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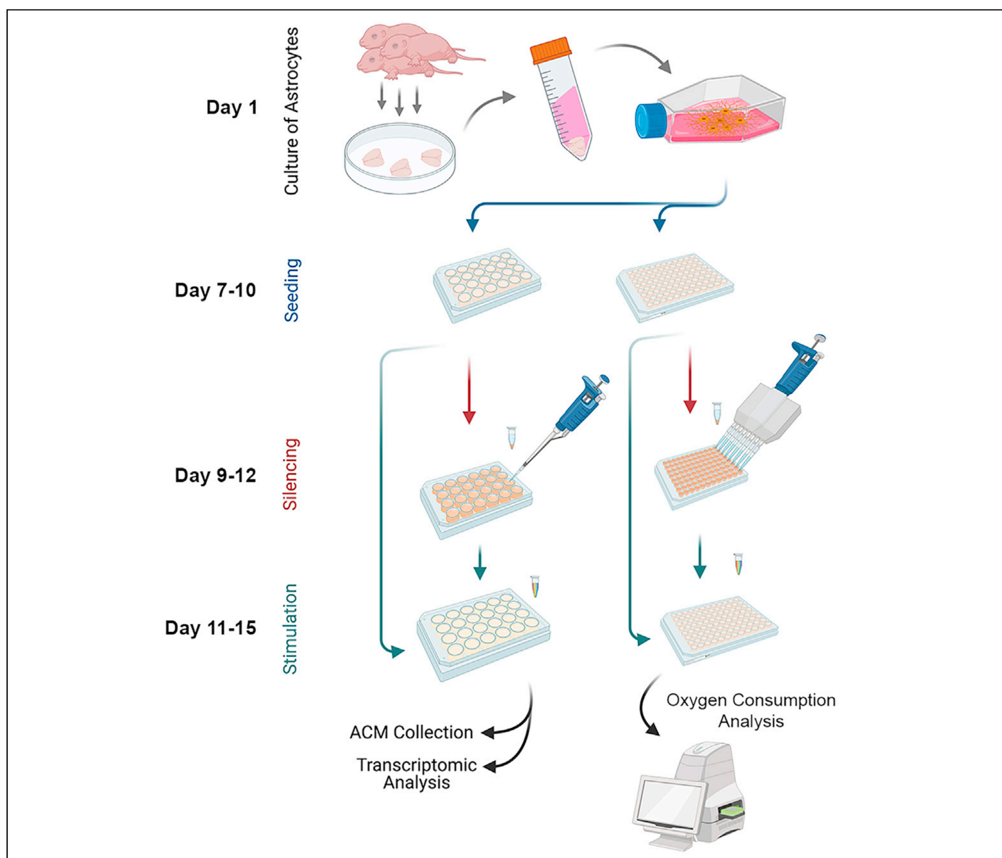
Protocol

Protocol for *in vitro* analysis of pro-inflammatory and metabolic functions of cultured primary murine astrocytes



Robust protocols are required to investigate *in vitro* the molecular mechanisms that control astrocyte metabolism and pro-inflammatory activities. In the present protocol, we describe step by step the isolation and culture of primary murine astrocytes from neonatal brains, followed by their genetic manipulation with siRNA. We further describe cytokine activation of the cultured astrocytes for the analysis of their pro-inflammatory responses, and the oxygen consumption analysis to assess their metabolic function.

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Highlights

Primary murine astrocyte culture establishment

Effective knockdown studies on primary astrocytes *in vitro*

Stimulation with cytokines for downstream assays

Oxygen consumption analysis to assess metabolic function

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Protocol

Protocol for *in vitro* analysis of pro-inflammatory and metabolic functions of cultured primary murine astrocytes

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SUMMARY

Robust protocols are required to investigate *in vitro* the molecular mechanisms that control astrocyte metabolism and pro-inflammatory activities. In the present protocol, we describe step by step the isolation and culture of primary murine astrocytes from neonatal brains, followed by their genetic manipulation with siRNA. We further describe cytokine activation of the cultured astrocytes for the analysis of their pro-inflammatory responses, and the oxygen consumption analysis to assess their metabolic function.
For complete details on the use and execution of this protocol, please refer to Chao et al. (2019), Clark et al. (2021), and Rothhammer et al. (2018).

