

## Analysis of Monocyte Cell Fate by Adoptive Transfer in a Murine Model of TLR7-induced Systemic Inflammation

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**[Abstract]** Myeloid plasticity is a hallmark of the innate immune response to Toll-like receptor (TLR) activation. Here, we provide a protocol for monocyte cell fate tracking by adoptive transfer in the context of systemic inflammation induced by TLR7 activation, the principal innate immune receptor sensing viral RNA in mice. Defined monocyte subsets are isolated from the bone marrow of donor mice by cell sorting and adoptively transferred into the systemic circulation of congenic hosts, with or without concurrent activation of TLR7 via the topical application of the small molecule agonist, imiquimod, in a cream formulation that induces a systemic inflammatory response. Advantages are the precise definition of donor cell populations and resulting cell fate without the need for host conditioning in a model that recapitulates key aspects of the systemic inflammatory response to TLR7 stimulation.

**Keywords:** Aldara/Imiquimod, TLR7, Inflammation, Monocyte differentiation, Adoptive transfer, Flow cytometry.

**[Background]** The diversity of myeloid differentiation responses is an integral part of the adaptive immune response and is critical for tissue restoration and maintenance during inflammation and inflammatory dysregulation. Infectious agents, such as viruses, or tissue injury trigger an inflammatory response involving monocytes of the major (classic) subtype in mice, Ly6C<sup>hi</sup> monocytes (Bonnardel and Guillems, 2018). These monocytes originate from progenitor cells in the bone marrow (BM), circulate in peripheral blood (PB), and respond to tissue signals by differentiating into a spectrum of effector phagocytes: macrophages (MF), dendritic cells (DC), and monocytes with patrolling behavior (Hettinger *et al.*, 2013; Gamrekelashvili *et al.*, 2016; Bonnardel and Guillems, 2018; Arazi *et al.*, 2019; Chakarov *et al.*, 2019). Monocyte lineage studies are facilitated by *Cx3cr1*<sup>GFP/+</sup> reporter mice, in which monocytes and macrophages, but not granulocytes, express distinct GFP signals (Jung *et al.*, 2000).

Toll-like receptor 7 (TLR7) is a member of the family of pathogen sensors expressed on myeloid cells. TLR7 is the principal innate immune receptor sensing viral RNA in mice, a function homologous to TLR7/8 in humans (Diebold *et al.*, 2004). It is highly expressed in endosomes of myeloid cells, where viral RNA triggers the production of pro-inflammatory cytokines and type I interferons, causing local and systemic inflammation and the induction of an antiviral state (Iwasaki and Pillai, 2014). Importantly, myeloid TLR7 signaling is required for the efficient protection of mice against virulent RNA viruses (Kaminski *et al.*, 2012), and it is involved in immune responses to the influenza A virus and SARS-CoV2. The activation of TLR7 can also be induced by the small molecule TLR7 agonist, imiquimod (IMQ), an

imidazoquinoline derivative used clinically in a cream formulation as antiviral and antitumor therapies (van der Fits *et al.*, 2009). TLR7 stimulation induces cytokine production in both mouse and human patrolling monocytes and mediates the sensing and disposal of damaged endothelial cells by Ly6C<sup>lo</sup> monocytes (Cros *et al.*, 2010; Carlin *et al.*, 2013); however, chronic TLR7 stimulation drives the differentiation of Ly6C<sup>hi</sup> monocytes into specialized macrophages and causes the development of anemia (Akilesh *et al.*, 2019). Furthermore, systemic stimulation with TLR7 agonists induces the conversion of Ly6C<sup>hi</sup> monocytes into patrolling Ly6C<sup>lo</sup> monocytes in wildtype mice (Santiago-Raber *et al.*, 2011; Gamrekelashvili *et al.*, 2020).

Here, we provide a protocol to study monocyte cell fate under inflammatory conditions using adoptive transfer of monocyte subsets from genetically defined mouse strains, with the concurrent stimulation of host TLR7 via the topical application of IMQ in a cream formulation (Aldara). Ly6C<sup>hi</sup> monocytes from the bone marrow of *Cx3cr1*<sup>GFP/+</sup> CD45.2<sup>+</sup> mice are FACS-sorted and transferred into congenic CD45.1<sup>+</sup> mice by tail-vein injection, and donor-derived cells are identified by GFP and CD45.2 expression. The main distinguishing features in comparison with conventional bone-marrow reconstitution experiments are the precise definition of donor cell populations and resulting cell fate without the need for host conditioning. The protocol is transferrable to other progenitor or mature cell populations if suitable numbers can be retrieved from the donors. Furthermore, key aspects of the systemic response to TLR7 stimulation seen in viral models are recapitulated in a virus-free model.

