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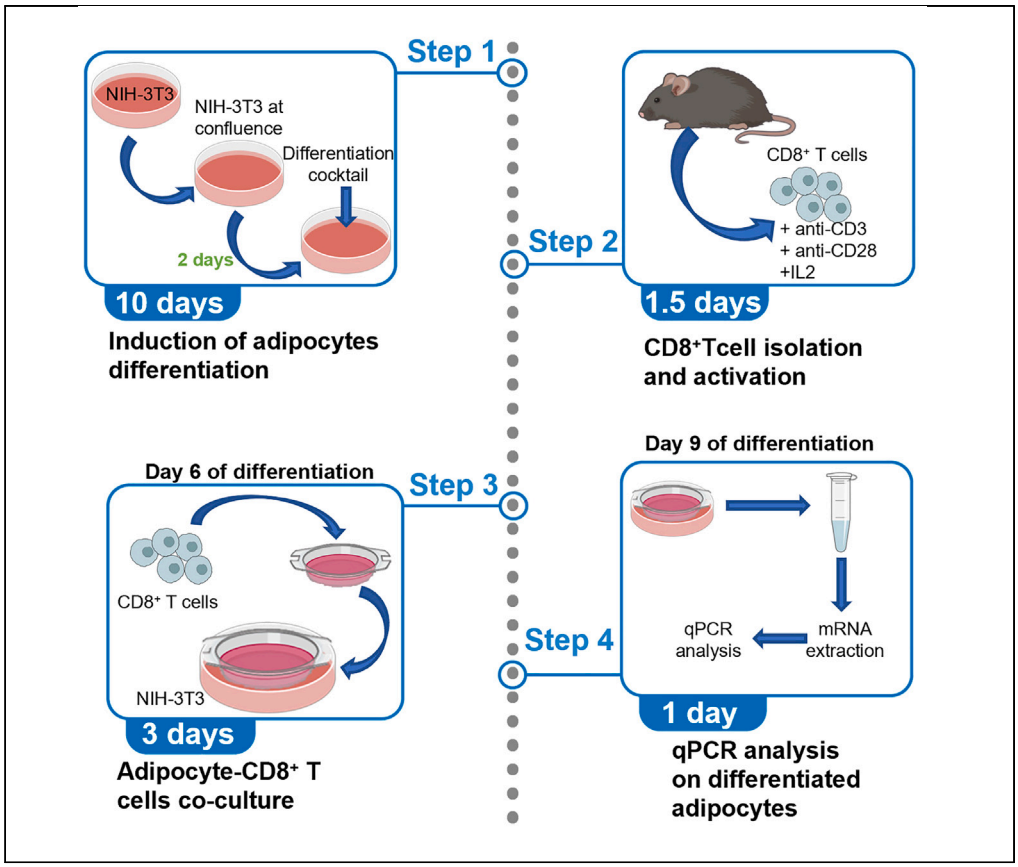
# Protocol to evaluate the impact of murine MCT1-deficient CD8$^+$ T cells on adipogenesis




The infiltration of activated T cells, such as CD8$^+$ effector, in metabolic tissues plays a crucial role for the initiation and propagation of obesity-induced inflammation. Given the pivotal role of lactate transporter monocarboxylate transporter 1 (MCT1) in immune cell activation, we present a protocol for the isolation and activation of CD8$^+$ T lymphocytes selectively lacking MCT1. We describe steps for the induction of adipocyte differentiation, CD8$^+$ T isolation and activation, and adipocyte-CD8$^+$ T cell co-culture. We then detail qPCR analysis on differentiated adipocytes.

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

Chiara Macchi, Annalisa Moregola, Giuseppe Danilo Norata, Massimiliano Ruscica

chiara.macchi@unimi.it (C.M.)
annalisa.moregola@unimi.it (A.M.)
massimiliano.ruscica@unimi.it (M.R.)

## Highlights

Protocol to co-culture murine NIH-3T3 adipocytes and CD8$^+$ T cells

Steps for isolation of CD8$^+$ T lymphocytes from lymph nodes and spleen of mice

Investigating the impact of MCT1 deficiency in CD8$^+$ T cells on adipogenesis

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# Protocol to evaluate the impact of murine MCT1-deficient CD8$^+$ T cells on adipogenesis

Chiara Macchi,[1,4,*] Annalisa Moregola,[1,4,*] Giuseppe Danilo Norata,[1,2] and Massimiliano Ruscica[1,3,5,*]

[1]Department of Pharmacological and Biomolecular Sciences "Rodolfo Paoletti," Università degli Studi di Milano, 20133 Milan, Italy

[2]SISA Center for the Study of Atherosclerosis, Bassini Hospital, Via M. Gorki 50, Cinisello Balsamo, 20092 Milan, Italy

