bio-protocol

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Flow Cytometry Analysis of SIRT6 Expression in Peritoneal Macrophages

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Abstract

The sirtuin 6 has emerged as a regulator of acute and chronic immune responses. Recent findings show that SIRT6 is necessary for mounting an active inflammatory response in macrophages. In vitro studies revealed that SIRT6 is stabilized in the cytoplasm to promote tumor necrosis factor (TNFα) secretion. Notably, SIRT6 also promotes TNFα secretion by resident peritoneal macrophages upon lipopolysaccharide (LPS) stimulation in vivo. Although many studies have investigated SIRT6 function in the immune response through different genetic and pharmacological approaches, direct measurements of in vivo SIRT6 expression in immune cells by flow cytometry have not yet been performed. Here, we describe a step-by-step protocol for peritoneal fluid extraction, isolation, and preparation of peritoneal cavity cells, intracellular SIRT6 staining, and flow cytometry analysis to measure SIRT6 levels in mice peritoneal macrophages. By providing a robust method to quantify SIRT6 levels in different populations of macrophages, this method will contribute to deepening our understanding of the role of SIRT6 in immunity, as well as in other cellular processes regulated by SIRT6.

Keywords: SIRT6, Inflammation, Macrophages, Peritoneal cavity cells, Flow cytometry

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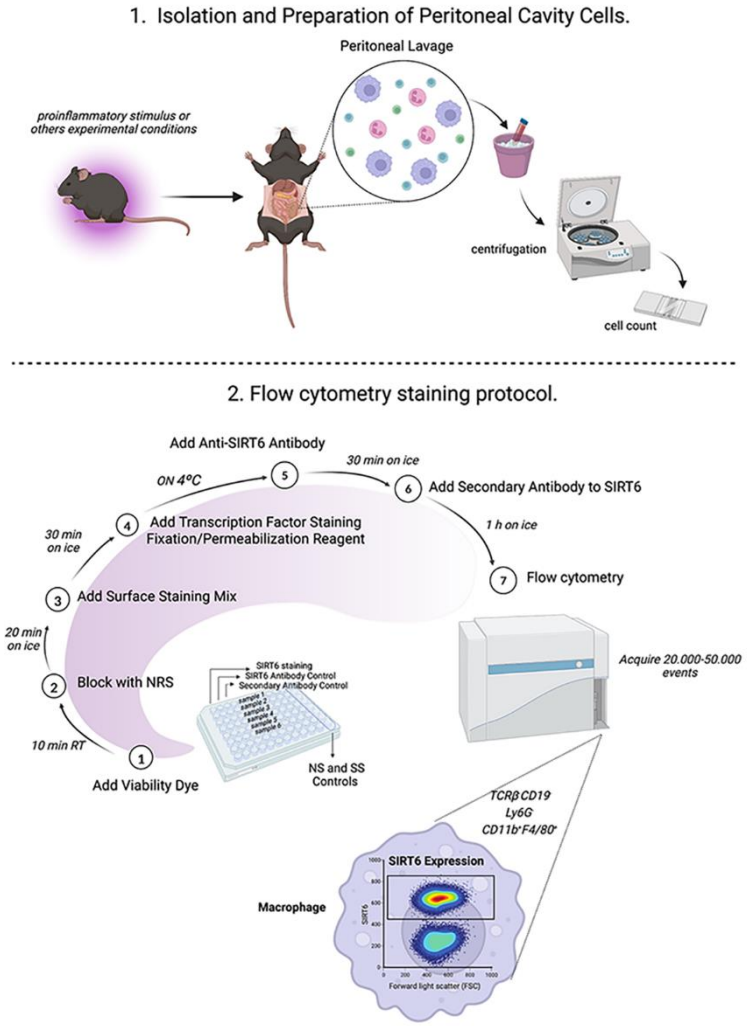
Graphical abstract:



Background

Sirtuins, NAD^+-dependent deacetylases, have been implicated in many biological processes, including inflammation. In the context of the inflammatory response, SIRT6 in particular has attracted attention due to its apparently coexisting and opposite pro- and anti-inflammatory activities (Kawahara et al., 2009; Xiao et al., 2012; Jiang et al., 2013; 2016). Initially, and similarly to other sirtuins, SIRT6 was proposed to inhibit inflammation by silencing NFκB and c-Jun–dependent transcription. Through its deacetylating enzymatic activity on Ac-H3K9, SIRT6 indirectly regulates cytokine expression (Kawahara et al., 2009; Bauer et al., 2012; Xiao et al., 2012). Recently, it has been reported that SIRT6 exerts pro-inflammatory functions through its ability to remove fatty acid groups from proteins in the cytoplasmic compartment. In particular, SIRT6 promotes tumor necrosis factor (TNFα) secretion by the demyristoylation of pro-TNFα in fibroblasts and macrophages during lipopolysaccharide(LPS)-mediated acute inflammatory response (Jiang et al., 2013; Jiang et al., 2016; Bresque et al., 2022). In addition, SIRT6 is actively regulated during acute inflammation in vivo, and its inhibition dampens TNFα secretion, reducing LPS-induced septic shock, obesity-induced systemic inflammation, and progression of experimental autoimmune encephalomyelitis (EAE) (Sociali et al., 2017; Ferrara et al., 2020; Bresque et al., 2022).

Most studies focusing on the role of SIRT6 in inflammation have been performed in vitro and have not measured changes in its localization and expression during the inflammatory response. Recently, studies regarding conditions for SIRT6 ablation have been developed to assess SIRT6 relevance in chronic inflammation and its consequences on immune cell populations in vivo (Lee et al., 2017; Ferrara et al., 2020). The peritoneal cavity provides a large number of differentiated macrophages and is a useful tool for studying in vivo immune responses. Recently, we demonstrated, by using flow cytometry and immunofluorescence, that there was an increase in both SIRT6-positive cells and SIRT6 fluorescence intensity in CD11b+F4/80^{hi} peritoneal macrophages stimulated by LPS. Our work is the first to apply flow cytometry to quantify SIRT6 levels in vivo in macrophages isolated from LPS-stimulated mice peritoneum (Bresque et al., 2022). Thus, this protocol sheds light on this issue, providing a new tool to study SIRT6 expression in immune and potentially other cells. This flow cytometry protocol, which employs a commercially available anti-SIRT6 antibody, offers a robust method to quantify the levels of an intracellular protein and may be applied to a wide variety of samples, including macrophages from adipose tissue.

Materials and Reagents

1. Male C57BL/6 mice (bred and maintained at Institut Pasteur Montevideo animal facility-UBAL)
2. Falcon® 50 mL conical centrifuge tubes (Corning, catalog number: 352070)
3. 1.5 mL Eppendorf microcentrifuge tubes (CNWTC, catalog number: TYA10)
4. 96-well plates, V-bottom (Deltalab, catalog number: 900012.1)
5. 1 mL syringes with 26 G needle (BD, catalog number: 303176)
6. 5 mL syringes with 24 G needle (BD, catalog number: 302187)
7. Ketamine (Pharmaservice, Ripoll Vet, Montevideo, Uruguay)
8. Xylazine (Unimedical, Montevideo, Uruguay)
9. 95% ethanol (Drogueria Industrial Uruguaya, catalog number: 12702)
10. RPMI (GIBCO, catalog number: 61870-010)
11. FBS (GIBCO, catalog number: 10437-028)
12. BSA (Capricorn, catalog number: BSA-1U)
13. EDTA (Fluka, catalog number: 03620)
14. PBS (Sigma, catalog number: D1408)
15. 0.4% trypan blue solution (GIBCO, catalog number: 15250061)
16. Normal rat serum (NRS) (provided by URBE - Facultad de Medicina - UdelaR)
17. eBioscience™ FoxP3/Transcription Factor Staining Buffer set (Invitrogen, catalog number: 00-5523-00)
18. LIVE/DEAD fixable Far-Red dead cell stain kit (Thermo Fisher Scientific, catalog number: L10120)
19. APC anti-CD11b antibody (Millipore, catalog number: MABF520)
20. APCCy7 anti-CD19 antibody (BioLegend, catalog number: 115530)
21. APCCy7 anti-TCRβ chain antibody (BioLegend, catalog number: 109220)
22. APCCy7 anti-Ly-6G antibody (BioLegend, catalog number: 127624)
23. Brilliant Violet™ 510 anti-CD11b antibody (BioLegend, catalog number: 101245)
24. PE anti-F4/80 antibody (Millipore, catalog number: MABF1530)
25. Anti-SIRT6 antibody (Abcam, catalog number: ab191385)
26. Alexa Fluor™ 488 goat anti-rabbit IgG antibody (Invitrogen, catalog number: A11034)
27. Ketamine/xylazine anesthesia (see Recipes)
28. 70% ethanol (see Recipes)
29. RPMI supplemented with 0.2% FBS (see Recipes)
30. FACS buffer (see Recipes)
31. 15% normal rat serum (15% NRS) (see Recipes)

