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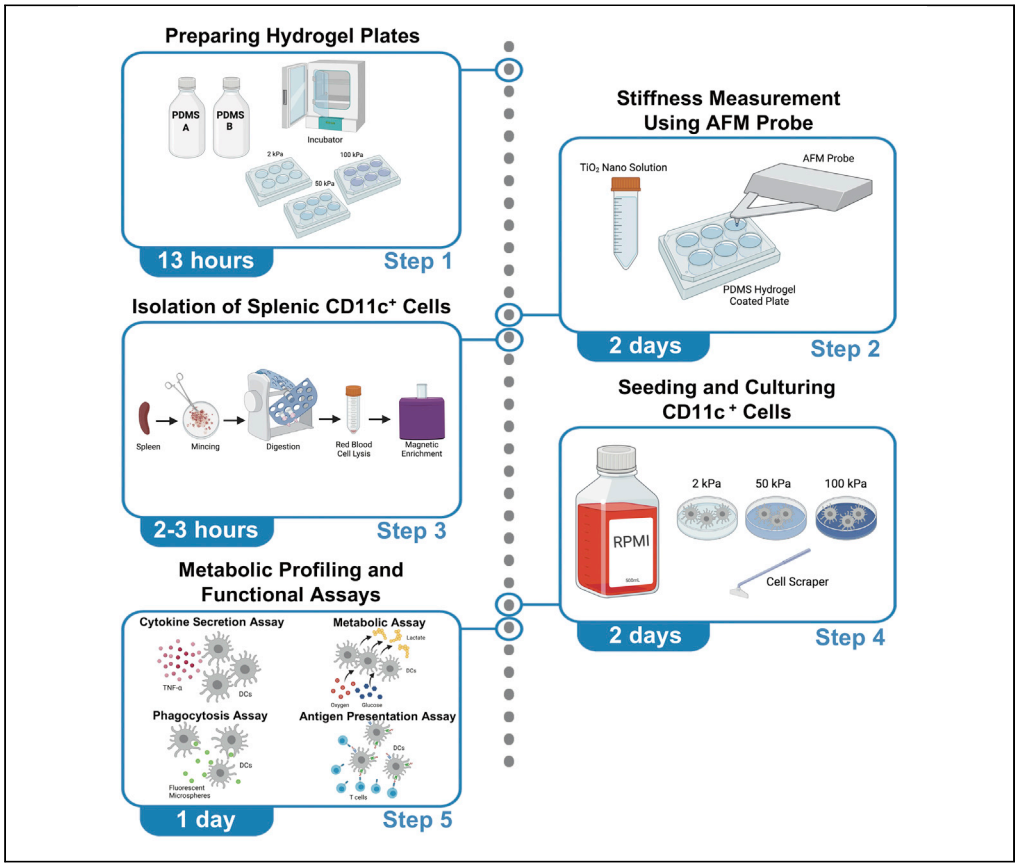
Protocol

PDMS hydrogel-coated tissue culture plates for studying the impact of substrate stiffness on dendritic cell function



The mechanical properties of polydimethylsiloxane hydrogels can be tuned to mimic physiological tensions, an underappreciated environmental parameter in immunology studies. We describe a workflow to prepare PDMS-coated tissue culture plates with biologically relevant substrate stiffness, and the use of these hydrogel plates to condition isolated primary splenic CD11c+ dendritic cells (DC). Finally, we suggest downstream applications to study the impact of substrate stiffness on DC function and metabolism. The protocol could be adapted to study other mechanosensitive immune cell subsets.

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Highlights

A protocol for using PDMS hydrogel plates to study mechanosensing in immune cells

Workflow for preparing and validating PDMS hydrogel-coated culture plates

Isolation and culture of tissue CD11c+ DCs for metabolic and functional analysis

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PDMS hydrogel-coated tissue culture plates for studying the impact of substrate stiffness on dendritic cell function

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SUMMARY

The mechanical properties of polydimethylsiloxane hydrogels can be tuned to mimic physiological tensions, an underappreciated environmental parameter in immunology studies. We describe a workflow to prepare PDMS-coated tissue culture plates with biologically relevant substrate stiffness, and the use of these hydrogel plates to condition isolated primary splenic CD11c+ dendritic cells (DC). Finally, we suggest downstream applications to study the impact of substrate stiffness on DC function and metabolism. The protocol could be adapted to study other mechanosensitive immune cell subsets.