BEFORE YOU BEGIN

Institutional permission and must be granted in advance. Work with your institution's Institutional Animal Care and Use Committee (IACUC) to get the necessary approvals and always follow their guidelines for pups euthanasia (see note 3).

Coating of flasks and plates with poly-L-lysin

Timing: 30 min

Note: Work under sterile conditions.

1. Coat 75 cm^2 flasks (T75) or plates with Poly-L-lysin. Add 5–8 mL of Poly-L lysine to each flask. For plates use the volumes stated in Table 1.
2. Incubate for at least 10 min at Room Temperature (20°C–24°C).

Table 1. Volumes of Poly-L lysine used to coat different sizes of plates

Culture plate	Volume per well of PLL for precoating
12 well	0.5 mL
24 well	0.25 mL
48 well	0.2 mL
96 well Seahorse	0.1–0.05 mL

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Alternatives: incubate at 37°C to ensure maximal coating or 4°C overnight (ON, 16 h).

3. Remove Poly-L-Lysine. Wash once with PBS or HBSS and let the flask dry while proceeding with next steps.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Poly-L-lysine	Sigma-Aldrich	# P4707
DPBS no calcium no magnesium	Gibco	# 14190144
HBSS no calcium no magnesium	Gibco	# 14170112
75 cm2/250mL Flasks	Falcon	# 353136
0.25% Trypsin-EDTA	Gibco	# 25200072
70 micron Nylon Cell Strainers	Fisherbrand	# 22-363-548
DMEM F12 with Glutamax	Gibco	#10565042
DMEM no glucose no glutamine	Gibco	#A1443001
OptiMEM	Gibco	#31985070
Fetal Bovine Serum - heat inactivated (FBS-HI)	Gibco	#10-438-026
Penicillin-Streptomycin (10,000 U/mL) (P/S)	Gibco	#15140122
HEPES 1M	Gibco	#15630080
N1 Medium Supplement	Sigma-Aldrich	#N6530
Glucose Solution 200 g/L	Gibco	#A2494001
MEM Non-Essential Amino Acids Solution (NEAA)	Gibco	#11140050
Bovine Albumin Fraction V (7.5% solution)	Gibco	#15260037
INTERFERin	Polyplus Transfection	#409
Buffer RLT	Qiagen	#79216
recombinant mouse IFNg	R&D Systems	#485-MI-100
recombinant mouse TNFa	R&D Systems	#410-MT-010
recombinant mouse IL1b	R&D Systems	#401-ML-005
recombinant mouse IFNb	R&D Systems	#8234-MB-010/CF
recombinant human TGFb	Miltenyi Biotec	#130-095-067
recombinant human TGFa	R&D Systems	#239-A-100
recombinant mouse VEGF-B	R&D Systems	#751-VE-025
recombinant mouse IL-17	R&D Systems	#421-ML-025/CF
Complement Component C1q Native Protein	MyBioSource	#MBS143105
recombinant mouse IL-10	R&D Systems	#417-ML-005
recombinant GM-CSF	PeproTech	#315-03
XF DMEM medium, pH 7.4	Agilent Technologies	103575-100
XF 1.0 M Glucose solution	Agilent Technologies	103577-100
XF 100 mM Pyruvate solution	Agilent Technologies	103578-100
XF 200 mM Glutamine solution	Agilent Technologies	#103579-100
Oligonucleotides		
SMARTpool siRNAs	Dharmacon	N/A
ON-TARGETplus Non-targeting Pool	Dharmacon	D-001810-10
Critical commercial assays		
Seahorse XF Cell Mito Stress Test Kit	Agilent Technologies	#103015-100
CyQUANT Cell Proliferation Assay Kit, for cells in culture	Thermo Fisher Scientific	#C7026
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J 1–3 days old mouse pups	The Jackson Laboratory	#000664
Other		
Conical 50 and 15 mL tubes	Falcon	#352196 #352070
70 μm cell strainers	Corning	#431751
Serological pipets	Greiner Bio-One	#606180 #607180