## **Materials and Reagents**

1. LS columns (Miltenyi Biotec, catalog number: 130-042-401)
2. 0.5 ml and 1.5 ml microfuge tubes (Sarstedt, catalog numbers: 72.699 and 72.690.001)
3. 15 ml tubes (Greiner Bio-One, CELLSTAR, catalog number: 188261)
4. 1 ml syringes with 25 G and 27 G needles (B. Braun, Injekt-F 9166033V, Sterican 4657705)
5. K<sub>3</sub>EDTA-containing blood collection tubes (Microvette 100 µl; Sarstedt, catalog number: 20.1278)
6. 96-well V-bottomed plates (Greiner Bio-One, catalog number: 651101)
7. 6-well plates (Merck, Biochrom, catalog number: TPP92406)
8. Cell strainers (Greiner Bio-One, catalog numbers: 542040 [40 µm], 542000 [100 µm])
9. Nylon mesh (Heidland, catalog number: 03-70/41)
10. Anti-biotin microbeads (Miltenyi Biotec, catalog number: 130-090-485)
11. Propidium iodide (Merck, Sigma-Aldrich, catalog number: 81854-25MG, Stock 1 mg/ml in PBS, 1:12,000 final)
12. Histopaque 1083 (Merck, Sigma-Aldrich, catalog number: 10831-100ML)
13. Red blood cell lysis buffer (RBC lysis buffer; BioLegend, catalog number: 420301)
14. Imiquimod (Meda, Aldara, 5% cream, catalog number: PZN-00111981)
15. Vaseline (Winthrop GmbH, catalog number: PZN-02726847)
16. Depilating cream (Silk & Fresh depilating cream)

17. Bepanthen eye ointment (Bayer vital GmbH, catalog number: PZN-01578675)
18. Ketamine (Ketamine, 100 mg/ml; CP-Pharma, catalog number: 1202)
19. Rompun (Xylavet, 20 mg/ml; CP-Pharma, catalog number: 1205)
20. Midazolam (Midazolam-Actavis 1 mg/ml; Actavis, catalog number: PZN-03831807)
21. Isofluran (Isofluran CP 1 ml/ml; CP-Pharma, catalog number: 1214)
22. Heparin 5000 U/ml (Heparin-Natrium, 25000; Ratiopharm, catalog number: PZN-03029843)
23. Cell counting slides (Thermo Fisher Scientific, catalog number: C10228)
24. PBS (Merck, Sigma-Aldrich, catalog number: D8537)
25. Antibodies (Tables 1 and 2)

**Table 1. Antibodies and fluorescent dyes for the sorting of inflammatory monocytes**

Antibody/Dye	Clone	Company	Cat. No.	Final Dilution
CD16/32 (TruStain FcX)	93	BioLegend	101319	1:200
CD3 PE	17A2	BioLegend	100205	1:400
Ter119 PE	Ter119	BioLegend	116208	1:800
CD45R/B220 PE	RA3-6B2	BD Pharmingen	553089	1:800
Ly6G PE	1A8	BioLegend	127608	1:800
CD19 PE	6D5	BioLegend	115507	1:400
CD49b PE	DX5	BD Pharmingen	553858	1:200
NK1.1 PE	PK136	BioLegend	108707	1:200
CD90.2 PE	Thy1.2	BD Pharmingen	553005	1:800
CD11b Pacific Blue	M1/70	BioLegend	101224	1:200
Ly6C PE-Cy7	HK1.4	BioLegend	128018	1:1400

**Table 2. Antibodies and fluorescent dyes for donor cell enrichment and flow cytometry**

Antibody/Dye	Clone	Company	Cat. No.	Final Dilution
CD16/32 (TruStain FcX)	93	BioLegend	101319	1:200
CD3 Biotin	17A2	BioLegend	100243	1:400
Ter119 Biotin	Ter119	BioLegend	116203	1:400
CD45R/B220 Biotin	RA3-6B2	BioLegend	103203	1:400
Ly6G Biotin	1A8	BioLegend	127603	1:400
CD19 Biotin	6D5	BioLegend	115504	1:400
CD45.1-Biotin	A20	BioLegend	110703	1:200
CD117 APC-Cy7	2B8	BioLegend	105825	1:100
CD11b Pacific Blue	M1/70	BioLegend	101224	1:200
Ly6C PE-Cy7	HK1.4	BioLegend	128018	1:2800
F4/80 BV650	BM8	BioLegend	123149	1:200
CD11c BV605	N418	BioLegend	117334	1:400
I-A/I-E BV510	M5/114.15.2	BioLegend	107635	1:400
NK1.1 Biotin	PK136	BioLegend	108704	1:400
CD45.2 Alexa700	104	BioLegend	109821	1:200
CD43 PerCP-Cy5.5	S7	BD Pharmingen	562865	1:400
Streptavidin-PE-Dazzled594		BioLegend	405247	1:400

