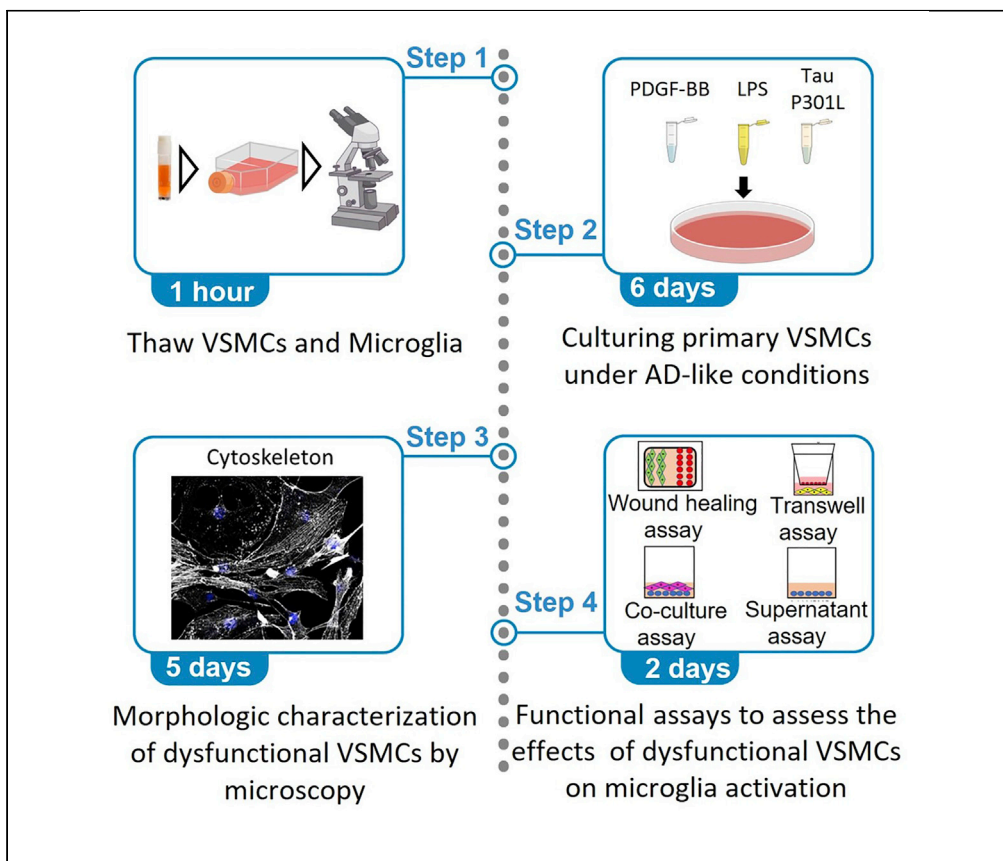


## Protocol

Protocol to assess the effects of dysfunctional human vascular smooth muscle cells on other brain cells using *in vitro* models of Alzheimer's disease



Here, we present a protocol to culture primary human vascular smooth muscle cells (VSMCs) under Alzheimer's disease (AD)-like conditions, including steps for morphological characterization with microscopy. We then describe functional assays, including wound healing, transwell, coculture, and supernatant assays, to evaluate the effect of dysfunctional VSMCs on the induction of the AD-associated microglial phenotype. Our approach can be applied to assess the effects of dysfunctional VSMCs on other cerebral cell lines including pericytes, astrocytes, and neurons under AD-like conditions *in vitro*.

Karla Lucia F. Alvarez, Jorge Aguilar-Pineda, Karin J. Vera-Lopez, Christian L. Lino Cardenas

clinocardenas@mgh.harvard.edu (C.L.L.C.)  
kalvarezf@ucsm.edu.pe (K.L.F.A.)

### Highlights

Detailed protocol to induce dysfunctional human VSMCs

Characterization of dysfunctional human VSMCs by PCR and fluorescence microscopy

Effects of dysfunctional human VSMCs on the Alzheimer-associated microglia phenotype

Applicable to study VSMC effects on other brain cells under AD-like conditions *in vitro*

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## Protocol

Protocol to assess the effects of dysfunctional human vascular smooth muscle cells on other brain cells using *in vitro* models of Alzheimer's disease

Karla Lucia F. Alvarez,<sup>1,3,\*</sup> Jorge Aguilar-Pineda,<sup>1</sup> Karin J. Vera-Lopez,<sup>1</sup> and Christian L. Lino Cardenas<sup>1,2,3,4,\*</sup>

<sup>1</sup>Laboratory of Genomics and Neurovascular Diseases, Vicerrectorado de investigacion, Universidad Catolica de Santa Maria, Arequipa, Peru

<sup>2</sup>Cardiovascular Research Center, Cardiology Division, Massachusetts General Hospital, Boston, MA 02114, USA

<sup>3</sup>Technical contact

<sup>4</sup>Lead contact

\*Correspondence: [clinocardenas@mgh.harvard.edu](mailto:clinocardenas@mgh.harvard.edu) (C.L.L.C.), [kalvarezf@ucsm.edu.pe](mailto:kalvarezf@ucsm.edu.pe) (K.L.F.A.)  
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## SUMMARY

Here, we present a protocol to culture primary human vascular smooth muscle cells (VSMCs) under Alzheimer's disease (AD)-like conditions, including steps for morphological characterization with microscopy. We then describe functional assays, including wound healing, transwell, coculture, and supernatant assays, to evaluate the effect of dysfunctional VSMCs on the induction of the AD-associated microglial phenotype. Our approach can be applied to assess the effects of dysfunctional VSMCs on other cerebral cell lines including pericytes, astrocytes, and neurons under AD-like conditions *in vitro*.