For complete details on the use and execution of this protocol, please refer to Chakraborty et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps to make polydimethylsiloxane (PDMS) hydrogel-coated plates with elastic modulus of 2 kPa, 50 kPa, and 100 kPa which mimic physiological and pathological tissue stiffnesses (Table 1) (Wells, 2013). The substrate stiffness is then validated by nanoindentation using atomic force microscopy. The protocol further explains the process to isolate and culture primary splenic $CD11c^+$ DCs on hydrogel plates for downstream applications.

Spleens for isolating $CD11c^+$ DCs were obtained from C57BL/6 mice. The mice were maintained in a pathogen-free, temperature-controlled, and 12 h light and dark cycle environment at the Toronto Medical Discovery Tower animal research facility and the University of Alberta Health Sciences Laboratory Animal Services mouse barrier facility. All the mice used were sex-matched and age-matched (8–10 weeks old) littermate males and/or females. All animal experiments were approved by the Animal Care Committee at the University Health Network and the University of Alberta.

Table 1. Elastic moduli of biological tissues

Elastic modulus	Tissue	References
<1 kPa	Soft mucosa, brain (0.05–0.5 kPa)	(Flanagan et al., 2002)
2–5 kPa	Adipose tissue, lymph nodes	(Flanagan et al., 2002; Samani et al., 2003; Leung et al., 2013)
0–12 kPa	Lung	(McGee et al., 2008)
12 kPa	Cardiomyocytes, skeletal muscle	(Engler et al., 2004)
3–16 kPa	Spleen, endothelium	(Pawluś et al., 2016; Arda et al., 2011; Le Master et al., 2018)
5–27 kPa	Bone marrow	(Jansen et al., 2015)
40 kPa	Inflamed lymph nodes, high-grade invasive ductal carcinoma tumor (breast cancer)	(Meng et al., 2020; Samani et al., 2007)
20–50 kPa	Cirrhotic liver	(Umut Ozcan et al., 2011)
> 50 kPa	Cartilage, skin, bone	(Liang and Boppart, 2010; Choi et al., 2013; Tilleman et al., 2004)
35–70 kPa	Fibrotic scar in cardiac tissue	(Engler et al., 2008)

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dow Corning Sylgard 527 silicone dielectric gel (Part A and B)	Ellsworth	Cat#1696742
Fibronectin	Sigma-Aldrich	Cat#F1141-2 mg
Phosphate buffered saline (PBS)	Wisent	Cat#311-011-CL
Collagenase I	Sigma-Aldrich	Cat#C0130-1 g
Dulbecco's Modified Eagle Medium (DMEM)	Wisent	Cat#319-005-CL
NH_4Cl	Alfa Aesar	Cat#40193-A1
$KHCO_3$	Alfa Aesar	Cat#40195-36
EDTA	Bio Basic Canada Inc.	Cat#6381-92-6
FBS	Life Technologies	Cat#12484028
RPMI-1640	Wisent	Cat#350-005-CL
Penicillin/streptomycin	Gibco	Cat#15140-122
L-glutamine	Wisent	Cat#609-065-EL
2-mercaptoethanol	Sigma-Aldrich	Cat#M3148-100 mL
Trypan Blue	Gibco	Cat#15250-061
1H, 1H, 2H, 2H-perfluorooctyltriethoxysilane (13F)	Sigma-Aldrich	Cat#667420
3-(Trimethoxysilyl)propyl methacrylate (MSMA, 98%)	Sigma-Aldrich	Cat#440159
Tetraethyl orthosilicate (TEOS >98%)	Sigma-Aldrich	Cat#131903
Titanium tetraisopropoxide (TTIP, ≥97%)	Sigma-Aldrich	Cat#87560
Isopropanol alcohol (IPA)	Sigma-Aldrich	Cat#190764
Dipentaerythritol penta-/hexa-acrylate (DPHA, regent grade)	Sigma-Aldrich	Cat#407283
2-Hydroxy-2-methylpropiophenone (HMPP, 97%)	Sigma-Aldrich	Cat#405655
Critical commercial assays		
CD11c MicroBeads UltraPure, Mouse	Miltenyi Biotec	Cat#130-125-835
MACS LS Columns	Miltenyi Biotec	Cat#130-042-401
Experimental models: Organisms/strains		
Mouse: C57BL/6J	The Jackson Laboratory	Strain#000664
Other		
50 mL Conical tube	FroggaBio	Cat#TB50-500
15 mL Conical tube	FroggaBio	Cat#TB15-500
1.5 mL microcentrifuge tubes	Sarstedt	Cat#72.690.300
10 mL Serological pipette	Sarstedt	Cat#86.1254.001
6-well plate	Falcon	Cat#353046
24-well plate	Falcon	Cat#353047
40 μm cell strainer	Corning	Cat#C431750
Tissue culture cell scraper 25 cm	Sarstedt	Cat#83.1830
Hybridization Incubator	Tyler Research	N/A
UV Stratalinker 2400	Stratagene	N/A
Spectrolinker XL-1000 UV Crosslinker	Spectronics Corporation	N/A

