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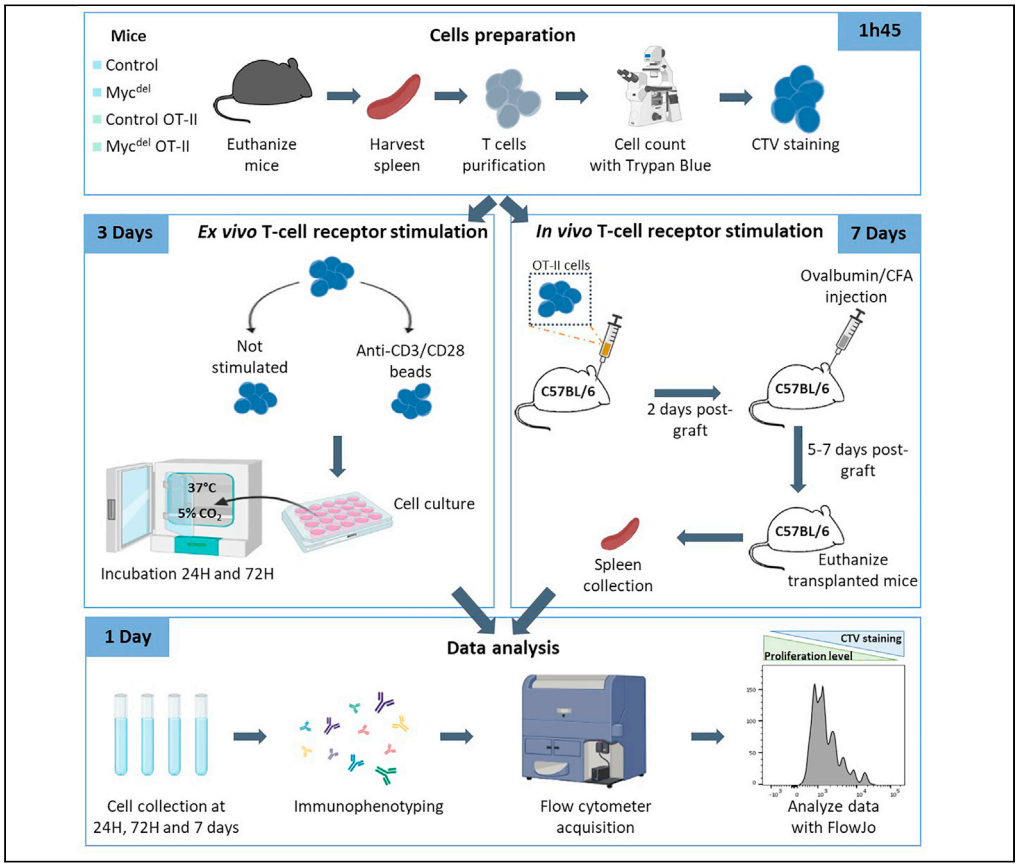
Protocol

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



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.

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Highlights

Isolation and enrichment of T cells from mouse spleen

Ex vivo T cell receptor stimulation with anti-CD3/CD28 antibodies

In vivo T cell receptor stimulation using ovalbumin-OT-II mouse model

Analysis of TCR-induced T cell proliferation

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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. $Myc^{flox/flox}$ 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 Vα2/Vβ5.1 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^{del}' corresponds to CD4-Cre X ROSA26-LSL-eYFP X $Myc^{flox/flox}$ mouse; and 4) 'Myc^{del} OT-II' corresponds to Myc^{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 Vβ5 PE (1:400)	BD Pharmingen	Cat#: 553190, RRID: AB_394698
TCR Vα2 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#: 21875-034
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
GlutaMax	Life Technologies	Cat#: 25030-024
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#: T8154
Ionomycin	Life technologies	Cat#: I24222
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		
Mycflox/flox mice	Andreas Trumpp (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
C57BL/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://www.bdbiosciences.com/
Other		
6-well plate	Falcon	Cat#: 353046
48-well plate	Falcon	Cat#: 353078
5 mL polystyrene tubes	Falcon	Cat#: 352054
15 mL tubes	Sarstedt	Cat#: 62.554.502
50 mL tubes	Sarstedt	Cat#: 62.547.254
70 μm cell strainer	Sarstedt	Cat#: 83.3945.070