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

## BEFORE YOU BEGIN

We have shown that dysfunctional VSMCs can play a detrimental role on AD pathogenesis (Aguilar-Pineda et al., 2021). The protocol below details the steps to establish functional assays that can be used to evaluate the effect of dysfunctional VSMCs on other brain cells using *in vitro* systems to mimic AD-like conditions for the screening of new drug treatments.

⚠ **CRITICAL:** All sterile procedures should be performed in a Class II biological safety cabinet under standard aseptic technique.

## Prepare cell cultures

⌚ **Timing:** 1 h

**Note:** Obtain primary human VSMCs from neural crest origin (carotid artery) and growth medium from Cell Applications company. Obtain primary human microglia cells and growth medium from ScienCell company.

1. Thaw primary human VSMCs in a water bath at 37°C for 5 min and resuspend in 10 mL of warmed growth medium (37°C).
2. Place the cells in pre-treated plates (Nunc™ OmniTray™ Single-Well Plate) and maintained at 37°C with 5% CO<sub>2</sub> in a humidified chamber.



3. After 5 h, discard old medium and feed cells with 10 mL of fresh growth medium to eliminate any excess of DMSO.
4. Thaw primary human microglia cells in a water bath at 37°C for 5 min.
5. Then, resuspend microglia cells in 5 mL of warm growth medium (37°C) and centrifuged at 180 g for 3 min. Discard the supernatant without breaking the cell pellet.
6. Resuspend microglia cells with 2 mL of growth media (37°C) and seed the cell pellet in pre-treated plates (Nunc™ OmniTray™ Single-Well Plate) with 10 mL of growth medium and maintain at 37°C with 5% CO<sub>2</sub> in a humidified chamber.
7. Allow VSMCs or microglia cells to become proliferative (90% confluence) before beginning passaging and experimentations.

**Note:** Before using trypsin and PBS bring them to room temperature (20°C–22°C) before starting to culture cells.

△ **CRITICAL:** To avoid loss of cellular identity use human primary VSMCs from passage 1–10. Microglia cells were used at passage 2–5.

**Note:** Prepare solution stock of the neurotoxins listed in the “materials and equipment” section and prepare several aliquots of them to conserve bioactivity. Store them at –20°C to avoid degradation.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, peptides, and recombinant proteins</b>		
Recombinant PDGFBB	Sigma-Aldrich	Cat#GF149
Recombinant Tau P301L	rPeptide	Cat#T1014-1
LPS	Sigma-Aldrich	Cat#L9143
D-PBS	Sigma-Aldrich	BSS-1005
Formaldehyde 37%	Sigma-Aldrich	F8775-500ML
Culture medium ready-to-use with FBS and antibiotics (VSMCs)	Cell Applications	Cat#311-500
Culture medium (Microglia)	ScienCell Research Laboratories	Cat# 1901
Trypsin	Thermo Scientific	Cat# 25200072
Trizol	Thermo Scientific	Cat# 10296010
DAPI	Thermo Scientific	Cat#D1306
Opti-MEM	Thermo Fisher Scientific	Cat# 31985062
Donkey serum	Abcam	Cat# ab7475
<b>Critical commercial assays</b>		
CellTracker Green CMFDA	Thermo Scientific	Cat#C7025
CellTracker Deep Red Dye	Thermo Scientific	Cat#C34565
CytoSelect™ 24-Well Wound Healing Assay	Ibid	Cat#CBA-120-5
miRNeasy kit	QIAGEN	Cat#217084
Nucblue live cell stain	Thermo Fisher Scientific	Cat#R37605
Applied Biosystems™ SYBR Green™ PCR Master mix	Applied Biosystems	Cat# A25741
Invitrogen™ ActinGreen™ 488 ReadyProbes™ Reagent (AlexaFluor™ 488 phalloidin)	Invitrogen	Cat# R37110
Molecular Probes™ ProLong™ Diamond Antifade Mountant with DAPI	Molecular Probes	P36962
<b>Antibodies</b>		
iNOS, dilution 1:400	Abcam	Cat# ab15323
MHCII, dilution 1:100	Santa Cruz biotechnology	Cat#sc59322
IL-6, dilution 1:100	Santa Cruz biotechnology	Cat# sc-57315
Secondary antibody AF488 , dilution 1:400	Thermo Fisher Scientific	Cat#A32790

