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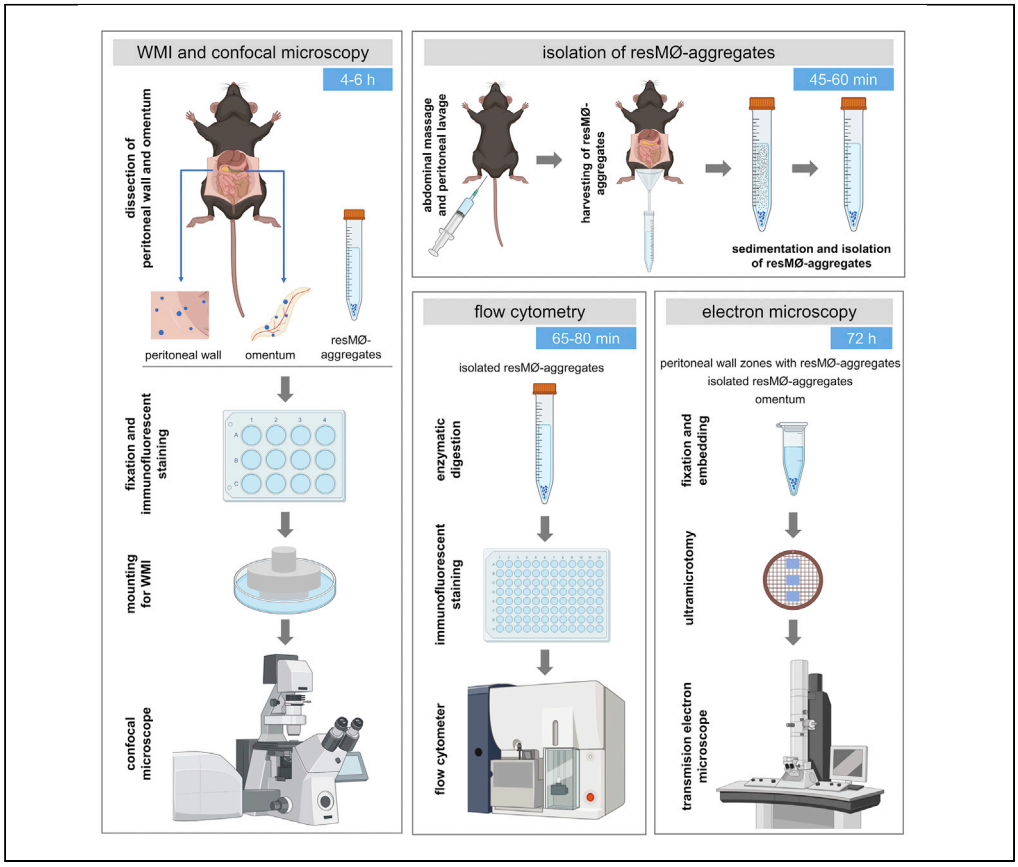
Protocol

Whole-mount immunofluorescence imaging and isolation of mesothelium-bound immune cell aggregates during mouse peritoneal inflammation



Resident peritoneal macrophages (resMØs) are crucial for repairing peritoneal injuries and controlling infections by forming mesothelium-bound resMØ-aggregates in the peritoneal wall and omentum. Here we present a protocol to analyze these structures in mouse models of peritoneal inflammation. We describe the dissection, fixation, immunofluorescent staining, and mounting of whole peritoneal wall and omentum samples and subsequent confocal microscopy imaging of resMØ-aggregates. We also detail the steps to isolate resMØ-aggregates for additional studies, including flow cytometry and electron-microscopy-based analysis.

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

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Highlights

Imaging of mouse peritoneal wall and omentum by whole-mount confocal microscopy

Isolation of mesothelium-bound resMØ-aggregates formed during peritoneal inflammation

Processing of resMØ-aggregates for flow cytometry and electron microscopy

Can be adapted to imaging of the thoracic cavity

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Whole-mount immunofluorescence imaging and isolation of mesothelium-bound immune cell aggregates during mouse peritoneal inflammation

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SUMMARY

Resident peritoneal macrophages (resMØs) are crucial for repairing peritoneal injuries and controlling infections by forming mesothelium-bound resMØ-aggregates in the peritoneal wall and omentum. Here we present a protocol to analyze these structures in mouse models of peritoneal inflammation. We describe the dissection, fixation, immunofluorescent staining, and mounting of whole peritoneal wall and omentum samples and subsequent confocal microscopy imaging of resMØ-aggregates. We also detail the steps to isolate resMØ-aggregates for additional studies, including flow cytometry and electron-microscopy-based analysis.

For complete details on the use and execution of this protocol, please refer to Vega-Pérez et al. (2021).[1]

BEFORE YOU BEGIN

The protocol below describes the specific steps for imaging mesothelium-bound immune cell aggregates, hereafter resMØ-aggregates (for resident peritoneal macrophage aggregates), formed in the peritoneal wall or omentum during peritoneal inflammation[1–4] by whole mount immunofluorescence (WMI), i.e., in whole peritoneal walls and omenta, combined with confocal microscopy, and to isolate these aggregates to perform additional analyses. Examples of expected outcomes included in the present protocol refer to the bacterial infection model described by our group,[1] based on the intraperitoneal injection of the *Escherichia coli* strain M6L4. This protocol can also be used for imaging the surface of other peritoneal organs, such as the mesentery, spleen, gut or ovary, as well as the inner wall of the thoracic cavity.

Institutional permissions

All the experiments were approved by the Animal Care and Use Committee of the Centro Nacional de Biotecnología-CSIC, Madrid. Ethical approvals from the relevant institutions are required prior to starting this procedure.

Establishing a mouse model of peritoneal inflammation

The experimental model used to analyze the innate immune system response to peritoneal inflammation relied on a single intraperitoneal inoculum of the *E. coli* M6L4 strain in C57BL/6