Equipment

1. Surgical instruments (mayo and iris scissors straight and mosquito hemostatic and dissecting forceps)
2. Refrigerated centrifuge (Eppendorf, model: 5804R), Rotor A-2-DWP
3. Inverted microscope (Nikon, model: Eclipse TS100)
4. Hemacytometer (Bright-Line™, catalog number: Z359629)
5. Attune™ NxT flow cytometer (Invitrogen, catalog number: A24858)

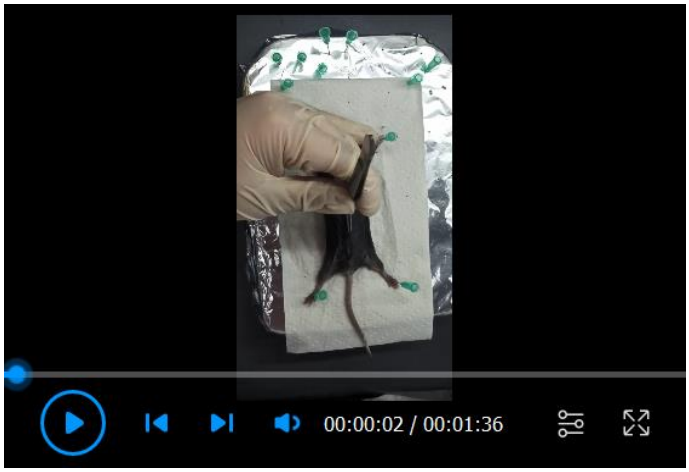
Software

1. Attune™ NxT Software (Invitrogen)
2. FlowJo (LLC, FlowJo_Vx.0.7)

Procedure

A. Isolation and preparation of peritoneal cavity cells (refer to explicative video to follow steps 5–10)

1. Anesthetize the mouse via subcutaneous injection of 500 µL of ketamine/xylazine (see Recipes) using a 1 mL syringe with a 26 G needle.
2. Euthanize the mice by cervical dislocation.
3. Place the mouse on its back on the Styrofoam block.
4. Spray the mouse with 70% ethanol (see Recipes). Using dissecting forceps and mayo scissors, grab and cut the external skin of the peritoneum.
5. Separate the skin until approximately 3 cm of the peritoneum is exposed.
6. Pull up the peritoneum skin with dissecting forceps and elevate it (see Video 1).
7. Inject 4 mL of RPMI supplemented with 0.2% FBS in the peritoneal cavity using a 5 mL syringe with a 24 G needle. Insert the needle gently in the area held with dissecting forceps.
8. After injection, clamp the area with hemostatic forceps and gently massage the peritoneum to release cells.
9. Using iris scissors, perform an incision in the peritoneum wall in the area above the clamp. Holding the hemostatic forceps, flip the mouse face down in a 50 mL Falcon tube. Alternatively, peritoneal lavage collection could be performed using a plastic Pasteur pipette.
10. Once there, open the clamp, release, and collect peritoneal fluid.
11. Centrifuge the peritoneal fluid collected at 500 × *g* for 10 min at 4 °C.
12. Discard the supernatant and resuspend cells in 1 mL of RPMI supplemented with 0.2% FBS.
13. Count viable cells using trypan blue (1:2 dilution) and a hemacytometer.