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
1 mL syringe	Terumo	Cat#: SS+01H1
2.5 mL syringe	Terumo	Cat#: SS-02SE1
30G needle	Fisher Scientific	Cat#: 15391557
EasySep Magnet	StemCell	Cat#: 18000
Hemocytometer	KOVA International	Cat#: 87144F
Microscope	Nikon Eclipse	TS100
Centrifuge	Eppendorf	Cat#: G5810R
Microcentrifuge	Eppendorf	Cat#: G5415R
Flow cytometer	BD Biosciences	FACS Canto II
Isofluorane (Vetflurane)	Centravet	n/a
Xylazine (Rompun 2%)	Centravet	n/a
Ketamine (Imalgen 1000)	Centravet	n/a

MATERIALS AND EQUIPMENT

PBS1×

Reagent	Final concentration	Amount
Dulbecco's Phosphate Buffered Saline (DPBS) 10×	1 X	100 mL
dH_2O	n/a	Up to 1 L
Total	**n/a**	**1 L**

Store at 4°C for up to one month.

PBS1×/2%FBS

Reagent	Final concentration	Amount
Fetal Bovine Serum	2%	8 mL
PBS1×	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×	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
dH_2O	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.			

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: The amount of antibody mentioned in the above tables are for 5 samples containing each up to 1 × 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×/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×/2%FBS, and dilacerate the spleen with the piston of a syringe. (Add PBS1×/2%FBS to facilitate the dilaceration).
6. Adjust the volume to 20 mL for each tube with PBS1×/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.