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tissue Culture Plates	Corning	#3596 #3526
Centrifuge	N/A	N/A
Incubator	N/A	N/A
Shaker with controlled temperature	VWR	#76407-108
XFe96 Seahorse analyzer	Agilent Technologies	N/A

MATERIALS AND EQUIPMENT

Alternatives: The list of reagents described in the key resources table are the ones tested and available in our laboratory. Equivalent chemicals, plasticware, media, from different vendors may be suitable alternatives and can be tested by interested users.

Note: Always prewarm the media to 37°C before adding it to the cell cultures. All media can be prepared and stored at 4°C for up to 6 months. Avoid long periods of incubation of media bottles at 37°C to prevent the loss of properties. It is recommended aliquoting the media to avoid repeated heating of large volumes.

Complete DMEM F12		
Reagent	Final concentration	Amount
DMEM F12	1×	435 mL
FBS-H1	10%	50 mL
P/S 10,000 U/mL	100 U/mL	5 mL
HEPES 1M	100 mM	5 mL
NEAA 100×	1×	5 mL
Total	n/a	500 mL

N1- DMEM F12		
Reagent	Final concentration	Amount
DMEM F12	1×	475 mL
N1 Medium Supplement 100×	1×	5 mL
BSA Fraction V 7.5%	0.075%	5 mL
P/S 10,000 U/mL	100 U/mL	5 mL
HEPES 1M	100 mM	5 mL
NEAA 100×	1×	5 mL
Total	n/a	500 mL

Low Glucose- DMEM		
Reagent	Final concentration	Amount
DMEM no glucose no glutamine	1×	98 mL
Glucose 200 g/L	1 mM	0.09 mL
P/S 10,000 U/mL	100 U/mL	1 mL
HEPES 1M	100 mM	1 mL
Total	n/a	100 mL

STEP-BY-STEP METHOD DETAILS

Culture of astrocytes

Timing: 7–10 days

This protocol describes how to obtain a pure culture of neonatal astrocytes from brains from mouse pups 1–3 days old. Older mice could be use but the yield of the process is reduced significantly. We recommend using 6–8 brain cortices per T75 flask to be cultured. Numbers and surface area can be adjusted if the researcher choses to use whole brains or older mice.

Note: Culture of astrocytes from adult mouse brain has been reviewed elsewhere (Sun et al., 2017). Please refer to this same publication for micrographs of postnatal astrocyte cultures similar to the ones described here.

Alternatives: Whole brains instead of cortices could be used. However, Astrocytes from different brain regions are heterogeneous, which may lead to increased variability in the results across experiments.

Note: Always work under sterile conditions.

1. Prepare sterile dissection tools and place them into 70% Ethanol.
2. Prepare one 50 mL tube with 10–15 mL of 4°C HBSS per Flask to be cultured, place them on ice.
3. Prepare 10 cm petri dishes with 5–8 mL of 4°C HBSS and place them on ice.
4. Anesthetize pups.
5. Euthanize each pup by decapitation. Gently extract the brain by performing two lateral incisions at the back of the head. Avoid touching the brains and gently drop them facing up into the petri dishes.

