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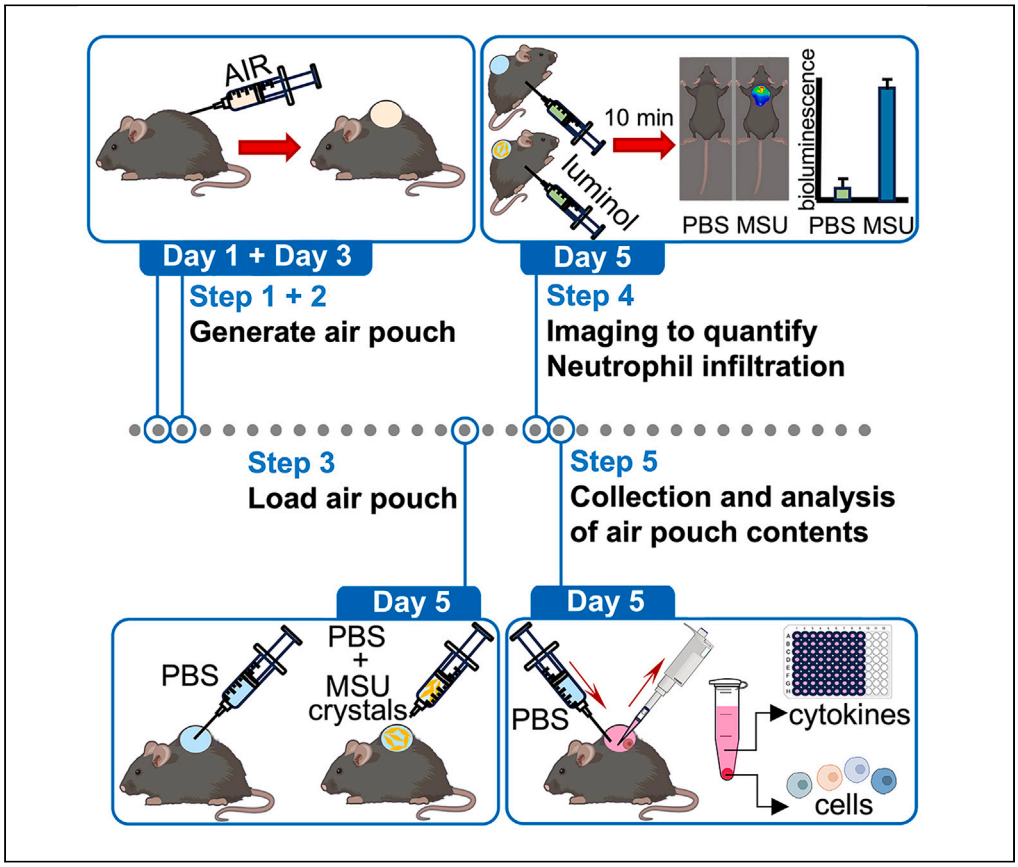
Protocol to create a murine subcutaneous air pouch for the study of monosodium urate crystal-induced gout



Monosodium urate (MSU) crystal deposition in articular joints and bursal tissue causes acute joint inflammation, which is a hallmark of gout. Here, we describe the steps necessary to create a subcutaneous air pouch on the back of mice that resembles this bursa-like space with a synovial lining-like membrane. We then detail the injection of MSU crystals into this pouch, which induces a localized inflammatory response reminiscent of gout and approaches to quantify the inflammatory response.

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

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Highlights

Protocol for generating a subcutaneous air pouch in mice

Instructions for *in vivo* imaging of MPO activity in the subcutaneous air pouch

Instructions for obtaining lavage fluid from the subcutaneous air pouch

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Protocol to create a murine subcutaneous air pouch for the study of monosodium urate crystal-induced gout

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SUMMARY

Monosodium urate (MSU) crystal deposition in articular joints and bursal tissue causes acute joint inflammation, which is a hallmark of gout. Here, we describe the steps necessary to create a subcutaneous air pouch on the back of mice that resembles this bursa-like space with a synovial lining-like membrane. We then detail the injection of MSU crystals into this pouch, which induces a localized inflammatory response reminiscent of gout and approaches to quantify the inflammatory response.

For complete details on the use and execution of this protocol, please refer to Devi et al. (2023),[1] de Almeida et al. (2022),[2] and Ratsimandresy et al. (2017).[3]

BEFORE YOU BEGIN

The protocol below describes the steps required to generate the murine subcutaneous air pouch on the back of C57BL/6J mice[4] to model localized inflammatory responses induced by MSU crystals[5] that are reminiscent of gout.[6] The air pouch has been also used in BALB/c mice,[7,8] and has been used to study rheumatoid arthritis,[9] infection,[7] and cancer.[10] The murine air pouch is created by injecting sterile air subcutaneously on the dorsal region of the mice. The formation of the space/pouch induces the migration of fibroblast and mononuclear cells together with the generation of blood vessels in the membrane of the subcutaneous air pouch and therefore allows the study and manipulation of cellular and inflammatory response in a local environment. The time estimates listed in this protocol may vary depending on the number of animals and the experience and skill of the investigators.