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Experimental models: Cell lines</b>		
Human carotid Vascular Smooth Muscle Cells	Cell Applications	Cat#3514K-05a
Human microglia	ScienCell Research Laboratories	Cat#1900
<b>Oligonucleotides</b>		
Forward primer COL6A3	Sigma-Aldrich	ATGAGGAAACATCGGCACTTG
Reverse primer COL6A3	Sigma-Aldrich	GGGCATGAGTTGTAGGAAAGC
Forward primer MMP9	Sigma-Aldrich	TGTACCGCTATGGTTACTCTG
Reverse primer MMP9	Sigma-Aldrich	GGCAGGGACAGTTGCTTCT
Forward primer GJA1	Sigma-Aldrich	GGTGA CTGGAGCGCCTTAG
Reverse primer GJA1	Sigma-Aldrich	GCGCACATGAGAGGCGCACATGAGAGATTGGGA
Forward primer MMP2	Sigma-Aldrich	TGACTTTCTTGGATCGGGTCG
Reverse primer MMP2	Sigma-Aldrich	AAGCACCATCAGATGACTG
Forward primer COL1A1	Sigma-Aldrich	GAGGGCCAAGACGAAGACATC
Reverse primer COL1A1	Sigma-Aldrich	CAGATCACGTCATCGCACAAAC
Forward primer MYH10	Sigma-Aldrich	TGGTTTTGAGGCAGCTAGTATCA
Reverse primer MYH10	Sigma-Aldrich	AGTCCTGAATAGTAGCGATCCTT
Forward primer CNN1	Sigma-Aldrich	CTGTCAGCCGAGGTTAAGAAC
Reverse primer CNN1	Sigma-Aldrich	GAGGCCGTCCATGAAGTTGTT
Forward primer MYH11	Sigma-Aldrich	CGCCAAGAGACTCGTCTGG
Reverse primer MYH11	Sigma-Aldrich	TCTTTCCCAACCGTGACCTTC
Forward primer TAGLN	Sigma-Aldrich	AGTGCAGTCCAAAATCGAGAAG
Reverse primer TAGLN	Sigma-Aldrich	CITGCTCAGAATCACGCCAT
Forward primer ACTA2	Sigma-Aldrich	AAAAGACAGCTACGTGGGTGA
Reverse primer ACTA2	Sigma-Aldrich	GCCATGTTCTATCGGGTACTTC
Forward primer SMTN1	Sigma-Aldrich	CCCTGGCATCCAAGCGTTT
Reverse primer SMTN1	Sigma-Aldrich	CTCCACATCGTTCATGGACTC
<b>Software and algorithms</b>		
Leica Application Suite X software	Leica Geosystems	Version LAS X
ImageJ software	National Institutes of Health	<a href="https://imagej.nih.gov/ij/">https://imagej.nih.gov/ij/</a>
<b>Other</b>		
Trans-well plates	Corning	Cat#354578
QuantStudio™ 5 Real Time PCR machine	Thermo Fisher Scientific	Cat#A34322
8-well microscopy slides	Corning	Cat# 354118
Sterile conical tube	CLS430829	Cat# CLS430829
Inverted microscope	Leica Microsystems	DM IL LED
Leica TCS SP8 confocal microscopy	Leica Microsystems	TCS SP8
Nunc 6-Well Plate, Round	Thermo Fisher Scientific	Cat#140675
Nunc™ OmniTray™ Single-Well Plate	Thermo Fisher Scientific	Cat#165218

**MATERIALS AND EQUIPMENT**

**PDGF-BB (20 µg/mL)**

Reconstitute 10 µg of PDGF-BB in 500 µL of phosphate buffered saline, aliquot (10–20µL) and store at –20°C for 6 months or +4°C for 1 week. Avoid repeated freeze-thaw.

**Recombinant Tau P301L (100 µg/mL)**

Reconstitute 50 µg of recombinant P301L in 500 µL of Opti-MEM, aliquot (50–100µL) and store at –20°C for long term (6 months) to avoid multiple freeze/thaw cycles.

**LPS (5 mg/mL or 1 mg/mL)**

Reconstitute 10 mg of LPS in water (5 mg/mL) or cell culture medium (1 mg/mL) yielding a hazy, faint yellow solution. Aliquot (10–20 µL) and store at –20°C for long term (8 months). Avoid multiple freeze/thaw cycles.

#### PDGF-BB-cell culture medium and working solutions

Reagent	Final concentration	Amount
Cell culture medium (ready to use)	n/a	10 mL
PDGF-BB (20 µg/mL)	60 ng/mL	30 µL
<b>Total</b>	<b>n/a</b>	<b>10 mL</b>

Store at –20°C for 6 months

#### Tau P301L-cell culture medium and working solutions

Reagent	Final concentration	Amount
Cell culture medium (ready to use)	n/a	10 mL
Tau P301L (100 µg/mL)	40 ng/mL	4 µL
<b>Total</b>	<b>n/a</b>	<b>10 mL</b>

Store at –20°C for 6 months

#### LPS-cell culture medium and working solutions

Reagent	Final concentration	Amount
Cell culture medium (ready to use)	n/a	10 mL
LPS (5 mg/mL)	2.5 µg/mL	5 µL
<b>Total</b>	<b>n/a</b>	<b>10 mL</b>

Store at –20°C for 8 months

#### Neutralizing Solution and working solutions

Reagent	Final concentration	Amount
Cell culture medium ready-to-use; containing 10 % fetal bovine serum	n/a	20 mL
<b>Total</b>	<b>n/a</b>	<b>20 mL</b>

Store at 4°C for 4 months

#### Equipment setup

Cells were visualized with the Leica TCS SP8 confocal microscopy station and pictures were digitized with the Leica Application Suite X software. Two-dimensional and white-light images were analyzed using ImageJ software.

### STEP-BY-STEP METHOD DETAILS

#### Culturing human primary VSMCs under AD-like conditions

- ⌚ Timing: About 9 days
- ⌚ Timing: 1 day for step 1
- ⌚ Timing: About 8 days for step 2
- ⌚ Timing: About 6 days for step 3

This step contains information on how to culture human primary VSMCs using three experimental AD-like conditions *in vitro*. First condition: PDGF-BB a potent myogenic cytokine which has been found to be increased in brains of patients with AD (Barroeta-Espar et al., 2019). Second condition: the mutant Tau P301L which is associated with dementia and neurogenerative tauopathies (Lathuilière et al., 2017). And third condition: LPS which is a lipopolysaccharide associated with amyloid plaques formation (Lee et al., 2008).

### 1. Human primary VSMCs detachment

- a. Aspirate the medium from the culture plates.
- b. Add 5 mL of PBS and gently swirl it to remove traces of serum from the culture medium.
- c. Add 5 mL of Trypsin/EDTA solution.
- d. Remove 4 mL of the solution immediately.
- e. Monitor the trypsinization progress at room temperature under an inverted microscope.

**Note:** If rounded cells are attached to the cell surface, release them by hitting the side of the plate against your palm until most of the cells are detached.

- f. Add 5 mL of neutralizing Solution and transfer the solution into a 50 mL sterile conical tube.