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mice, at a 1 × 10^7 cfu sublethal dose, in 0.2 mL of PBS. Analyses were performed during the early phase of the response, i.e., at 0–18 h post infection.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
*Anti-CD11b, PE-Cy7-conjugate, clone M1/70 (1:3000)	eBioscience	Cat # 25-0112-81 RRID: AB_469587
*Anti-CD11c, APC-Cy7-conjugate, clone HL3 (1:100)	BD Biosciences	Cat # 561241 RRID: AB_10611727
*Anti-CD16/CD32, purified, clone 2.4G2 (1:100)	BD Biosciences	Cat # 553142 RRID: AB_394657
*Anti-CD19, PE-conjugate, clone 1D3 (1:400)	BD Biosciences	Cat # 553786 RRID: AB_395050
*Anti-CD45, FITC-conjugate, clone 30-F11 (1:200)	BioLegend	Cat # 103108 RRID: AB_312973
*Anti-CD45, Pacific Blue-conjugate, clone 30-F11 (1:800)	BioLegend	Cat # 103126 RRID: AB_493535
*Anti-CD45R/B220, Pacific Blue-conjugate, clone RA3-6B2 (1:100)	BioLegend	Cat # 103227 RRID: AB_492876
*Anti-CD90.2, PE-conjugate, clone 53-2.1 (1:500)	BD Biosciences	Cat # 553006 RRID: AB_394545
*Anti-F4/80, APC-Cy7-conjugate, clone BM8 (1:100)	BioLegend	Cat # 123118 RRID: AB_893477
*Anti-F4/80, FITC-conjugate, clone BM8 (1:100)	eBioscience	Cat # 11-4801-82 RRID: AB_2637191
*Anti-MHC II (I-A/I-E), APC-conjugate, clone M5/114.15.2 (1:1000)	eBioscience	Cat # 17-5321-81 RRID: AB_469454
*Anti-I-A/I-E, FITC-conjugate, clone 2G9 (1:500)	BD Biosciences	Cat # 553623 RRID: AB_394958
*Anti-ICAM-1, FITC-conjugate, clone 3E2 (1:400)	BD Biosciences	Cat # 553252 RRID: AB_394734
*Anti-Ly6C, PerCP-Cy5.5-conjugate, clone AL-21 (1:100)	BD Biosciences	Cat # 560525 RRID: AB_1727558
*Anti-Ly6G, PE-conjugate, clone 1A8 (1:200)	BD Biosciences	Cat # 551461 RRID: AB_394208
*Anti-Tim4, BV421-conjugate, clone 21H12 (1:1000)	BD Biosciences	Cat # 742773 RRID: AB_2741037
**Anti-CD19, Alexa Fluor 647-conjugate, clone 6D5 (1:500)	BioLegend	Cat # 115522 RRID: AB_389329
**Anti-F4/80, Alexa Fluor 488-conjugate, clone BM8 (1:500)	BioLegend	Cat # 123120 RRID: AB_893479
**Anti-F4/80, Alexa Fluor 594-conjugate, clone BM8 (1:500)	BioLegend	Cat # 123140 RRID: AB_2563241
**Anti-F4/80, Alexa Fluor 647-conjugate, clone BM8 (1:1000)	BioLegend	Cat # 123122 RRID: AB_893480
**Anti-Fibrinogen, rabbit polyclonal (1:1000)	Agilent Dako	Cat# A008002, RRID: AB_578481
**Anti-Ly6G, Alexa Fluor 647-conjugate, clone 1A8 (1:500)	BioLegend	Cat # 127609, RRID: AB_1134162
**Anti-Podoplanin, Alexa Fluor 488-conjugate, clone 8.1.1 (1:1000)	BioLegend	Cat # 127406 RRID: AB_2161930
**Anti-Tim4, Alexa Fluor 647-conjugate, clone 21H12 (1:500)	BD Biosciences	Cat # 564177 RRID: AB_2647750
**Anti-ZO-1, Alexa Fluor 594-conjugate, clone ZO1-1A12 (1:500)	Invitrogen	Cat # 339194 RRID: AB_2532188
**Goat anti-rabbit Ig, Alexa Fluor 546-conjugate (1:1000)	Thermo Fisher	Cat # A11035 RRID: AB_143051
**Goat-anti-rabbit Ig, Alexa Fluor 647-conjugate (1:1000)	Thermo Fisher	Cat # A21245 RRID: AB_2535813

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli M6L4 strain	Dr. G. Núñez (University of Michigan Medical School) Zeng et al.[5]	N/A
Chemicals, peptides, and recombinant proteins		
1,4-Diazabicyclo[2.2.2]octane	Sigma-Aldrich	Cat # D27802
Acetone	VWR	Cat # 83683.230
BDMA (benzyl dimethylamine)	TAAB	Cat # B008
Bovine serum albumin	Sigma-Aldrich	Cat # A7030
Bovine serum albumin - low fatty acid	Sigma-Aldrich	Cat # A8806
DDSA (dodecenyl succinic anhydride) EM grade	TAAB	Cat # D027
Dulbecco's Modified Eagle's Medium (DMEM)-low glucose	Sigma-Aldrich	Cat # D6046
EDTA	Merck	Cat # 108418
Epon 812	TAAB	Cat # T026
DAPI	Sigma-Aldrich	Cat # 28718-90-3
DNase I	Roche	Cat # 10104159001
FBS (fetal bovine serum)	Sigma-Aldrich	Cat # F7524
Glutaraldehyde 25%	TAAB	Cat # G002
Glycerol 85%	Sigma-Aldrich	Cat # 1040941000
HEPES	Merck	Cat # H3375
HEPES buffer 1 M	Biowest	Cat # LO180-500
Liberase TL	Roche	Cat # 05401020001
MNA (methyl nadic anhydride)	TAAB	Cat # M012
Mowiol 4-88	Merck	Cat # 91381
NaH_2PO_4	Merck	Cat # 106346
Na_2HPO_4	Merck	Cat # 106586
NaCl	Merck	Cat # 116224
Osmium tetroxide 2%	TAAB	Cat # O018/1
Paraformaldehyde 4% aqueous solution, EM grade	Electron Microscopy Sciences	Cat # 157-4
Paraformaldehyde 16% aqueous solution, EM grade	Electron Microscopy Sciences	Cat # 15700
Sodium cacodylate trihydrate	Sigma-Aldrich	Cat # C0250
Toluidine blue	Sigma-Aldrich	Cat # T-3260
Tris base	Roche	Cat # 03573826001
Triton X-100	Sigma-Aldrich	Cat # X100
Uranyl acetate	Electron Microscopy Sciences	Cat # 22400
Critical commercial assays		
BD Cytofix/cytoperm kit	BD Biosciences	Cat # 554714
eBioscience Foxp3/Transcription Factor Staining Buffer Set	Thermo Fisher	Cat # 00-5523-00
FITC Annexin V Apoptosis Detection Kit with 7-AAD	BioLegend	Cat # 640922
Experimental models: Organisms/strains		
Mouse: C57BL/6J (8–10-week-old females)	Charles River	Strain code: 632
Software and algorithms		
Fiji	ImageJ Schindelin et al.[6]	https://imagej.net/Fiji
LAS X	Leica	Htttps://www.leica-microsystems.com/products/microscope-software
FlowJo X	FlowJo	https://www.flowjo.com/solutions/flowjo
Prism 8	GraphPad Software	http://www.graphpad.com/scientific-software/prism/

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
1.5 mL eppendorf tubes	Eppendorf	Cat # 0030121023
15 mL Falcon conical polypropylene tubes	Falcon	Cat # 352096
12-well plates, not treated	Falcon	Cat # 351143
5 mL polypropylene tubes	Corning	Cat # 352002
50 mL Falcon conical polypropylene tubes	Falcon	Cat # 352070
60-mm Petri dishes	Falcon	Cat # 353004
96-well plates, flat-bottom, not treated	Fisher Scientific	Cat # 267578
96-well plates, V-bottom, not treated	Fisher Scientific	Cat # 249570
CO_2 euthanasia chamber	Plexx	Cat # TT-8200
Dissection board	Fisher Scientific	Cat # 36-119
Glass funnel 55 mm	Fisher Scientific	Cat # 11572423
Forceps, 45° angled serrated, 16 cm	FST	Cat # 11080-02
Forceps, standard straight serrated, 13 cm	FST	Cat # 11000-13
Forceps, fine straight serrated, 13 cm	FST	Cat # 11008-13
Magnetic stirrer	IKA	Cat # 3622000
µ-Dish 35 mm dishes with No. 1.5 ibidi Polymer Coverslip	ibidi	Cat # 81156
Mouse lab animal cages (Eurostandard type II L)	Tecniplast	Cat # 1284L
Needles 25 G	BD Biosciences	Cat # 300600
200 µL P200 micropipette	Gilson	Cat # FA10005M
Scissors, straight blunt, 11 cm	FST	Cat #14074-11
Scissors, straight sharp, 8.5 cm	FST	Cat #14084-08
Solid aluminum cylindrical weights	This paper	supplied on demand
Syringes 10 mL	BD Biosciences	Cat # 305959
BD LSR II Flow Cytometer	BD Biosciences	N/A
Roker 2D Digital rocking shaker	IKA-Werke GmbH	Cat # 4003000
Jeol 1011 Transmission Electron microscope	Jeol Ltd.	N/A
Leica DM IL LED phase contrast inverted microscope	Leica microsystems	N/A
Leica EM UC6 Ultramicrotome	Leica microsystems	N/A
Leica DM 2500 Microscope	Leica microsystems	N/A
Leica TCS SP8 Confocal Microscope	Leica microsystems	N/A
Leica Stellaris 5 Confocal Microscope	Leica microsystems	N/A

Note: *Antibodies for Flow Cytometry; **Antibodies for Confocal Microscopy.