Note: Always follow your institution's IACUC guidelines for anesthesia and euthanasia procedures

6. Using magnification system if needed, gently separate the cortices from the rest of the brain. Add cortices to the 50 mL tubes with HBSS previously prepared
7. Repeat steps 5 and 6 until done with all the pups
8. Once Cortices have been pooled in the 50 mL tubes (5–8/tube) spin them down 400 or 300 × *g* (RCF) for 5 min at 4°C
9. Remove the supernatant carefully to avoid moving the cortices.
10. Add 5 mL of 0.25% Trypsin-EDTA, pipette up and down at least 6 times with a 5 or 10 mL pipet to gently disaggregate the tissue.
11. Incubate for 15 min at 37°C in a cell culture incubator.
12. Add 10 mL of Complete DMEM/F12 to stop the reaction. Invert the tube
13. Place a 70 micron cell strainer on top of a new 50 mL tube and filter the suspension by gravity flow. Help the flow through with a syringe plunger if necessary.
14. Centrifuge the flow-through at 400 × *g* for 5 min
15. Remove supernatant
16. Resuspend pellet in 12–15 mL of Complete DMEM/F12.
17. Add to the precoated T75 flask
18. Incubate at 37°C 5% CO_2 and 85%–95% humidity.
19. Change media the next day or the latest 48 h later
20. Replace media every 2–3 days
21. Astrocytes will be confluent and rid of debris by day 7–10.

Note: Media can be replaced every 2–3 days for around a month and the cells will still be fine. Old cultures will just have more microglia growing on them (see next steps).

Alternatives: Instead of using Complete DMEM, one can use N1-DMEM to avoid presence of FBS which could impact astrocyte biology. Astrocytes typically grow twice as slow in N1-DMEM and we recommend starting with more cell density (Like 10–12 cortices per T75 flask).

Table 2. Astrocyte optimal seeding densities

Culture plate	Volume of media per well	Number of cells
12 well	1.5 mL	4–5 × 10^5
24 well	1 mL	2–2.5 × 10^5
48 well	0.5 mL	10^5
96 well Seahorse	0.1–0.2 mL	30,000–40,000

Continue to step 34 for astrocyte silencing or directly to step 40 for astrocyte stimulation.

Expect to have a confluent culture after about 2 or 3 weeks of culture. Change media as described above.

Seeding of astrocytes

Timing: 3 h hands on, 24–48 h total

Once cells are confluent and uniform, healthy and without major debris, they can be seeded in multi well plates for downstream analyses. Please refer to Table 2 for cell numbers to be used in each type of multi well plate.

22. Shake T75 flask for 30 min 180–200 rpm at 37°C

Note: If the shaker doesn't have CO_2 influx, it is convenient to seal the lid of flasks so they can keep the CO_2 levels inside.

23. Remove media and add 10 mL of fresh medium
24. Shake for 1.5–2 h more at 200 rpm at 37°C. Check under the microscope for the detachment of microglia.
25. Remove media
26. Rinse astrocytes once with HBSS
27. Add 4 mL of 0.25% Trypsin-EDTA and shake them at 37°C checking them every 2 min until monolayer has detached.
28. Add 10 mL of complete DMEM/F12 to stop the reaction
29. Pipet thoughtfully and transfer to a 50 mL tube.
30. Centrifuge at 400 × *g* for 5 min
31. Resuspend the pellet in 10 mL of Complete DMEM/F12 and count the cells
32. Seed the cells according to numbers of Table 2
33. Incubate at 37°C, 5% CO_2 and 85%–95% humidity for 24 or 48 h until the cells look flat again.

Note: For problems with cell attachment refer to troubleshooting 1.

Note: For problems with culture contamination refer to troubleshooting 2.

Note: Media from steps 23 and 25 will contain microglia; if you want to use them for downstream analyses, we recommend you purify them using positive selection for CD11b+ cells or any other marker of your choice. You could do that by FACS or magnetic beads-based separation.

Silencing of astrocytes

Timing: 30 min hands on, total from 8 h to 3 days.

In this section we describe how to silence primary murine astrocytes with siRNAs.

Table 3. Knockdown of astrocytes

Culture plate	Opti-MEM volume	INTERFERin volume	Amount of siRNA	Volume of DMEM per well
24 well	100 μL	2 μL	0.6 pmoles	500 μL
48 well	75 μL	1 μL	0.3 pmoles	300 μL
96 well Seahorse	50 μL	0.5 μL	0.17 pmoles	100 μL

Note: Always use the microscope to confirm that cells are healthy and attached before starting the protocol. User should determine the best siRNA sequence for their target molecule of interest. As a general guidance, we used DHARMACON smart pools.