**Note:** Examine the plate under a microscope. If there are >20% cells left in the plate, repeat steps b–f.

- g. Centrifuge the conical tube at 220 × g for 5 min.
- h. Aspirate the supernatant from the tube without disturbing the cell pellet.
- i. Flick the tip of the conical tube with your finger to loosen the cell pellet.
- j. Resuspend the cells in 5 mL of Smooth Muscle Cell Growth Medium by gently pipetting the cells to break up the clumps.
- k. Count the cells with a hemocytometer.

### 2. Generation of dysfunctional VSMCs and gene expression

- a. Seed 300–400 × 10<sup>5</sup> cells (60% confluency) per well in a 6-well plate.

**Note:** Seeding cells in a 6 well plate is important to obtain 6 experimental replicates per treatment.

- b. Place the cell culture plate in a standard incubator at 37°C and let cells to sit and acclimate for 24 h.
- c. Replace with 2 mL of fresh growth medium containing PDGFBB, Tau P301 or LPS. Change fresh growth medium containing neurotoxins every 24 h for 3 days.
- d. Remove the culture media and wash the plate three times with 2 mL of PBS.
- e. After PBS aspiration, add 750 μL QIAzol Lysis Reagent (included in miRNeasy kit) per well and mix extensively by pipetting all over the plate.
- f. Let sit for 10 min at room temperature before proceeding RNA extraction. Follow the manufacturer's instructions of the miRNeasy kit (<https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en>).
- g. Use 100 ng of total RNA as the starting template for cDNA synthesis.
- h. Synthesize cDNA via reverse transcription following the manufacturer's instructions of your preference ([https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2F%2FTFS-Assets%2F%2FLSG%2Fmanuals%2FsuperscriptIV\\_VILO\\_master\\_mix\\_UG.pdf](https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2F%2FTFS-Assets%2F%2FLSG%2Fmanuals%2FsuperscriptIV_VILO_master_mix_UG.pdf)).
- i. Use 100–150 ng of cDNA to perform RT-qPCR.
- j. Dilute the cDNA samples (1:5 ratio) with molecular grade water.
- k. Thaw the gene-specific primers (COL6A3, MMP9, GJA1, MMP2, COL1A1, MYH10, CNN1, MYH11, TAGLN, SMTN1, and ACTA2) on ice and prepare the reaction mix.
- l. Follow RT-qPCR protocol according to manufacturer's instructions of the Applied Biosystems™ SYBR Green™ PCR Master mix ([https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2F%2FTFS-Assets%2F%2FLSG%2Fmanuals%2F100031508\\_PowerUp\\_SYBRgreen\\_QRC.pdf](https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2F%2FTFS-Assets%2F%2FLSG%2Fmanuals%2F100031508_PowerUp_SYBRgreen_QRC.pdf)).
- m. Turn on the Real time PCR machine 30 min prior to running the qPCR

- n. Follow the recommended qPCR program.
- o. Analyze gene expression by quantitative PCR (qPCR) on a SYBR green system.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	2 min	40
Denaturation	94°C	15 s	40 cycles
Annealing	60°C	1 min	
Extension	72°C	10 s	
Hold	10°C	–	

**Note:** The fold change in gene expression can be calculated relative to the endogenous GAPDH (encoding glyceraldehyde-3-phosphate dehydrogenase) control using the ddCT method.

**Note:** Dysfunctional VSMCs should show increased expression of the following synthetic markers: COL6A3, MMP9, GJA1, MMP2, COL1A1, MYH10. And decreased expression of the following contractile markers: CNN1, MYH11, TAGLN (SM22- $\alpha$ ), SMTN1, ACTA2 (Lino Cardenas et al., 2018a, 2018b, 2019).

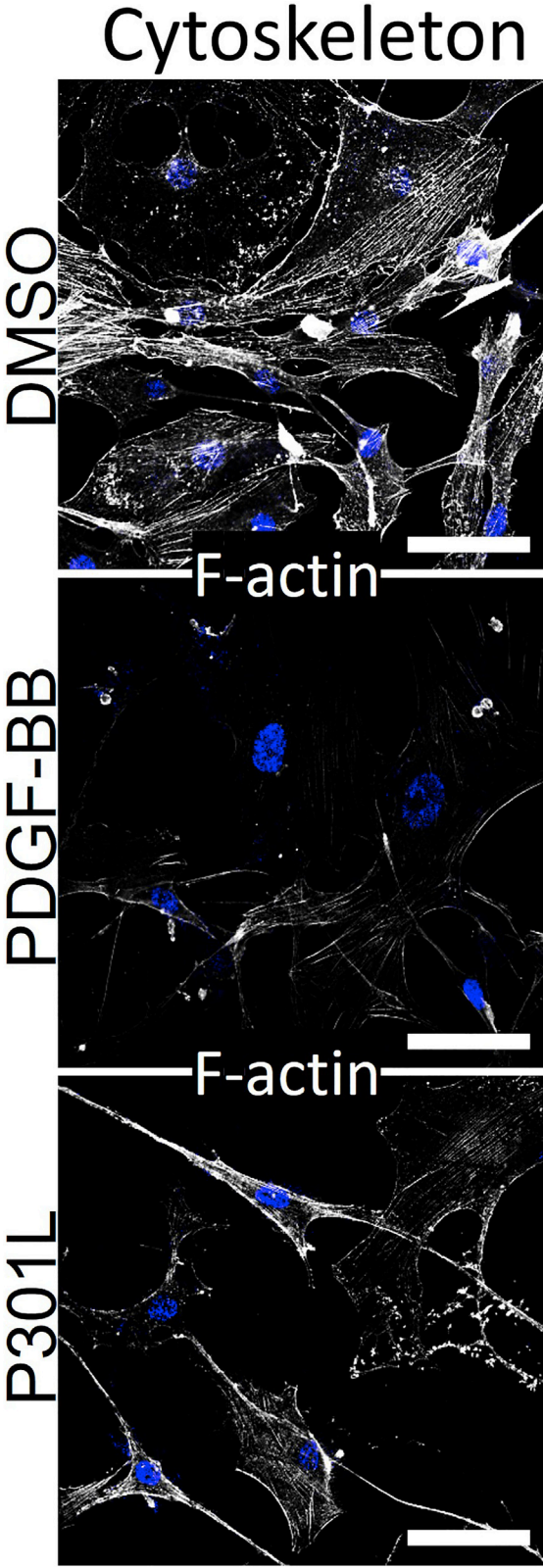
3. Morphologic characterization of dysfunctional VSMCs by microscopy
  - a. Seed enough cells in an 8-well microscopy slides until obtain 50% confluency and allow them to acclimate for 24 h before initiate treatment.
  - b. Replace with 2 mL of fresh growth medium containing PDGFBB, Tau P301 or LPS. Change fresh growth medium containing neurotoxins every 24 h for 3 days.
  - c. Wash twice with 1 mL of PBS and fix cells with 4% formaldehyde for 20 min.
  - d. Permeabilize the cells with 0.1% triton X-100 in PBS for 15 min.
  - e. Wash twice with 1 mL of PBS.
  - f. Add 2 drops of phalloidin reagent per mL of media and incubate for 30 min.
  - g. Wash with 2 mL of PBS twice and use mounting media containing DAPI (Diamond Antifade Mountant with DAPI).
  - h. Visualize cells with the Leica TCS SP8 confocal microscopy station on 40 $\times$  or 63 $\times$  optical and digitized pictures with the Leica Application Suite X software. Two-dimensional and white-light images should be analyzed using ImageJ software.