[3]Department of Cardio-Thoracic-Vascular Diseases - Foundation IRCCS Cà Granda Ospedale Maggiore Policlinico, Milan, Italy

[4]Technical contact

[5]Lead contact

*Correspondence: chiara.macchi@unimi.it (C.M.), annalisa.moregola@unimi.it (A.M.), massimiliano.ruscica@unimi.it (M.R.)
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## SUMMARY

**The infiltration of activated T cells, such as CD8$^+$ effector, in metabolic tissues plays a crucial role for the initiation and propagation of obesity-induced inflammation. Given the pivotal role of lactate transporter monocarboxylate transporter 1 (MCT1) in immune cell activation, we present a protocol for the isolation and activation of CD8$^+$ T lymphocytes selectively lacking MCT1. We describe steps for the induction of adipocyte differentiation, CD8$^+$ T isolation and activation, and adipocyte-CD8$^+$ T cell co-culture. We then detail qPCR analysis on differentiated adipocytes. For complete details on the use and execution of this protocol, please refer to Macchi et al.[1]**

## BEFORE YOU BEGIN

The protocol below primarily focuses on an *in vitro* co-culture between CD8$^+$ T cells, specifically lacking monocarboxylate transporter 1 (MCT1), and murine adipocytes (NIH-3T3 cells).

1. Prepare all the buffers required for the protocol: RPMI-10, MACS buffer, complete DMEM for culturing NIH-3T3 pre-adipocytes and maintain them sterile at 4°C.
2. Generation of the animal model (http://www.informatics.jax.org/reference/J:328998).

Breed mice carrying floxed alleles of *Slc16a1* (termed *Slc16a1*$^{f/f}$) on the C57BL/6 genetic background with CD4$^{cre+}$ mice (kindly given by Prof. Marelli-Berg, Queen Mary University of London, UK) for specific deletion of *Slc16a1* in both CD4$^+$ and CD8$^+$ T lymphocytes.

3. Since the aim of this protocol is to assess late stages of adipogenesis, it is necessary that the pre-adipocytes are already in an advanced phase of the differentiation process when the lymphocytes are ready for the co-cultured.
   Differentiation of NIH-3T3 pre-adipocytes in mature adipocytes[2] (Figure 1A).
   a. Seed 6-well plates with NIH-3T3 preadipocytes at 100,000 cells per well. Use Dulbecco's Modified Eagle's Medium (DMEM) – high glucose supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin-Streptomycin (10,000 U/mL) (complete DMEM). Use 2 mL of medium for each well.
   b. Let cells to grow at 37°C with 5% of $CO_2$ until they reach confluence.
   c. Leave NIH-3T3 preadipocytes to grow for further 2 days (T0), after which the differentiation into mature adipocytes can be induced.