26. Flow cytometry buffer (FCS-EDTA-PBS, see Recipes)

27. Cell collection buffer (20% FCS-PBS, see Recipes)

28. Heparin-PBS (250 U/ml) for collection of peripheral blood (see Recipes)

29. RBC lysis buffer (see Recipes)

## **Equipment**

1. Hair shaver (Aesculap Isis GT420, Type HS61)
2. Caliper (Pariere, 8030/R)
3. Mouse heat pad (Beurer, HK25, Type P10)
4. Centrifuge (Hermle, Z383K)
5. Table-top centrifuge (VWR, Micro Star 17R)
6. Flow cytometer (Beckton Dickinson, LSR-II)
7. Cell sorter (Beckton Dickinson, FACS Aria Fusion)
8. Quadro MACS separation unit (Miltenyi Biotec, catalog number: 130-090-976)
9. Automated cell counter (Thermo-Fisher Scientific, Invitrogen, Countless II FL, AMQAF1000)
10. Automated blood counter (Scil Animal Care Company, Scil Vet ABC)

## **Software**

1. FlowJo (version 10.6.2, FLOWJO LLC, <https://www.flowjo.com>)

2. Prism (version 7.04, GraphPad, <https://www.graphpad.com>)
3. Adobe Illustrator (version 25.0, <https://www.adobe.com>)

## Procedure

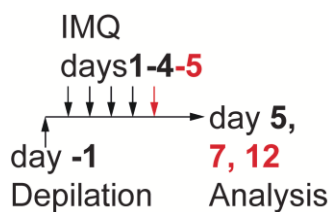
### A. Induction of systemic inflammation using imiquimod

#### Notes:

1. *Experimental procedures can only be performed after approval by the local Animal Care Committee.*
2. *Perform experiments with 8-12-week-old mice. Reporter mice are B6.129P-Cx3cr1<sup>tm1Litt</sup>/J, obtained from the Jackson Laboratory (Jung et al., 2000; Gamrekelashvili et al., 2016 and 2020).*
3. *IMQ-treated mice must be kept separately from the Vaseline (Sham) group.*
4. *Mix blood thoroughly with Heparin-PBS or K<sub>3</sub>EDTA to avoid coagulation.*
5. *Pre-warm RBC lysis buffer at room temperature to achieve efficient lysis.*

#### 1. Induction of systemic inflammation using IMQ (Figure 1)

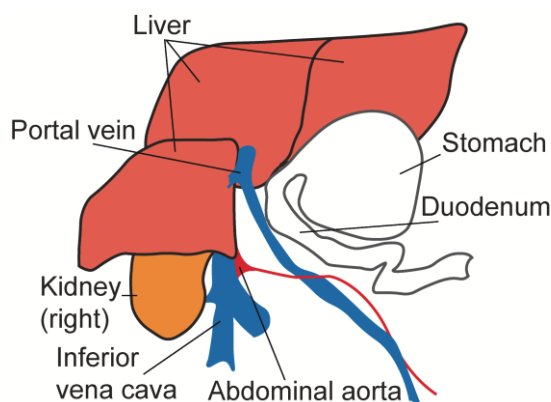
- a. On Day -1, weigh the mice, record the weight, and intraperitoneally inject Ketanest (Esketamin 80 mg/kg), Rompun (Xylazin 2.5 mg/kg), and Dormicum (Midazolam 2.5 mg/kg) in 0.9% NaCl solution to anesthetize the mice (injection volume 10 µl/g mouse weight, using a 1-ml syringe and 27 G needle).
- b. Apply a drop of Bepanthen eye ointment from the tube to the eye to avoid dry eyes, and test paw reflexes to make sure the mouse is sleeping.
- c. Shave the back (appr. 3 cm<sup>2</sup>) of the mice with an electric shaver and apply the depilating cream.
- d. Keep the mice for appr. 2 min on a heat pad and wipe away the depilating cream with a spadel.
- e. Wash and rinse the back of the mice with warm water to remove the remaining depilating cream.
- f. Keep the mice on the heat pad until awake and then transfer to cages with *ad libitum* access to food and water.
- g. Two days later (Day 1), weigh the mice, record the weight, and place each mouse separately in a glass jar with appr. 200-300 µl isofluran for 10-15 s.
- h. Place each dizzy/sleepy mouse on the heat pad, measure the thickness of the ear using a caliper, and quickly apply 50 mg IMQ (Aldara) cream or an equal amount of Vaseline to the back and the right ear using a stainless-steel spadel.
- i. Repeat the same procedure for 3-4 consecutive days (Figure 1).