34. Mix INTERFERin reagent with the desired siRNAs in Opti-MEM following Table 3.
35. Incubate the mix 10 min at room temperature.
36. Change the media of the astrocytes to fresh new media (Complete DMEM or N1-DMEM) following volumes on Table 3.
37. Add the mix of INTERFERin, siRNAs and Opti-MEM dropwise to the astrocyte's wells and incubate at 37°C.

If viability of astrocytes is critically reduced, go to troubleshooting 3.

38. After 48 h incubation, downstream analysis can be performed (see following sections)
39. Knockdown efficiency can be confirmed by qPCR or western blot, it should be detected from 24 to 96 h after transfection.

Note: If target molecule expression is not reduced, go to troubleshooting 4.

Note: In our experience, astrocytes are quite hard to transfect or transduce. Researchers could explore the possibility of transducing with lentiviruses or electroporating or nucleofecting the astrocytes if the above transfection with siRNAs does not end in the expected results.

Proceed to the following section or oxygen consumption analysis section as desired.

Cytokine stimulation and Astrocyte Conditioned Media collection

Timing: 30 min hands on, total from 8 h to 3 days.

In this section we describe how to stimulate primary murine astrocytes with cytokines *in vitro* and how to proceed with gene expression analysis and/or Astrocyte Conditioned Media (ACM) collection.

Researchers can choose to do the following steps after seeding the astrocytes (step 33) or after silencing (step 39).

40. When cells are ready, change the media to fresh media (Complete DMEM or N1-DMEM) with the desired final concentration of the required cytokines. See Table 4 as a guidance for concentrations (Chao et al., 2019; Rothhammer et al., 2018; Sanmarco et al., 2021)

Note: This is general guidance, but researchers must set up the optimal condition for specific cytokines and combinations. Proceed to step 41 or 43

Note: On use of small molecules or chemical inhibitors: If the researcher needs to treat astrocytes with some sort of drug, we recommend doing that 30 min prior the cytokine stimulation

Table 4. Cytokines for astrocytes stimulation

Cytokine	Final concentration
mouse IFNγ	100 ng/mL
mouse TNF	5–50 ng/mL
mouse IFNβ	500 IU/mL
mouse IL1β	10–100 ng/mL
human TGFβ	5 ng/mL
human TGFα	0.1 ng/mL
mouse VEGF-B	10 ng/mL
mouse IL-17	10 ng/mL
mouse C1q	100 ng/mL
mouse IL-10	100 ng/mL

(Chao et al., 2019; Clark et al., 2021) and keep the drug in the media after cytokine addition if the drug effects are reversible or if the incubation time is going to be long.

41. Incubate at 37°C for 6–48 h for gene expression analysis.
42. Wash the cells gently once with HBSS, aspirate HBSS and lysate the cells with Buffer RLT (Qiagen), store at −20 or −80°C for later RNA extraction (Qiagen RNeasy kit) or proceed directly with RNA extraction and desired gene expression analysis (RT-qPCR…).
43. For ACM collection, incubate the cells at 37°C with media with cytokines for 8 h to ON.
44. Wash the cells gently at least twice with HBSS to get rid of any trace of cytokines.
45. Replace with fresh media without cytokines and incubate at 37°C for 2 days.
46. Collect the ACM to be used from downstream studies like Neurotoxicity assay or measurement of cytokine production by ELISA.

Note: ACM media can be aliquoted and frozen at −80°C, however for certain downstream procedures it is better to use it fresh.

Oxygen consumption analysis

Timing: 3 h and 1 more hour on a following day hands on. Total 24–48 h

In this part of the protocol, we describe oxygen consumption analysis performed on primary murine astrocytes with a XFe96 Seahorse analyzer (Agilent Technologies).

⚠ CRITICAL: Change astrocyte media to Low Glucose-DMEM

47. Incubate at 37°C for 24 or 48 h (this a starvation step)
48. Stimulate the astrocytes, if needed, following steps 40 and 41.