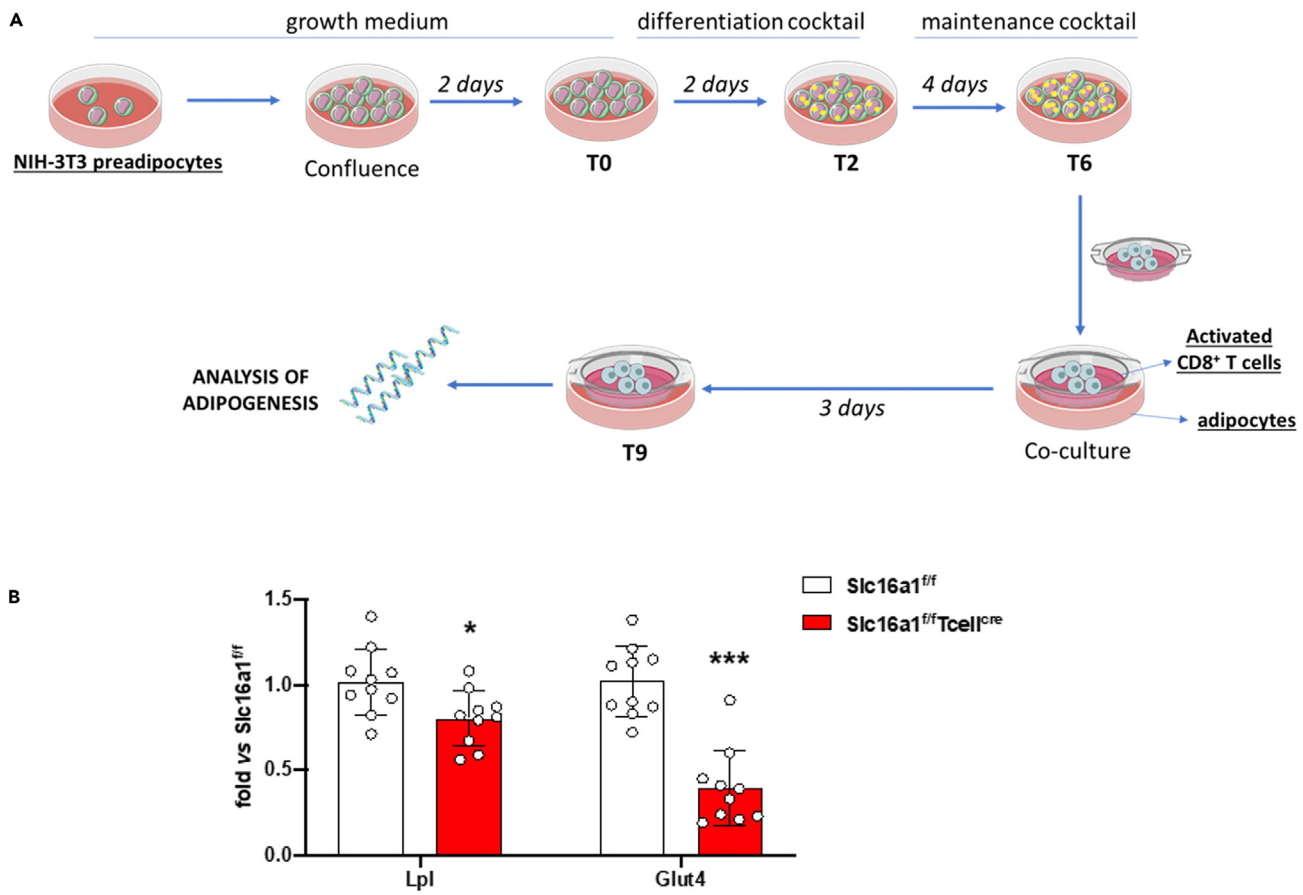


**Figure 1. Step-by-step description of adipogenesis analysis in adipocytes co-cultured with activated CD8⁺ T cells**

(A) Schematic representation of the co-culture timeline between activated CD8⁺ T cells and NIH-3T3 differentiated murine adipocytes. T0 represents the timepoint the differentiation of NIH-3T3 into mature adipocytes is induced.

(B) Example of results obtained by co-culturing activated CD8⁺ T lymphocytes with NIH-3T3 differentiated murine adipocytes. CD8⁺ T lymphocytes were isolated from Slc16a1$^{f/f}$Tcell$^{cre}$ mice selectively lacking MCT1 in CD8⁺T cells and their counterpart Slc16a1$^{f/f}$ mice. After activation, CD8⁺T cells were co-cultured with mature adipocytes. The gene expression of the adipogenic genes, Lpl and Glut 4 is reported.[1] n= 5 per group. Glut4, glucose transporter type 4; Lpl, lipoprotein lipase; Slc16a1, solute carrier family 16 member 1 (monocarboxylic transporter 1). *p< 0.05 and ***p< 0.001 (as assessed by Student's t-test).

d. Replace the cell growth medium with a differentiation cocktail composed of complete DMEM (high glucose) containing insulin (10 μg/mL), dexamethasone (1μM), 3-isobutyl-1- methylxanthine - IBMX - 0.5 mM) for further 2 days (T2).
   i. Prepare insulin by a stock solution of 5 mg/mL in HCl 0.1M; dexamethasone by a stock solution of 5mM in EtOH 100%; IBMX by a stock solution of 250 mM in KOH 0.35M.
e. Replace the differentiation medium with the differentiation-maintenance one, consisting of complete DMEM (high glucose) containing insulin (10 μg/mL).
   i. Let the cells grow in this medium until day 6 (T6).
f. At day 6 co-culture adipocytes with CD8⁺ T cells.

*Note:* This step (f) will last until day 9 (T9) which corresponds to the end of the differentiation protocol.

## Institutional permissions

All animal procedures were performed in accordance with the guidelines from directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by the Ethical Committee (Authorization 780/2016 to MR).

