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TITLE:

Detecting Migration and Infiltration of Neutrophils in Mice

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neutrophil, migration, arthritis, immunohistochemistry, air punch, chemotaxis

SUMMARY:

Here, we present three methods to assess neutrophil migration and infiltration both in vivo and in vitro. These methods can be used to discover promising therapeutics targeting neutrophil migration.

ABSTRACT:

Neutrophils are a major member of the innate immune system and play pivotal roles in host defense against pathogens and pathologic inflammatory reactions. Neutrophils can be recruited to inflammation sites via the guidance of cytokines and chemokines. Overwhelming infiltration of neutrophils can lead to indiscriminate tissue damage, such as in rheumatoid arthritis (RA). Neutrophils isolated from peritoneal exudate respond to a defined chemoattractant, N-formyl-Met-Leu-Phe (fMLP), in vitro in Transwell or Zigmond chamber assays. The air pouch experiment can be used to evaluate the chemotaxis of neutrophils towards lipopolysaccharide (LPS) in vivo. The adjuvant-induced arthritis (AA) mouse model is frequently used in RA research, and immunohistochemical staining of joint sections with anti-myeloperoxidase (MPO) or anti-

neutrophil elastase (NE) antibodies is a well-established method to measure neutrophil infiltration. These methods can be used to discover promising therapies targeting neutrophil migration.

INTRODUCTION:

Neutrophils are the most abundant white blood cell and account for 50−70% of the whole white blood cell population in humans[1]. Neutrophils are one of the primary responders during acute inflammation. Neutrophils can be recruited to inflammation sites via the guidance of cytokines and chemokines released by tissue-resident cells[2,3,4], which is mediated by the interactions between cell adhesion molecules on the surface of neutrophils and vascular endothelium cells[5]. Neutrophils are fundamental to host defense and play a role in pathologic inflammatory reactions due to their powerful capacity to damage tissue via the release of reactive oxygen species (ROS) and other tissue-damaging molecules[3,6].

Previous studies have described several neutrophil isolation protocols from mice or humans. Oh et al. demonstrated a density gradient separation method to isolate human neutrophils from whole human blood[7]. However, the isolation of sufficient neutrophils from mouse blood is difficult because of the small blood volume. Alternatively, large numbers of pure and viable mouse neutrophils can be elicited from mouse peritoneal fluid, and these purified neutrophils can be used ex vivo to examine several aspects of cellular functions ex vivo, including neutrophil infiltration, migration, chemotaxis, oxidative burst, cytokine and neutrophil extracellular trap (NET) production[8]. Transwell assays[9] or Zigmond chamber assays[10,11] can be used to evaluate neutrophil migration in vitro. The air pouch model is used to evaluate the migration and infiltration of neutrophils in vivo. The subcutaneous air pouch model is a convenient in vivo animal model to study the migration of inflammatory cells.

Traditionally, neutrophils were considered as pathogen eliminators in acute phases of inflammation. However, recent findings have shown that neutrophils are complicated cells that perform a significant variety of specialized functions. Neutrophils can regulate many processes such as acute injury and repair, tumorigenesis, autoimmune response, and chronic inflammation[12,13]. Neutrophils also modulate adaptive immune responses and can regulate B cells and T cells[14,15]. Substantial shortage of neutrophils leads to mortality or severe immunodeficiency in humans and neutrophil depletion in mice leads to fatality, while excessive activation or recruitment of neutrophils in organs causes several immune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)[6]. Neutrophils are the most abundant cells in the synovial fluid of RA patients. Neutrophils produce excessive amounts of myeloperoxidase (MPO) and neutrophil elastase (NE) via degradation, which exacerbates cartilage erosion. MPO is a peroxidase enzyme mainly expressed in the granules of neutrophils[16]. NE is associated with articular cartilage destruction[17]. MPO and NE could be used to evaluate the status of neutrophil migration and infiltration in the tissue of RA patients.

This article provides three conventional methods to evaluate the migration of normal neutrophils induced both in vivo and in vitro, as well as the infiltration of pathological neutrophils in a mouse joint-specific inflammation model.

PROTOCOL:

All experimental procedures were reviewed and approved by the Beijing University of Chinese Medicine Animal Care and Use Committee.

NOTE: C57BL/6 mice (7-8 weeks old) were used.

1. Neutrophil isolation

1.1. Acquisition of peritoneal exudate cells

1.1.1. Prepare fresh 10% proteose peptone solution in ddH_2O. Calculate the volume needed according to the number of mice.

NOTE: Set the number of mice as N, (2N+1) mL of solution must be dissolved and filtered in advance.

1.1.2. Spray the workspace with 70% ethanol. Use an insulin injector to draw 1 mL of peptone solution and discharge bubbles.

1.1.3. Conduct the first intraperitoneal injection of 1 mL of peptone solution per mouse.

1.1.3.1. Grab the mouse in a head-down position with one hand. Disinfect the injection spot with alcohol-soaked cotton balls.

NOTE: The preferred injection position lies in the lateral aspect of the lower left or right quadrant of the abdomen.

1.1.3.2. Infuse the reagents rapidly. Inject 1 mL into the peritoneal cavity of each mouse with the insulin injector.

NOTE: The angle between the needle and the skin should be approximately 15−30° to avoid injuring the intestine or other organs.

1.1.4. Allow inflammatory response to develop overnight. After 12 h, conduct the second injection in the same manner as the first injection.

1.1.5. Three hours after the second injection, anesthetize mice with 5% isoflurane for 5 min in a gas anesthesia chamber at a speed of 2 L/min. Remove the anesthetized mice from the chamber and sacrifice them by cervical dislocation.

NOTE: All the following steps should be processed in a tissue culture hood.

1.1.6. Spray the mouse with 70% ethanol. Lay the mouse on a sterile plastic pad and fix the limbs with needles.

1.1.7. Use a sterile set of surgical tools to make a horizontal incision (~1 cm) in the middle of the lower abdomen. Lift the skin of the upper abdomen with forceps and cut along the midline of the abdomen and expose the intact peritoneal wall.

1.1.8. Inject 5 mL of sterile RPMI-1640 complete medium into the abdominal cavity with a 30 G x 1/2" needle. Insert the needle through the peritoneal wall with the beveled edge of the needle facing up and inject the entire volume.

1.1.9. Shake the pad horizontally for 5 min. Massage the abdomen gently several times.

1.1.10. Inject a 23 G x 1 1/4" needle into the lateral space of the abdomen. Extract the abdominal liquid (~5 mL) and collect it in a 50 mL centrifuge tube.

NOTE: Place the tubes on ice as soon as possible in case of neutrophil activation.

1.1.11. Inject another 5 mL of complete medium and repeat the procedure to remove the remaining cells from the peritoneum. Pool the peritoneal fluid in the 50 mL centrifuge tube.