**Figure 1. Experimental set-up for the induction of inflammation**

## 2. Mouse preparation and analysis of systemic inflammation

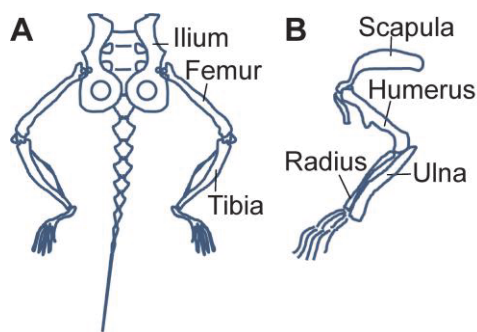
- a. On Day 5, 7, or 12, anesthetize the mice by intraperitoneal injection with Ketanest (Esketamin 80 mg/kg), Rompun (Xylazin 2.5 mg/kg), and Dormicum (Midazolam 2.5 mg/kg) in 0.9% NaCl solution (injection volume 10 µl/g mouse weight, using a 1-ml syringe and 27 G needle).
- b. Check the footpad reflex to confirm that the mouse is sleeping.
- c. Fix the sleeping mice to the pad, open the abdomen, and collect the blood from the inferior vena cava using a 1-ml syringe with a 25 G ×5/8" needle (Braun Melsungen) (Figure 2):
  - i. 300 µl blood (PB) in 100 µl Heparin-PBS (250 U/ml) for cell analysis.
  - ii. 50 µl blood in K<sub>3</sub>EDTA-containing tubes for blood counts.
  - iii. The remaining blood without heparin for serum collection and analysis.



**Figure 2. Schematic representation of the mouse abdominal situs, ventral view.** After opening the abdominal cavity of deeply anesthetized mice, collect blood from the inferior vena cava.

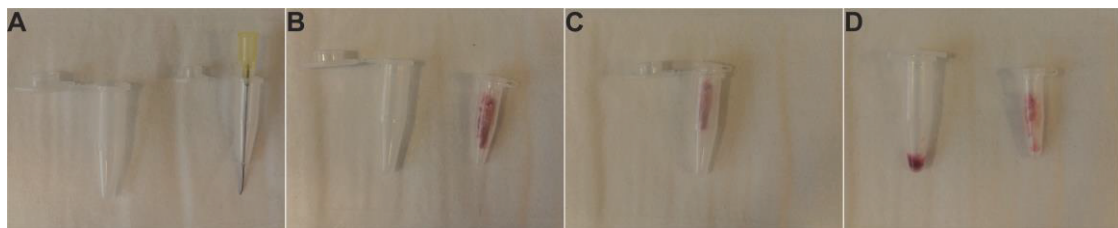
- d. Euthanize the mice, open the abdomen, excise the spleen (Spl), and place in PBS on ice.
- e. Cut and remove the skin from the hind limb and collect the hind limb with the muscles on a paper towel.
- f. Carefully remove the muscles using scissors. Clean the rest of the muscles and soft tissue with a paper towel and isolate the bones [one tibial (Tibia) and one femoral (Femur) bone (Figure 3)].





**Figure 3. Schematic representation of the bones of the mouse hindlimb (A) and forelimb (B).** The Ilium, Femur, Tibia, and Humerus are collected for bone-marrow isolation and analysis.

- g. Pre-weigh the 1.5-ml Eppendorf collection tube of the custom-made bone-marrow (BM) isolating device (Figure 4). Cut one end of the bone, place in a 0.5-ml tube with a hole in the bottom, and collect the BM by centrifugation of the isolating device in a table-top centrifuge at  $5,000 \times g$  for 20 s.



**Figure 4. Assembly and use of the custom-made BM isolating device.** A. Perforation of a 0.5-ml tube with a 21 G needle for BM isolation. B. Bones cleaned from muscles and placed in a 0.5-ml tube. C. Assembly for centrifugation. D. Isolated BM in a 1.5-ml collection tube after centrifugation.