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Purified anti-mouse CD3 antibody | BioLegend | Cat: 102102; RRID: AB_312659 |
| Purified anti-mouse CD28 antibody | BioLegend | Cat: 102102; RRID: AB_312867 |
| Anti-mouse CD69 APC-Cy7 antibody | BD Pharmingen | Cat: 561240 RRID: AB_10611852 |
| Chemicals, peptides, and recombinant proteins | | |
| 3-isobutyl-1-methylxanthine (IBMX) | Merck (Sigma-Aldrich) | Cat: I5869 |
| Dexamethasone | Merck (Sigma-Aldrich) | Cat: D1756 |
| Insulin | Merck (Sigma-Aldrich) | Cat: I6634 |
| eBioscience™ 1X RBC Lysis Buffer | Thermo Fisher Scientific | Cat: 00-4333-57 |
| Human recombinant IL-2 | PeproTech | Cat: #GMP200-02 |
| Critical commercial assays | | |
| EasySep™ mouse CD8+ T cell isolation kit | STEMCELL | Cat: #19853 |
| RNeasy Mini Kit (50) | Qiagen | Cat: 74104 |
| Experimental models: Cell lines | | |
| Mouse: NIH-3T3-L1 | ATCC | CL-173 |
| Experimental models: Organisms/strains | | |
| Mouse: Slc16a1$^{flox/flox}$<br>For breeding;<br><br>- Age: 7 weeks<br>- Sex: male and female | Sonveaux Pierre | N/A |
| Mouse: CD4$^{cre+}$<br>For breeding;<br><br>- Age: 7 weeks<br>- Sex: male and female | Marelli-Berg Federica | N/A |
| Mouse: Slc16a1$^{flox/flox}$ selectively lacking MCT1 in CD8$^{+}$T cells<br>For CD8$^{+}$ T lymphocytes isolation:<br><br>- Age: 28 weeks<br>- Sex: male | N/A | N/A |
| Oligonucleotides | | |
| Primers: Cebpα<br>Fw: GAACAGCTGAGCCGTGAACT<br>Rev: TAGAGATCCAGCGACCCGAA | Metabion | https://wop.metabion.com |
| Primers: Cebpδ<br>Fw: GAACCCGCGGCCTTCTAC<br>Rev: GAAGAGTTCGTCGTGGCACA | Metabion | https://wop.metabion.com |
| Primers: Cidea<br>Fw: CACGCATTTCATGATCTTGG<br>Rev: CCTGTATAGGTCGAAGGTGA | Metabion | https://wop.metabion.com |
| Primers: Glut4<br>Fw: GCTCTGACGATGGGGAACC<br>Rev: GCCACGTTGCATTGTAGCTC | Metabion | https://wop.metabion.com |
| Primers: Lep<br>Fw: CAAGCAGTGCCTATCCAGA<br>Rev: AAGCCCAGGAATGAAGTCCA | Metabion | https://wop.metabion.com |
| Primers: Lpl<br>Fw: TCGGGCCCAGCAACATTATC<br>Rev: TGGTCAGACTTCCTGCTACG | Metabion | https://wop.metabion.com |
| Primers: Pparδ<br>Fw: TCTCCCAGAATTCCTCCCCT<br>Rev: GAGCTTCATGCGGATTGTCC | Metabion | https://wop.metabion.com |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| Primers: Pparγ<br>Fw: TGTGAGACCAACAGCCTGAC<br>Rev: AAGTTGGTGGGCCAGAATGG | Metabion | https://wop.metabion.com |
| Primers: Ppargc1α<br>Fw: CATTTGATGCACTGACAGATGGA<br>Rev: GTCAGGCATGGAGGAAGGAC | Metabion | https://wop.metabion.com |
| Primers: Rpl13a<br>Fw:GCGCCTCAAGTGGTGTTGGAT<br>Rev: GAGCAGCAGGGACCACCAT | Metabion | https://wop.metabion.com |
| Software and algorithms | | |
| GraphPad-Prism8 | GraphPad | https://www.graphpad.com/scientific-software/prism |
| Novoexpress | Agilent | https://www.agilent.com/en/product/research-flow-cytometry/flow-cytometry-software/novocyte-novoexpress-software-1320805 |
| Bio-Rad CFX Manager | Bio-Rad | https://www.bio-rad.com/it-it/sku/1845000-cfx-manager-software?ID=1845000 |
| Other | | |
| Counting chamber, Fast Read® 102 | VWR | Cat: 630-1893 |
| Costar® 6-well cell culture plates (Product number: 3516) | Merck (Sigma-Aldrich) | Cat: 3516 |
| Costar® 24 mm Transwell® with 0.4 μm Pore Polycarbonate Membrane Insert, Sterile | Corning | Cat: 3412 |
| Maxima First Strand cDNA Synthesis Kit for RT-qPCR | Thermo Fisher | Cat: K1642 |
| Maxima SYBR Green/Fluorescein qPCR Master Mix (2X) | Thermo Fisher | Cat: K0242 |
| EasySep Magnet | STEMCELL | Cat: #18000 |

## MATERIALS AND EQUIPMENT

PCR cycling conditions are showed in Figure 2.

*Alternatives:* For the real time PCR procedure, we use the instrument CFX Connect Real-Time System (Bio-Rad) although a different real-time PCR detection system can be used.