1.1.12. Centrifuge the pooled peritoneal fluid at 400 x *g* for 10 min at room temperature (RT).

1.1.13. Discard the supernatant. Resuspend the cells in 1 mL of RPMI-1640 complete medium.

NOTE: Do not vortex to avoid neutrophil activation.

1.2. Isolation of neutrophils

1.2.1. Add 4 mL of freshly prepared 70.2% density gradient medium (e.g., Percoll) in a 15 mL centrifuge tube.

1.2.2. Carefully overlay 4 mL of freshly prepared 54.8% density gradient medium on the 70.2% density gradient medium slowly along the edge of the tube with sharp pipette tips in the 15 mL centrifuge tube.

NOTE: Exercise caution to avoid disturbing the interface between the 54.8% density gradient medium and 70.2% density gradient medium.

1.2.3. Carefully overlay the 1 mL peritoneal cell suspension on top of the 54.8% density gradient medium layer slowly with sharp pipette tips (**Figure 1A**).

NOTE: Exercise caution to avoid disturbing the interface between the cell suspension and 54.8% density gradient medium.

1.2.4. Centrifuge at 1,500 x *g* for 30 min at 22 °C without braking.

1.2.5. Collect the neutrophils at the interface of the 54.8% density gradient medium and 70.2% density gradient medium layers (**Figure 1B**) to a new tube.

1.2.6. Add 1 mL of RPMI-1640 complete medium to the collected cells and carefully resuspend cells by gently pipetting several times. Centrifuge at 100 x *g* for 10 min at RT and carefully remove the supernatant.

1.2.7. Repeat the wash step (step 1.2.6) once.

1.2.8. Add 0.5 mL of culture medium to the pellet and resuspend cells by gently pipetting several times. Take a 50 µL aliquot to count the cells using an automatic hematology analyzer.

2. Neutrophil migration assay

2.1. Measure neutrophil migration by Transwell assay[9] or Zigmond chamber assay as previously described[10,11].

3. Air pouch assay

3.1. First air injection

3.1.1. On day 0, anesthetize mice with 5% isoflurane for 3 min in a gas anesthesia chamber at a speed of 2 L/min, and maintain the anesthesia of each mouse in a single breathing unit with 2% isoflurane at a speed of 0.5 L/min.

3.1.2. Use a 0.22 µm filter attached to a 5 mL syringe to obtain a 3 mL volume of sterilized air.

3.1.3. Lift the back skin of the anesthetized mouse with tweezers and subcutaneously inject 3 mL of sterilized air using a 26 G x 3/8" needle.

3.1.4. After treatment, remove the mice from the breathing unit. Monitor the mice to ensure they are alive until they start to move around.

3.2. Second air injection

3.2.1. On day 3, inject an additional 3 mL of sterilized air into the previously established air pocket to sustain the air pouch as described in section 3.1.

3.3. Treatment

3.3.1. On day 6, 6 h before sacrifice, inject different treatments into the air pouch. Inject 1 mL of

phosphate-buffered saline (PBS) as a negative control. Inject 1 mL of 1 µg/mL LPS as the positive control to induce local inflammation.

3.3.2. Anesthetize and sacrifice mice as described in step 1.1.5. Prepare wash buffer according to **Table of Materials**.

3.3.3. For each air pouch, wash the air pouch with 1 mL of wash buffer and collect the inflammatory exudate in a 15 mL centrifuge tube. Wash the air pouch with 2 mL of wash buffer 2x and collect the inflammatory exudate in the same centrifuge tube.

3.3.4. Centrifuge at 100 x *g* for 10 min at RT. Discard the supernatant and resuspend cells in 1 mL of wash buffer. Count the cells to quantify the neutrophil ratio using the automatic hematology analyzer.

NOTE: See representative results in **Figure 2**.

4. Induction of the adjuvant-induced arthritis (AA) mouse model

4.1. Suspend complete Freund's adjuvant (CFA) by vortexing at least 5 s, then draw 100 µL of suspension into an insulin injector.

4.2. Anesthetize mice as described in step 3.1.1.

4.3. Mark the chosen paw and inject 20 µL of CFA into the ankle joint space. Inject 20 µL of suspension into four periarticular spots on the chosen paw (80 µL in total).

4.4. Remove mice from the breathing unit and put the processed mice in a new chamber. Monitor mice to ensure that they are breathing until they regain the ability to move.

4.5. Every 3 days, assess the joint diameter by measuring the ankle joint diameter using a pocket thickness gauge (**Figure 3A**).

4.6. Every 3 days, assess arthritis severity by arthritis scoring criterion (**Figure 3C**): 0, normal, no evidence of erythema and swelling; 1, the mildest arthritis, erythema and mild swelling confined to the tarsals or ankle joint; 2, moderate arthritis, erythema and mild swelling extending from the ankle to the tarsals; 3, severe arthritis, erythema and moderate swelling extending from the ankle to metatarsal joints; 4, the most severe arthritis, erythema and severe swelling encompass the ankle, foot and digits, or ankylosis of the limb.

5. Immunohistochemical staining of joint sections

5.1. Joint isolation

5.1.1. Sacrifice the mouse in section 4 using cervical dislocation after anesthesia with isoflurane.

Spray the mouse with 70% ethanol.

5.1.2. Remove the skin and part of the muscle from the hind leg with tweezers and scissors. Spray the joint with 70% ethanol and remove the rest of the muscles using a paper towel.

5.1.3. Fix the ankle joint in 4% paraformaldehyde for 2 days at RT. Decalcify the joint in 10% EDTA for 1 month at RT and change the medium weekly.

5.1.4. Embed the tissue in paraffin and prepare 4-µm-thick tissue sections.

5.1.4.1. Place tissue in a marked mold with certain volume of liquid paraffin. Cool briefly.

5.1.4.2. Set the thickness at 4 µm and cut slices on a microtome. Float sections in a 43 °C water bath.

5.1.4.3. Mount sections onto slides and dry overnight. Preserve the slides at RT.

5.2. Safranin O and fast green staining of joint sections

NOTE: The following staining steps are conducted at RT.

5.2.1. Place the slides from step 5.1.4.3 in a rack and perform the following washes to rehydrate at RT: xylene for 5 min (3x), 100% ethanol for 2 min (2x), 95% ethanol for 2 min (2x), 70% ethanol for 2 min, and 50% ethanol for 15 min. Wash in running tap water for 10 min.

5.2.2. Stain in 0.1% fast green solution for 5 min. Rinse in 1% acetic acid for 10 s.

5.2.3. Stain in 0.1% safranin O staining solution for 20 min. Immerse the slides in the following washes: 95% ethanol for 2 min (2x), 100% ethanol for 2 min (2x), and xylene for 2 min (2x).

5.2.4. Mount the tissue sections and observe the tissues under a microscope.

NOTE: See representative images in **Figure 4A**.