Video 1. Peritoneal Lavage.

Note: Intraperitoneal anesthesia is not recommended due to its inflammatory effect on peritoneal cells. It is important to anesthetize mice before cervical dislocation to facilitate precise manipulation and avoid blood infiltration in the peritoneal cavity. To prevent damage on peritoneal cavity organs and avoid collection of blood or other fluids with peritoneal fluid, it is important to be very careful when injecting, clamping, and cutting the peritoneum. The use of a 24 G needle for the peritoneal lavage is crucial. A bigger needle will damage the peritoneum and the organs in the cavity. At least 1 min of peritoneal mice massage is required to recover as many cells as possible from the peritoneal cavity. The technique used for the peritoneal lavage ensures the complete recovery of the volume injected in the peritoneal cavity and, therefore, maximum cell recovery. With this protocol, at least 5 × 10^6 cells per animal will be recovered. After animal sacrifice, all procedures should not exceed 5 min to maximize cell viability. After collection, it is important to maintain peritoneal cavity cells on ice. For optimal peritoneal cell counting, it is recommended to use a final 1:20 dilution. Dilute the cells 1:10 in RPMI supplemented with 0.2% FBS and then dilute 1:2 with trypan blue.

B. Flow cytometry staining protocol

1. Use three wells for each sample to be analyzed: one for SIRT6 staining and the others for SIRT6 antibody control and secondary antibody control (see detailed control information in Table 1). For Single Stain (SS) and No Stain (NS), mix the remaining cells from all samples and plate them. In all cases, plate 2 × 10^5 cells in each well.
2. Centrifuge the cells at 500 × *g* for 2 min.
3. Aspirate and discard supernatant with a multichannel pipette and wash with 100 µL of PBS. Alternatively, the supernatant could be decanted by inversion of the plate.
4. Centrifuge cells at 500 × *g* for 2 min.
5. Discard supernatant and resuspend all sample wells in 10 µL of fixable viability dye (see Table 2). For SS and NS wells, resuspend in 10 µL of PBS.
6. Incubate the plate 10 min at room temperature, protected from light.
7. Add 15 µL of 15% NRS (see Recipes) to each well (including SS and NS) and resuspend the cells.
8. Incubate 20 min on ice protected from light.
9. Add 25 µL of surface staining mix (see Table 2) to each sample well and resuspend cells. For SS wells, add the antibody selected for each fluorophore [e.g., for PE SS, use PE Anti-F4/80 (see Table 2 for each SS)]. For NS wells, add and resuspend in 25 µL of FACS buffer (see Recipes).
10. Incubate 30 min on ice protected from light.
11. Centrifuge cells at 500 × *g* for 2 min.
12. Discard supernatant and wash with 100 µL of FACS buffer.
13. Repeat steps 11 and 12.