MATERIALS AND EQUIPMENT

Aluminum weights

In-house designed solid aluminum weights are used for pressing peritoneal wall or omentum samples against the bottom of µ-Dish 35 mm dishes, for optimal confocal microscopy imaging (see Figures 1A and 1B). We can provide these weights on-demand at cost price; alternatively these weights can be ordered from the machining company Erosimar (Madrid, Spain. https://www.erosimar.com).

PBS

Reagent	Final concentration	Amount
NaH_2PO_4	1.8 mM	0.25 g
Na_2HPO_4	8.3 mM	1.2 g
NaCl	150 mM	8.77 g
double distilled water (ddH_20)	N/A	1,000 mL
Total	**N/A**	**1,000 mL**

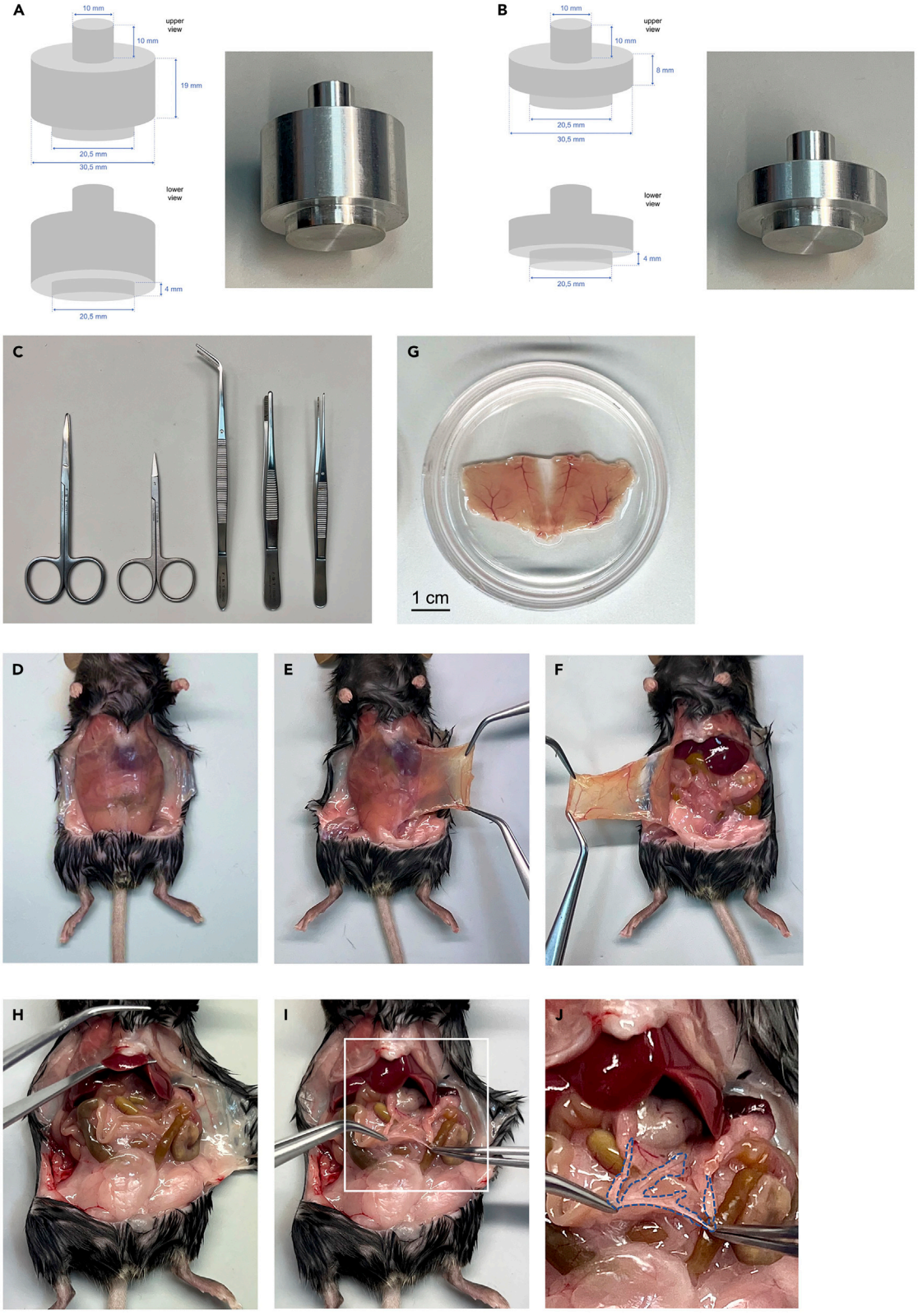

A
10 mm
upper view
10 mm
19 mm
20,5 mm
30,5 mm
lower view
4 mm
20,5 mm
B
10 mm
upper view
10 mm
8 mm
20,5 mm
30,5 mm
lower view
4 mm
20,5 mm
C
G
1 cm
D
E
F
H
I
J

Figure 1. Preparation of peritoneal wall and omentum samples for WMI and confocal microscopy studies

(A) Description of the aluminum weight used for WMI and confocal microscopy imaging of peritoneal wall samples.

(B) Description of the aluminum weight used for WMI and confocal microscopy imaging of omentum samples.

(C) Surgical tool set used for this protocol including (from left to right) straight blunt 11 cm scissors, straight sharp 8.5 cm scissors, 45° angled serrated 16 cm forceps, standard straight serrated 13 cm forceps and fine straight serrated 13 cm forceps.

(D) Incision in the skin in the abdominal area.

(E and F) Dissection of the peritoneal wall.

(G) Peritoneal wall flattened and stretched on the bottom of the 60-mm Petri dish.

(H–J) Dissection of the omentum. (I) Magnification of the area marked by a white lined square in (H).

Note: Adjust pH to 7.4, sterilize by autoclaving, and store at room temperature (20°C–22°C) for up to 3 months.

PBS 2% BSA		
Reagent	Final concentration	Amount
Bovine Serum Albumin (BSA)	2% w/v	2 g
PBS	N/A	100 mL
Total	**N/A**	**100 mL**

Note: Store at 4°C for up to 1 week.

PBS-EDTA-FBS		
Reagent	Final concentration	Amount
EDTA	5 mM	1.86 g
FBS	3% v/v	30 mL
PBS	N/A	970 mL
Total	**N/A**	**1,000 mL**

Note: Store at 4°C for up to 1 week.

Antibody staining solution		
Reagent	Final concentration	Amount
Bovine Serum Albumin (BSA)	2% w/v	0.2 g
DAPI	0.005 mg/mL	50 µL
PBS	N/A	10 mL
Total	**N/A**	**10 mL**

Note: Prepare before use.