Institutional permissions

Mice were maintained and all the steps were conducted according to the procedures approved by Cedars Sinai Medical Center committees on use and care of animals under protocol #IACUC008362, and adherence to the NIH guide for the care and use of laboratory animals. A prior approval from the relevant institutional animal ethical committee must be obtained for the performance of this experiment.

Reagent and equipment preparation

1. Prepare dissecting instruments, including scissors, surgical knives, and forceps, and sterilize them using appropriate methods.

2. Prepare instruments and tools, including isoflurane vaporizer, balance, centrifuge, vortex, electric shaver, Bioluminescence *in vivo* imager, 1 mL and 5 mL syringes, 25 gauge needles, and 0.22 μM syringe filters.
3. Prepare reagents, including MSU crystals, 1× PBS, luminol or XenoLight RediJect Inflammation probe.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DPBS	Corning	Cat#21-031-CV
MSU	InvivoGen	Cat#Tlrl-msu
Uric acid	Sigma-Aldrich	Cat# U262
Trypan blue solution 0.4%	Invitrogen	Cat#15250061
XenoLight RediJect Inflammation probe	PerkinElmer	Cat# 760536
Luminol sodium salt	Sigma-Aldrich	Cat# A4685
Experimental models: Organisms/strains		
Mouse: C57BL/6, any age and sex	The Jackson Laboratory	JAX#00066;RRID:IMSR_JAK:000664
Software and algorithms		
Living Image	PerkinElmer	N/A, RRID: SCR_014247
Other		
Isoflurane vaporizer	VetEquip	#911103
IVIS spectrum	PerkinElmer	N/A, RRID: SCR_020397
5 mL syringe	BD Medical	309646
1 mL syringe	BD Medical	309628
0.22 μm syringe filter	MilliporeSigma	SLGSM33SS
25 gauge needle	BD Medical	305127
Versapor-3000T 3 μm filter	Pall Life Sciences	82581H

STEP-BY-STEP METHOD DETAILS

Initial air pouch inflation

Timing: 90–120 min

1. Day 1: Select 12–16 weeks old mice, weigh the mice and divide them into 2 groups: treatment and control.

   Note: We usually use mice in this age range, where the air pouch can be easily inflated. For larger/heavier mice the volume of air injected may have to be adjusted to obtain an inflated air pouch, but we have not tested this in aged or obese mice.

2. Bring the mice to the animal procedure room.
   a. Organize your workspace.
   b. Anesthetize the mice using the method of choice.

   Note: We are using an isoflurane vaporizer from VetEquip (Figure 1).

3. Check that the mice are unconscious.

   Note: check with toe-pinch reflex.

4. Carefully and without much pressure shave the dorsal cervical/thoracic region of the mice using an electric shaver (Figure 2).

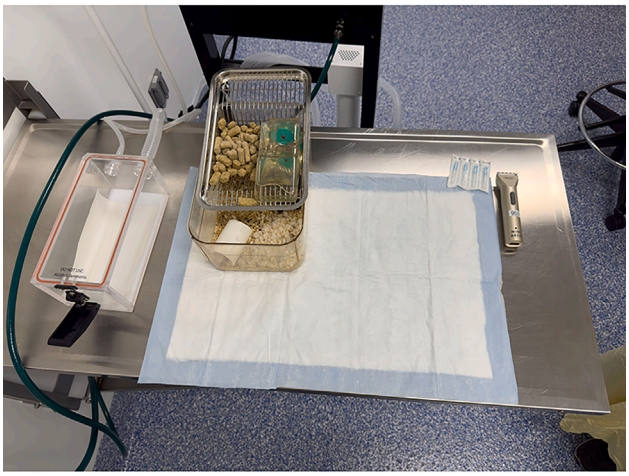

Figure 1. Organization of the workspace

⚠ CRITICAL: Taking great care to not damage and irritate the skin during shaving. Do not use a hair removal cream, as this causes significant skin inflammation which may affect your experiment and causes background when imaging inflammation.

5. Place a sterile 5 mL syringe, a sterile 0.22 μm syringe filter and a sterile 25 gauge needle in the biosafety cabinet.

   Note: You will need one set per mouse.

   Note: We thoroughly wipe all items brought under the biosafety cabinet with 70% ethanol.