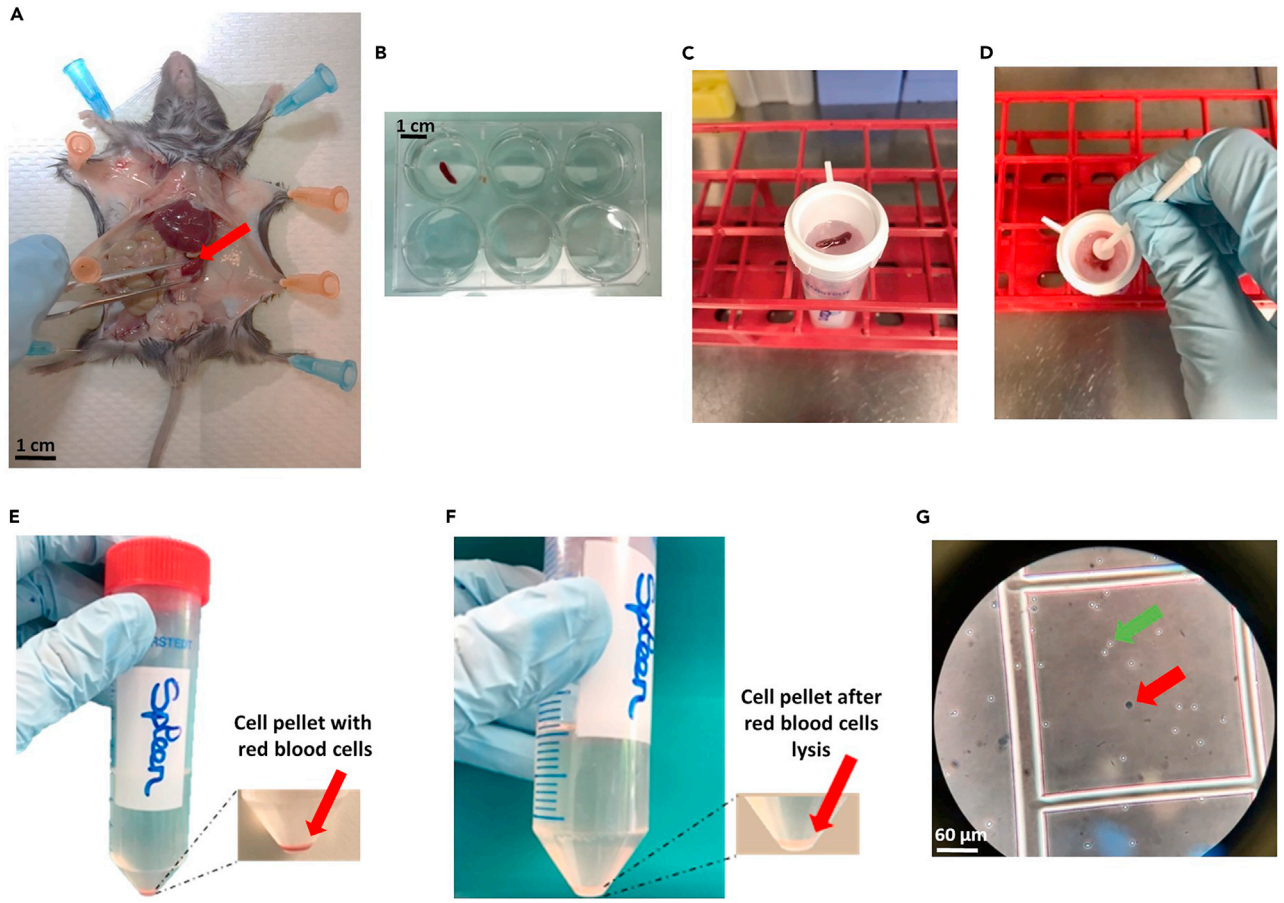

Figure 1. Spleen harvesting steps

(A) Euthanize mice according to institutional guidelines. Collect spleen using scissors and tweezers. The red arrow indicates the spleen.

(B) Place spleen in a 6-well plate containing few milliliters of PBS1×/2%FBS and keep at RT.

(C) Place spleen on a 70 µm cell strainer.

(D) Dilacerate the organ with the piston of a syringe.

(E) After centrifugation, red cell pellet indicates the presence of red blood cells.

(F) Following RBC lysis buffer, the cell pellet is depleted from red cells.

(G) Hemocytometer image of cells stained with trypan blue. The red arrow indicates a dead cell while the green arrow indicates a live cell. Cells were observed with a microscope (20× magnification). To calculate cell concentration we used this formula: [Number of cells in a small grid] × 9 × dilution factor × 10^4 = number of cells/mL.

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×/2%FBS and centrifuge (450 × *g*, 7 min, RT).
10. Remove supernatant and resuspend cell pellet with 10 mL of PBS1×/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×).
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 × *g* (7 min, RT).
14. Resuspend the pellet in PBS1×/2%FBS in order to be at 1 × 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 (StemCell™) which is designed to isolate T cells by negative selection and we follow the manufacturer's instructions 10000003744-PIS_01.pdf (stemcell.com). Below (steps 22–30) is the procedure to process 5 × 10^7 cells (from Myc^{del}, Myc^{del} OT-II or Control mice).

22. Transfer 5 × 10^7 cells into a FACS tube.
23. Add 25 µL of Normal Rat Serum.
24. Add 25 µL of EasySep™ Mouse T cell isolation cocktail.
25. Incubate 10 min at RT.
26. Vortex EasySep™ Streptavidin RapidSpheres™ 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 × 10^7 cells/mL in PBS1×.

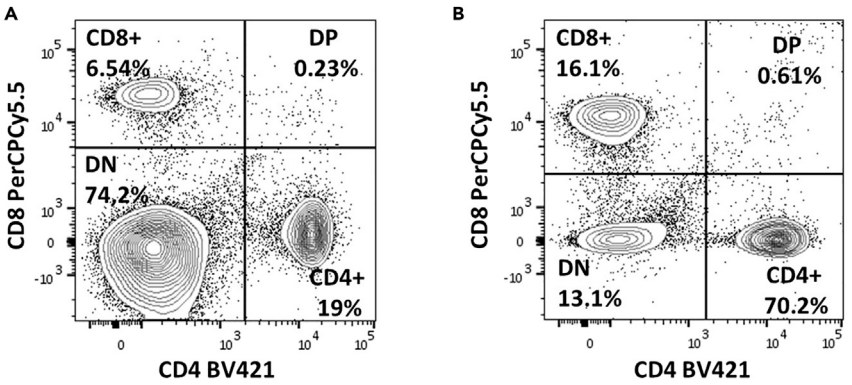

Figure 2. T cells enrichment

FACS plot showing the expression of CD4 and CD8. Percentages of cells in depicted gates are indicated.
(A) Before T cells enrichment.
(B) After T cells enrichment.

Note: 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.

33. Dilute CTV stock solution (5 mM) at 1:1000 with PBS1×and protect from light.
34. In a microcentrifuge tube, add 20 μL diluted CTV (5 μM) to 3 × 10^6 cells in 1 mL PBS1×.
35. Mix well by gentle pipetting.
36. Incubate at 37°C for 15 min (in the dark).
37. Wash cells twice with 500 μL of cold PBS1×. Spin cells (700 × *g*, 5 min, 4°C).
38. Resuspend cell pellet in complete RPMI (for *ex vivo* assays, step 39) or PBS1× (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.