⚠ CRITICAL: Make sure you still use Low Glucose DMEM

49. The day before performing the assay make sure you hydrate the sensor cartridge in Seahorse XF Calibrant.
50. Start Mito Stress assay with Seahorse XF Cell Mito Stress Test Kit (#103015-100, Agilent Technologies). Strictly follow the manufacturers' manual (Mito_Stress_Test_Kit_Guide). With this system, oxygen consumption rate (OCR) is quantified real-time after sequential addition of 2 mM Oligomycin, 1 mM FCCP and 5 mM Rotenone/Antimycin A.

Note: Preparation and performance of Seahorse analysis is very well explained in the Seahorse XF Cell Mito Stress Test Kit User Guide. Please refer to it and strictly follow the protocol.

51. Once Seahorse run is done carefully remove the plate from the machine
52. Aspirate media, wash cells once with HBSS and make sure under the microscope that the cells are still attached to the wells and looking healthy.

Note: If cells are no longer attached or look severely distressed, refer to troubleshooting 5.

53. Aspirate all the liquid and freeze the plate immediately at −80°C for at least over night before proceeding. Plates can be store for up to 4 weeks.
54. Thaw the cell plates at room temperature.
55. Quantify the cells using CyQUANT cell proliferation assay kit (#C7026, Invitrogen) following the protocol for Cell Number Determination (CyQUANT_manual). We recommend using a cell number standard curve with previously frozen aliquots of known astrocyte numbers. This is an easy and reliable method.

⚠ CRITICAL: Normalize OCR rate to cell number. This is crucial step to get reliable and reproducible results.

⚠ CRITICAL: A confluent and healthy monolayer is crucial for the good performance of the Seahorse OCR assay. We do not recommend performing the assay with patchy monolayers or unhealthy cells.

Alternatives: Likewise, we describe here for the XF Cell Mito Stress Test, other Agilent seahorse tests can be run following the same pre- and post-Seahorse analysis steps described here.

Alternatives: Normalization can be performed with other techniques like direct number of cells quantification by DAPI staining and imaging. However, normalization to protein amount (with BCA or Bradford assays for example) is extremely discouraged as the poly-L-lysine in the plate would interfere with measurements.

EXPECTED OUTCOMES

As mentioned above, we recommend plating 6–8 brain cortices per 75 cm^2 flask. That will give rise to roughly 5 to 6 million mature astrocytes per flask.

When using this protocol for siRNA-based knockdown, in most cases we achieved > 50% gene silencing as determined by mRNA levels (Figure 1); these levels were sufficient to affect astrocyte responses (Chao et al., 2019). However, for some genes this protocol did not result in significant knockdown.

We and others have shown that astrocytes can acquire a pro-inflammatory phenotype following activation with multiple cytokines including TNF, IFNγ, IL1β, C1q (Figure 2) (Chao et al., 2019; Clark et al., 2021; Liddelow et al., 2017; Rothhammer et al., 2016, 2018; Wheeler et al., 2019, 2020). Interestingly, some cytokines can also induce an anti-inflammatory astrocyte phenotype (Sanmarco et al., 2021). Thus, we encourage researchers to carefully choose cytokine of interest for their investigation.

LIMITATIONS

Researchers should consider that this protocol may result in some small microglia contamination in astrocytes cultures. Hence, we recommend the addition of a flow cytometry-based cell sorting step before astrocyte seeding to completely remove microglia. The caveat of this stage is that sometimes astrocytes will take longer to look good after seeding.

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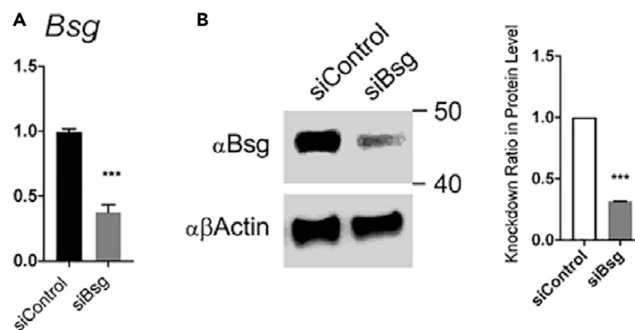

Figure 1. siRNA silencing efficiency example

(A and B) *Bsg* gene expression (A) and protein levels (B) after siRNA knockdown in astrocytes in vitro (unpaired t test, error bars represent SEM).