6. Carefully attach a sterile 0.22 μM syringe filter to a sterile 5 mL syringe.
   a. Draw in 5 mL volume of air within a biosafety cabinet.

   Note: This will reduce contaminants to obtain sterilized air for inflation of the sub cutaneous air pouch as shown.

7. Remove the syringe filter and replace with the sterile and capped 25 gauge needle and place aside.

   Note: Prepare one syringe per mouse (Figure 3).

8. Disinfect the shaved area with sterile 70% alcohol wipes.
9. Slightly and carefully lift the shaved skin in the dorsal region with tweezers to provide a space for injection
   a. Carefully insert the 25 gauge needle attached to the sterile air filled syringe for about 3/8 inch
   b. While still lifting the skin with forceps and slowly subcutaneously inject the entire 5 mL of the sterilized air.

   Note: This will inflate the air pouch (Figure 4).

   ⚠ CRITICAL: Make sure to inject air under the skin and not into the muscle.

10. Place the mouse back into the cage. Observe the mice until they are fully conscious and start moving.

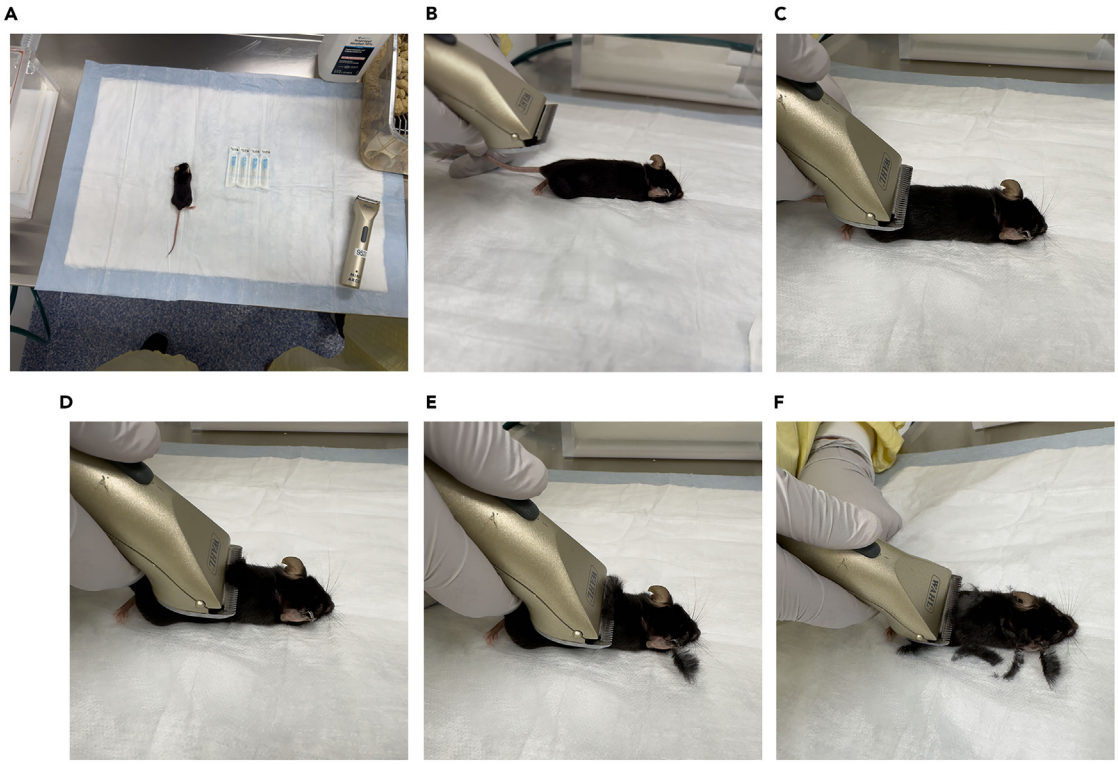

Figure 2. Shaving of mice
(A) Layout of our workspace after anesthetizing mice.
(B–F) Carefully shave mice with an electric shaver using several passes without nicking or otherwise damaging the skin.

Note: We single house mice after injection of the air pouch to prevent mice damaging the air pouch from grooming or fighting.

Note: The air pouch will create a cavity with a lining reminiscent to the synovium.

Re-inflation of the subcutaneous air pouch

Timing: 30–45 min

This step describes the re-inflation of the subcutaneous air pouch at day 3.

11. Day 3: After 3 days, the subcutaneous air pouch will be partially deflated, as notable by its smaller size.
12. Gently sterilize the air pouch with sterile 70% alcohol wipes as above.
13. Prepare a syringe filled with 3 mL of sterile air as above.
14. Carefully and very slightly lift the air pouch skin with forceps.