- h. Weigh the collection tube again, record the difference in weight to define the BM weight and place on ice.
- i. Resuspend BM in 10 ml PBS, filter through a 70- $\mu$ m nylon mesh, and wash by centrifugation at  $400 \times g$  for 7 min.
- j. Aspirate the supernatant, resuspend the cell pellet in 10 ml PBS, and repeat the filtration and washing step once more.
- k. Weigh the spleen, prepare a single-cell suspension by passing through a syringe plunger in a 6-well plate and filtering through a nylon mesh, and centrifuge at  $400 \times g$  for 7 min.
- l. To remove the erythrocytes, resuspend the PB and Spl pellets in 5 ml or 1 ml room-temperature RBC lysis buffer and incubate for 17 min or 1 min, respectively.
- m. Stop the lysis procedure by adding 10 ml PBS, filter the suspension through a nylon mesh, and centrifuge at  $400 \times g$  for 7 min.
- n. Repeat the washing-filtration-centrifugation step once more.
- o. Resuspend the Spl, BM, and PB in PBS (10 ml, 10 ml, or 5 ml, respectively) and count the

cells in an automated cell counter to define the absolute cell number.

- p. Resuspend the cells in FCS-EDTA-PBS buffer and place  $\leq 1 \times 10^6$  cells per sample in a 96-well V-bottomed plate for staining and subsequent flow cytometry analysis.

## B. Adoptive transfer of monocytes into IMQ-treated mice and monocyte differentiation analysis

### Notes:

1. *Experimental procedures can only be performed after approval by the local Animal Care Committee.*
2. *Perform experiments with 8-12-week-old mice. Use B6.SJL-Ptprca<sup>a</sup>Pepc<sup>b</sup>/BoyJ (CD45.1<sup>+</sup>) mice as recipients (Jackson Laboratory).*
3. *Collect no more than  $2 \times 10^6$  sorted monocytes in 2 ml collection buffer and carefully mix the collection tube every 20-30 min to avoid excess stress to the cells. This step enhances cell viability and ensures a high yield of donor cells for analysis after adoptive transfer.*
4. *For successful flow cytometry analysis of transferred cells, it is important to deplete recipient cells from recipient Spl and BM but not from peripheral blood.*
5. *It is critical to remove erythrocytes using density centrifugation with Histopaque 1083 and not RBC lysis for successful analysis of transferred donor cells.*
6. *Cell enrichment on magnetic columns must be performed with ice-cold FCS-EDTA-PBS buffer. Avoid excess air bubble and foam formation.*

### 1. Induction of systemic inflammation using IMQ

- a. Depilate and treat recipient CD45.1<sup>+</sup> mice on days 1-4 with IMQ as described in A and Figure 1.

### 2. Isolation and adoptive transfer of inflammatory monocytes on day 2

- a. Euthanize 4-5 donor CX<sub>3</sub>CR1-GFP<sup>+</sup> CD45.2<sup>+</sup> mice and isolate the bones (hips (Ilium), femoral (Femur), tibial (Tibia), and brachial (Humerus) bones from both sides, 8 bones/mouse total, Figure 3). Clean the muscles from the bones using scissors and a paper towel. Collect the cleaned bones in PBS and keep on ice.
- b. Dip the bones in 70% EtOH for 3-5 s and dry on EtOH-sterilized dry paper towel. Cut one end of the bone and put 4 bones/tube in 0.5-ml Eppendorf tubes with a hole in the bottom created using a 21 G needle (Figure 4).
- c. Close the tube and place in another 1.5-ml collection tube. Centrifuge this device in a tabletop centrifuge with a fixed-angle rotor at  $5,000 \times g$  for 20 s.
- d. Collect the bone marrow, resuspend in 50 ml PBS, filter through a 100- $\mu$ m sterile cell strainer, and centrifuge in a swinging-bucket rotor at  $400 \times g$  for 7-8 min to collect the pellet.
- e. Resuspend the cell pellet from 4-5 mice in 6-8 ml PBS, overlay 3-4 ml suspension on 3 ml Histopaque 1083 in 15-ml tubes, and centrifuge in a swinging-bucket rotor at  $420 \times g$  for 20 min without the brake.
- f. Collect the interphase (appr. 5 ml) containing the cells in a 15-ml tube, fill the tube with PBS,