5.3. Immunohistochemical staining to visualize neutrophils

5.3.1. Bake the paraffin sections for 2 h at 78 °C. Place the slides in a rack and perform the following washes to rehydrate at RT: xylene for 15 min (2x), 100% ethanol for 5 min (2x), 95% ethanol for 5 min, 80% ethanol for 5 min, H_2O for 3 min, and PBS for 3 min.

NOTE: Do not let slides dry at any time during this step.

5.3.2. Add one drop of permeabilization buffer to cover the tissue. Incubate sections in a humidity-controlled tray at 37 °C.

5.3.3. Rinse slides in PBS for 3 min (3x). Avoid rinsing the tissue directly.

5.3.4. Perform heat-induced antigen epitope retrieval using a pressure boiler.

5.3.4.1. Arrange slides in a rack. Immerse slides in the pressure boiler filled with retrieval buffer.

5.3.4.2. Put the pressure boiler on a microwave oven. Set the microwave oven at 600 W and heat the slides for 10 min.

5.3.4.3. After boiling, keep slides in the boiler to cool to 90 °C. Take out the slides and rinse them in PBS for 3 min (3x).

5.3.5. Quench endogenous peroxidase activity in freshly prepared 3% H_2O_2 at RT for 15 min. Rinse slides in PBS for 3 min (3x).

5.3.6. Outline a large circle around the sample with a hydrophobic pen, avoid touching the sample. Block with 3% bovine serum albumin (BSA) in a humidity-controlled chamber at 37 °C for 60 min.

5.3.7. Remove blocking solution. Add 50 µL of PBS-diluted primary antibody to each section quickly. Then, incubate the slides in a humidity-controlled tray at 4 °C overnight.

NOTE: Different dilution ratios are used for different antibodies (1:25 for MPO and 1:20 for NE).

5.3.8. On the second day, take out the tray and let it stand at RT for 30 min. Then, rinse the slides in PBS for 3 min (3x).

5.3.9. Add 50 µL of PBS-diluted secondary antibodies to the tissue.

NOTE: Different dilution ratios were applied: 1:1,000 for MPO and 1:1,500 for NE.

5.3.10. Incubate slides in a humidity-controlled tray at 37 °C for 30 min. Then, rinse the slides in PBS for 3 min (3x).

5.3.11. Develop in diluted 3,3’-diaminobenzidine (DAB) solution for 5 min. Keep an eye on the reaction in case of the development of a dark color. Rinse the slides in distilled water.

5.3.12. Counterstain the slides in hematoxylin for 10 s. Rinse the slides in tap water for 5 min.

5.3.13. Rinse in acid alcohol superfast differentiation solution for 3 s. Then rinse in tap water for 10 min.

5.3.14. Immerse the slides in the following washes at RT: 80% ethanol for 5 min, 95% ethanol for

5 min, 100% ethanol for 5 min, and xylene for 15 min (2x). Fix the coverslip with mounting solution. Observe the tissue under a microscope.

NOTE: See representative images in **Figure 4B**.

REPRESENTATIVE RESULTS:

Peritoneal exudate cells were collected from lavage fluid of mice. Cells were resuspended in 1 mL of RPMI-1640 complete medium, layered onto a two-step (54.8%/70.2%) discontinuous density gradient (**Figure 1A**), and centrifuged at 1,500 x *g* for 30 min. Neutrophils (≥95%, ~1 x 10^7 neutrophils/mouse) were recovered from the lower interface (**Figure 1B**).

Air pouch experiments were performed to investigate the neutrophil recruitment stimulated by LPS in vivo (**Figure 2A**). The leukocyte subsets in the air pouch exudates were measured (**Figure 2B**).

Neutrophil migration in RA was evaluated via the CFA-induced arthritis murine model. Compared with the control group, the AA group showed significant edema in the paw. In the AA group, the ankle joint diameter increased (**Figure 3B**) and the arthritis score rose consistently (**Figure 3D**).

Cartilage damage is the representative syndrome of RA, safranin O-fast green cartilage staining was performed to assess the cartilage damage in AA mouse. As shown in **Figure 4A**, CFA challenge induced a large amount of leukocyte infiltration, significant cartilage erosion and synovial hyperplasia. MPO and NE expression levels are representative markers of neutrophil infiltration. Immunohistochemical assays were performed to observe neutrophil infiltration in joints. MPO and NE expression was significantly upregulated in the joint section (**Figure 4B**).

FIGURE LEGENDS:

Figure 1: Neutrophil isolation. (**A**) Peritoneal exudate cells resuspended in 1 mL of RPMI-1640 complete medium were layered onto a two-step (54.8%/70.2%) discontinuous density gradient. (**B**) After centrifugation at 1,500 x *g* for 30 min, neutrophils (≥95%, ~1 x 10^7 neutrophils/mouse) were recovered from the lower interface.

Figure 2: Representative results of the air pouch assay. (**A**) Illustration of the air pouch assay. (**B**) Representative results of leukocyte subset infiltration in the air pouch assay. PBS: control; LPS: 1 μg/mL LPS. Data are presented as the mean ± SD.

Figure 3: Representative results of the adjuvant-induced arthritis (AA) mouse model. (**A**) The ankle joint diameter was measured using a pocket thickness gauge. (**B**) Joint swelling was assessed based on the ankle joint diameter (n = 7). (**C**) Pictures of each arthritis score. (**D**) The severity of arthritis was graded on a scale of 0−4 points (n = 7). Data are presented as the mean ± SD.

Figure 4: Representative results of safranin O-fast green cartilage staining and immunohistochemical assay of joint sections from control and AA mice. (**A**) Representative

results of safranin O-fast green cartilage staining of joint sections. (**B**) Representative results of the expression level of MPO and NE in the ankle joints.

DISCUSSION:

Detailed protocols of highly-purified neutrophils from peripheral blood[7], bone marrow and tissues[18] have been available for a long time. Here we adopt a method of isolating neutrophils from peritoneal fluid[19] in which mature neutrophils remain inactivated for further anti-inflammatory and antioxidant studies.

We used the air pouch experiment to explore the LPS-induced infiltration of neutrophils in vivo. This method has been proposed as a potential method for directly measuring cell infiltration in the general inflammatory environment in vivo.

It is noteworthy that the air pouch assay only demonstrates the neutrophil's function in an organ-nonspecific manner and removes several steps of the leukocyte recruitment cascade. Since neutrophil recruitment to specific organs can rely on different organ properties, adhesion molecules, and chemokines, exploring the neutrophil functional state in organ-specific conditions is of great importance for studying the role that neutrophils potentially play in certain diseases[3]. It has been suggested that endpoint models are required to investigate neutrophil infiltration. Therefore, performing immunohistochemical staining on the joint sections of mice in the AA model can provide an insightful perspective on the neutrophils in the joint space. According to our data, vast numbers of neutrophils are recruited into the joint tissue and serve as fundamental evidence for further research on interrupting the infiltration of neutrophils to treat RA. In addition, comprehensive assays, for example, safranin O-fast green staining, are required to evaluate the disease model for further study.