*Alternatives:* To determine $CD8^+$ T cells activation we use the Novocyte 3000 cytofluorimeter but any other cytofluorimeter can be used as long as it has the laser to read the fluorophore used for the staining. We use CD69 APC-Cy7 which requires the 640 nm laser for excitation and has an emission peak of 799 nm, but it is possible to change the antibody used with another one with proper characteristics readable by the instrument.

| **RPMI-10** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| RPMI 1640 | N/A | 429.5 mL |
| FBS (fetal bovine serum) | 10% | 50 mL |
| Sodium pyruvate (100mM) | 1 mM | 5 mL |
| HEPES (1M) | 10 mM | 5 mL |
| β-Mercaptoethanol (50mM) | 50 μM | 500 μL |
| Penicillin/Streptomycin | 1% | 5 mL |
| Glutamine (200mM) | 2 mM | 5 mL |
| **Total** | **N/A** | **500 mL** |
| Store at 4°C. Heat to 37°C before use. | | |

| MACS buffer: | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| PBS | N/A | 488 mL |
| FBS (fetal bovine serum) | 2% | 10 mL |
| EDTA (0.5M) | 2 mM | 2 mL |
| **Total** | **N/A** | **500 mL** |
| Store at 4°C for at maximum 30 days | | |

*Note:* Check before use if the buffer has floating particles. To overcome this issue, it is mandatory to filter the buffer with a sterile filter under the hood to be sure to use only sterile buffer.

| Complete DMEM for culturing NIH-3T3 pre-adipocytes: | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Dulbecco's Modified Eagle's Medium (DMEM) – high glucose | N/A | 440 mL |
| FBS (fetal bovine serum) | 10% | 50 mL |
| L-glutamine | 1% | 5 mL |
| Penicillin-Streptomycin (10,000 U/mL) | 1% | 5 mL |
| **Total** | **N/A** | **500 mL** |
| Store the medium at 4°C for at maximum 30 days. Heat to 37°C before use. | | |

## STEP-BY-STEP METHOD DETAILS

### $CD8^+$ T lymphocytes isolation from lymph nodes and spleen

Timing: 2.5 h

This section describes how to collect mice lymph nodes and spleen and how to process these tissues to obtain a uniform cell suspension for the isolation of $CD8^+$ T cells to be used for the co-culture with mouse adipocytes NIH-3T3 cells.

1. Collect the lymph nodes and spleen.
   a. Euthanize mouse with isoflurane (2%) or other approved methods.
   b. Open the abdomen of the mice by using surgical scissors and tweezers. It is important to lift the skin without opening the peritoneum.

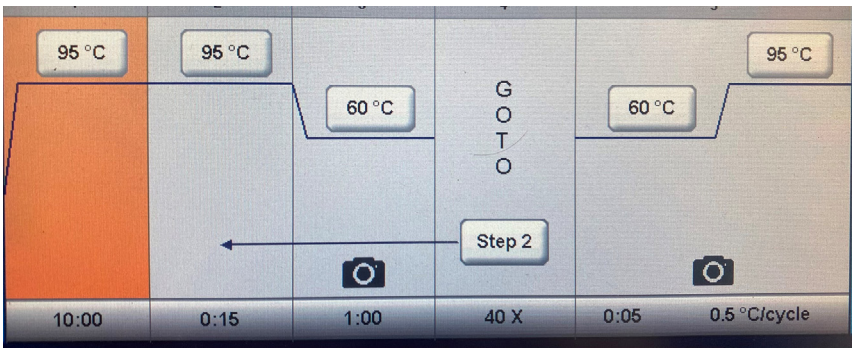


**Figure 2. PCR cycling conditions**
Initial denaturation (95°C for 10 min). Repeat 40 cycles of denaturation (95°C for 15 seconds) and annealing (60°C for 1 min).

i. Collect the two inguinal lymph nodes located in the subcutaneous adipose tissue of the inguinal left and right regions.
ii. Collect brachial and axillary lymph nodes located in the axillary left and right regions.
iii. Ensure to eliminate the surrounding adipose tissue.
iv. Place the organs collected in a 48-well plate containing 500μL of MACS buffer (PBS/2% FBS/2 μM EDTA) each well.[3]

c. Open the peritoneal cavity and collect the spleen which is located inside the rib cage on the left, above the stomach. Place the organ in the 48-well plate containing 500 μL of MACS buffer previously used also for lymph nodes.

2. Prepare a uniform cell suspension of primary lymphocytes from the lymph nodes and spleen.
   a. In a sterile biosafety cabinet, place a 70 μm cell strainer on top of a 50 mL tube.
   b. Mash the lymph nodes and spleen, through the strainer by using a 1mL syringe plunger and 10 mL MACS buffer.