Note: This is possible due to the partial deflation to stabilize the point of injection.

CRITICAL: Make sure to inject the air into the air pouch.

15. Re-inflate the subcutaneous air pouch by injecting an additional 3 mL of sterilized air into the air pouch.

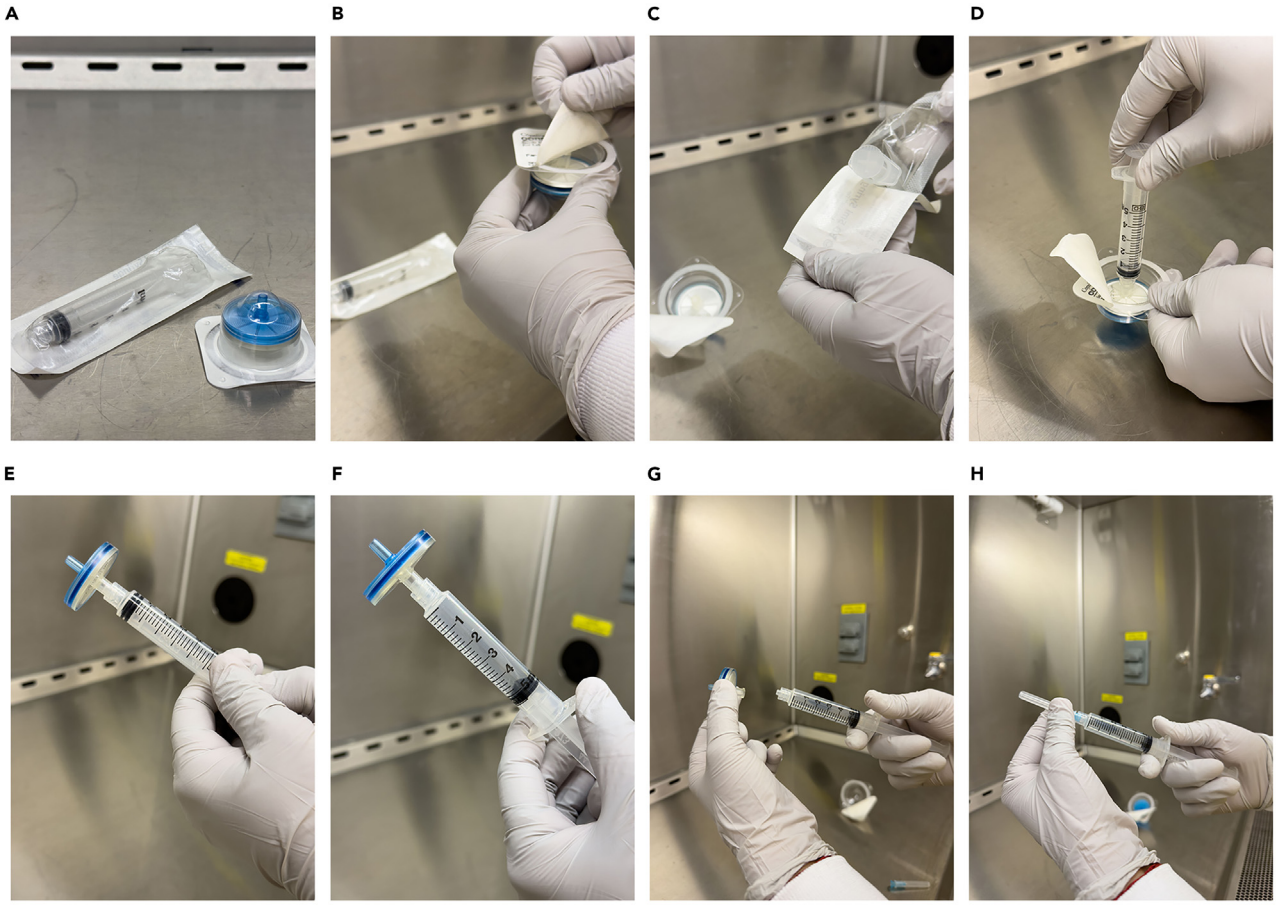

Figure 3. Preparation of sterile air

(A) You need a sterile packed 5 mL syringe, a sterile packed 0.22 μm syringe filter and a sterile 25 gauge needle placed in the biosafety cabinet.
(B and C) Open the filter and syringe and keep the filter in the plastic wrap.
(D) Connect the syringe to the filter by securing the filter through the plastic wrap.
(E and F) Draw 5 mL of air into the syringe.
(G) Remove the filter from the syringe.
(H) Connect a capped 25 gauge needle to the syringe and place aside.

Note: Inject into the top of the established air pouch (Figure 5).