Neutrophils are the major subset of infiltrating inflammatory cells and work as the first line of defense against invading pathogens or tissue injury[20,21]. If neutrophils infiltrate tissues in large numbers, high levels of cytokines and NETs are secreted, which together may overwhelm the protective mechanisms in tissues and lead to tissue damage. Tissue injury further stimulates neutrophil infiltration, thus forming a vicious cycle[22]. Interfering with cell migration by means of trapping activated cells in lymphoid organs has been proposed as an important therapeutic approach and has been applied in clinical trials with various side effects[23]. Discovering a novel treatment to concurrently regulate neutrophil migration and inflammatory activity is a promising strategy for treating inflammatory diseases in the future. Furthermore, if motility-regulating agents are combined, neutrophil-mediated drug delivery[24] can increase drug specificity, thus decreasing side effects.

However, further modifications can be combined to broaden the application of the above-mentioned assays. For example, in the AA mouse model, to obtain the overall impression of infiltration of inflammatory cells in the joint, researchers are encouraged to enzymatically digest joints to release the resident leukocyte populations and then apply flow cytometry to count the populations.

The protocols herein include three ways to assess neutrophil migration and infiltration. The application of these protocols is useful for discovering potential treatments for RA and other inflammatory diseases involving neutrophils.

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DISCLOSURES:

The authors have nothing to disclose.

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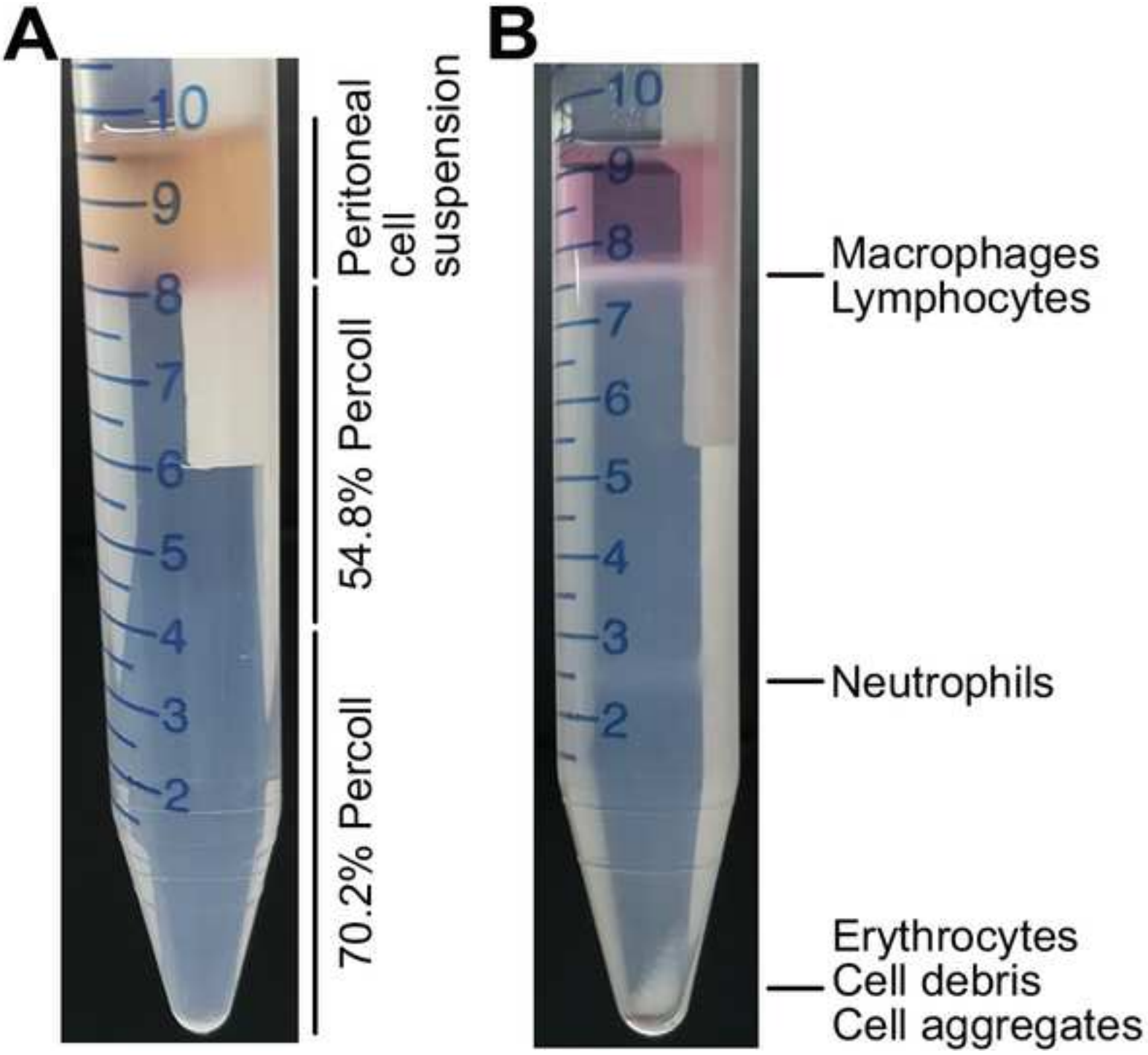