MATERIALS AND EQUIPMENT

Cell Culture Medium		
Reagent	Final concentration	Amount/Volume
RPMI-1640	N/A	500 mL
Penicillin/streptomycin	100 U/mL / 100 μg/mL	5 mL
L-glutamine	2 mM	5 mL
2-mercaptoethanol	50 μM	500 μL
FBS	10%	50 mL
Store at 4°C for up to 1 month.		

Magnetic-Activated Cell Sorting (MACS) Buffer		
Reagent	Final concentration	Amount/Volume
Phosphate buffered saline	N/A	500 mL
FBS	2%	10 mL
EDTA	1 mM	1 mL
Store at 4°C for up to 1 month.		

Ammonium-Chloride-Potassium (ACK) Lysis Buffer		
Reagent	Final concentration	Amount
NH_4Cl	150 mM	8.02 g
$KHCO_3$	10 mM	1 g
EDTA	0.1 mM	37.2 mg
Store up to 6 months if kept at 4°C		

TiO_2 Nano Solution		
Reagent	Final concentration	Amount
TTIP	47 mM	1 mL
IPA	3.7 M	20 mL
HCl	44 mM	50 mL
Ethanol (100%)	N/A	20 mL
Deionized Water	N/A	10 mL
Store at room temperature (20°C–22°C) for up to 1 month.		

TiO_2/SiO_2 Hybrid (MFTS)		
Reagent	Final concentration	Amount
TEOS	1.9 M	4 mL
MSMA	0.45 M	1 mL
13F	0.11 M	0.4 mL
TiO_2 Nano Solution	N/A	1 mL
IPA	1.4 M	1 mL
HCl	13 mM	2 mL
Store at room temperature (20°C–22°C) for up to 1 month.		

AFM Tip Modifier Solution		
Reagent	Final concentration	Amount/Weight ratio
MFTS	N/A	110
DPHA	N/A	1.67
HMPP	N/A	0.21
Store at room temperature (20°C–22°C) for up to 1 month in the dark.		

Preparation of reagents

Timing: 30 min

- Prepare cell culture media [RPMI-1640 supplemented with Penicillin (100 U/mL), Streptomycin (100 mg/mL), L-glutamine (2 mM), 2-mercaptoethanol (50 μM), and 10% heat-inactivated fetal bovine serum (FBS)]. Filter-sterilize through a 0.2 μm filter and store at 4°C.
- Prepare magnetic-activated cell sorting (MACS) buffer [Phosphate buffered saline supplemented with 2% heat inactivated FBS and Ethylenediaminetetraacetic acid (EDTA) (1 mM)]. Filter sterilize through a 0.2 μm filter and store at 4°C.
- Prepare Ammonium-Chloride-Potassium (ACK) lysis buffer [Ammonium Chloride (NH_4Cl), 150 mM, Potassium Bicarbonate ($KHCO_3$), 10 mM and EDTA (0.1 mM)] in 1 L Milli-Q H_2O. Dissolve all chemicals in 850 mL of Milli-Q H_2O. Adjust pH to 7.2–7.4. Add Milli-Q H_2O until the volume reaches 1000 mL. Filter-sterilize through a 0.2 μm filter and store at 4°C.
- Details on tip modifier solution preparation is described in steps 12–14.

