Annexin-V APC.
48. Acquire cells on a flow cytometer and analyze data using FlowJo software.
49. At 72H, repeat steps 42–48.

In vivo T cell receptor stimulation

© Timing: 7 days

Herein, we performed in vivo stimulation assays using OT-II mouse model. This model generates some CD4 T cells expressing OT-II TCR which recognizes chicken ovalbumin antigen (Barnden et al., 1998). The major steps are the following: at day 0, splenic T cells (from OT-II MYC-deficient or MYC-proficient mice) are harvested and engrafted in C57BL/6 recipient mice. After two days, T cells are stimulated through injection of ovalbumin (Day 2) and few (usually ranging from 3 to 5) days later, mice are euthanized for analysis (Day 7).

50. Resuspend CTV-labelled cells (from step 38 above) at 10 × 10⁶ cells/mL in PBS1× at RT and protect from light.
51. Sedate C57BL/6 recipient mice using isoflurane with a flow rate of 4 L/min for 3 min.

Alternatives: Mice sedation can be performed by an intraperitoneal injection of xylazine/ketamine anesthetic.

52. 100 μL of cells are i.v. injected (retro orbital injection) using an insulin syringe (or 1 mL syringe with 30 gauge needle).

Alternatives: Tail vein injection can be applied.

53. Place grafted mice under a heating lamp until they wake up, and then put them back in their cage.
54. Allow mice to recover (here we set 2 days for the recovery time).
55. After 2 days, anesthetize grafted mice as described in step 51.
56. Perform a subcutaneous injection in the ear of 100 μg of ovalbumin in the presence of complete Freund’s adjuvant (maximum volume of injection: 100 μL) (Figure 3).
57. Place injected mice under a heating lamp until they wake up.
58. 3–5 days post-immunization, euthanize grafted mice (as described in step 1) for analysis.
59. Collect the cervical lymph nodes, trimming away any non-specific tissue (like fat) and place the lymph nodes in PBS1×/2%FBS at RT.
60. Put a 70 μm cell strainer on a 50 mL conical tube.
61. Place lymph nodes on the cell strainer, which was previously moistened with PBS1×/2%FBS, and dilacerate with the piston of a syringe.
62. Adjust the volume to 10 mL with PBS1×2%FBS.
63. Centrifuge cells (450 x g, 7 min, RT). Discard supernatant.
64. Resuspend cell pellet with 5 mL of PBS1×2%FBS.
65. Count cells as described in steps 11 and 12.
66. Transfer 10^6 cells into a FACS tube.
67. Add 5 μL of antibodies cocktail n-4 and FACS Buffer up to 100 μL for each sample. Incubate for 30 min at 4°C, in the dark.
68. Add 2 mL PBS1× and centrifuge (650 x g, 3 min, 4°C). Discard supernatant.
69. Resuspend cell pellet in 100 μL of FACS buffer.
70. Acquire cells on a flow cytometer and analyze files using FlowJo software.

Alternatively: Our mouse models bred on C57BL/6 background harbor ROSA26-LSL-eYFP tracking system, thus we can easily distinguish cells of interest from host wild type C57BL/6 cells. As an alternative to ROSA26-LSL-eYFP, two distinct strains of C57BL/6 mice can be used to differentiate host cells from injected cells: typically CD45.1 or CD45.2 C57BL/6 mice strains.

EXPECTED OUTCOMES
This protocol aims to investigate T cell response upon TCR stimulation. With physiological T cells, ex vivo stimulation with anti-CD3/CD8 antibodies, induces cell surface expression of activation-marker genes, such as CD69, and also cell growth and a burst of proliferation. Those responses to TCR stimulation can be monitored by flow cytometry (Figure 4). Typically, cell proliferation is assessed using CFSE-based reagent like CTV (Figure 4D). Then, impact on T cell response can be investigated according to different factors. Herein we analyzed the impact of Myc inactivation and we found that MYC-deficient T cells do not proliferate despite CD69 expression (Figure 4). These ex vivo results can be further validated by in vivo assays in which T cells are stimulated with a specific antigen. We used ovalbumin to activate T cells that harbor OT-II TCR. As expected, physiological OT-II+ T cells proliferate upon ovalbumin injection whereas MYC-deficient OT-II cells do not expand.
Moreover, in vivo analysis allows the investigation of T helper (Th) cell polarization. Herein, we injected ovalbumin with complete Freund’s adjuvant, that favors Th1/Th17 polarization. However, alternative adjuvants such as Alum (aluminum hydroxide) support Th2 polarization (Vasilakos et al., 2000).

LIMITATIONS

In vivo assays require an animal facility, competent staff to take care of mouse models and to perform experimental procedures on mice. These assays also depend on the availability of TCR transgenic mice. Herein, we used OT-II mice that can be stimulated by ovalbumin, if the mice possess another type of transgenic TCR, the stimulation should be performed with the corresponding antigen.

We did not identify any limitation for ex vivo assays. By itself, these assays do not require any specific expertise and can be easily performed by beginners. Moreover, besides basic equipment needed
for molecular/cellular biology (such as centrifuge, microscope, flow cytometer), ex vivo assays do not necessitate any particular material.

**TROUBLESHOOTING**

**Problem 1**
CTV labelling is insufficient (at 24H, a mean fluorescence intensity below $10^4$ is considered as insufficient) (step 33).

**Potential solution**
Only use freshly diluted solution of CTV.

Increase the final concentration of CTV or test several CTV concentrations to obtain the optimal labeling of your cells.

**Problem 2**
Insufficient T cells enrichment (fall short of 85%) (step 30 b).

**Potential solution**
The problem may result from a counting error. Underestimating the cell count can impact the efficiency of purification. You may increase by 10% the recommended amount of antibody cocktail and beads.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact. Dominique Payet Bornet (payet@ciml.univ-mrs.fr).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
This study did not generate/analyze datasets and code.

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**AUTHOR CONTRIBUTIONS**
C.G., J.Q., M.N., M.L., D.P.B., and C.M. performed the experiments. C.G., C.M., and D.P.B. wrote the paper. All authors read and approved the final manuscript.
DECLARATION OF INTERESTS
The authors declare no competing interests.

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