Preparation of MSU crystals

Timing: 1 day

Note: Alternatively, MSU crystals are commercially available (see key resources table), but we usually prepare it in the laboratory because of the larger quantities required for *in vivo* injection.

16. Weigh 2 g of uric acid.
17. Add 300 mL of 0.01 M NaOH (need to be freshly prepared with 70°C deionized H_2O).
18. Adjust the pH to 7.1–7.2.
19. Filter solution through a 0.2 μm filter to remove any undissolved uric acid.
20. Incubate at RT by slowly and continuously stirring for 16–24 h.
21. Then add 2/3 of the volume of absolute ethanol while continue to stir.

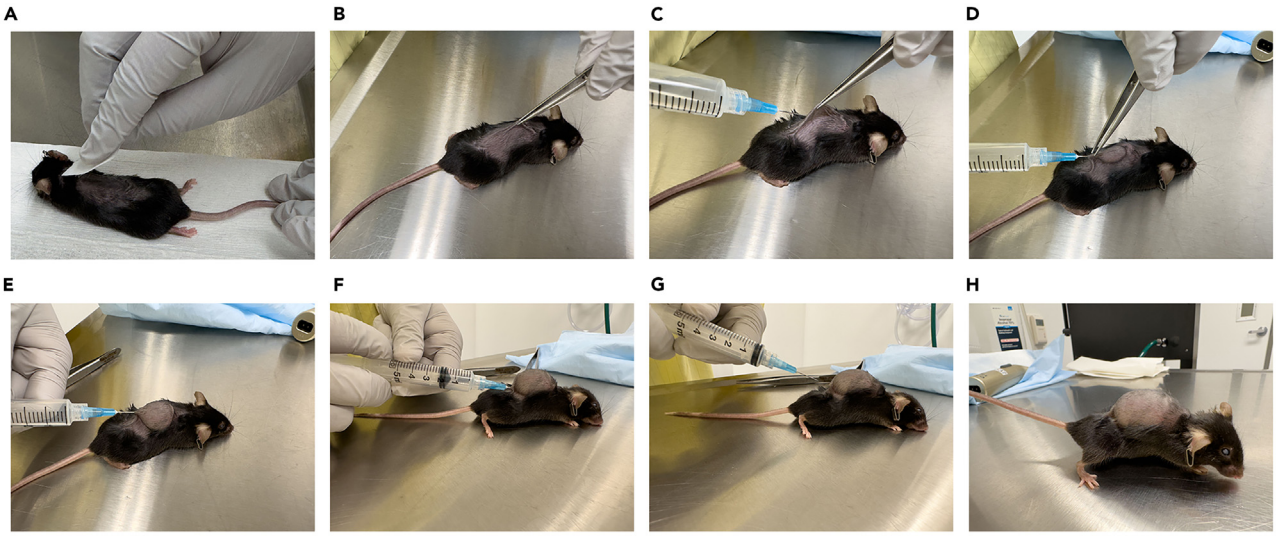

Figure 4. Inflation of the subcutaneous air pouch
(A) Sterilizing the shaved area with alcohol wipes.
(B) Slightly lift the shaved skin in the dorsal region with forceps.
(C) Position the needle in a flat angle close to parallel to the mouse, which aids s.c. injection.
(D) Carefully insert the 25 gauge needle attached to the sterile air filled syringe for about 3/8 inch below the lifted skin while still lifting the skin with forceps and slowly start injecting the sterile air.
(E–G) Continue to slowly inject the 5 mL of sterile air while monitoring the growing air pouch.
(H) The finished subcutaneous air pouch created with 5 mL of sterile air.

22. Filter solution with a 3 μm filter to recover the crystals and discard the solution.
23. Transfer the membrane with the crystals to a petri dish.
    a. Rinse crystals twice with absolute ethanol.

    Note: This will sterilize the crystals.

    b. Let the crystals air dry inside the biosafety cabinet under UV-light.

    Note: This will maintain crystal sterility.

    Note: Alternatively, the crystals can also be autoclaved before resuspending in PBS.

    Note: Alternatively, the crystals can also be centrifuged for collection and between washing steps at 500 x g for 10 min and dried.

24. Weigh an empty 50 mL sterile conical tube for subtraction below.
25. Transfer the crystals from the petri dish to 50 mL sterile conical tube inside the biosafety cabinet.
26. Weigh the tube containing the crystals and subtract the weight of the empty tube to obtain the dry weight of the crystals.
27. Resuspend crystals in sterile PBS at 100 mg/mL.
28. Remove 10 μL of the crystal-containing solution
    a. Sonicate on ice for 20 min at 200–300 W using a microtip (5 s on followed by 5 s off).

    Note: This will disperse the crystals for a homogenous suspension

    b. Check crystals on a slide under a microscope for the needle-like shape (Figure 6A).