39. Resuspend cells in complete RPMI at a cell concentration of 3 × 10^6 cells/mL
40. In a 48-well plate, dispatch 10^6 cells of each sample in 2 wells:
    a. Well #1: 'Not stimulated'.
    b. 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% CO_2 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 × 10^6 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.

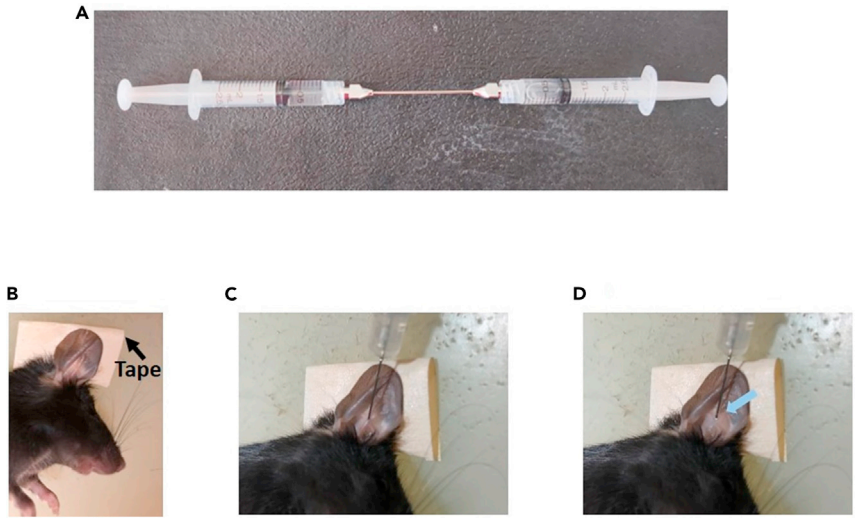

Figure 3. Ovalbumin/CFA injection

(A) Preparation of ovalbumin-CFA mixture. Two syringes containing ovalbumin and complete Freund's adjuvant are linked together by a connector. The two solutions are mixed by pressing several times the plunger of the two syringes.

(B) The ear of the sedated mouse is immobilized with a tape.

(C) Subcutaneous injection to the ear. Place the needle of the syringe subcutaneously.

(D) Inject ovalbumin-CFA mixture by pushing the plunger of the syringe. The blue arrow shows swelling due to correct injection.

62. Adjust the volume to 10 mL with PBS1×/2%FBS.
63. Centrifuge cells (450 × *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 × *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.