   ***Note:*** the percentage of $CD8^+$ T cells obtained from the cell suspension (derived both from lymph nodes and spleen) is less than 10%.

   c. Centrifuge the cells at 500 *g* for 5 min, aspirate the supernatant with the vacuum and resuspend the pellet in 1 mL of eBioscience™ 1X RBC Lysis Buffer. RBC or red blood lysis buffer is used for the lysis of erythrocytes in single-cell suspension. Leave the suspension at 4°C for 5 min.
   d. Bring up the suspension to a volume of 10 mL by using MACS buffer. This step allows us to wash the cells. Centrifuge the suspension for 5 min at 500 *g*.

   ***Note:*** If there is a need to pool out lymph nodes and spleen collected from more than one mouse, then increase the volume of lysis buffer, and wash the cells with at least 3 times the volume of the lysis buffer used.

   e. Aspirate the supernatant and resuspend the cells in 10 mL of MACS buffer.
   f. Count the cells in the counting chamber by using a 1:20 dilution in Trypan blue.

   ***Note:*** Whether the number of cells is too high to be counted in the squares of the chamber, it is necessary to increase the dilution to limit count errors.

3. Isolate $CD8^+$ T lymphocytes with negative selection beads present in the EasySep™ mouse $CD8^+$ T cell isolation kit.
   a. Centrifuge cells at 500 *g* for 5 min.
   b. Resuspend the cells in MACS buffer in order to achieve a concentration of $1 \times 10^8$ cells per mL and then transfer samples in a 5 mL sterile polystyrene round bottom tube.

   ***Note:*** For the isolation protocol, we usually use the EasySep™ magnet with which the isolation can be performed with 2 mL as a maximum volume of cells. If you have more than $2 \times 10^8$, divide the sample for two isolations, in two different tubes.

   c. Add rat serum 50 μL/mL and 50 μL/mL of isolation cocktail supplied with the kit. This cocktail is done by biotinylated monoclonal antibodies against non-$CD8^+$ T cells dissolved in PBS and 0.1% BSA. Mix briefly and incubate 10 min at 22°C.
   d. Vortex the beads vial present in the kit for 10 s and add a concentration of 125 μL/mL to each sample.
   e. Vortex rapidly and leave the tubes for 5 min at 22°C.
   f. Fill in the tube with MACS buffer until reaching a volume of 2.5 mL.
   g. Place the tube into the magnet and incubate for 2.5 min.

h. Pour the enriched cells by inverting the magnet and collect the $CD8^+$ T cells in a new 15 mL tube.
i. Count the enriched cells with Trypan Blue with a 1:10 dilution.
j. Resuspend the cells in RPMI-10 medium in order to achieve a cell concentration of $2\times10^6$ cells/mL.
k. $CD8^+$ T cells are ready for cells activation.

*Note:* To assess $CD8^+$ T cell activation, save an aliquot of your cells ($2\times10^5$ cells are sufficient) to perform the cytometry staining the same day of the isolation (flow cytometry staining and evaluation of activation is described in the next paragraph 2 "Activation assessment").

### $CD8^+$ T lymphocyte activation

Timing: 25 h

This section explains in detail how to activate $CD8^+$ T lymphocytes *in vitro* and how to assess their activation through flow cytometry.

4. *In vitro* activation.
   a. Coat a 12-well plate with 500 µL per well anti-CD3 (0.5 µg/mL) and CD28 (2.5 µg/mL) diluted in PBS, for 1 h at 37°C.[4]

⚠ CRITICAL: The coating must be performed in PBS and not in MACS buffer because the presence of fetal bovine serum (FBS) can reduce the efficiency of anti-CD3 and anti-CD28 coating.

   b. Aspirate the coating and plate $2\times10^6$ of isolated $CD8^+$ T lymphocytes in each well ensuring to reach a final volume of 2.5 mL of complete RPMI medium (R10; RPMI plus 10% FBS, glutamine, HEPES, 2-ME, sodium pyruvate and antibiotics) preheated at 37°C.
   c. Add interleukin (IL)-2 at a concentration of 25 U/mL and leave the cells to be activated for 24 h at 37°C in the presence of 5% $CO_2$.
5. Harvesting of $CD8^+$ T cells.
   a. After 24h of incubation, collect $CD8^+$ T cells from the 12-well plate into a 15 mL tube. Use a 1 mL pipette.

*Note:* if you have many wells to collect, use a 50 mL tube.

   b. Add 1 mL of MACS buffer to the well and pipette again to be sure to collect all the cells.

*Note:* to ensure that all cells are collected before throwing away the plate, add 1 mL of PBS to the well and check at the microscope to see if the plate is free of cells. Otherwise pipette again and add the solution to the previous collected cells.

   c. Centrifuge the 15 mL tube containing the cells at 500 *g* for 5 min.
   d. Aspirate the supernatant and resuspend the pellet with MACS buffer to allow the cells to be counted. Use Trypan Blue, as described above.
   e. Centrifuge the 15 mL tube containing the cells at 500 *g* for 5 min.
   f. Resuspend the counted cells in R10 complete medium to achieve a $2\times10^6$ cells every 1.5 mL.
   g. $CD8^+$ T cells are now ready to be co-cultured with adipocytes.
6. Activation assessment: the staining with anti-CD69 APC-Cy7, an early activation marker, allows us to evaluate changes in cell physical parameters.