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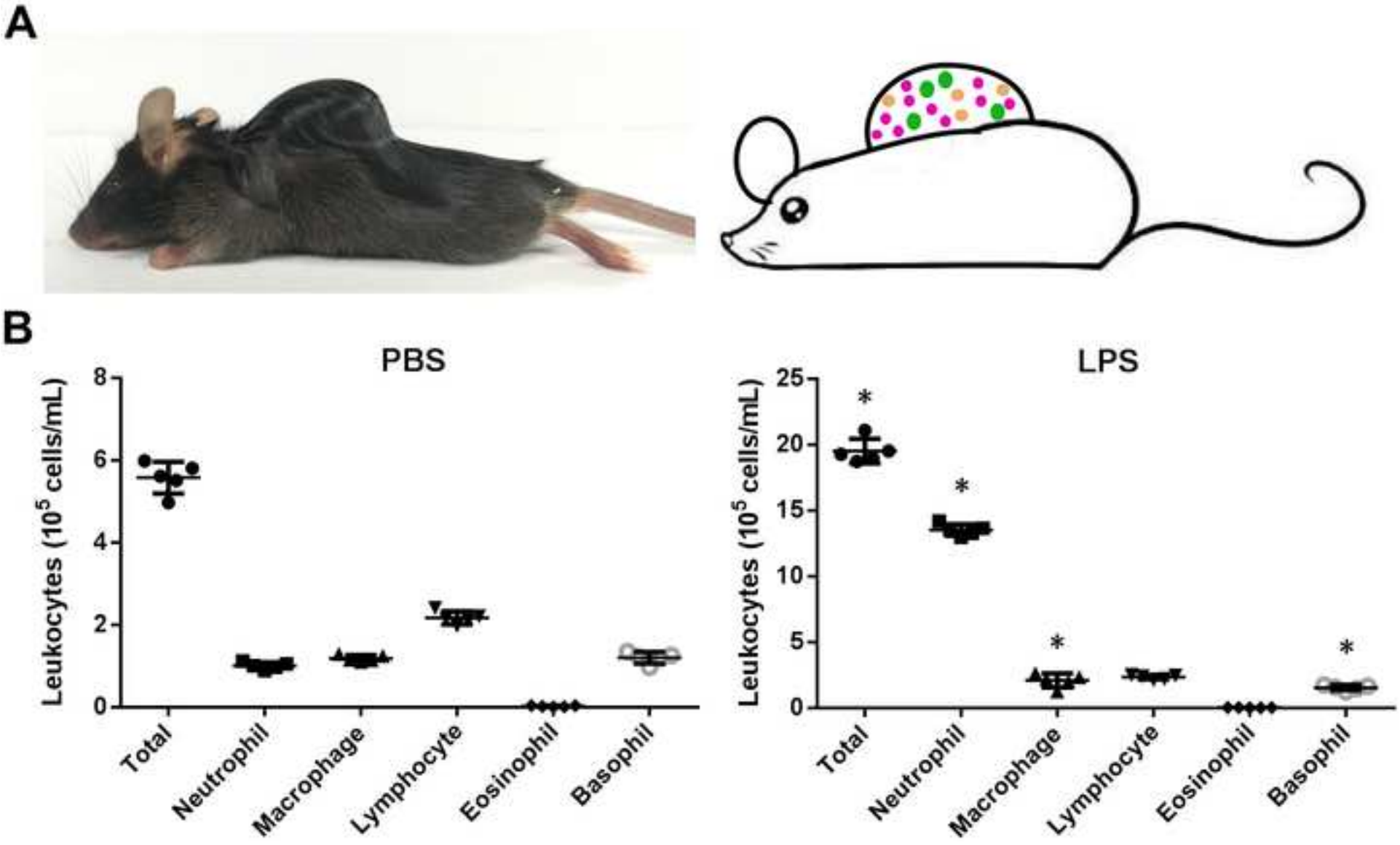

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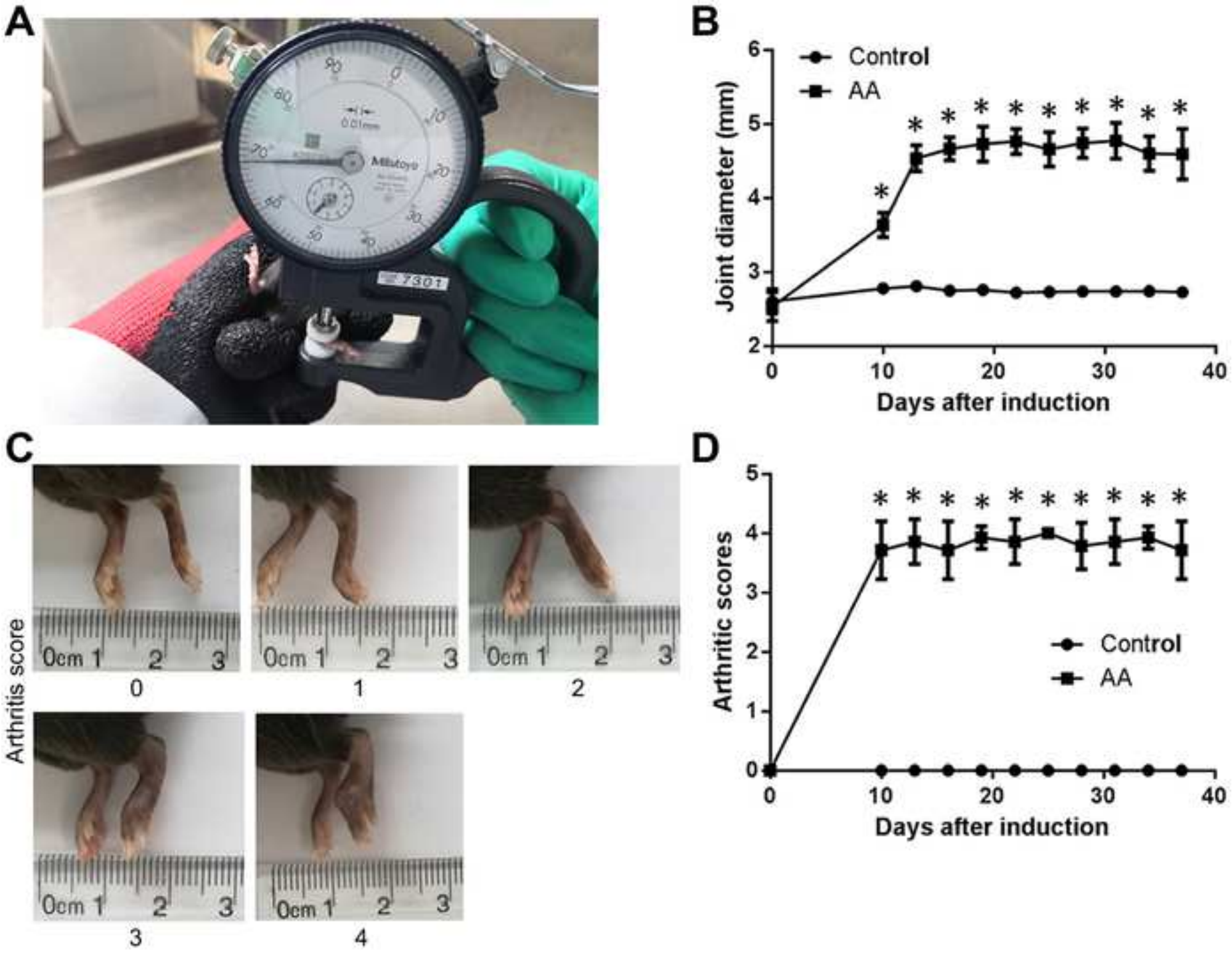