Mowiol-based WMI mounting solution		
Reagent	Final concentration	Amount
Mowiol 4-88	10% w/v	2.4 g
Glycerol 85%	25% v/v	6 mL
Tris 0.2 M stock	100 mM	12 mL
ddH_2O	N/A	6 mL
1,4-Diazabicyclo[2.2.2] octane	2.5% w/v	0.6 g
Total	**N/A**	**25 mL**

Note: Mix 2.4 g Mowiol 4-88, 6 mL Glycerol 85%, 12 mL Tris 0.2 M and 6 mL ddH_2O in a 50 mL Falcon tube in a magnetic stirrer overnight (12–16 h). Heat at 70°C in a waterbath for 10 min to fully dissolve Mowiol. Clarify by centrifugation at 5,000 g for 15 min at 4°C. Transfer the supernatant to a new 50 mL Falcon tube, add 0.6 g 1,4-Diazabicyclo[2.2.2] octane and mix gently by inversion of the tube. Store at −20°C in 2 mL single-use aliquots for up to 6 months.

Tris 0.2 M stock: Dissolve 2.42 g Tris Base in 100 mL ddH_2O and adjust pH to 8.5. Store at room temperature (20°C–22°C) up to 3 months.

Enzymatic digestion solution		
Reagent	Final concentration	Amount
Liberase TL 2.5 mg/mL stock	0.05 mg/mL	10 µL
DNase I 2.5 mg/mL stock	0.25 mg/mL	50 µL
HEPES 1 M	50 mM	25 µL
PBS BSA-low fatty acid (10×) stock	0.01 mg/mL	50 µL
DMEM-low glucose	N/A	365 µL
Total	**N/A**	**500 µL**

Note: Prepare before use.

Liberase TL 2.5 mg/mL stock: Dissolve 5 mg Liberase TL in 2 mL ddH_2O. Store at −20°C in 50 µL single-use aliquots for up to 6 months.

DNase I 2.5 mg/mL stock: Dissolve 2.5 mg DNase I in 1 mL ddH_2O. Store at 4°C for up to 1 week.

PBS BSA-low fatty acid (10×) stock: Dissolve 1 g BSA-low fatty acid in 10 mL PBS. Store at 4°C for up to 1 week.

Fixation solution for electron microscopy		
Reagent	Final concentration	Amount
Paraformaldehyde 16%	1.5%	4.7 mL
Glutaraldehyde 25%	1.5%	3 mL
HEPES 0.4 M stock	0.15 M	18.8 mL
ddH_2O	N/A	23.5 mL
Total	**N/A**	**50 mL**

Note: Store at 4°C for up to 24 h.

HEPES 0.4 M stock: Dissolve 9.53 g HEPES in 100 mL ddH_2O. Adjust pH to 7.2 and store at 4°C for up to 3 months.

⚠ CRITICAL: Paraformaldehyde and glutaraldehyde are toxic; wear a lab coat, gloves and safety googles. Work in a chemical safety hood and dispose waste in accordance with local regulations.

HEPES 0.15 M buffer

Dissolve 3.57 g HEPES in 100 mL ddH_2O. Adjust pH to 7.2 and store at 4°C for up to 3 months.

Cacodylate buffer 0.1 M

Dissolve 0.64 g sodium cacodylate trihydrate in 15 mL ddH_2O. Adjust pH to 7.4 and store at 4°C for up to 3 months.

Cacodylate buffer 0.2 M

Dissolve 1.28 g sodium cacodylate trihydrate in 15 mL ddH_2O. Adjust pH to 7.4 and store at 4°C for up to 3 months.

Osmium tetroxide solution

Mix 2 mL osmium tetroxide 2% and 2 mL cacodylate buffer 0.2 M. Prepare before use.

⚠ CRITICAL: Osmium tetroxide is acutely toxic; wear a lab coat, gloves and safety googles. Work in a chemical safety hood and dispose waste in accordance with local regulations.

Uranyl acetate solution

Dissolve 0.2 g uranyl acetate in 10 mL ddH_2O. Store at 4°C for up to 3 months.

⚠ CRITICAL: Uranyl acetate is radioactive and highly toxic; wear a lab coat, gloves and safety googles. Work in a chemical safety hood behind a protective screen and dispose waste in accordance with local regulations.

Epoxy resin

Reagent	Final concentration	Amount
Epon 812	48%	2.4 g
DDSA EM grade	19%	0.95 g
MNA	33%	1.65 g
BDMA	3%	0.15 g
Total	N/A	5 g

Note: Using a precision balance, add sequentially with a Pasteur pipette 2.4 g 812 Epon resin, 0.95 g DDSA EM grade, 1.65 g MNA and 0.15 g BDMA in a 50 mL Falcon tube. Mix gently in a magnetic stirrer at room temperature (20°C–22°C) until the solution is completely homogenized. Centrifuge at 15,000 g for 5 min to remove air bubbles. Prepare before use.

STEP-BY-STEP METHOD DETAILS

Whole mount immunofluorescence and confocal microscopy imaging of resMØ-aggregates

Timing: 4–6 h/mouse

This step describes how to carry out the dissection, fixation, immunofluorescent staining and mounting of whole peritoneal wall and omentum samples to perform WMI/confocal microscopy studies.

1. Dissection and fixation of the peritoneal wall.
   a. Euthanize mice by CO_2 inhalation.
   b. Place the mouse on its back on a dissection board.
   c. Spray the abdomen with 70% ethanol.
   d. Using straight blunt scissors (see Figure 1C, showing the surgical tool set used) make an incision in the skin in the abdominal area, starting in the lower abdominal midline, without penetrating the abdominal wall, and extend the incision until the ribcage.
   e. Extend the incision to the hind and forelegs (Figure 1D).
   f. Dissect the whole peritoneal wall using straight sharp scissors and 45° angled serrated forceps (Figures 1E and 1F).
   g. Transfer the peritoneal wall to a 60-mm Petri dish.