**Note:** Dysfunctional VSMC should show decrease in F-actin fibers (green fluorescence) and loss of cytoskeleton integrity as assessed by Phalloidin staining (Figure 1).

### Functional assays to evaluate the effect of dysfunctional VSMCs on microglia cells activation

- ⌚ Timing: About 10 days for step 4
- ⌚ Timing: About 7 days for step 5
- ⌚ Timing: About 7 days for step 6
- ⌚ Timing: About 8 days for step 7

Functional assays are useful to investigate the effect of a given stimulus on the behavior of a cell population, including cell migration, invasion, proliferation, and survival. Example of these assays are the wound healing assay and the transwell assay. Herein, we use these assays to evaluate the effect that



**Figure 1. Morphological changes in the cytoskeleton of dysfunctional VSMCs**  
Scale bar: 20  $\mu$ m



dysfunctional VSMCs could have on the migration capability of microglial cells. We also evaluated the paracrine VSMC-mediated activation of microglial cells with a co-culture and supernatant assays.

#### 4. Wound healing assay

- a. Adjust cellular concentration of VSMCs or microglia to  $0.5 \times 10^6$  cells/mL using cell culture media (Cat#311-500 to VSMCs and Cat# 1901 to Microglia).
- b. Seed 250  $\mu$ L of VSMCs or microglia into the silicone insert (included in kit Cat#CBA-120-5).
- c. Stain VSMCs with CellTracker Green CMFDA and microglia cells with CellTracker Deep Red Dye overnight (16 h), following the manufacturer's instructions ([https://tools.thermofisher.com/content/sfs/manuals/celltracker\\_fluorescent\\_probes\\_man.pdf](https://tools.thermofisher.com/content/sfs/manuals/celltracker_fluorescent_probes_man.pdf)). For nuclear staining use NucBlue live cell stain, 2 drops per mL for 16 h.
- d. For VSMCs add fresh 100  $\mu$ L of medium containing neurotoxins as indicated above (Step two; generation of dysfunctional VSMCs). For microglia cells also add fresh 100  $\mu$ L growth normal medium containing neurotoxins. Maintain the cells for 72 h in the incubator at 37°C and 5% CO<sub>2</sub>.
- e. Using a sterile forceps remove the silicone insert from the plate well to begin the wound healing assay under normal growth medium.
- f. Visualize wells under a fluorescence microscope after 0, 12, 24, 48 h, focusing on the center of the wound field. Maintain the cells for 48 h in the incubator at 37°C and 5% CO<sub>2</sub> and image on 10 $\times$  until cells migrate to close the empty gap (Figure 2).

**Note:** 5 technical replicates per condition should be performed to achieve representative and significant data.

**Note:** Avoid bumping and moving the inserts.

**Note:** Carefully follow the manufacturer's instructions of the 24-well plate to ensure that the inserts are correctly oriented and have a firm contact with the bottom of the plate well (<https://www.cellbiolabs.com/sites/default/files/CBA-120-wound-healing-assay.pdf>).

**Note:** Avoid twisting the insert as this will damage the wound field.

#### 5. Transwell assay

- a. Seed  $0.2 \times 10^6$  VSMCs at the bottom and  $0.2 \times 10^6$  microglia cells on the top of a Transwell-12 plate. For VSMCs incubate with neurotoxins as indicated in the "generation of dysfunctional VSMCs".
- b. After 72 h, add 300  $\mu$ L fresh normal growth medium to VSMCs and label microglia cells overnight (16 h) with CellTracker Deep Red Dye followed by a change of 300  $\mu$ L fresh growth media.
- c. Position the insert containing microglia cells on top of VSMCs. Make sure that the insert merged in medium and maintain the cells for 16 h in the incubator at 37°C and 5% CO<sub>2</sub>.
- d. Remove the top insert and wash the bottom compartment with 300  $\mu$ L PBS before fixation with 300  $\mu$ L of 4% of formaldehyde for 10 min.
- e. Wash the cells three times with 300  $\mu$ L PBS.
- f. The migration of microglia cells should be quantified as the number of red cells migrated towards VSMCs. Imaging with fluorescent microscope at 10 $\times$  (Figure 3).