14. Discard supernatant and resuspend all cells in 100 µL of transcription factor staining fixation/permeabilization reagent (prepared as described by manufacturer).
15. Incubate overnight at 4 °C protected from light.
16. Centrifuge cells at 500 × *g* for 2 min.
17. Discard supernatant and wash with 100 µL of transcription factor staining permeabilization buffer (prepared as described by manufacturer).
18. Repeat steps 16 and 17.
19. Discard supernatant and resuspend cells in 20 µL of anti-SIRT6 antibody to each SIRT6 staining and secondary antibody control wells. For SIRT6 antibody control, SS, and NS, resuspend in 20 µL of transcription factor staining permeabilization Buffer.
20. Incubate 30 min on ice protected from light.
21. Centrifuge cells at 500 × *g* for 2 min.
22. Discard supernatant and wash with 100 µL of transcription factor staining permeabilization buffer (prepared as described by manufacturer).
23. Centrifuge cells at 500 × *g* for 2 min.
24. Discard supernatant and resuspend in 20 µL of conjugated secondary antibody to SIRT6 staining wells and SIRT6 control wells of each sample. For secondary antibody control, SS, and NS, add 20 µL of transcription factor staining permeabilization buffer.
25. Incubate 1 h on ice protected from light.
26. Discard supernatant and wash with 100 µL of transcription factor staining permeabilization buffer.
27. Centrifuge cells at 500 × *g* for 2 min.
28. Repeat steps 26 and 27.
29. Discard supernatant and resuspend all wells in 100 µL of FACS buffer.
30. Transfer the content of each well to Eppendorf tubes.
31. Add up to 400 µL of additional FACS buffer to each tube.
32. Acquire between 2×10^4 and 5×10^4 events per sample in the flow cytometer.

Note: For SS and NS, one well per fluorophore used in staining (SS conditions) and one additional well for NS condition are necessary. The transfer of the content of each well to appropriate tubes is only necessary if using a flow cytometer without a plate handler. The additional FACS buffer volume needed in each tube depends on the flow cytometer used. For Attune™ NxT flow cytometer, 200 µL were added to each sample, and 400 µL were added to SS and NS conditions.

Table 1. Antibody controls description.
Summary of the used staining and utility of SIRT6 antibody and secondary antibody controls. If wanted, an isotype control for SIRT6 antibody can be added.

Antibody Control	Viability staining	Surface staining mix	Anti-SIRT6 antibody staining	Secondary antibody staining	Utility
SIRT6 antibody control	Yes	Yes	No	Yes	Identification of conjugated secondary antibody signals for non-specific binding targets.
Secondary antibody control	Yes	Yes	Yes	No	Identification of SIRT6 antibody noise signal without addition of conjugated secondary antibody. Visualization of possible spectral overlap.

Table 2. Reagent preparations for each staining step.
Detailed information to prepare all the reagents used. For each staining step, the table shows the antibody used, the initial dilution at which the antibody was prepared, the final dilution once added to the wells, and the diluent used. Note that for the surface staining mix there are two alternatives for the dump gate composition [1) or 2)], depending on the inflammatory conditions of the assay.

	Initial dilution (prepared)	Final dilution (added to wells)
Fixable viability dye staining		
- LIVE/DEAD Far-Red	1:500	1:500
Diluted in PBS 1×		
Surface staining		
Surface mix:		
- Brilliant Violet 510 anti-CD11b	1:150	1:300
- PE anti-F4/80	1:100	1:200
- APCCy7 Dump Gate:		
1) with inflammatory stimuli:		
APCCy7 anti-CD19	1:200	1:400
APCCy7 anti-TCRβ	1:200	1:400
APCCy7 anti-Ly6G	1:200	1:400
2) without inflammatory stimuli:		
APCCy7 anti-CD19	1:200	1:400
Diluted in FACS buffer		
Single Stain (SS):		
- APC anti-CD11b (for Far-Red SS)	1:100	1:200
- Brilliant Violet 510 anti-CD11b	1:150	1:300
- PE anti-F4/80	1:100	1:200
- APCCy7 anti-CD19	1:200	1:400
- FITC anti-CD11c (for Alexa Fluor 488 SS)	1:100	1:200
Diluted individually in FACS buffer		
Intracellular/intranuclear SIRT6 staining		
- Anti-SIRT6	1:200	1:200
- Conjugated secondary antibody (Alexa Fluor 488 goat anti-rabbit)	1:200	1:200
* Diluted individually in the transcription factor staining permeabilization buffer.		