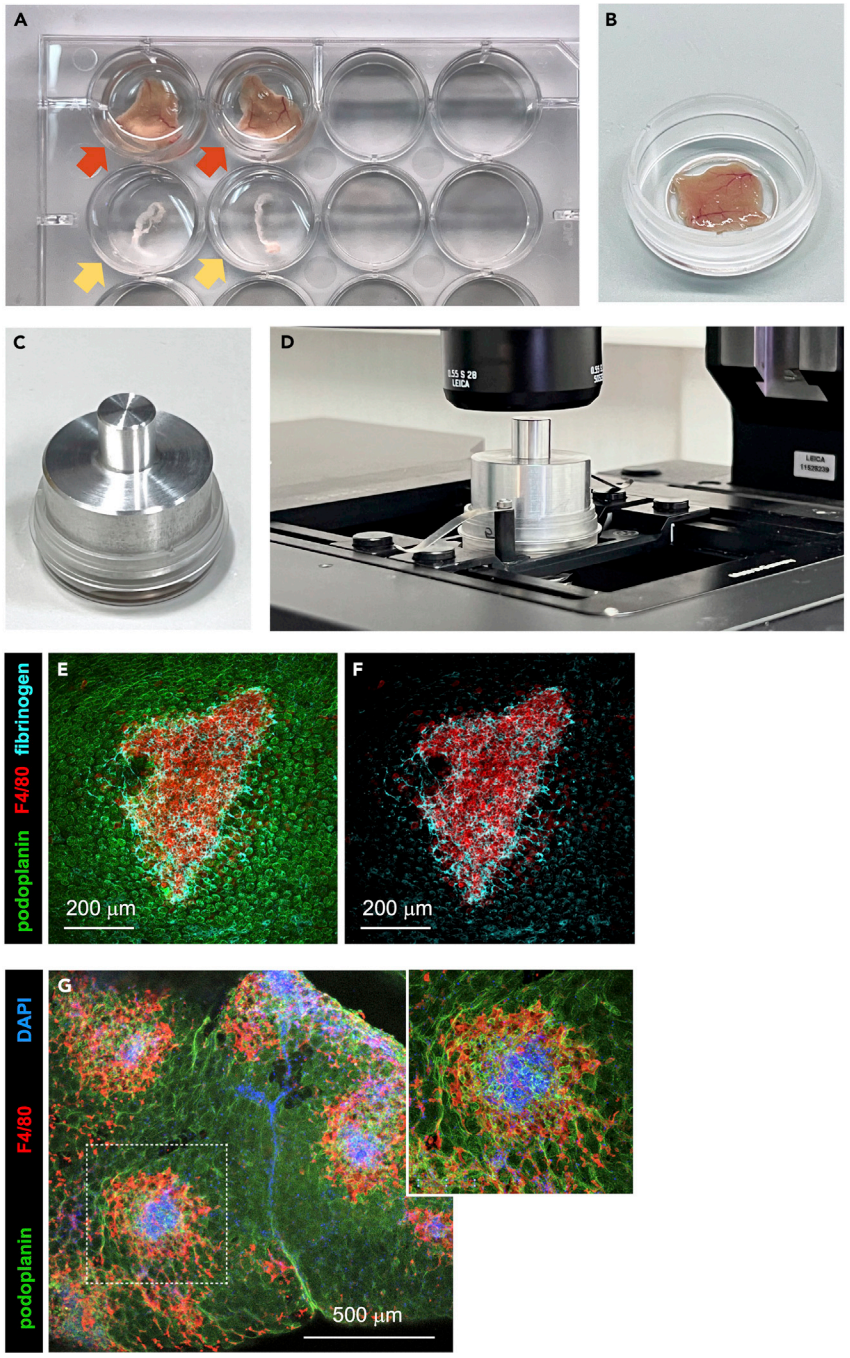


Figure 2. Immunofluorescence staining of whole peritoneal wall and omentum samples

(A) Peritoneal wall (red arrows) and omentum (yellow arrows) samples transferred to wells of a 12-well plate to carry out the immunofluorescence staining process.

(B) Peritoneal wall sample transferred to a µ-Dish 35 mm dish with No. 1.5 ibidi Polymer Coverslip containing 300 µL of mowiol-based WMI mounting solution.

(C) Peritoneal wall sample mounted in a µ-Dish 35 mm dish with No. 1.5 ibidi Polymer Coverslip, with 300 µL of mowiol-based WMI mounting solution, and a peritoneal wall aluminum weight on top.

(D) Peritoneal wall sample mounted in a µ-Dish 35 mm dish with No. 1.5 ibidi Polymer Coverslip with aluminum weight on top, placed on the stage of a confocal microscope.

(E and F) WMI/confocal microscopy imaging of a resMØ-aggregate in the peritoneal wall, at 4 h after *E. coli* infection, after immunofluorescent staining with Alexa Fluor 488-conjugated anti-podoplanin (mesothelium), Alexa Fluor

Figure 2. *Continued*
594-conjugated anti-F4/80 (resMØs), and unconjugated anti-fibrinogen (fibrin network) followed by Alexa Fluor 647-conjugated secondary antibody. (E) podoplanin, F4/80 and fibrinogen staining; (F) F4/80 and fibrinogen staining. Images were acquired on a multispectral Leica TCS SP8 confocal microscope and analyzed using ImageJ software.
(G) Low magnificaction confocal microscopy image of the omentum, at 4 h after *E. coli* infection, showing the distribution of resMØ-aggregates after immunofluorescent staining with Alexa Fluor 488-conjugated anti-podoplanin (mesothelium), Alexa Fluor 594-conjugated anti-F4/80 (resMØs), and DAPI (nuclei). Insert: enlargement of the area marked in G with a white dotted line. Images were acquired on a multispectral Leica SP8 confocal microscope and analyzed using ImageJ software. Figures F and G reprinted with permission from Vega-Pérez at al.[1]

⚠ CRITICAL: Make sure that the side of the peritoneal wall facing the peritoneal cavity is placed upwards during immunofluorescence staining.

h. Flatten and stretch out the peritoneal wall on the bottom of the 60-mm Petri dish using standard straight serrated forceps (See Figure 1G).
i. Add 5 mL of 4% paraformaldehyde solution and incubate for 5 min at 4°C.
j. Wash twice in PBS for 5 min.
k. Cut the peritoneal wall along the midline in two halves and trim the edges so each can fit in a well of a 12-well plate.

2. Dissection and fixation of the omentum.
   a. Follow the steps 1a to 1e described above.
   b. Using 45° angled serrated and fine serrated forceps, dissect the omentum by lifting the liver up, and isolating the omentum from the adipose tissue associated to pancreas, stomach and spleen (Figures 1H–1J; Methods video S1).
   c. Transfer the omentum to into a well of a 12-well plate containing 1 mL of 4% paraformaldehyde solution and incubate for 5 min at 4°C.
   d. Wash twice in PBS for 5 min.

Note: Fixed peritoneal wall or omentum samples can be kept in PBS overnight (12–16 h), at 4°C, before proceeding with immunofluorescence staining, although this can compromise the correct detection of some antigens as well as tissue integrity.

⚠ CRITICAL: Paraformaldehyde is hazardous; wear gloves, work in chemical safety hood and dispose waste in accordance with local regulations.

3. Immunofluorescence staining of whole peritoneal wall and omentum samples.

Note: All staining steps are performed in 12-well plates (Figure 2A) under continuous agitation using a rocking shaker, in the dark.

   a. Incubate each sample in 1 mL PBS 2% BSA for 30 min at 4°C for blocking unspecific binding of primary antibodies.
   b. Incubate each sample in 1 mL of fluorophore-conjugated antibodies, diluted as indicated in the key resources table, in antibody staining solution, for 2–4 h at 4°C.
   c. Wash twice in PBS for 5 min.

Note: When using primary unconjugated antibodies, incubate the samples with unconjugated antibodies, diluted as indicated in the key resources table, in PBS 2% BSA for 2 h at 4°C, wash twice in PBS for 5 min and incubate with secondary antibodies in antibody staining solution, for 2 h at 4°C. For staining of intracellular molecules incubate the samples with 0.1% Triton X-100 for 15 min at 4°C prior to incubation with antibodies.

Note: A complete list of the antibodies used in our WMI studies, and the corresponding working dilutions, is included in the key resources table. If using antibodies distinct from those listed in this protocol, the working dilution and time of incubation should be defined on the basis of titration experiments.

4. Mounting samples for confocal microscopy.
   a. Transfer peritoneal wall or omentum samples to μ-Dish 35 mm dishes with No. 1.5 ibidi Polymer Coverslip, containing 300 μL of mowiol-based WMI mounting solution (Figure 2B).

   Note: The surface of the peritoneal wall or omentum to be observed under the microscope should face the bottom of the dish. Place the samples carefully to avoid the formation of bubbles.

   b. Place an aluminum weight, for either peritoneal wall or omentum samples, on top of each sample, to flatten and press the samples against the bottom of the dish (Figure 2C).

   Note: The weight has to be kept on top of the samples during imaging (Figure 2D). These samples are ready to be analyzed by confocal microscopy.

   Note: An example of a standard staining combination allowing the detection of resMØ-aggregates in the peritoneal wall would include Alexa Fluor 488-conjugated anti-podoplanin (mesothelium), Alexa Fluor 594-conjugated anti-F4/80 (resMØs), and unconjugated anti-fibrinogen (fibrin network) followed by Alexa Fluor 647-conjugated secondary antibody and DAPI (Figures 2E and 2F). Low magnification confocal microscopy imaging allows to explore the distribution of resMØ-aggregates, as shown in Figure 2G. For additional information related to the analysis of resMØ-aggregates by WMI, please refer to Vega-Pérez et al.[1]

   ⚠ CRITICAL: The use of aluminum weights significantly improves the quality of WMI/confocal microscopy imaging.