Alternatives: 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

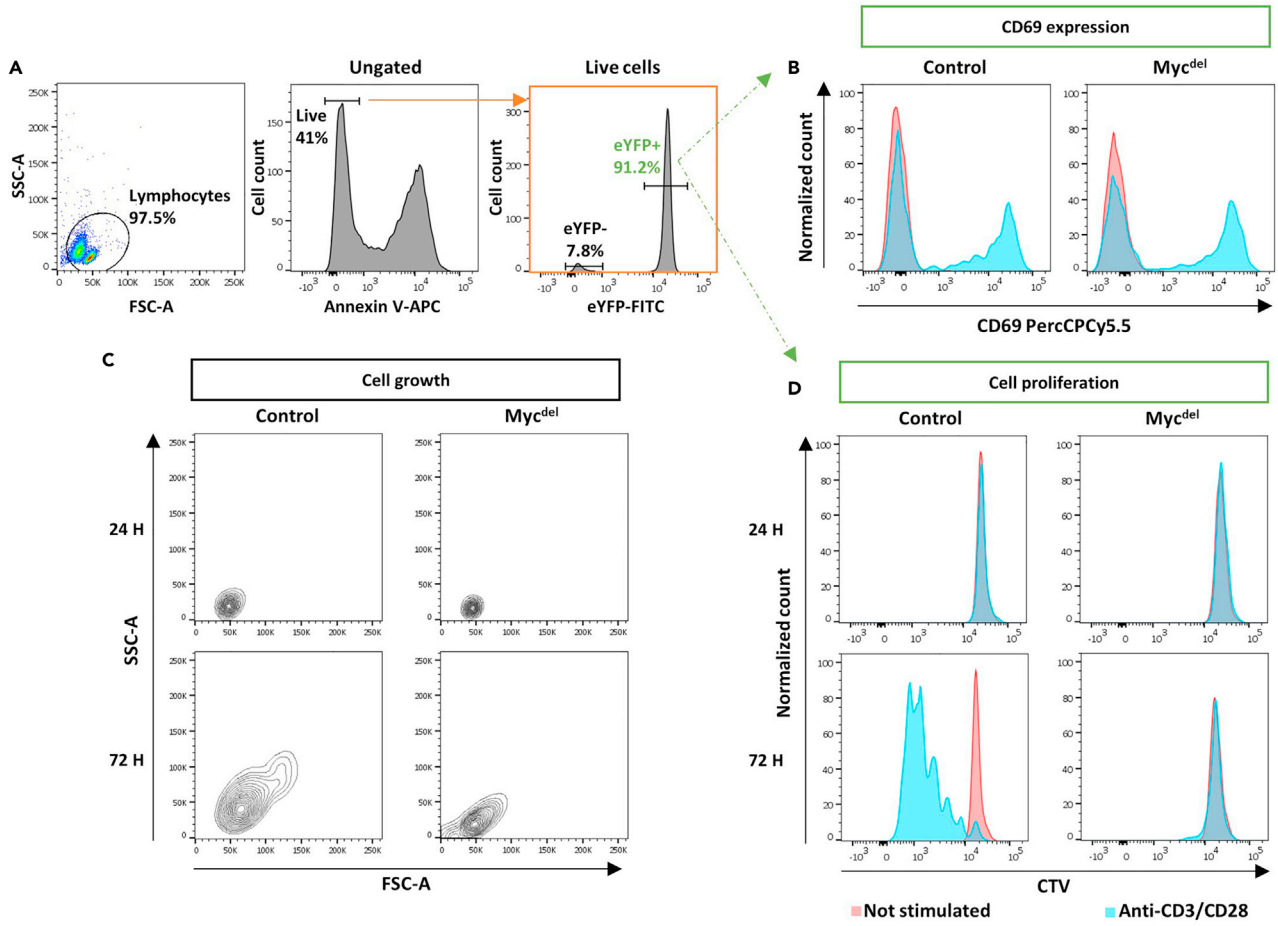

Figure 4. *Ex vivo* stimulation assays

(A) Gating strategy to select eYFP$^+$ cells population.

(B) Analysis of CD69 surface expression 24H post-stimulation was performed using eYFP$^+$ cells from Control and Myc^{del} mice.

(C) SSC/FSC analysis of eYFP$^+$ cells at 24H and 72H post-stimulation.

(D) Analysis of proliferation of eYFP$^+$ cell performed at 24H and 72H post-stimulation. Decrease of CTV staining indicates cell proliferation.

(B and D) Red histograms correspond to unstimulated cells and blue histograms to anti-CD3/CD28 stimulated cells.