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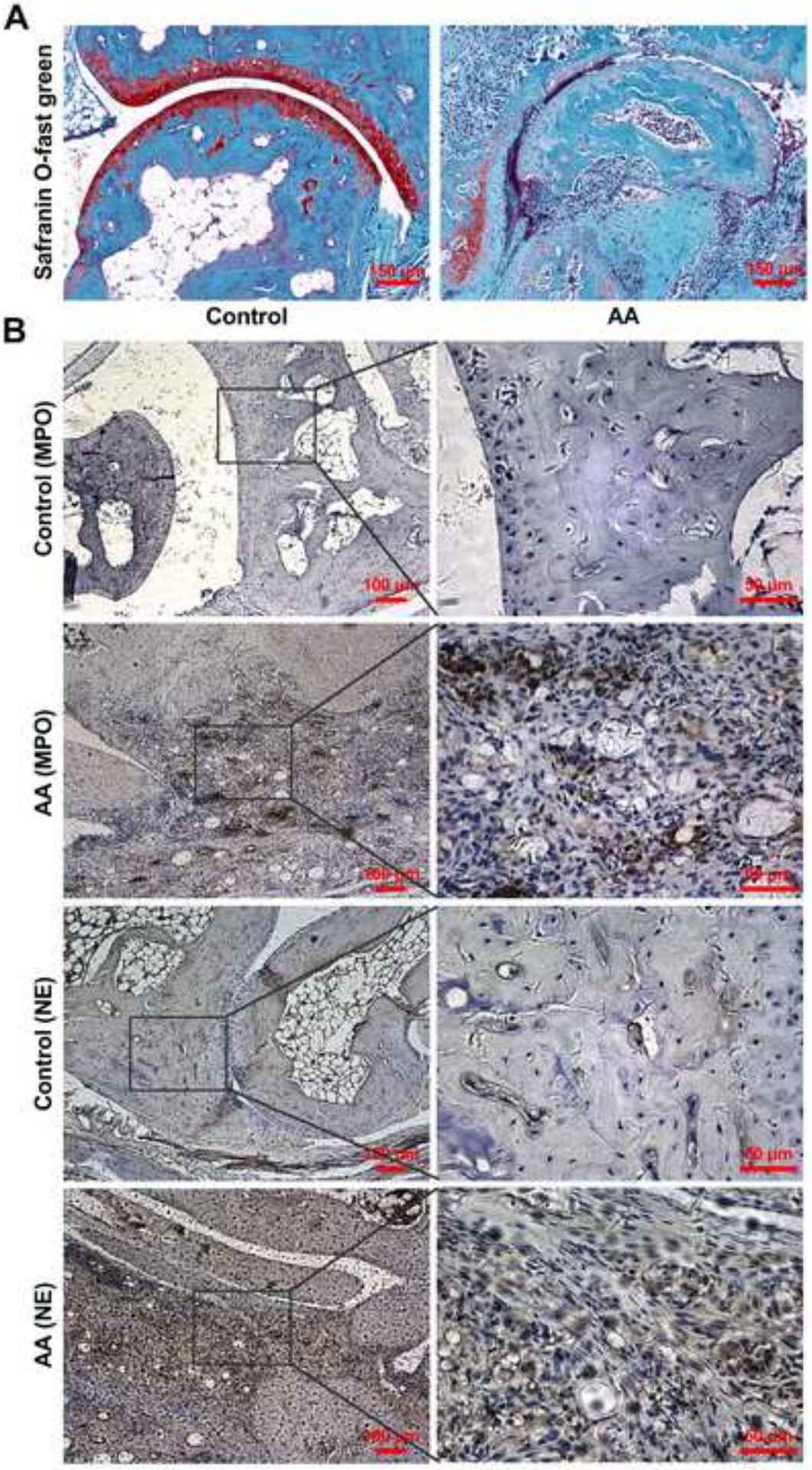

Name of Material/Equipment	Company	Catalog Number
0.1% Fast Green Solution	Solarbio	8348b
0.1% Safranin O Staining Solution	Solarbio	8348a
0.5 M Ethylenediaminetetraacetic acid solution (EDTA), pH 8.0	Sigma	324506
100% Ethanol	Beijing Chemical Works	
100% Methanol	Beijing Chemical Works	
15 mL Conical Polypropylene Centrifuge Tube	Falcon	14-959-53A
23 G x 1 1/4" Needle	BD	305120
26 G x 3/8" Needle	BD	305110
3% bovine serum albumin (BSA)		
3% H_2O_2		
3,3'-diaminobenzidine (DAB) kit	ZSGB-BIO	ZLI-9018
30 G x 1/2" Needle	BD	305106
30% H_2O_2	Beijing Chemical Works	
5 mL Syringe	BD	Z683574
50 mL Conical Polypropylene Centrifuge Tube	Falcon	14-432-22
50% Ethanol		

54.8% Percoll		
70% Ethanol		
70.2% Percoll		
80% Ethanol		
95% Ethanol		
Acid Alcohol Superfast Differentiation Solution	Beyotime	C0165S
ANTIBODIES		
Anti-Myeloperoxidase Antibody	Abcam	ab208670
Anti-Neutrophil Elastase Antibody	Abcam	ab21595
Automatic Hematology Analyzer	Sysmex	XS-800i
Bovine Serum Albumin (BSA)	VWR	0332-100G
Complete Freund's Adjuvant, 10 mg/ml	sigma	1002036152
Cover Slip	CITOGLAS	10212432C
Dial Thickness Gauge	Mitutoyo	7301
Eppendorf Microtubes, 1.5 mL	Sigma	Z606340
Foetal Bovine Serum (FBS) Premium	PAN	P30-1302
Gas Anesthesia System	ZS Dichuang	ZS-MV-IV
Goat Anti-Rabbit IgG H&L (HRP)	PPLYGEN	C1309
Hank's Balanced Salt Solution (HBSS)	Biological Industries, Beth HaEmek, Israel	02-016-1A
Hematoxylin Staining Solution	ZSGB-BIO	ZLI-9609
Lipopolysaccharide (LPS)	Sigma	L3012
MEDIA AND SUPPLEMENTS		
Modified Safranin O-fast Green FCF Cartilage Stain Kit	Solarbio	G1371
N-formyl-Met-Leu-Phe (fMLP)	Sigma	47729

Penicillin Streptomycin Solution, 100×	Invitrogen	1514022
Percoll	GE Healthcare	10245207
Permeabilization Buffer		
Phosphate Buffer Saline (PBS), 1×		
Phosphate Buffer Saline (PBS), 10×		
PLASTIC WARES AND EQUIPMENTS		
POWDER		
Proteose Peptone	Oxoid	1865317
Retrieval Buffer		
Retrieval Buffer A Stock for IHC		
Retrieval Buffer B Stock for IHC		
Roswell Park Memorial Institute (RPMI)-1640 medium	Sigma	R8758
RPMI-1640 Complete Medium		
Shu Rui U40 Disposable Sterile Insulin Injection Needle 1 mL	BD	328421
Slide	CITOGLAS	10127105P-G
SOLUTION		
Stock Isotonic Percoll (SIP)		
Wash Buffer in Air Pouch Assay		
Xylene	Beijing Chemical Works	

Comments/Description

Transfer 20 mg of fast grene FCF in one vial into another 100 mL beaker.Add 20 mL of H_2O into the beaker and dissolve the stain by stirring to make 0.1% fast green solution, and filter it using a Nalgene PES 75mm filter

Transfer 20 mg of safranin O stain in one vial into a 100 ml beaker. Add 20 mL of H_2O into the beaker and dissolve the stain by stirring to make 0.1% safranin O staining solution, and filter it using a Nalgene PES 75mm filter

Sterile

Dissolve 0.3 g BSA in 10 mL PBS
Mix 1 mL 30% H_2O_2 with methanol with 9 mL methanol

Mix 500 mL 100% ethanol with 500 mL dH_2O

Mix 2.74 mL SIP with 2.26 mL 1×PBS, stand still

Mix 700 mL 100% ethanol with 300 mL dH_2O

Mix 3.51 mL SIP with 1.49 mL 1×PBS, stand still

Mix 800 mL 100% ethanol with 200 mL dH_2O

Mix 950 mL 100% ethanol with 50 mL dH_2O

This is the secondary antibody used in the immunohistochemical staining.