STEP-BY-STEP METHOD DETAILS

Preparing hydrogel plates

Timing: 13 h

The sequence of steps below details how to prepare PDMS hydrogel coated plates with elastic modulus of 2 kPa, 50 kPa, and 100 kPa. Special care is taken to uniformly mix the hydrogel components to ensure consistent gel stiffness.

1. Calculate the amounts of reagents needed to prepare the hydrogel according to the proportions indicated below (calculations have been given for a total of 10 g gel as an example). (Table 2)

Table 2.

	A:B	Amount of part A (g)	Amount of part B (g)	Total weight of gel (g)
2 kPa gel	6:5	5.45	4.55	10
50 kPa gel	3:10	2.3	7.7	10
100 kPa gel	3:20	1.3	8.7	10

2. Weigh part A and part B of gel into a 50 mL conical tube. Mix thoroughly.

⚠ CRITICAL: Ensure that the A:B mixture is uniformly mixed by inverting the conical tube repeatedly for at least 3 min. Using this strategy, a homogeneous mixture is created with consistent gel stiffness.

Note: Prepare 10 g of hydrogel mix for a single 24-well plate and 12 g for a single 6-well plate. Prepare hydrogel mix according to the number of plates required.

3. Coat the wells of the plate using a 10 mL serological pipette. Ensure that the hydrogel solution fully covers the bottom of the plate. We estimate a minimum of 0.4 mL of gel to coat one single well in a 24-well plate, and 2 mL for one well in a 6-well plate.
4. Allow the hydrogel coated plate(s) to cure overnight (8–10 h) at 60°C in an incubator.

⚠ CRITICAL: Maintaining a consistent temperature during incubation is important to avoid any disturbance in the polymerization process.

5. Sterilize the hydrogel coated plates by ultraviolet irradiation for at least 15 min using a UV Stratalinker. The plates can be stored at room temperature (20°C–22°C) for up to two months.
6. On the day of assay, coat the hydrogel plates with the desired extracellular matrix proteins (e.g.,1 μg/mL fibronectin in PBS). Allow the proteins to adhere for 4 h at 37°C followed by two-washes with PBS.

⚠ CRITICAL: Once the hydrogel plates are coated with extracellular matrix proteins, it should always remain hydrated with PBS after washing prior to seeding with cells.

7. The plates are ready to be seeded with cells upon removal of PBS.

Stiffness measurement using AFM probe

Timing: 5 h

The following series of steps are taken to validate the elastic moduli of the prepared hydrogels via nanoindentation on a JPK NanoWizard II (JPK Instruments) atomic force microscope (AFM). AFM tips are first modified to facilitate the nanoindentation measurement on very soft gels. This process occurs once (3–4 h), and a successfully modified tip can be reused for future measurements. All nanoindentations are taken in ten replicates for each hydrogel, once, to confirm the stiffness (1 h). The hydrogels are prepared following a specific protocol, and stiffness measurements need not be repeated.

8. Alter the tip shape of a silicon nitride probe (0.06–0.10 N/m) with a titanium dioxide-based tip modifier solution.
   a. Spread the coating on a clean glass slide and create tiny streaks in a crisscross pattern.
   b. Load the tip in the AFM and, without submerging the tip, dab it in a streak of coating.
   c. Cure the coated AFM tip under UV-C (254 nm) at 120 mJ/cm^2 for 5 min utilizing a UV crosslinker (Spectrolinker XL-1000, Spectronics Corporation).
   d. Prop up the tip on its side under a microscope and image to estimate the radius of curvature of the indenter. (Figure 1A: Modified AFM tip)
9. Perform ten nanoindentations per PDMS gel sample (fibronectin-coated) in a phosphate buffer saline solution using the modified AFM tip in contact mode. (Figure 1B: Probe touching the 50 kPa gel surface). Follow the standard nanoindentation operating procedure described in the instrument manual.
10. Set the applied force to 20 nN for measurements on both gels, achieving indentation depths ranging from 100-1500 nm.
11. Fit the force curves using the Hertzian model for a spherical indenter to obtain the stiffness values. (Figure 1C: Force curve on 50 kPa gel)

Tip modifier solution preparation

Timing: 2 days

The following are steps to prepare a viscous, hydrophobic, and UV crosslink sensitive TiO_2 nano solution for ideal AFM indenter modification. This method needs only be completed once to prepare a series of modified tips.