- and wash by centrifugation at  $400 \times g$  for 7 min.
- g. Resuspend the pellet in 600  $\mu$ l FCS-EDTA-PBS and add 3  $\mu$ l Fc block (TruStain FcX). Incubate the tube at 4°C for 12-15 min.
- h. Without washing, add PE-labeled anti-mouse CD3- (1.5  $\mu$ l), CD19- (1.5  $\mu$ l), B220- (0.75  $\mu$ l), Ly6G- (0.75  $\mu$ l), Ter119- (0.75  $\mu$ l), CD90.2- (0.75  $\mu$ l), NK1.1- (3  $\mu$ l), CD49b- (3  $\mu$ l); anti-mouse CD11b-Pacific Blue (3  $\mu$ l), and anti-mouse Ly6C-PE-Cy7 (0.43  $\mu$ l) antibodies (Table 1) to the 600  $\mu$ l cell suspension and incubate for an additional 15 min.
- i. Wash the cells twice in 10 ml buffer, filter through a 40- $\mu$ m cell strainer, resuspend in 2 ml FCS-EDTA-PBS buffer, and sort Lin<sup>+</sup>CD11b<sup>+</sup>CX<sub>3</sub>CR1-GFP<sup>lo</sup>Ly6C<sup>hi</sup> cells. Collect  $\sim 2 \times 10^6$  sorted cells in 2 ml ice-cold 20% FCS-PBS and mix the collection tube every 20-30 min during sorting.
- j. Immediately after sorting, wash the sorted cells twice with 10 ml PBS each, resuspend in PBS, and intravenously inject  $0.7 \times 10^6$ - $2 \times 10^6$  cells per mouse in 100  $\mu$ l volume into the tail vein using a 1-ml syringe and 27 G needle. To dilate the tail vein and facilitate injection, prewarm the tail in warm water.
3. Isolation and analysis of donor-derived CD45.2<sup>+</sup> monocytes from CD45.1<sup>+</sup> recipients 3 days after adoptive transfer.
  - a. Euthanize recipient mice in deep narcosis, collect blood in 200  $\mu$ l Heparin-PBS, collect the spleen and 8 bones (hips, femoral, tibial, and brachial bones).
  - b. Prepare a single-cell suspension from Spl by passing through a 5-ml syringe into a 6-well plate, resuspending in 4 ml PBS, and filtering through a 70- $\mu$ m nylon mesh.
  - c. Isolate BM as described in Figure 4, resuspend in 4 ml PBS, and filter through a 70- $\mu$ m nylon mesh.
  - d. Dilute the blood (PB) with PBS to 7 ml.
  - e. Load Spl, BM, and PB separately onto 2.5 ml Histopaque 1083 in 15-ml tubes and centrifuge in a swinging-bucket rotor at  $420 \times g$  for 20 min without the brake.
  - f. Collect the interphase (appr. 5ml) containing the cells in a clean 15-ml tube, fill up the tube with PBS, and wash once by centrifugation at  $400 \times g$  for 7 min.
  - g. Resuspend the cell pellet in 100  $\mu$ l (BM, Spl) or 50  $\mu$ l (PB) FCS-PBS-EDTA buffer supplemented with Fc block (1:200 dilution) and incubate at 4°C for 15 min. Without washing the Fc block, add biotin-labeled antibodies consisting of anti-mouse CD45.1, CD3, CD19, B220, Ly6G, and Ter119 to the final dilution indicated in Table 2, but do not add the biotinylated anti-mouse NK1.1 at this stage.
  - h. Quickly vortex the tube and incubate at 4°C for 15 min.
  - i. Fill the tubes with 5 ml FCS-EDTA-PBS buffer and centrifuge at  $400 \times g$  for 8 min.
  - j. Wash PB once more with 5 ml FCS-EDTA-PBS buffer and transfer the pellet to a 96-well V-bottomed plate for further staining.
  - k. Resuspend the Spl and BM pellets in 240  $\mu$ l FCS-EDTA-PBS buffer, add 60  $\mu$ l anti-biotin microbeads, and incubate at 4°C for 15 min. Fill the tubes again with FCS-EDTA-PBS buffer