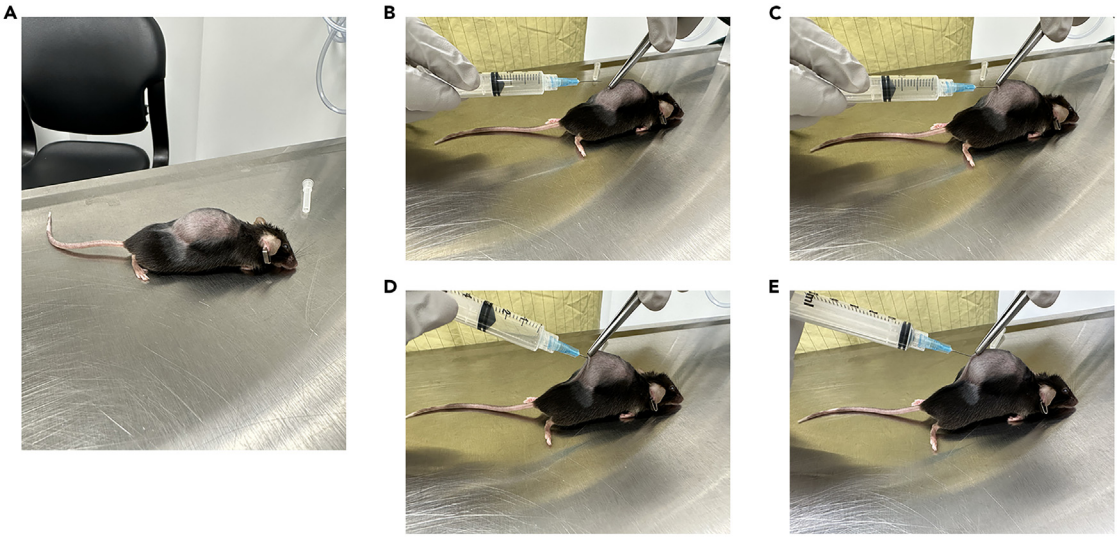

Figure 5. Re-inflation of the subcutaneous air pouch
(A) The partially deflated subcutaneous air pouch at day 3.
(B) Very slightly lift the skin of the air pouch with forceps.
(C) Position the needle in a flat angle close to parallel to the mouse at the top of the air pouch where one can lift the skin.
(D) Carefully insert the 25 gauge needle attached to the sterile air filled syringe for about 3/8 inch below the lifted skin into the air pouch while still holding the skin with forceps to stabilize the injection point and slowly start injecting the sterile air.
(E) Continue to slowly inject the 3 mL of sterile air while monitoring the growing air pouch.

Note: The size of the crystals will be variable and larger crystals seem to be more effective than smaller crystals. We are not aware of a study measuring the length of the MSU crystals that are taken up by macrophages, but the silica crystals that are taken up by macrophages are between ~0.8 μm and ~4.5 μm in length with a median length of 1.65 μm.[11]

29. Crystals can be stored at 4°C for 6 month and on −20°C for at least 1 year.

⚠ CRITICAL: If you do not observe the needle like shape then repeat the preparation and carefully check the pH and extend the stirring time to 24–48 h. In our experience, if the crystals do not precipitate within this time frame, then any longer incubation will not produce any usable crystals. The needle like shape of the crystals is essential for cellular uptake.

MSU crystal injection into the subcutaneous air pouch

Timing: 30–45 min

This step describes the injection of MSU crystals into the subcutaneous air pouch at day 5.

30. Day 5: Prepare 3 mg/mL of monosodium urate (MSU) in 1 mL of sterile 1× PBS and 1 mL of sterile 1× PBS as a negative control.

⚠ CRITICAL: MSU crystals do not dissolve and remain as a precipitate. Rigorous mixing or sonication of the solution just before drawing it into the syringe is important for reproducible results.

31. Anesthetize the mice.
32. Inject the MSU crystals (3 mg in 1× PBS) into the established air pouch.

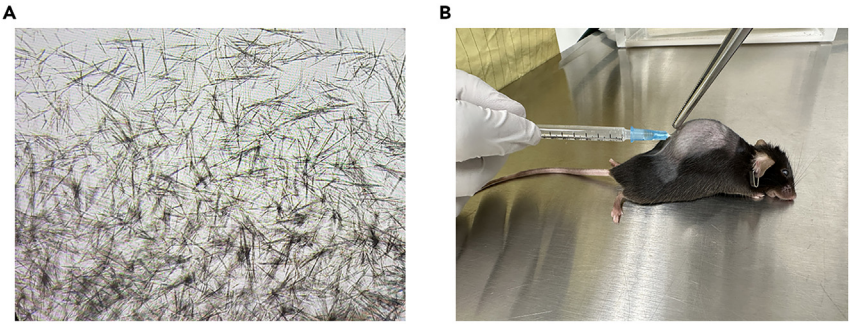

Figure 6. MSU crystal injection into the subcutaneous air pouch
(A) A preparation of MSU crystals prepared according to our protocol and viewed under a microscope.[7,8]
(B) Injection of MSU crystals into the subcutaneous air pouch using a 1 mL syringe, stabilizing the pouch with forceps.