(Figure 5). 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

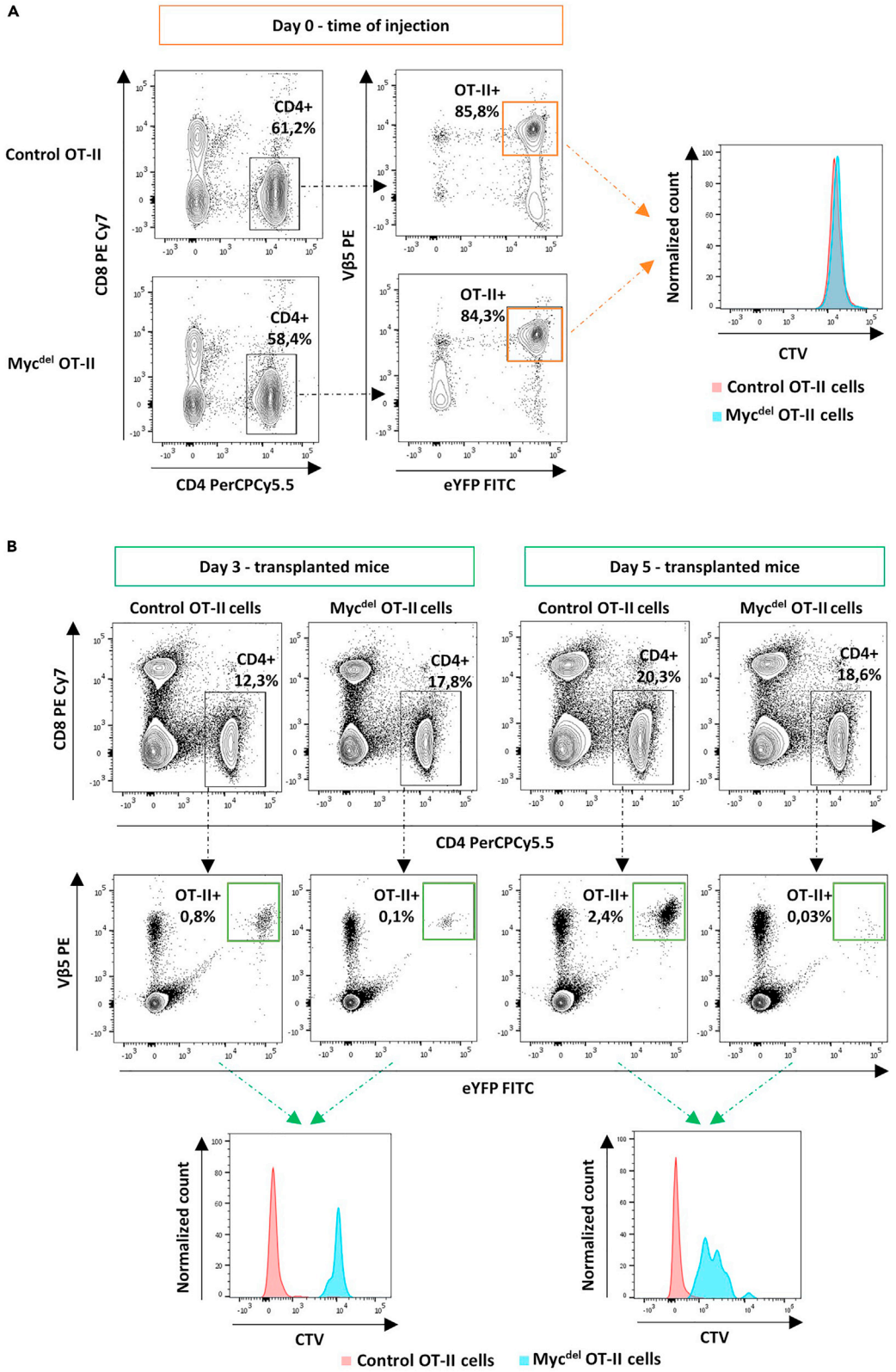
A
Day 0 - time of injection
Control OT-II
Myc^del OT-II
CD8 PE Cy7
CD4+
61,2%
CD4+
58,4%
CD4 PerCPCy5.5
Vβ5 PE
OT-II+
85,8%
OT-II+
84,3%
eYFP FITC
Normalized count
CTV
Control OT-II cells
Myc^del OT-II cells
B
Day 3 - transplanted mice
Day 5 - transplanted mice
Control OT-II cells
Myc^del OT-II cells
CD8 PE Cy7
CD4+
12,3%
CD4+
17,8%
CD4+
20,3%
CD4+
18,6%
CD4 PerCPCy5.5
Vβ5 PE
OT-II+
0,8%
OT-II+
0,1%
OT-II+
2,4%
OT-II+
0,03%
eYFP FITC
Normalized count
CTV
Control OT-II cells
Myc^del OT-II cells

Figure 5. *In vivo* stimulation assays

(A) T cells from Control OT-II (top panels) and Myc^{del} OT-II (bottom panels) spleens were purified and labeled with CTV. FACS showing CD4 and CD8 expression (left panels). $CD4^+$ gate was further analyzed for OT-II and eYFP expression (right panels). The histogram displays CTV staining of $OT\text{-}II^+eYFP^+$ cells from Control OT-II (red) and Myc^{del} OT-II (blue).

(B) FACS analysis of lymph nodes from mice transplanted with Control OT-II and Myc^{del} OT-II cells 3 and 5 days post-ovalbumin injection. Top panels: CD4 and CD8 expression. Bottom panels: OT-II and eYFP expression in $CD4^+$ cells. The histograms display CTV staining of $OT\text{-}II^+eYFP^+$ cells from Control OT-II (red) and Myc^{del} OT-II (blue).

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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