Sterile

Density gradient medium
Mix 100 µL Triton X-100 with 1 L dH_2O to get 0.01% Triton X-100

Mix 90% ddH_2O with 10% (v/v) 10×PBS, autoclaved

Disslove 16 g NaCl, 0.4 g KCl, 2.88 g $Na_2HPO_4.2H_2O$, 0.48 g KH_2PO_4 (anhydrous) in 200 mL ddH_2O, adjust pH 7.4, autoclaved

Mix 18 mL retrieval buffer A with 82 mL retrieval buffer B, add dH_2O to 1000 mL, adjust pH to 6.0
Dissolve 4.2 g citric acid ($C_6H_5O_7.H_2O$) in 200 mL dH_2O

Dissolve 5.88 g trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_20$) in 200 mL dH_2O

RPMI-1640 medium is supplemented with 10% FBS and 1% penicillin/streptomycin.

Mix 90% (v/v) of percoll with 10% (v/v) 10×PBS, stand still for 20 min
Dilute 0.5M EDTA to 10mM with HBSS

Rebuttal Letter

Dear Editors and Reviewers:

We appreciate the comments from the reviewers and the opportunity to improve the quality of our manuscript (Submission No: JoVE60543).

We have carefully addressed all the issues raised by the reviewers, and we have tried to improve this revision, we wish it could meet the high standards of the reviewers and this journal. The modifications are highlighted in red in the revised version of the manuscript.

Protocol text need to be featured in the video is highlighted in yellow.

Our point-by-point responses to reviewers' critiques are included below in blue.

I am looking forward to hearing from you.

Sincerely,

Guangrui Huang, Ph.D.

Associated Professor of Microbiology and Biochemical Pharmacy

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Tel: +86-10-64286526 Fax: +86-10-64286526

E-mail: hgr@bucm.edu.cn (Guangrui Huang).

Reviewers' comments:

Reviewer #2:

Manuscript Summary:

This is a very interesting work, however, the resolution of the histological zones could be better.

Response: We agree with the Reviewer and we have replaced revised Figure 4 with high resolution pictures accordingly.

Minor Concerns:

I think that the figure 1 was not necessary included

Response: We agree with the Reviewer and we have deleted Figure 1.

Reviewer #4:

Manuscript Summary:

This manuscript describes three frequently used methods for evaluating the migration of normal neutrophils induced both in vivo and in vitro, as well as the infiltration of pathological neutrophils in a mouse joint-specific inflammation model.

The manuscript clearly sets out its objective and describes the protocols in a very straight forward manner with enough detail to repeat the procedures. The advantages of using these procedures and manners in which they can be modified to broaden their applications are nicely discussed. These methods are important methods for those working in the field and I would enjoy seeing the published videos.

Minor Concerns:

Perhaps I missed this, but it isn't clear to me what High-solution Buffer A/ Low solution buffer B and Retrieval buffers A/B are. Are these buffers purchased ready, if not can authors provide the recipes?

Response: We are sorry for our carelessness. The recipes have been included in the **Material** lists. For clarity, we have replaced “high-solution buffer A” with “70.2% Percoll” and replaced “low solution buffer B” with “54.8% Percoll”.

Reviewer #5:

Manuscript Summary:

The manuscript contains the methodologic details to evaluate the capacity of murine neutrophils to in vivo migrate in the inflammatory sites, by using the "air pouch assay" and the "adjuvant-induced arthritis". Even if no particular originality and novelty can be observed, the manuscript contains important technical information that can be useful for other researchers in the field. However, a more detailed and updated introduction is

needed.

Response: We do appreciate Reviewer's suggestion. We have added "Traditionally, neutrophils were considered as pathogen eliminators in acute phases of inflammation. However, recent findings have demonstrated that neutrophils are complicated cells capable of a significant array of specialized functions. Neutrophils are able to regulate many processes such as acute injury and repair, tumorigenesis, autoimmune response, and chronic inflammation[12,13]. Neutrophils also modulate adaptive immune responses and can regulate B cells and T cells[14,15]. Substantial shortage of neutrophils leads to mortality or severe immunodeficiency in humans and depletion of neutrophils in mice also leads to fatality. While excessive activation or recruitment of neutrophils in organs causes several immune diseases, such as RA and systemic lupus erythematosus (SLE)[6]." in the Introduction section at line 73-81.

Major Concerns:

A Figure related to protocol 1. peritoneal neutrophils and also some comments on the level of activation can be useful to understand the number and the state of neutrophils that can be obtained with this protocol.

Response: We agree with the reviewer. New Figure 1 has been added to illustrated neutrophil isolation.

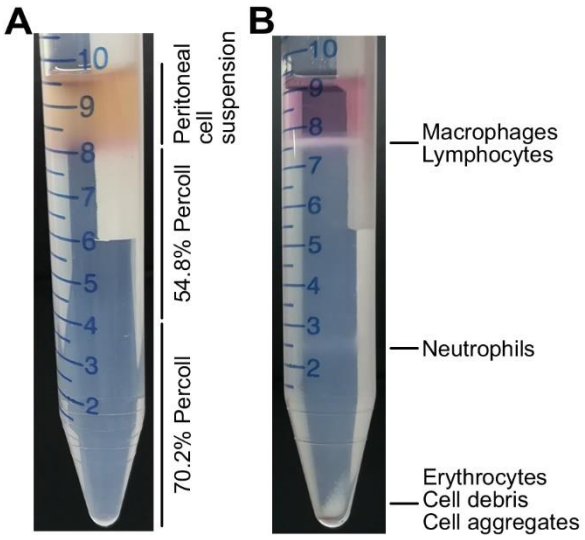

Figure 1: Neutrophil isolation. (A) Peritoneal exudate cells were collected from lavage fluid

of mice. Cells were resuspended in 1 ml of RPMI-1640 complete medium, layered onto a two-step (54.8%/70.2%) discontinuous Percoll gradient. (B) After centrifuged at 1500 ×g for 30 min at 22 °C, neutrophils (≥ 95%, approximately 1×107 neutrophils/mouse) were recovered from the lower interface.