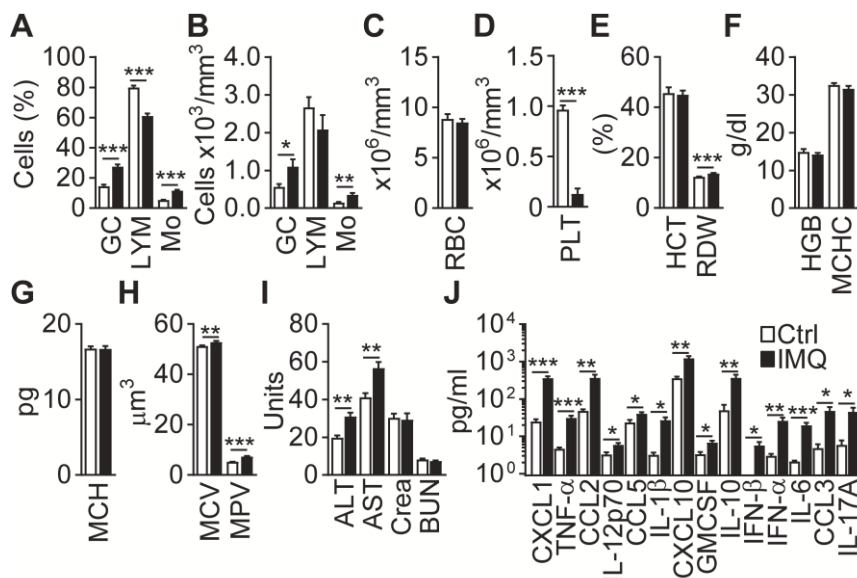
to wash the excess beads and centrifuge at  $400 \times g$  for 7-8 min.

- l. Place the LS columns in the Quadro MACS separation unit and equilibrate with ice-cold FCS-EDTA-PBS buffer (1 ml buffer per column).
- m. Resuspend the Spl and BM pellets in 2 ml FCS-EDTA-PBS buffer and load onto the pre-equilibrated LS columns.
- n. Wash the columns twice with 2 ml FCS-EDTA-PBS buffer, collect the flowthrough, pellet by centrifugation at  $400 \times g$  for 7 min, transfer to a 96-well V-bottomed plate, and stain in 30  $\mu$ l volume with the appropriate antibody cocktail (Table 2).
- o. Resuspend the cells after staining and wash in FCS-EDTA-PBS buffer. Add propidium iodide (PI 1 mg/ml stock in PBS, 1:12,000 final dilution) prior to acquisition and acquire flow cytometry data for further analysis.

## Data analysis

### A. Analysis of IMQ-induced systemic inflammation

1. Measure the mouse weight, ear thickness, blood count, coagulation, inflammatory cytokine levels, liver and kidney function, and myeloid cell composition of the BM, PB, and Spl, and plot the data as described in Figures 5A-5J in this manuscript and in Figure 3E, Figure 4G, Figure 3 – figure supplement 1A, Figure 4 – figure supplement 1C in Gamrekashvili *et al.* (2020).



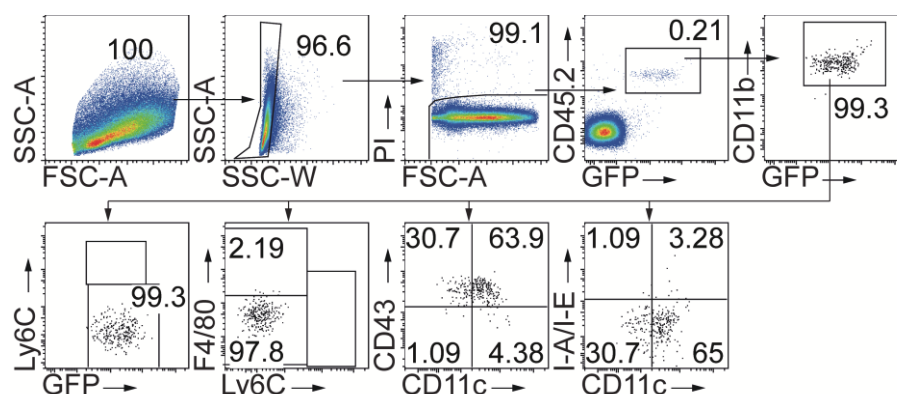
**Figure 5. IMQ induces systemic inflammation.** (A-H) Blood parameters in Ctrl (Vaseline) or IMQ-treated mice (GC – granulocytes, LYM – lymphocytes, Mo – monocytes, RBC – red blood cells, PLT – platelets, HCT – hematocrit, RDW – red blood cell distribution width, HGB – Hemoglobin, MCHC – mean corpuscular hemoglobin concentration, MCH – mean corpuscular hemoglobin, MCV – Mean corpuscular volume, MPV – mean platelet volume). (I) ALT/AST, creatinine (Crea) and blood urea nitrogen (BUN) in IMQ-treated mice. (J) Cytokine and

chemokine concentrations in Ctrl or IMQ-treated mouse sera. (A-J) Data are pooled from 3-4 experiments,  $n = 4-9$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; two-tailed unpaired Student's  $t$ -test.