Data analysis

1. Perform fluorophore compensation using SS acquisition data.
2. Apply compensation matrix to all samples.
3. Select population of interest by morphology (Figure 1A). In this step, exclude events corresponding to cellular debris from the gate.
4. Select single cells events (Figure 1B).
5. Select live cells (negative cells for viability dye staining) (Figure 1C). Cell viability should be around 80%–90%.

6. Select the negative population for the staining used for the dump gating (Figure 1D).
7. Select CD11b positive cells (Figure 1E).
8. Within CD11b positive cells, there are three populations defined by the expression of F4/80: negative (F4/80^{-}), low (F4/80^{lo}), and high (F4/80^{hi}) (Figure 1F). In our case, F4/80^{lo} and F4/80^{hi} populations were analyzed, since these are considered different macrophage populations (Bain et al., 2016).

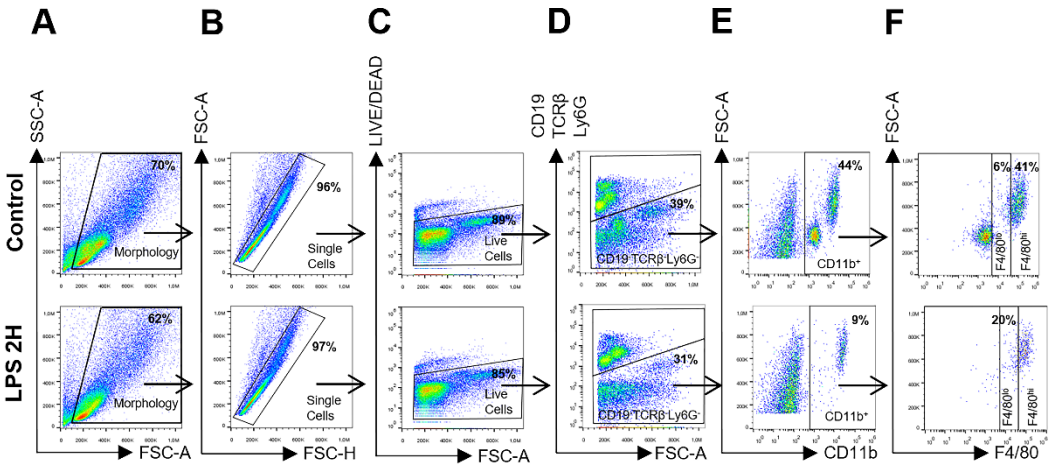


Figure 1. Gating strategy for definition of macrophage populations.
Figure shows the definition of the population of interest by morphology (A), selection of single cells (B) and live cells (C), exclusion of different lineages with dump gating (D), posterior selection of CD11b positive cells (E), and identification of two macrophage populations (F4/80^{lo} and F4/80^{hi}) within these cells (F). SIRT6 expression was analyzed in these two populations. Arrows indicate the population selected for the next step of the gating strategy. The percentage of the selected population is shown in each graph.
Image was extracted and modified from the original research article (Bresque et al., 2022). In this experiment, mice were injected with LPS or PBS (control), and SIRT6 expression in the peritoneal cells was assessed using the protocol described previously.

9. Select the population of macrophages of interest (CD11b^{+}F4/80^{lo} and CD11b^{+}F4/80^{hi}) in control samples. Define SIRT6 positive population as the gate above all cells observed in SIRT6 antibody and secondary antibody controls; this gate must be the same in both controls. For each control, you may allow up to 1% of cells inside the SIRT6 positive cells gate (Figure 2A). Repeat this step individually for all samples, since the fluorescence of the controls may vary between samples and/or conditions.
10. Apply the SIRT6 positive gate defined in SIRT6 stained replicates for each sample (Figure 2B).
11. Repeat steps 9 and 10 for all the populations of macrophages analyzed.
12. For data analysis, use a percentage of SIRT6 positive cells and SIRT6 geometric mean fluorescence intensity (GMFI) within SIRT6 positive cells. Use GMFI as a measurement of SIRT6 expression levels within this population.
13. Statistical analysis is made as described in the original research article (Bresque et al., 2022).