Reprinted with permission from Chao et al. (2019).

The protocol described here only applies to neonatal mice astrocytes, which are easier to culture and more broadly used in the field. Adult astrocytes can be also cultured from adult brain after myelin removal, but cultures typically take around 3 weeks to be established.

TROUBLESHOOTING

Problem 1

Cells did not attach or only a few did, and they do not look flat (step 33).

Potential solution

Probably Poly-L lysine coating did not work or was insufficient. There is not a real solution at this step of the protocol and unfortunately your cells are lost at this point. Next time incubate the Poly-L lysine for longer period of time.

Another possibility is that the cells have died due to contaminations (see problem 2).

As a general practice, if you are planning to treat your cells with some sort of drug, wait till they are seeded and attached before initiating treatment.

Problem 2

Cells are contaminated right after the seeding or during some time of the culture (step 33).

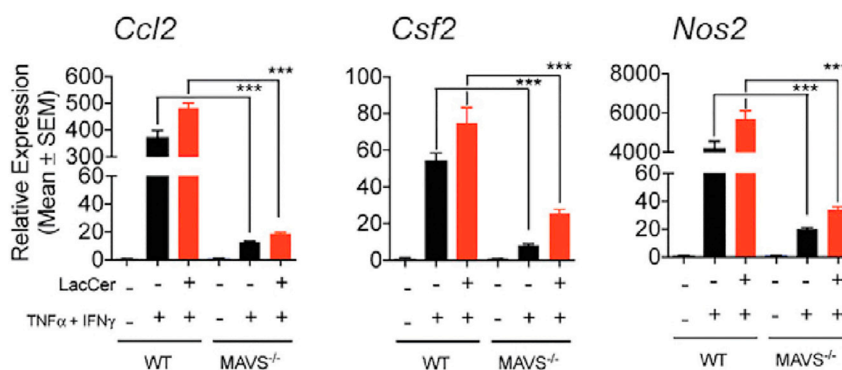

Figure 2. Astrocytes stimulation example

mRNA expression determined by qPCR of proinflammatory genes after different stimuli including TNF and IFNg cytokines. (unpaired t test, compared to corresponding condition between WT and MAVS−/− astrocytes, error bars represent SEM).

Reprinted with permission from Chao et al. (2019).

Potential solution

Discard the culture immediately and decontaminate the incubator. We also recommend discarding the media used to culture those specific samples to avoid possible cross contaminations.

If cells were contaminated right after their isolation, consider next time improve your sterile manipulation immersing the dissection tools in 70% ethanol. The use of a tissue culture hood is recommended at every step.

Problem 3

Viability is drastically reduced after siRNA treatment (step 37).

Potential solution

Replace the media after 4 h of incubation with siRNAs/INTERFERin mix. This hopefully will maximize cell viability. We never experienced reduced viability, but knockdown of certain molecules can be harmful to astrocytes.

Problem 4

Gene or Protein of interest expression is not reduced after siRNA treatment (step 39).

Potential solution

Change to an alternative siRNA sequence for the same gene or try to adjust the silencing window.

Problem 5

Cells did suffer or did not survive after Seahorse assay (step 53).

Potential solution

Try to optimize cell seeding density as well as how long you wait to perform the assay after seeding the cells. Make sure next time you coat the seahorse plate with fresh Poly-L lysine. If possible, coat the plates ON at 4°C.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Francisco J. Quintana, fquintana@rics.bwh.harvard.edu.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

C.G.V. and F.J.Q. wrote the manuscript. F.J.Q. designed and supervised the study and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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