- Analyze the flow cytometry data using the Flowjo software. Calculate the relative frequency of myeloid cell subsets [for a definition of the subsets, see Figure 3E, Figure 3 – figure supplement 2, Supplementary File 1 in Gamrekelashvili *et al.* (2020)] in live cells after the exclusion of doublets, export the numerical data to Excel, and calculate the absolute cell numbers per mg BM, mg Spl, or  $\mu$ l blood.
- Transfer the data to GraphPad Prism, plot the values as the mean and standard error of the mean, and calculate the statistical significance using an unpaired two-tailed Student's  $t$ -test. Define  $P < 0.05$  as statistically significant and indicate on the graphs.

#### B. Analysis of adoptively transferred monocytes using flow cytometry

- Analyze the flow cytometry data using the Flowjo software. Calculate the relative frequency of myeloid cell subsets in live CD45.2<sup>+</sup>CD11b<sup>+</sup>CX<sub>3</sub>CR1-GFP<sup>+</sup> donor cells after the exclusion of doublets and CD45.1<sup>+</sup> recipient cells, export the numerical data into GraphPad Prism, and plot the values as the mean and standard error of the mean, as shown in Figures 5F and 5G in Gamrekelashvili *et al.* (2020). Calculate the statistical significance using an unpaired two-tailed Student's  $t$ -test. Define  $P < 0.05$  as statistically significant and indicate on the graphs.
- Create representative flow cytometry plots, as shown in Figure 6, depicting the gating strategy, surface phenotype, and frequency of donor-derived cells. Initially, define the cells according to size and granularity (FSC-A vs. SSC-A plot), exclude doublets (SSC-W vs. SSC-A plot) and dead (PI<sup>+</sup>) cells (FSC-A vs. PI plot), and gate donor cells (CD45.2<sup>+</sup>GFP<sup>+</sup>). As a next step, define monocytes (CD11b<sup>+</sup>GFP<sup>+</sup> population) in donor cells and create subsequent plots showing the expression of Ly6C, F4/80, CD11c, CD43, and I-A/I-E as markers of monocyte subsets.



**Figure 6. Adoptively transferred classic monocytes differentiate into patrolling monocytes.** Flow cytometry plots showing the gating strategy and analysis of donor cells 3 days after adoptive transfer of CD45.2<sup>+</sup> classic monocytes in the spleen of IMQ-treated CD45.1<sup>+</sup> recipients. Donor-derived monocytes (CD45.2<sup>+</sup>CD11b<sup>+</sup>CX<sub>3</sub>CR1-GFP<sup>+</sup>Ly6C<sup>hi</sup>F4/80<sup>lo/-</sup>CD11c<sup>-</sup>

CD43<sup>lo/-</sup>I-A/I-E<sup>lo/-</sup> cells) no longer show a classic monocyte phenotype in recipient mice but instead show a phenotype consistent with patrolling monocytes (CD45.2<sup>+</sup>CD11b<sup>+</sup>CX<sub>3</sub>CR1-GFP<sup>+</sup>Ly6C<sup>lo/-</sup>F4/80<sup>lo</sup>CD11c<sup>lo</sup>CD43<sup>hi</sup>I-A/I-E<sup>lo/-</sup>).

## **Recipes**

1. FCS-EDTA-PBS buffer  
FCS 20 ml (2% final)  
EDTA (0.5 M stock in PBS, pH 7.2) 4 ml (2 mM final)  
PBS 1,000 ml
2. Sorted cell-collection buffer (20% FCS-PBS buffer)  
FCS 10 ml  
PBS 40 ml  
Filter through a 0.22-µm filter and keep sterile
3. Heparin-PBS (250 U/ml) for collection of peripheral blood  
Heparin stock 5,000 U/ml 50 µl  
PBS 950 µl  
Take 100 µl heparin-PBS for ≤ 500 µl blood.
4. RBC lysis buffer  
RBC lysis buffer (10×) 1 ml  
Deionized water 9 ml

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## **Competing interests**

The authors declare no competing financial or non-financial interests.

## **Ethics**

All animal experiments described in this protocol were approved by the local Animal Welfare Board (LAVES, Lower Saxony, Animal Studies Committee, TVA 16/2251, 18/2777, 2018/221).

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