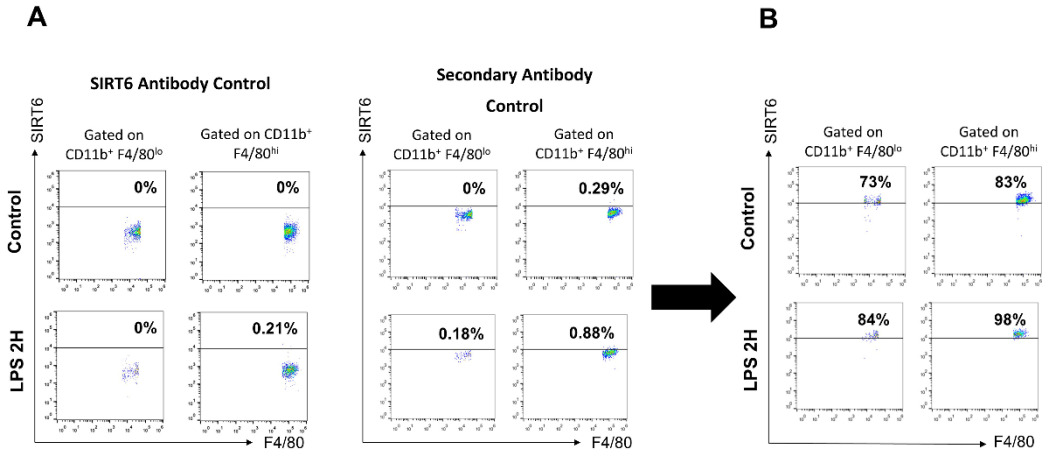


Figure 2. Gating strategy for definition of SIRT6 positive cells.
(A) Definition of SIRT6 positive cells using antibody controls (SIRT6 antibody and secondary antibody) in macrophage populations (F4/80^{lo} and F4/80^{hi}). SIRT6 positive gate is above all events in both antibody controls; up to 1% of events in this gate are allowed for antibody controls. (B) SIRT6 positive gate applied to SIRT6 stained samples (application of gate represented by black arrow). The percentage of SIRT6 positive cells is shown inside the SIRT6 positive gate. Image was extracted and modified from the original research article (Bresque et al., 2022). In this experiment, mice were treated with LPS or PBS (control), and SIRT6 expression was assessed using the protocol described previously.

Recipes

1. **Ketamine/xylazine anesthesia (calculated based on 30 g mice)**
   Ketamine (50 mg/mL), 200 mg/kg, 120 µL
   Xylazine (20 mg/mL), 24 mg/kg, 40 µL
   ddH_2O, 340 µL
   Total: 500 µL

2. **70% ethanol**
   95% ethanol, 737 mL
   ddH_2O, 263 mL
   Total: 1,000 mL

3. **RPMI supplemented with 0.2% FBS**
   FBS 0.2%, 2 mL
   RPMI, 998 mL
   Total: 1,000 mL

4. **FACS buffer**
   BSA 0.1%, 1 g
   2 mM EDTA, 20 mL (prepared for 100 mM EDTA)
   ddH_2O, 980 mL
   Total: 1,000 mL

5. **15% normal rat serum (15% NRS)**
   Normal rat serum (NRS), 2.25 mL

FACS buffer, 12.75 mL
Total: 15 mL

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Competing interests

CE and MB declare on behalf of all the authors that there are no conflicts of interest related to this manuscript.

Ethics

All mice used in this study (male C57BL/6) were bred and maintained at the Institut Pasteur de Montevideo Animal Facility Unit (UBAL). The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Institut Pasteur de Montevideo (CEUA, protocol numbers 70153-000839-17, 003-19, and 006-19). All the studies described were performed according to the methods approved in the protocol and following all international guidelines and legal regulations.

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