In Vitro Vascular Permeability Assay (96-Well)

Catalog No. ECM642

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Not for use in diagnostic procedures.

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Introduction

A fundamental requirement for the physiological performance of organs is the formation of diffusion barriers that separate and maintain compartments of different structure. The endothelial cell lining of the internal vasculature defines a semi-permeable barrier between the blood and the interstitial spaces of the body. This barrier is composed of intercellular adherens, tight, and gap junction complexes, as well as desmosomes [1]. Junction substructure components such as connexins, integrins, cadherins, catenins, occludins, desmoplakins, selectins, and platelet endothelial cell adhesion molecule-1 (PECAM-1) all act as interface regulators for paracellular permeability of ions, nutrients, therapeutic agents, and macromolecules [2, 3]. Endothelial cell adhesive characteristics provide strength and stability for neighboring cells and the cellular cytoskeleton by interacting with actin and myosin contractile filaments [4, 5]. Junctional molecules also influence cell signaling and trigger responses that are translated into cell morphology changes and physiological angiogenesis [6, 7].

A multitude of vasoactive cytokines, growth factors, and signal modulators react with endothelial cell substructural components to control permeability. Vascular endothelial growth factor (VEGF), interleukin-1 alpha and beta (IL-1α and IL-1β), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) have been shown to increase endothelial monolayer permeability [8, 9, 10, 11]. Thrombin stimulation of cytoskeletal signaling pathways has been shown to manipulate cell permeability [12]. Lipopolysaccharide (LPS) induces junction barrier loss and cell detachment by activating protein tyrosine kinases (PTKs) and caspase cleavage reactions [13]. In contrast, junctional adhesion molecule (JAM) decreases permeability by initiating cell adhesion [14] and angiopoietin-1 (Ang-1) can protect endothelial barrier function through regulation of junctional complexes [15, 16].

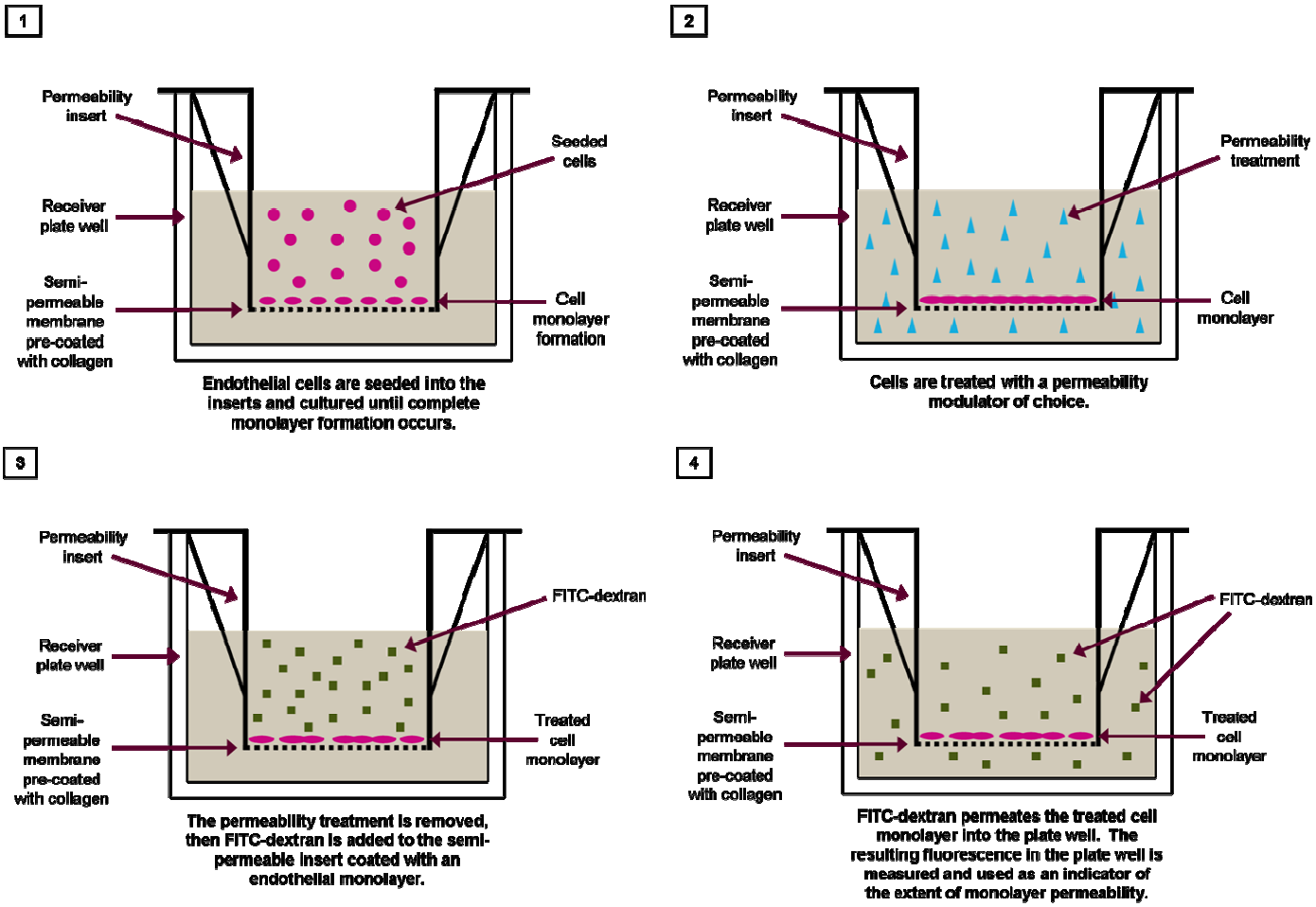
Disruptions of the barrier integrity are manifested as microvascular hyperpermeability, which is associated with many systemic disease states. Pathological angiogenic disease states include heart disease, diabetes, cancer, stroke, hypertension, arthritis, and Alzheimer's [1, 17, 18]. Increases in tissue permeability may be caused by weak, hemorrhaging vessels that become oedematous, and intensifies with irregular fluid flow through the vessels [17]. Expanding the knowledge of endothelial junction behavior and the agents that influence that behavior will lead to new therapies for controlling endothelial permeability.