*Note:* If you want to evaluate the activation of the $CD8^+$ T cells, you must compare the cells before the induction of activation with the activated ones, and you need to perform the same staining for not activated cells the day of the isolation and 24 h post activation ($2\times10^5$ cells are sufficient for the staining).

a. Transfer $2\times10^5$ cells in a 96 well plate (V-bottom).
b. Centrifuge the plate at 800 *g* for 5 min.
c. Discard the supernatant and resuspend the pellet with 50 μL MACS buffer containing 0.5 μL of anti-CD69 antibody per well.
d. Incubate for 30 min at 4°C in the dark.
e. Add 150 μL of MACS buffer to each well to wash the cells.
f. Centrifuge 5 min at 800 *g*.
g. Repeat steps "e" and "f" once more.
h. Resuspend each well with 100 μL of MACS buffer and transfer cells to a 5 mL tube.
i. Bring each sample to a final volume of 250 μL with MACS buffer.
j. Acquire the samples with the cytofluorimeter: compare median of the forward scatter, the side scatter and CD69 fluorescence intensity between basal and activated cells (Figure 3).

*Note:* During the activation process, the dimension (cell size) and complexity (cell granularity) of $CD8^+$ T cells are raised. These features translate into a shift in the median fluorescence intensity read in the forward scatter (a physical parameter that refers to the cell size) and the side scatter (an index of cell granularity or complexity). The expression of cluster of differentiation (CD)69, a marker of early activation, increases rapidly after activation leading to a higher fluorescence that is detected in cytofluorimetric analysis if compared to a $CD8^+$ T cell not stimulated.

### Co-culture between activated $CD8^+$ T lymphocytes and murine NIH-3T3 cells

Timing: 3 days

This section describes how to co-culture differentiating NIH-3T3 cells with $CD8^+$ T lymphocytes to evaluate the impact of the MCT1 expression in $CD8^+$ T lymphocytes on late stages of adipogenesis.

7. Co-culture (Figure 1A).
   a. At day 6 (T6) of the differentiation protocol, put a transwell insert and add on the insert $2\times10^6$ activated $CD8^+$ T cells, in a 1.5 mL volume, in each well of the 6-well plate.
   b. Let the co-culture to growth for further three days, until the last day of the preadipocytes differentiation into mature adipocytes (T9).
   c. Remove the transwell inserts and harvest cells from each well of the 6-well plate

8. RNA isolation
   a. Extract RNA through a spin column isolation method.

   *Note:* To isolate RNA, we usually use the RNeasy Mini Kit (Qiagen), but any other kit that allows for a good yield of high-quality RNA can be used.

   b. Perform a reverse transcription to synthesize cDNA.

   *Note:* To obtain cDNA, we usually reverse transcribe 1 μg/μL of RNA with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo fisher).[5] Any other kit which ensures a good efficiency in cDNA synthesis can be used. If the RNA yield is low, reverse transcribe 0.5 μg/μL of RNA.

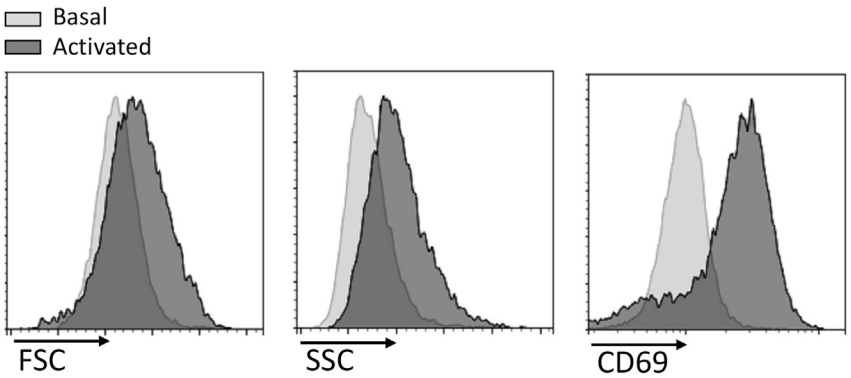


**Figure 3. Flow cytometry analysis of activated CD8$^+$ T cells**

The Left panel shows the comparison of forward scatter median fluorescence intensity between basal and activated cells.