Note: The procedure is as described for the sterile air using a 1 mL syringe.

33. Inject 1 mL 1× PBS as a negative control into a separate mouse.
    a. Using forceps to slightly grab the air pouch skin as above.

Note: This will stabilize the injection point (Figure 6B).

Note: MSU crystals are commercially available but can also be easily prepared,[1,2] especially considering the larger quantities necessary for injection into the air pouch. Ensure the proper crystal size is present in your preparation, as crystal size affects the inflammatory response by affecting efficacy of phagocytosis.[11]

Note: It is necessary to anesthetize mice, as the air pouch makes it impossible to properly restrain mice for injection.

Note: Injection of 3 mg MSU crystals usually results in a potent inflammatory response unless the quality of the MSU crystal preparation is poor (e.g. only some needle like crystals are present with the majority of crystals showing other elongated or roundish morphology). Increasing the injected MSU crystal quantity to 5–10 mg can help to nevertheless elicit an inflammatory response.

34. Place the mice back into the cage.
    a. Observe mice until they are fully conscious and active.
    b. Return the mice into the holding room for an additional 4 h or the time point under investigation.

Note: 4 h MSU crystal exposure allows to measure monocyte and neutrophil infiltration into the air pouch and analysis of inflammatory cytokines.

In vivo imaging of MPO activity in the subcutaneous air pouch

Timing: 90–120 min

This step describes the quantification of neutrophil infiltration into the subcutaneous air pouch by *in vivo* bioluminescence imaging.

35. 4 h post MSU crystal injection, prepare a 50 mg/mL stock solution of XenoLight RediJect Inflammation probe or luminol by dissolving in sterile 1× PBS.

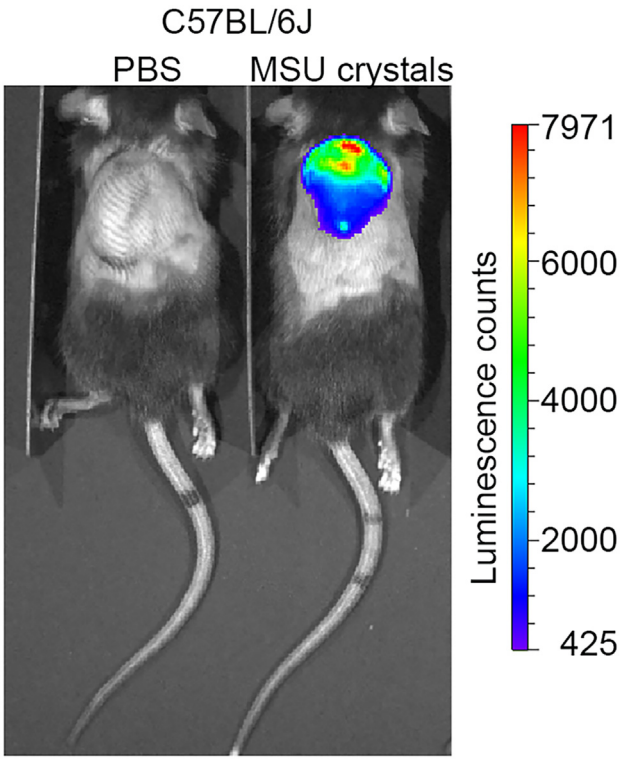

Figure 7. *In vivo* imaging of MPO activity in the subcutaneous air pouch

C57BL/6J mice were injected with PBS or 3 mg/mL MSU crystals as described above, 4 h later injected with luminol (200 mg/kg). MPO bioluminescence was imaged 10 min later with a 5 min exposure time in an IVIS Spectrum instrument, showing a representative example.

Note: XenoLight RediJect Inflammation probe is a convenient readymade solution for measuring neutrophil MPO activity, but identical results can be obtained using luminol (5-amino-2,3- dihydro-1,4-phthalazinedione). Luminol is specific for neutrophil MPO activity and does not detect eosinophil peroxidase activity or macrophage NADPH oxidase activity.[12,13]

36. Prepare 1 mL syringes with 25 gauge needles containing above solution.
37. Anesthetize mice with isoflurane and intraperitoneally (i.p) inject XenoLight RediJect Inflammation probe (200 mg/kg)

Note: Luminol (200 mg/kg) works identically and is much less expensive.