12. Prepare the TiO_2 nano solution.
   a. Mix 1 mL of TTIP with 20 mL IPA to form a clear solution.
   b. Slowly add the solution to 50 mL of aqueous HCl (pH 1.2), under vigorous stirring.
   c. Transfer the solution to a 100 mL Teflon-lined hydrothermal reactor and heat at 160°C for 24 h.

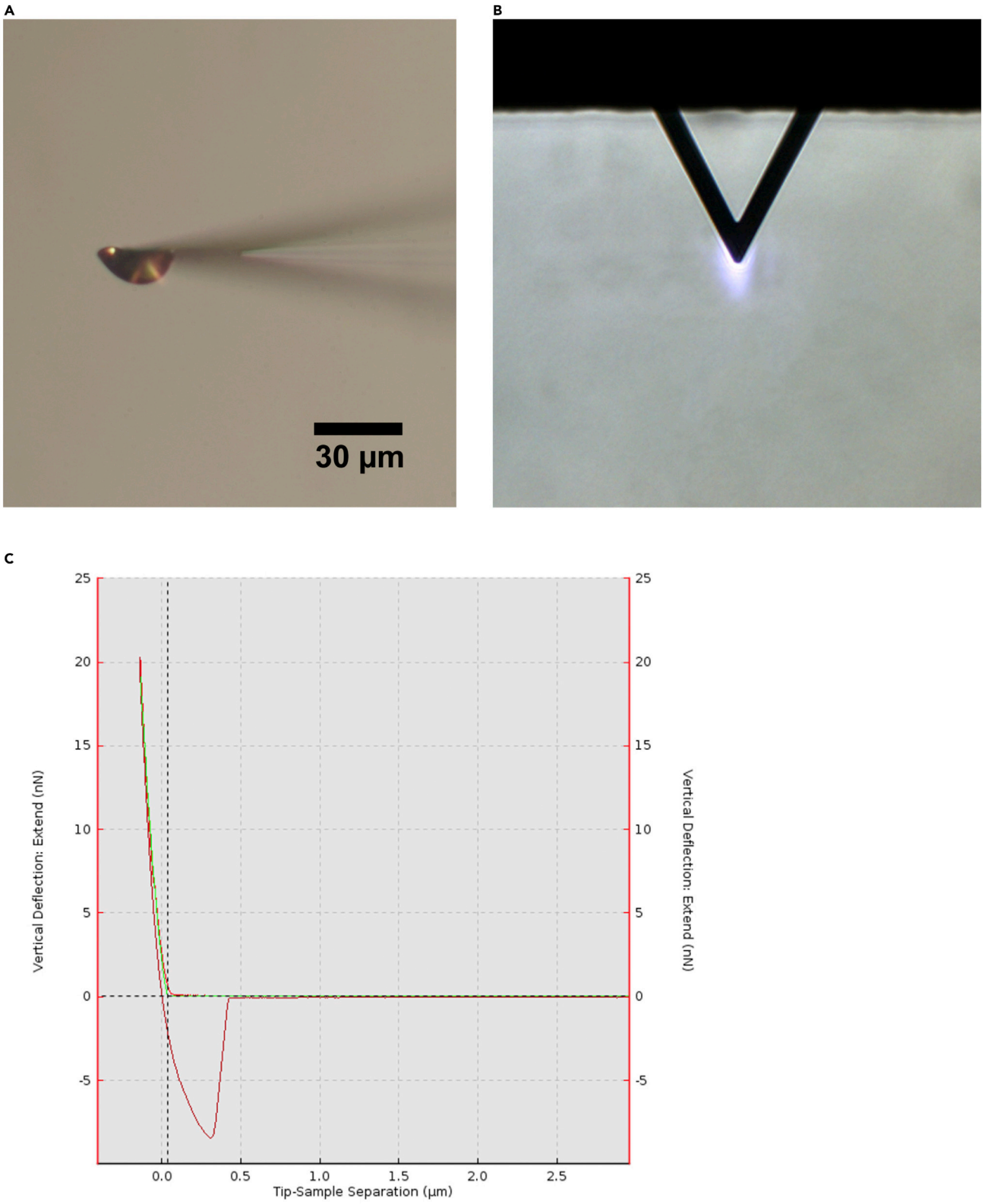
A
30 μm
B
C
25
20
15
10
5
0
-5
0.0
0.5
1.0
1.5
2.0
2.5
Tip-Sample Separation (μm)
Vertical Deflection: Extend (nN)
Vertical Deflection: Extend (nN)