Isolation of resMØ-aggregates

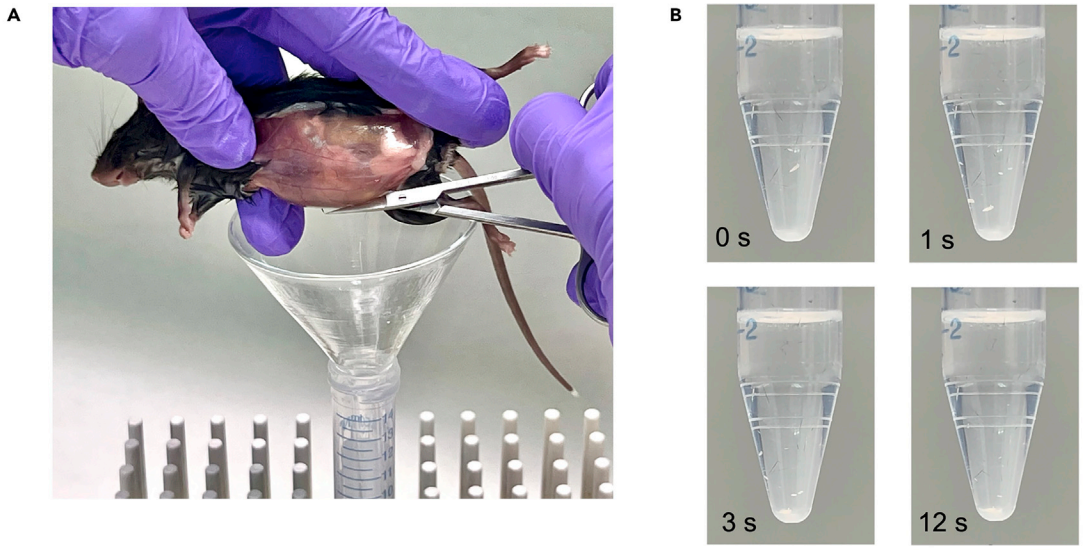
Timing: 45–60 min/mouse

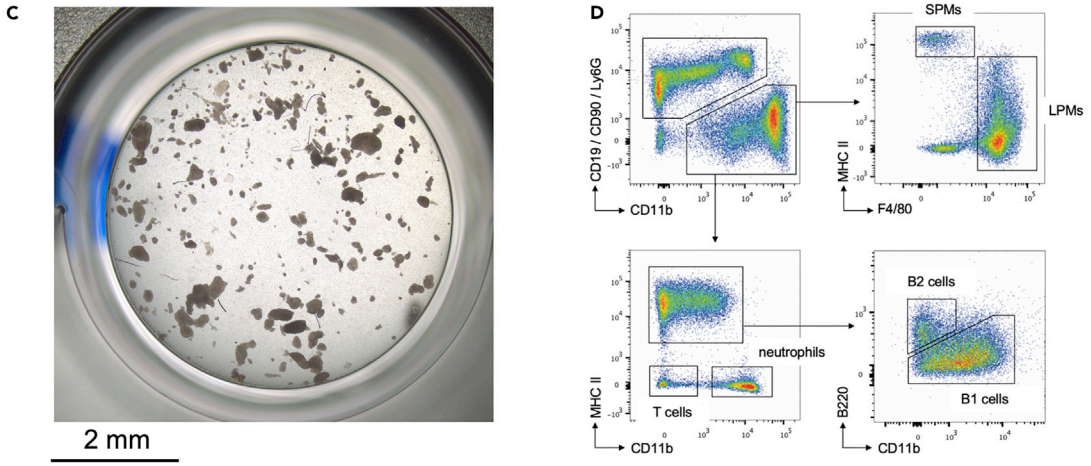
This step describes how to isolate mesothelium-bound resMØ-aggregates formed during inflammatory processes in different organs of the peritoneal cavity, particularly in the peritoneal wall and omentum. resMØ-aggregates induced by *E coli* infection can be easily isolated during the first 6 h post infection while, from 18 h post infection, resMØ-aggregates start to disaggregate and are more difficult to isolate. Isolated resMØ-aggregates can be subsequently analyzed by flow cytometry or electron microscopy, as detailed below, or be subjected to other studies.

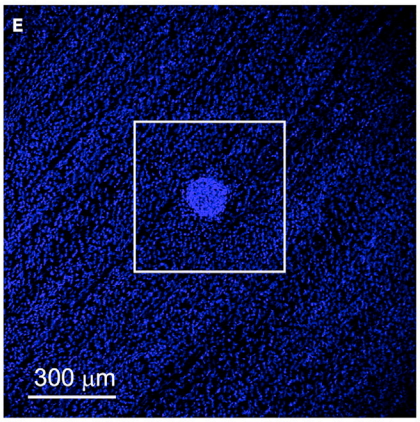
5. Follow steps 1a to 1c described above.
6. Make an incision in the skin in the abdominal area, starting in the lower abdominal midline, without penetrating the abdominal wall, and extend the incision until the neck.
7. Extend the incision to the hind and forelegs and remove all the abdominal and thoracic skin.
8. Inject 6–9 mL of cold PBS in the peritoneal cavity at the lower left quadrant of the abdomen, using a 10 mL syringe with a 25 G needle.

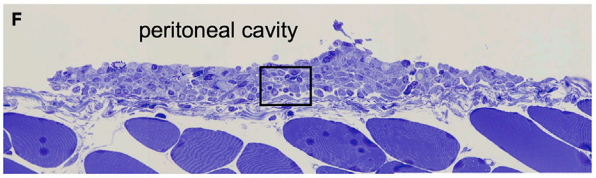
   Note: The volume of PBS to be injected has to be adjusted to the age/size of the mice used. For 8–10-week-old C57BL/6 females 8 mL were injected.

9. Massage the abdomen vigorously to detach resMØ-aggregates from the mesothelium (Methods video S2).


A
B
0 s
1 s
3 s
12 s


C
2 mm
D
SPMs
LPMs
CD19 / CD90 / Ly6G
CD11b
MHC II
F4/80
neutrophils
T cells
B2 cells
B1 cells
B220


E
300 µm


F
peritoneal cavity
50 µm

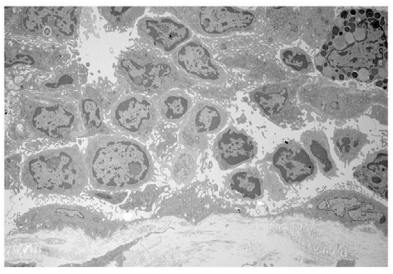

G
10 µm

Figure 3. Flow cytometry and electron microscopy analysis of isolated resMØ-aggregates

(A) Harvesting of resMØ-aggregates by making an incision in the peritoneal wall, after injecting 8 mL PBS intraperitoneally and performing a peritoneal massage.

(B) Sedimentation of resMØ-aggregates harvested at 4 h after *E. coli* infection; the time after starting of the sedimentation process is indicated.

(C) Phase contrast microscopy image of resMØ-aggregates isolated at 4 h after *E. coli* infection, and transferred into a well of a flat-bottom 96-well plate.

(D) Flow cytometry analysis of resMØ-aggregates isolated at 4 h after *E. coli* infection, after immunofluorescent staining with PE-Cy7-conjugated anti-CD11b, APC-Cy7-conjugated anti-F4/80, APC-conjugated anti-MHC II, PE-conjugated anti-Ly6G, PE-conjugated anti-CD19, Pacific Blue-conjugated anti-B220 and PE-conjugated anti-CD90. Data were acquired on a Becton Dickinson LSR II flow cytometer and analyzed using FlowJo X software.