Line 196: the grade arthritis severity should be better described: what the authors intend as mild, moderate, severe, the most severe? Figures related to different grading can be very useful to reproduce the protocol. In addition, picture showing the measurements with pocket gauge can be very useful too.

Response: We do appreciate Reviewer's suggestion. We have added "4.7. Every 3 days, assess arthritis severity by arthritis scoring criterion. 0, normal, no evidence of erythema and swelling; 1, the mildest arthritis, erythema and mild swelling confined to the tarsals or ankle joint; 2, moderate arthritis, erythema and mild swelling extending from the ankle to the tarsals; 3, severe arthritis, erythema and moderate swelling extending from the ankle to metatarsal joints; 4, the most severe arthritis, erythema and severe swelling encompass the ankle, foot and digits, or ankylosis of the limb." at line 206-211.

We have added pictures of each arthritis score and measurement of ankle joint diameter using a pocket thickness gauge to revised Figure 3.

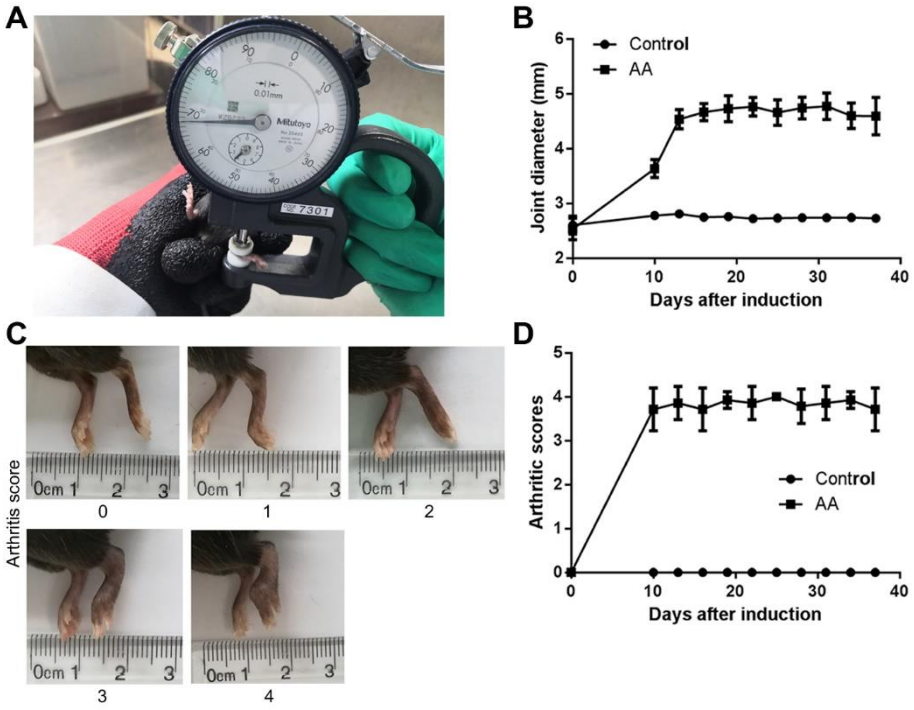

Figure 3: Representative results of the adjuvant-induced arthritis (AA) mouse model. (A)

Measurement of ankle joint diameter using a pocket thickness gauge (B) Joint swelling was assessed by measuring the ankle joint diameter (n=7). (C) Pictures of each arthritis score. (D) The severity of arthritis was graded on a scale of 0-4 points (n=7). Data are presented as the mean ± SD.

Minor Concerns:

Line 85: please, indicate the solution used to dissolve proteose peptone, which, I assume, may represent the negative control (please, specify).

Response: Done as advised.

Line 130: please indicate what high-isolation buffer A is.

Response: The recipe has been included in the **Material** lists.

Line 132: please indicate what low-isolation buffer A is.

Response: The recipe has been included in the **Material** lists.

Line 137: please indicate the volume of peritoneal cell suspension.

Response: Done as advised.

Line 177: wash buffer is not specified in the list.

Response: The wash buffer has been included in the **Material** lists as “wash buffer in air pouch assay”.

Line 186: in which solution is suspended CFA? Final concentration?

Response: CFA was purchased from sigma, which can be used directly. However, when stored at 4 °C, precipitation usually occurs in CFA solution and suspending is recommended before using. We have added “by vortex at least 5 seconds” at line 197.

Line 190: please, specify which joint (ankle? knee?)

Response: Done as advised.

Line 235: please specify what retrieval buffer is.

Response: The recipe has been included in the **Material** lists.

Line 287: it is not clear which parts of the joints are originated Figure B. A minor magnification where Figure B derives can be very useful.

Response: We do appreciate Reviewer’s suggestion. We have revised the Figure 4 as advised.

Line 297: statistics is missing

Response: We have revised Figure 2 as advised.

Line 303: statistics is missing

Response: We have revised Figure 3 as advised.

Line 307: as already mentioned it is not clear the origin of Figure B

Response: Done as advised.

Line 315: no anti-inflammatory or antioxidant studies are shown in peritoneal neutrophils

Response: This study focused on the migration and infiltration of neutrophils. We have used peritoneal neutrophils to investigate the anti-inflammatory and/or antioxidant effects of tanshinone IIA, celastrol, triptolide and emodin on arthritis. Our published articles are listed as follows:

1. Zhang S, Huang G, Yuan K, et al. Tanshinone IIA ameliorates chronic arthritis in mice by modulating neutrophil activities. Clin Exp Immunol. 2017 Oct;190(1):29-39.
2. Yuan K, Huang G, Zhang S, et al.Celastrol alleviates arthritis by modulating the inflammatory activities of neutrophils. Journal of Traditional Chinese Medical Sciences. 2017 Jan; 4(1):50-58.
3. Huang G, Yuan K, Zhu Q, et al. Triptolide inhibits the inflammatory activities of neutrophils to ameliorate chronic arthritis. Mol Immunol. 2018 Sep;101:210-220.
4. Zhu M, Yuan K, Lu Q, et al. Emodin ameliorates rheumatoid arthritis by promoting neutrophil apoptosis and inhibiting neutrophil extracellular trap formation. Mol Immunol. 2019 Aug;112:188-197.

The Name of Material/equipment needs some revision. For example, high and low solutions A and B as well as retrieval buffer A and B and wash buffer need to be specified.

Response: We are sorry for our carelessness. The recipes have been included in the **Material** lists. For clarity, we have replaced “high-solution buffer A” with “70.2% Percoll” and replaced “low solution buffer B” with “54.8% Percoll”.

Comments lack the end of the sentence: The secondary antibody used in the immunohistochemical staining……what?

Response: We are sorry for our carelessness. We have corrected the error.

An accurate editing of English is suggested throughout the manuscript.

Response: Done as advised.

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Author(s): Qingyi Lu, Kai Yuan, Haixu Jiang, Lu Zhao, Hesong Wang, Wei Wang, Guangrui Huang, Anlong Xu

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