Figure 1. Stiffness measurement using AFM probe

(A) A silicon nitride AFM probe modified with a spherical indenter made from a UV-cured titanium dioxide-based hydrophobic coating material.

(B) The AFM modified probe in contact with the uncoated 50 kPa gel surface.

(C) The Hertzian model fit (green) on a measured gel force curve (red).

d. Centrifuge the solution (15,000×*g*, 1 min) and clean the precipitate with 20 mL of absolute ethanol, followed by drying at 70°C for 2 h.

e. Disperse the powder in 10 mL of deionized water to complete the TiO_2 nano solution.

13. Making a surface functionalized TiO_2/SiO_2 hybrid (MFTS).

a. Mix TEOS (4 mL), MSMA (1 mL), 13F (0.4 mL), TiO_2 nano solution (from step 12, 1 mL), IPA (1 mL), and aqueous HCl (2 mL, pH 1.2) and vigorously stir for 24 h to form a homogenous solution.

14. Tip modifier solution preparation.

a. Mix MFTS (from step 13), DPHA, and HMPP (a photo-initiator) with the following weight ratio to form the final, clear solution: 110/1.67/0.21 (MFTS/DPHA/HMPP).

Isolation of splenic $CD11c^+$ cells

Timing: 2–3 h

The goal of the following steps is to digest mouse spleens and isolate the splenic DCs.

15. Obtain a single cell suspension via collagenase digestion of the spleen.

a. Place the spleen in a 5 cm plastic dish.

b. Mince the spleen into 1 mm pieces using curved scissors.

c. Transfer the minced tissue into a 1.5 mL microcentrifuge and add Collagenase 1-containing DMEM to a final volume of 500 μL at a final concentration of 45 U/mL.

d. Allow digestion to take place at 37°C for 45 min under continuous agitation (50 rpm).

e. After digestion, vortex the sample for 20 s and use repeated pipetting to thoroughly homogenize the tissue.

f. Pass the homogenized sample through a 40 μm cell strainer placed over a 50 mL conical tube. Rinse off the strainer with additional volumes of cell culture medium. Proceed with centrifugation at 400 g for 5 min to pellet the single cell suspension.

Note: Pre wet the cell strainer with 1 mL cell culture medium before adding the samples. If large pieces of the spleen are visible in the strainer, then use the plunger of a 1 mL syringe to mash it and then add 10 mL of cell culture medium to wash the cell strainer.

g. Perform red blood cell lysis.

i. Resuspend cells with 3 mL of ACK lysis buffer and allow lysis to occur on ice for 3 min.

ii. Stop lysis by adding > 5 volumes of PBS and centrifuge the cells at 400×g for 5 min. Aspirate supernatant and resuspend the cells in 2 mL appropriate buffer for cell isolation.

16. Magnetic enrichment of $CD11c^+$ cells. Please refer to the manufacturer's protocol (https://www.miltenyibiotec.com/DE-en/shop/comMiltenyiDatasheet/product?productId=54985).

Seeding and culturing $CD11c^+$ cells on a 24-well hydrogel plate

Timing: 2 days

The goal of these steps is to seed and culture isolated $CD11c^+$ cells on PDMS hydrogel coated plates for further downstream analyses.