(E) WMI/confocal microscopy image of the peritoneal wall after DAPI staining, allowing the detection of resMØ-aggregates at 4 h after *E. coli* infection.

(F) Semithin section of the peritoneal wall at 4 h after E. coli infection, showing a resMØ-aggregate. Toluidine blue staining.

(G) Electron microscopy image of the area marked by a black lined square in (F). Figures F and G reprinted with permission from Vega-Pérez at al.[1]

10. Make an incision in the peritoneal wall starting in the lower abdominal midline and extend the incision until the ribcage (Figure 3A).
11. Collect the peritoneal lavage containing the resMØ-aggregates directly into a 15 mL Falcon tube using a glass funnel (Figure 3A).
12. Place the tube on ice and leave resMØ-aggregates to sediment for 5 min (Figure 3B; Methods video S3).
13. Remove the supernatant carefully, leaving 0.5 mL with resMØ-aggregates at the bottom of the tube.
14. Add carefully 5 mL of cold PBS.
15. Repeat steps 12 and 13 as needed in order to obtain a resMØ-aggregate preparation as clean as possible of free cells.

Note: Check the quality of the preparation under an inverted phase contrast microscope after transferring the resMØ-aggregates into a well of a flat-bottom 96-well plate (Figure 3C).

⚠ CRITICAL: Use a 200 µL micropipette with tips in which the front end has been cut off to transfer isolated resMØ-aggregates, in order to avoid their disruption.

16. Keep isolated resMØ-aggregates in 1 mL PBS in a 15 mL Falcon tube.

⚠ CRITICAL: The intensity/strength of the abdominal massage is critical to harvest resMØ-aggregates properly. An insufficient massage will not detach resMØ-aggregates while a too vigorous massage may disintegrate resMØ-aggregates.

Processing of isolated resMØ-aggregates for flow cytometry

Timing: 20 min/mouse

This step describes how to disaggregate isolated resMØ-aggregates by enzymatic digestion to obtain a cell suspension containing the cells forming these aggregates, which can be subsequently analyzed by flow cytometry.

17. Centrifuge the isolated resMØ-aggregates preparation obtained in step 16 at 500 g for 5 min at 4°C in a 15 mL Falcon tube.
18. Remove the supernatant carefully.
19. Add 500 µL of the enzymatic digestion solution and incubate in a waterbath at 37°C for 5 min.
20. Disrupt resMØ-aggregates mechanically by gentle pipetting using a 200 µL micropipette.

Note: Check resMØ-aggregate disruption under an inverted phase contrast microscope, after transferring the cell suspension into a well of a flat-bottom 96-well plate.

21. Add 10 mL PBS-EDTA-FBS to stop the enzymatic digestion.

22. Centrifuge at 500 g for 5 min at 4°C.
23. Remove the supernatant and resuspend in 500 μL PBS-EDTA-FBS.

Note: resMØ-aggregates isolated during the first 60 min post-infection can be disaggregated without enzymatic digestion. In this case avoid steps 17–19 and 21.

Note: From 1.5×10^5 to 2.5×10^5 cells are usually obtained from resMØ-aggregates isolated at 4 h after *E. coli* infection of a 8–10-week-old C57BL/6 female.

Immunofluorescent staining of resMØ-aggregate cell suspensions for flow cytometry

Timing: 45–60 min

This step describes how to process resMØ-aggregate cell suspensions to be analyzed by flow cytometry. Immunofluorescent staining is performed in 96-well V-bottom plates in the dark.

24. Transfer the cells obtained in step 23 into a 96-well V-bottom plate at $\geq 10^5$ cells/well.
25. Centrifuge the 96-well plate at 500 g for 5 min at 4°C.
26. Discard the supernatant, resuspend the cells in 30 μL of anti-CD16/32 antibody to block Fc receptors, and incubate for 15 min at 4°C.
27. Wash with 100 μL PBS-EDTA-FBS per well and centrifuge at 500 g for 5 min at 4°C.
28. Discard the supernatant, resuspend the cells in 30 μL of fluorophore-conjugated antibodies, diluted as indicated in the key resources table in PBS-EDTA-FBS, and incubate for 15 min at 4°C.
29. Wash with 100 μL PBS-EDTA-FBS per well and centrifuge at 500 g for 5 min at 4°C.
30. Discard the supernatant, resuspend in 200 μL PBS-EDTA-FBS and transfer the cells to 5 mL polypropylene tubes. These samples are ready to be analyzed in a flow cytometer.

Note: A complete list of the antibodies used in our flow cytometry studies of resMØ-aggregate cells, and the corresponding working dilutions, is included in the key resources table. An example of a standard staining combination allowing the detection by flow cytometry of the main cell subsets composing resMØ-aggregates at 4 h after *E. coli* infection, i.e., LPMs (large resident peritoneal macrophages), SPMs (small peritoneal macrophages), B1 cells, B2 cells, T cells and neutrophils would include PE-Cy7-conjugated anti-CD11b, APC-Cy7-conjugated anti-F4/80, APC-conjugated anti-MHC II, PE-conjugated anti-Ly6G, PE-conjugated anti-CD19, Pacific Blue-conjugated anti-B220 and PE-conjugated anti-CD90 (Figure 3D). For additional information related to the analysis of resMØ-aggregates by flow cytometry, please refer to Vega-Pérez et al.[1]

Processing of resMØ-aggregates for electron microscopy imaging

Timing: 72 h

This step describes how to process isolated resMØ-aggregates, or peritoneal wall zones harboring resMØ-aggregates, for electron microscopy studies.

31. Processing isolated resMØ-aggregates for electron microscopy.

Note: Removal of the different reagents described below has to be performed using a Pasteur pipette and avoiding centrifugation. All the steps should be carried out in a chemical safety hood; process carefully to avoid damaging resMØ-aggregates.

a. Transfer isolated resMØ-aggregates obtained in step 16 into a 1.5 mL eppendorf tube.

b. Add carefully 0.5 mL of the Fixation Solution for Electron Microscopy, and incubate overnight (12–16 h) at 4°C.
c. Remove the Fixation Solution and wash (x2) with 1 mL HEPES 0.15 M buffer for 5 min at 4°C.
d. Remove HEPES 0.15 M buffer and wash (x4) with 1 mL cacodylate buffer 0.1 M for 5 min at 4°C.
e. Remove cacodylate buffer 0.1 M and incubate with 0.5 mL osmium tetroxide solution for 1 h at 4°C in the dark.
f. Remove osmium tetroxide solution and wash (x4) with 1 mL cacodylate buffer 0.1 M for 5 min at 4°C.
g. Remove cacodylate buffer 0.1 M and wash (x2) with 1 mL ddH_2O for 5 min at 4°C.
h. Remove ddH_2O and incubate with 0.5 mL uranyl acetate solution for 60 min at 4°C in the dark.
i. Remove uranyl acetate solution and wash (x2) with 1 mL ddH_2O for 5 min at 4°C.
j. Remove ddH_2O and incubate with 1 mL 70% acetone for 10 min at 4°C.
k. Remove 70% acetone and incubate with 1 mL 90% acetone for 10 min at 4°C.
l. Remove 90% acetone and incubate with 1 mL 100% acetone for 10 min at 4°C.
m. Exchange 100% acetone and incubate for 10 min at room temperature (20°C–22°C).
n. Incubate in 1 mL 1:1 epoxy resin/100% acetone for 30 min at room temperature (20°C–22°C).