The central panel comparison of forward scatter median fluorescence intensity between basal and activated cells. The right panel comparison of CD69 median fluorescence intensity between basal and activated cells.

c. Perform a qPCR analysis to evaluate the expression of key genes involved in the adipogenesis, e.g., Cebpα (CCAAT/enhancer binding protein), Cebpδ, Cidea (cell death inducing DFFA like effector a), Glut4 (glucose transporter type 4), Lep (leptin), Lpl (lipoprotein lipase), Ppar (peroxisome proliferator-activated receptor) δ, Pparγ, Ppargc1α (Pparg coactivator 1 alpha). RPL (ribosomal protein L) 13a can be used as a housekeeping.

*Note:* To perform a qPCR analysis, we usually use Maxima SYBR Green/Fluorescein qPCR Master Mix (2X) with a final concentration of primers of 300nM allowing a good efficiency (e.g, between 90% and 110% [6]). Any other kit ensuring a good DNA detection and analysis together with a high PCR specificity and sensitivity can be used. It is mandatory to test primer efficiencies before performing the experiments.

*Note:* the *in vitro* adipogenesis is composed by two phases, each of which is characterized by the activation of different genes. Therefore, the day of the differentiation protocol in which to start the co-culture should depends on which stage of adipogenesis needs to be analyzed. Co-culturing activated CD8$^+$ T cells at day 6 of the differentiation protocol allows to evaluate the possible effect on the second phase of adipogenesis, by studying the expression of genes such as GLUT4 and LPL (Figure 1).

## EXPECTED OUTCOMES

The outcome of the present protocol is to give an *in vitro* tool to evaluate the potential crosstalk between CD8$^+$ T cells, isolated from mice carrying a specific deletion of *Slc16a1* in CD8$^+$ T lymphocytes, and adipocytes. Indeed, the expression of cellular lactate transporter MCT1 (known as Slc16a1) increases during immune cell activation to cope with the metabolic reprogramming.[7] During an obesogenic diet, while adipose tissue expands via adipocyte hypertrophy or via the formation of new adipocytes through differentiation of resident precursors,[8] the accumulation of pro-inflammatory CD8$^+$ T cells in metabolic tissues seems to be crucial for the initiation of obesity-induced inflammation.[9] The evaluation of the effects of activated CD8$^+$ T cells on adipogenesis could thus simplify the *in vitro* study of the immune-adipose tissue axis in both health and pathological conditions, such as obesity. The expected outcome is a down-regulation of adipogenic genes driven by CD8$^+$ T lymphocytes selectively lacking MCT1.[1]

## LIMITATIONS

This protocol must be interpreted within a series of limitations. We activate CD8$^+$ T cells with anti-CD3 and anti-CD28 but this is not the only protocol that can be used. It is interesting to activate T

lymphocytes by using PMA and ionomycin. Since this method of activation is stronger, the timing of stimulation before co-culturing $CD8^+$ T cells with adipocytes has to be reduced.[10] Moreover, it is important to note that besides NIH-3T3 cells, other cellular models of preadipocytes could be used, such as C3H/10T1/2. In this case, the correct differentiation protocol must be used to obtain mature adipocytes at the end of the experiment.[11] Lastly, the entire protocol is set up on animal *in vivo* and *in vitro* models. If the effect of human $CD8^+$ T lymphocytes on human preadipocytes needs to be investigated, then several technical changes are required.

## TROUBLESHOOTING

### Problem 1

After lysis of erythrocytes with RBC lysis buffer the pellet after centrifugation is still red (step 2b).

### Potential solution

Repeat the step. Add again 1 mL of RBC lysis buffer and leave the suspension at 4°C for 5 min. Bring up the suspension to a volume of 10 mL with MACS buffer. Centrifuge the suspension for 5 min at 500 × *g* and resuspend the pellet with 10 mL of MACS buffer.

### Problem 2

Co-culturing NIH-3T3 cells at day 6 of the differentiation with $CD8^+$ T lymphocytes does not affect adipogenesis (step 7).

### Potential solution

It may be that the effect is not in the last stages of the process but in the first ones. To test whether this hypothesis is correct the co-culture could be started at day (T0) of the differentiation protocol. After 72h the RNA could be collected and the expression of adipogenic genes assessed. In this case an effect is expected on regulators of the early phase of adipogenesis, such as PPARγ.

## RESOURCE AVAILABILITY

### Lead contact

Massimiliano Ruscica massimiliano.ruscica@unimi.it.

### Materials availability

MCT1 mouse lines generated in this study have been deposited to https://www.informatics.jax.org/reference/J:328998.

### Data and code availability

For complete details on data and codes, please refer to Macchi et al.[1]

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## AUTHOR CONTRIBUTIONS

C.M. and A.M. performed most of the experiments and drafted the manuscript. M.R. and G.D.N. critically contributed to both the study design and the review of the manuscript for important intellectual input. M.R. and G.D.N. conceived and wrote the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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