38. After 10 min, expose the mice to an *in vivo* imaging system capable of capturing *in vivo* bioluminescence (Figure 7).

Note: We use an IVIS Spectrum instrument from Perkin Elmer using a 5 minute exposure time and quantification of images with the Living Image software (Perkin Elmer). Any other bioluminescent detection equipment can be used for this approach or neutrophil infiltration and inflammatory responses can be quantified as described below.

Collection of lavage fluid from the subcutaneous air pouch

Timing: 90–120 min

This step describes the collection of air pouch lavage fluid and cells for analysis.

39. After imaging, sacrifice the mice by the method approved by your IACUC.
40. With a scissor, make a small incision on the dorsal region of the air pouch skin.
    a. Hold the corners of the skin with the help of forceps.
    b. Puncture the air pouch membrane with a needle or a scalpel.

Note: The incision should be deep enough to just expose the apex of the air pouch membrane.

41. Inject 2 mL of 1× PBS with a syringe.
    a. Wash the air pouch by massaging the air pouch.
    b. Collect the lavage fluid with a 1 mL pipette into a 15 mL centrifuge tube.

Note: Pipet up and down to wash the air pouch.

    c. Keep the tube on ice.

⚠ CRITICAL: Crystals aggregate and clump inside the air pouch and using a syringe to recover the lavage fluid frequently results in a clogged needle and consequently a poor recovery. We therefore make this small incision and use a 1 mL pipette for lavage fluid collection.

42. Collect the lavage cells by centrifugation at 1200 rpm and 4°C for 10 min.
43. Separate the lavage supernatant for cytokine analysis or store at −80°C for later use.
44. Resuspend the lavage cells in 1 mL of sterile 1× PBS.

Note: Calculate the total number of cells by mixing 50 μL of cells with 50 μL of Trypan Blue.

45. Cells are now ready for analysis by flow cytometry or any other assay.

Note: We usually observe a 50 to 80-fold increase in total recovered cell numbers in response to MSU injection (e.g. PBS control: 10,000 cells vs MSU: 50,0000–80,0000 cells).[1,2]

Optional: Dissect the air pouch lining for immunohistochemistry of infiltrating leukocytes.[2]

EXPECTED OUTCOMES

A successful procedure results in a properly inflated subcutaneous air pouch, which remains inflated by day 5 without causing adverse effects to the animal. Inflammatory responses, such as caused by MSU crystal injection, remain largely localized to the air pouch.

The number of infiltrated neutrophils and MPO activity is much higher in mice after MSU crystal injection into the air pouch compared to mice injected with PBS. Similarly, much higher inflammatory cytokine and leukotrienes levels are present in air pouch lavage fluids after MSU injection.

LIMITATIONS

Injection of larger volume of air is painful to mice. Therefore, one cannot repeatedly inflate the air pouch or convert it into a chronic model of inflammation. The protocol requires anesthesia and animals are sacrificed at the end of the study.

TROUBLESHOOTING

Problem 1

The air pouch does not inflate (related to Step1 and Step 2).

Potential solution

The likely reason is that the injection is not subcutaneous (s.c.) and rather into the muscle or punctured the air pouch at a second site during re-inflation.

Problem 2

The air pouch does completely deflate by day 3 (related to Step 1).

Potential solution

The likely reason is that the injection damaged the skin.

Problem 3

Extensive background inflammation in control mice, especially when imaging mice (related to Step 4 and Step 5).

Potential solution

The likely reason is that the procedure caused an injury. We noticed that when shaving is not done carefully, it easily causes skin inflammation present for several days. In particular, usage of a hair removal creme causes skin inflammation and gentle shaving with an electric shaver is preferred.

Problem 4

No or very limited inflammation is observed after MSU crystal injection (related to Step 3).

Potential solution

The likely reason is that the MSU crystal preparation is of poor quality. Check the crystals for having a needle like morphology rather than being short, fragmented and globular. Clumping of crystals also can increase the size and morphology resulting in poor phagocytosis. Crystals do not dissolve and remain as a precipitate. Mixing or sonicating the solution just before drawing it into the syringe is important. Another reason could be that the MSU crystals were not injected into the air pouch, but into the muscle or the air pouch was punctured during injection at a second site and the MSU crystal solution did not enter the air pouch.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Andrea Dorfleutner (andrea.dorfleutner@cshs.org).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to and will be answered by the technical contact, Savita Devi (savita.hcu@gmail.com)

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate and analyze datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

C.S. and A.D. conceived this project. S.D. planned and performed the experiments, and all authors wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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