**Note:** 5 technical replicates per condition should be performed to achieve representative and significant data.

#### 6. Co-culture assay

- a. Seed  $0.2 \times 10^6$  VSMCs on a 12-well plate followed by incubation with neurotoxins as indicated in the section "generation of dysfunctional VSMCs". In parallel, seed  $0.2 \times 10^6$  microglia on a 12-well plate under normal growth conditions (final volume 500  $\mu$ L).

- b. Label VSMCs with CellTracker Deep Red Dye and microglia with Nucblue live cell stain (2 drops per mL) overnight (16 h) followed by a change of 500  $\mu$ L of fresh growth media.
- c. Trypsinize VSMCs, resuspend cell in normal growth media and count cells.
- d. Then seed  $0.2\text{--}0.3 \times 10^6$  VSMCs on top of microglia under normal growth conditions for 48 h.
- e. Wash 3 times with PBS and fix cells with 4% of formaldehyde for 10 min, follow by immunostaining of MHC-II or desire cell marker of activated microglia. Imaging (40 $\times$ ).

**Note:** Primary antibody against (MHC-II) should be used at 1:100 dilution overnight (16 h) at 4 $^{\circ}$ C, and secondary AF488 at 1:400 dilution (45 min, room temperature). This combination may allow the use of 3 fluorophores (Figure 4).

## 7. Supernatant assay to evaluate the activation of healthy microglia

### Cell culture supernatant assay

- a. Seed  $0.2 \times 10^6$  VSMCs on a 12-well plate and incubate them with 2 mL of neurotoxins or fresh normal media for microglia ( $0.2 \times 10^6$ ) as indicated in the section "generation of dysfunctional VSMCs".
- b. Add 2 mL of fresh media and incubate for 24 h
- c. Carefully aspirate supernatant into a sterile 1.5 mL centrifuge tube. Spin down at 1000  $\times$  g for 2 min and use 500  $\mu$ L of the supernatant to stimulate microglia (from step a) for 24 h in the incubator at 37 $^{\circ}$ C and 5% CO<sub>2</sub>.
- d. Wash three times with 1 mL of PBS and fix cells with 4% of formaldehyde for 10 min.
- e. Permeabilize cells with 0.5% of triton-x for 10 min and blocked with donkey serum at 10% for 1h followed by primary antibody (IL-6) incubation at 4 $^{\circ}$ C overnight (16 h).
- f. Use desire secondary antibody and incubate 45 min at room temperature.
- g. Mount slides with diamond mounting medium containing DAPI.
- h. Visualize cells with the Leica TCS SP8 confocal microscopy station and digitize pictures with the Leica Application Suite X software. Analyze two-dimensional and white-light images and quantify them using ImageJ software (Figure 5).
- i. Evaluate microglia activation by immunofluorescence staining of desire markers (Example: Iba1, MHC-II, IL-6, iNOS)

**Note:** Take 4 micrographs per well/condition using a fluorescence microscope (40 $\times$ –60 $\times$ )

### EXPECTED OUTCOMES

A successful generation of dysfunctional VSMCs should be characterized by the expression of COL6A3, MMP9, GJA1, MMP2, COL1A1, MYH10 and down regulation of SM22 $\alpha$ , CNN1 and MYH11. Also, dysfunctional VSMCs should present dramatic morphological changes in the cytoskeleton as shown in Figure 1. The effect of these cells on migration, invasion, and proliferation of microglia cells can be evaluated through functional assays (Figures 2, 3, 4, and 5). To have reproducible results regarding cell migration is important to use a commercial kit to create a wound field with a defined gap (Figure 2).

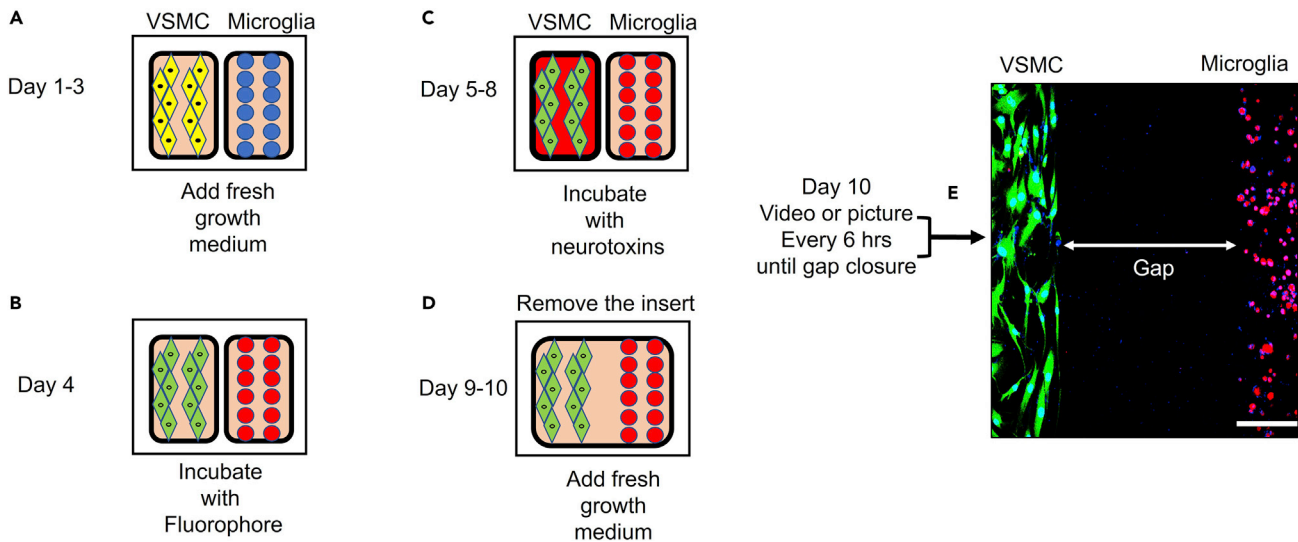
### LIMITATIONS

The generation of dysfunctional VSMCs was tested with cells derived from primary human carotid artery smooth muscle cells. The efficiency of this protocol was not tested with VSMCs isolated from different donors.