Note: Epoxy resin should be prewarmed at 40°C.

o. Incubate in 1 mL epoxy resin for 2 h at room temperature (20°C–22°C).
p. Exchange epoxy resin and incubate for 3 h at room temperature (20°C–22°C).
q. Transfer to shape molds, add epoxy resin and incubate for 48 h at 60°C. These samples are ready to be processed by ultramicrotomy.

32. Isolation of zones of the peritoneal wall harboring resMØ-aggregates.
    a. Follow steps 1a to 1f.
    b. Transfer the peritoneal wall to a 60-mm Petri dish containing 5 mL of the Fixation Solution for Electron Microscopy, and incubate overnight (12–16 h) at 4°C.
    c. Remove the Fixation Solution and wash twice with 5 mL PBS for 5 min at 4°C.
    d. Cut the peritoneal wall in two halves and trim the edges so each can fit in a well of a 12-well plate.
    e. Remove the PBS and incubate with 5 mL DAPI at 1:200 in PBS for 15 min at 4°C in the dark.
    f. Remove DAPI and wash with PBS for 5 min at 4°C.
    g. Transfer the peritoneal wall samples to μ-Dish 35 mm dishes with No. 1.5 ibidi Polymer Coverslip containing 300 μL of PBS.

⚠ CRITICAL: Do not use mowiol-based WMI mounting solution in this step.

    h. Place an aluminum weight for peritoneal wall on top of each sample.
    i. Analyze the samples by confocal microscopy to locate resMØ-aggregates, which can be identified as spots with a high density DAPI staining (Figure 3E), and choose zones harboring one or several noticeable resMØ-aggregates.
    j. Once spotted under the microscope by the DAPI staining, remove the aluminum weight carefully; the selected area can be then located by the laser beam. Next determine the position of the piece of the peritoneal wall to be dissected using as reference the position and morphology of the vasculature of this area.
    k. Dissect carefully small pieces (~2 × 2 mm) of peritoneal wall containing the zones defined in the previous step.

33. Processing of peritoneal wall zones harboring resMØ-aggregates for electron microscopy.

a. Transfer the peritoneal wall pieces obtained in step 32j into a 1.5 mL eppendorf tube.
b. Follow steps 31c to 31q. These samples are ready to be processed by ultramicrotomy. Semithin sections of the selected areas (Figure 3F) will allow to confirm the presence of resMØ-aggregates prior to perform electron microscopy studies (Figure 3G).

EXPECTED OUTCOMES

As pointed out above, during peritoneal inflammatory reactions caused by peritoneal damage or infection, resMØ-aggregates, which are crucial for repairing peritoneal injuries and controlling infections, are formed predominantly in the peritoneal wall and omentum. However, they can also develop in the mesothelial surface of other peritoneal organs, i.e., the mesentery, gonadal fat and intestine and, thus, the number of resMØ-aggregates detectable in the peritoneal wall or omentum is variable. At 4 h after *E. coli* infection, the number of resMØ-aggregates detectable in the peritoneal wall or the omentum is variable and usually ranges from a few (2–3) until many (10–15) in both locations.

On the other hand, the size of resMØ-aggregates formed in the peritoneal wall during the first hours after *E. coli* infection usually ranges from 100 to >800 μm. The structure and composition resMØ-aggregates formed in response to other inflammatory reactions may differ and therefore needs to be investigated.

As indicated above, from 1.5×10^5 to 2.5×10^5 cells are usually obtained after disaggregation of resMØ-aggregates isolated at 4 h after *E. coli* infection of an 8–10-week-old C57BL/6 female. However, the number of cells obtainable from resMØ-aggregates can differ depending on the age, sex, time of analysis and, particularly, on the experimental model of peritoneal aggression being analyzed.

LIMITATIONS

This protocol describes how to proceed for imaging resMØ-aggregates, formed in the peritoneal wall or omentum during peritoneal inflammation, by WMI combined with confocal microscopy. Although, as pointed out above, this protocol can be used for imaging other peritoneal organs in which resMØ-aggregates can form, such as mesentery, spleen, gut or ovary, as well as the inner wall of the thoracic cavity, it might be necessary in order to optimize the imaging in these organs to set up some steps of our protocol, and to modify the weight of the aluminum weights.

Examples of expected outcomes regarding the composition, kinetics and estimation of the number of resMØ-aggregates, described in the present protocol, refer to our experiments of peritoneal *E. coli* infection, but these parameters might differ depending on the peritoneal inflammation model used.

Similarly, as described in our protocol, resMØ-aggregates can be isolated to perform additional studies. However the possibility of isolating resMØ-aggregates is limited, in our *E. coli* infection model, to a time frame between around 1 h and 18 h after infection, determined by the composition, adhesion to the mesothelium and onset of the resMØ-aggregate disruption process. Time restrictions for isolating resMØ-aggregates may differ depending on the experimental model used.

As pointed out above, in our peritoneal *E.coli* infection model, resMØ-aggregates are preferentially formed in the peritoneal wall and omentum, but can also form in other peritoneal organs. Consequently, resMØ-aggregates isolated as described in this protocol might potentially contain resMØ-aggregates formed in all these locations and therefore, when analyzing them by flow cytometry, electron microscopy or other techniques, it is important that to take into account that the composition resMØ-aggregates may differ depending on the organ in which they are formed.

TROUBLESHOOTING

Problem 1

Mounting samples for confocal microscopy (step 4).

Potential solution

Commercial mounting media exist as an alternative to the homemade mowiol-based WMI mounting solution described in this protocol, but we recommend using of the homemade mowiol-based WMI mounting solution due to its superior optical quality. As pointed out above the use of aluminum weights to flatten peritoneal wall and omentum samples significantly improves the quality of imaging by WMI and confocal microscopy, and therefore their use is strongly recommended. On the other hand, special care should be taken during the processing of peritoneal wall and omentum samples to prevent them from excessive bending and twisting, respectively.

Problem 2

Isolating resMØ-aggregates (steps 9–15).

Potential solution

resMØ-aggregates are particularly fragile and therefore special care has to be taken when performing the abdominal massage, and when manipulating them, to preserve their integrity.

Problem 3

Processing isolated resMØ-aggregates for electron microscopy (step 31).

Potential solution

As pointed out above, resMØ-aggregates are particularly fragile and, therefore, special care has to be taken when manipulating them along all the steps of processing for electron microscopy to preserve their integrity.

Problem 4

Isolation of zones of the peritoneal wall harboring resMØ-aggregates (step 32).

Potential solution

As indicated above, the number of resMØ-aggregates detectable in the peritoneal wall or omentum is variable, ranging from a few (2–3) until many (10–15) at 4 h after *E. coli* infection. Consequently, in some cases it may be difficult to spot zones of the peritoneal wall containing resMØ-aggregates to be dissected and processed for electron microscopy, and thus it may be necessary to analyze a number of samples to find a convenient zone.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Carlos Ardavín (ardavin@cnb.csic.es).

Materials availability

Aluminum weights used WMI and confocal microscopy imaging can be provided on-demand at cost price. Alternatively these weights can be ordered from the machining company Erosimar (Madrid, Spain. https://www.erosimar.com).

Data and code availability

This study did not generate new unique datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102079.

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AUTHOR CONTRIBUTIONS

C.A. designed research, designed aluminum weights for WMI, analyzed data, and wrote the manuscript. M.F., A.V.-P., and A.G.-G. performed experiments, analyzed data, and contributed to review and editing of the manuscript. N.A.-L. performed experiments and contributed to review and editing of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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