17. Enumerate the magnetically isolated CD11c$^+$ cells.
18. Seed cells at a density of 1.3 × 10^6 cells/mL in supplemented RPM-I cell culture medium into fibronectin/hydrogel-coated plates.
19. Culture the CD11c$^+$ cells with stimuli of interest for appropriate lengths of time.
20. At end of culture, collect culture supernatant and harvest cells from the well with a cell scraper for the 50 kPa conditions for downstream applications [see (Chakraborty et al., 2021)].

Note: Do not scrape the surface of the 2 kPa hydrogel plates as they are fragile. Instead, use repeated gentle pipetting to dislodge the cells from the 2 kPa hydrogel plates.

Metabolic profiling of CD11c$^+$ cells

Timing: 1 day

The following steps describe assays that can be used to assess the metabolic activity of isolated CD11c$^+$ cells.

21. After conditioning the magnetically isolated CD11c$^+$ cells on PDMS hydrogels, a number of downstream assays can be performed to metabolically profile these cells. Some examples are described in (Tsai et al., 2018; Chakraborty et al., 2021), including Agilent Seahorse extracellular flux analysis, metabolic pathway gene and protein expression profiling, and nutrient uptake assays.
22. Metabolic pathways altered by PDMS hydrogel stiffness can be validated by co-culturing CD11c$^+$ cells on PDMS hydrogels in the presence of specific metabolic agonists or antagonists, as described in (Chakraborty et al., 2021).
23. Additional valuable insights can be gained from analyzing the cell culture supernatants for nutrient depletion and metabolite production, using approaches such as metabolomic footprinting assay.

Note: Since the hydrogel conditioned CD11c$^+$ cells will come in contact with different surface tension during isolation and assay preparation, we recommend working quickly, avoiding prolonged storage, and keeping all conditions consistent to minimize potential confounding effects.

EXPECTED OUTCOMES

Using the above protocol, we routinely obtain a soft gel resembling viscous liquid at 2 kPa and a firm, elastic gel at 50 kPa. We also observe visibly different phenotypes for some adherent cell types cultured on 2 kPa vs 50 kPa substrates under the microscope. The 50 kPa or plastic substrate induces cell spreading, while the 2 kPa substrate induces cell rounding. We note that in general stiff substrate promotes pro-inflammatory function and upregulates the metabolic activity of DCs compared to soft substrate (Chakraborty et al., 2021).

LIMITATIONS

For the hydrogel preparation, a homogeneous mixture is critical for obtaining consistent gel stiffness. Incomplete mixing will result in inconsistent curing and gel stiffness. This protocol is optimized for fibronectin-coating. Coating of PDMS hydrogels with other extracellular matrix proteins will require individual optimization. Stiffness measurement using AFM can be difficult with soft gels, which are sticky and may give errors in reading. Coating with fibronectin also creates a non-sticky shield between the tip and the gel. Therefore, repeated attempts are encouraged. Modifying the AFM tip successfully and taking the nanoindentation measurements can take up to 4–5 h to complete. Lastly, inconsistent techniques during cell isolation can give less than optimal cell viability, yield and purity. All procedures involving live cell manipulation need to be carried out on ice, unless

specified by the protocol. Cell viability and purity need to be confirmed via flow cytometry after each assay. A further limitation of CD11c-positive selection is the inclusion of $CD11c^+$ macrophages in the final purified cell product, which should be taken into account during data interpretation.

TROUBLESHOOTING

Problem 1

The hydrogels do not set or are inconsistent in texture (steps 2–4).

Potential solution

Ensure that accurate amounts of the respective reagents are weighed out and mixed. Ensure thorough mixing of the hydrogel reagents by inverting the tube containing the reagents for a set period (e.g., for a 50 mL conical tube, mix content for 5 min).

Problem 2

The hydrogels are hydrophobic (step 6).

Potential solution

Ensure that sufficient volume of cell culture media is added to the hydrogel-coated wells to ensure complete coverage.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sue Tsai at stsai@ualberta.ca.

Materials availability

The study did not generate new/unique reagents.

Data and code availability

This study did not generate new data sets/codes.

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AUTHOR CONTRIBUTIONS

M.L, K.C., N.K., and M.C., carried out methodology, investigation, and writing. C.G. A.S., D.A.W., and S.T. were responsible for supervision and resources. M.L., M.C., A.S., and S.T. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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