### TROUBLESHOOTING

#### Problem 1 (related to step 1: Culturing human primary VSMCs under AD-like conditions)

Poor viability of human VMCs after treatment with neurotoxins.



**Figure 2. Schematic workflow of the wound healing assay**  
Scale bar: 250  $\mu$ m

**Potential solution**

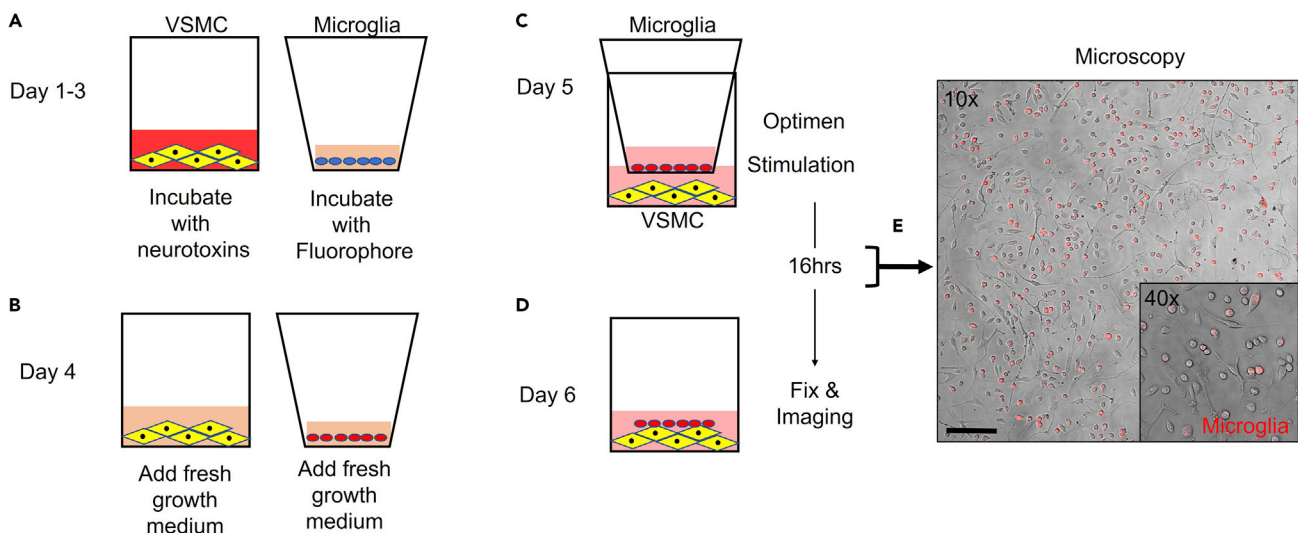
Primary human VSMCs are very sensitive cells. It is important to avoid the use of DMSO to reconstitute the neurotoxins. Other factors that could affect cell viability include the confluence of the culture and passage number. Maintain a confluence of 80% and use passages from 1 to 10.

**Problem 2 (related to steps 2 and 3: Culturing human primary VSMCs under AD-like conditions)**

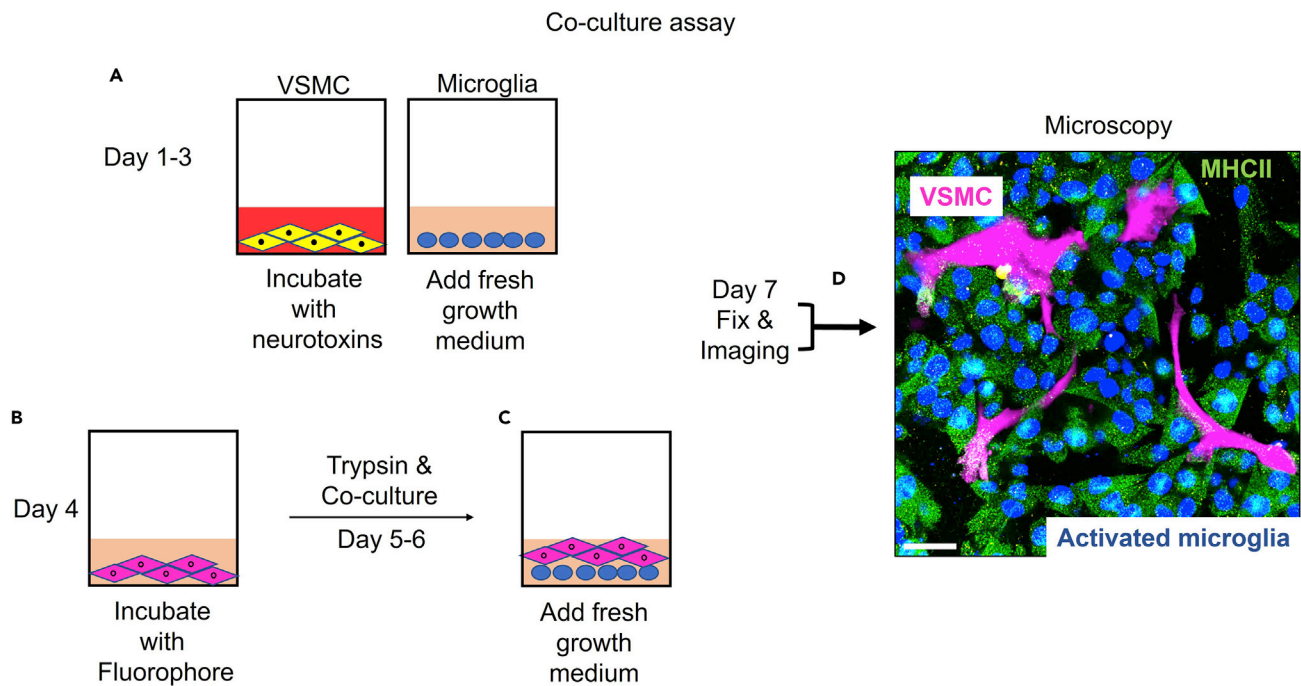
Synthetic gene expression and morphological changes in the cytoskeleton is not observed in neurotoxins-treated VSMCs.

**Potential solution**

VSMCs express two phenotypes *in vitro*. The synthetic phenotype is seen in response to stimulus like neurotoxins, while the contractile phenotype is the primary state of healthy VSMCs. This conversion



**Figure 3. Schematic workflow for the Transwell assay**  
Scale bar: 500  $\mu$ m



**Figure 4. Schematic workflow co-culture assay**

Scale bar: 20  $\mu$ m

could be affected by culture medium, time incubation with neurotoxins, and the passage number of cells. To avoid this issue make sure to use passage that are not older than 5–7, because VSMCs tend to lose their contractile identity in long culture periods. Make sure to store properly the neurotoxins as indicated above. We specially observed that the recombinant proteins could not induce neurotoxicity when store more than 3 months at  $-20^{\circ}\text{C}$ .

**Problem 3 (related to wound healing assay, step 4)**

Low quality of the wound field.

**Potential solution**

Reducing the liquid of the wells can improve the quality of the wound field. For an accurate analysis, collect a series of time-lapse images within 48 h of the experiment being started focusing on the same gap position during the entire experiment.

**Problem 4 (related to supernatant assay, step 7)**

Microglia cells are contaminated with VSMCs.

**Potential solution**

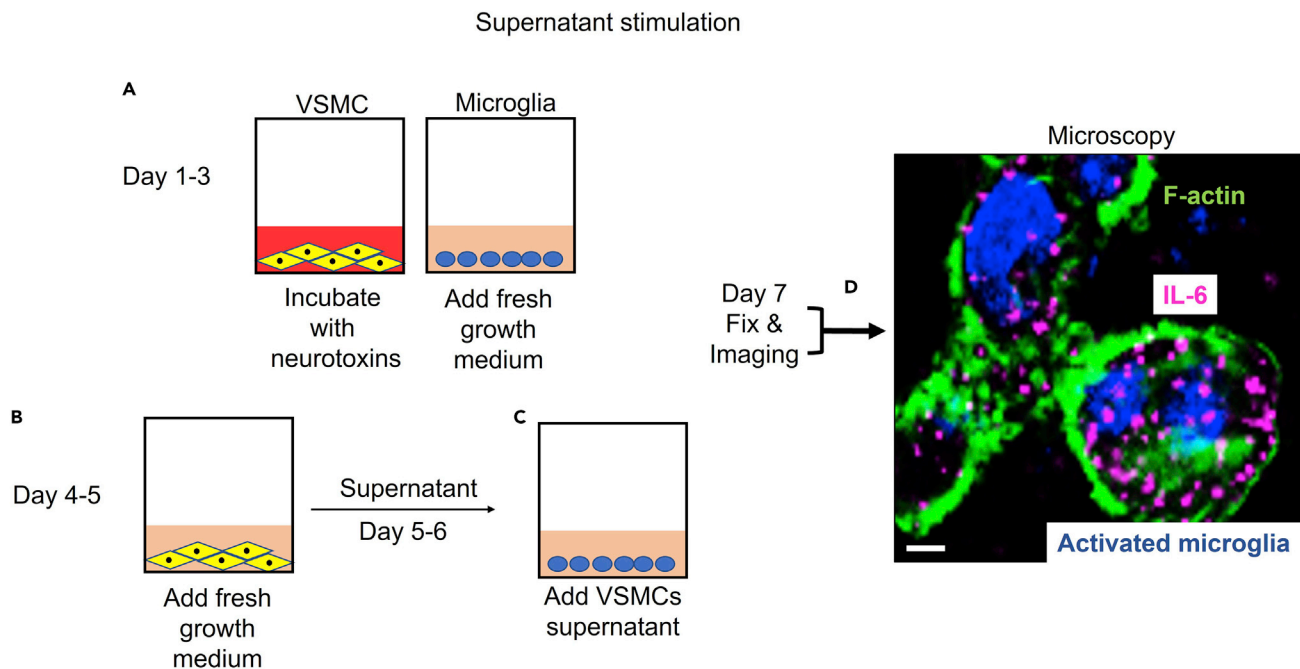
Filter the supernatant through a 0.2  $\mu$ m pore size.

**Problem 5 (related to steps 2 and 3: Culturing human primary VSMCs under AD-like conditions)**

Poor cell attachment of human VSMCs after passaging.

**Potential solution**

Ensure that all solutions are pre-warmed before use. As dead cells and debris will not attach, ensure the viability (greater than 90%) of the cells before dispensing them into plates.



**Figure 5. Schematic workflow supernatant assay**

Immunofluorescence staining shows microglia activation after incubation with supernatant from dysfunctional VSMCs. Activated microglial were characterized by morphologic changes and elevated expression of IL-6 at 100 × magnification, scale bars: 5 μm

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Christian L. Lino Cardenas ([clinocardenas@mgh.harvard.edu](mailto:clinocardenas@mgh.harvard.edu)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate or analyze any datasets or code.

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## AUTHOR CONTRIBUTIONS

C.L.L.C. conceived and designed the project; C.L.L.C., J.A.A.P., and K.L.F.A. did data curation; C.L.L.C. and K.L.F.A. wrote the manuscript; C.L.L.C., K.L.F.A., and K.J.V.L. reviewed and edited the manuscript; K.J.V.L. supervised the study and provided funding.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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