An essential ingredient of any *in vitro* permeability study is an intact, confluent cell monolayer. Endothelial cell monolayers cultured on semi-permeable membranes have been shown to form adherent and tight junctions [19]. The Millipore *In Vitro* Vascular Permeability Assay kit provides an efficient system for evaluating the effects of chemicals and drug compounds on endothelial cell adsorption, transport, and permeability.

Test Principle

The Millipore *In Vitro* Vascular Permeability Assay is performed in a 96-well receiver plate with 96 cell culture inserts connected in a single tray unit. The inserts contain 1 µm pores within a transparent polyethylene terephthalate (PET) membrane. Each insert has been pre-coated with an optimized concentration of type I rat-tail collagen. The high pore density membranes permit apical and basolateral access of cells to media and permeability molecules of interest.

Within Millipore's *In Vitro* Vascular Permeability Assay, endothelial cells are seeded onto the collagen-coated inserts. An endothelial monolayer forms in several days, which occludes the membrane pores. The cell monolayer is then treated with cytokines, growth factors, or other compounds of interest. After treatment, a high molecular weight FITC-Dextran is added on top of the cells, allowing the fluorescent molecules to pass through the endothelial cell monolayer at a rate proportional to the monolayer's permeability. The extent of permeability can be determined by measuring the fluorescence of the receiver plate well solution.



Application

Millipore's *In Vitro* Vascular Permeability Assay kit is ideal for measuring compounds that may disrupt or protect an endothelial monolayer. Each assay kit contains sufficient reagents for the evaluation of 96 samples. The Millipore *In Vitro* Vascular Permeability Assay is intended for research use only, not for diagnostic or therapeutic applications.

Kit Components

1. 96-Well Permeability Insert Assembly, Collagen-coated: (Part No. CS205315) One 96-well receiver tray containing 96 connected porous cell culture inserts pre-coated with type I rat-tail collagen.
2. Receiver Tray, 96-Well: (Part No. CS205311) Two 96-well plates.
3. FITC-Dextran Solution: (Part No. 90328) Two vials containing 250 µL.
4. Cell Stain: (Part No. 20294) One bottle containing 10 mL.
5. Black Plate, 96-Well Opaque: (Part No. CS205313) One 96-well plate.

Materials Not Supplied

1. Human umbilical vein endothelial cells (HUVEC) such as EndoGRO™ Human Umbilical Vein Endothelial Cells (Cat. No. SCCE001) or endothelial cell type of interest.
2. Endothelial cell Basal Medium. (Note: Phenol red should be avoided—it may interfere with fluorescence measurement and increase background fluorescence.)
3. Endothelial cell Growth Medium.
4. Vascular permeability factor (e.g., IL-1β, TNF-α, VEGF, Ang-1, etc.).
5. Cell detachment buffer (e.g., 0.05% trypsin).
6. Sterile phosphate-buffered saline (PBS) or Hank's balanced salt solution (HBSS) for washing of cells.
7. Sterile cell culture hood.
8. Pipettors (e.g., multichannel, repeater), liquid aspirators, etc. for handling of cells and liquid reagents.
9. Sterile plasticware (cell culture flasks, centrifuge tubes, pipettes, pipette tips, reagent reservoirs, etc. for handling of cells and liquid reagents).
10. CO_2 tissue culture incubator.
11. 70% ethanol for disinfection of plasticware, surfaces, etc.
12. Low speed centrifuge for cell harvesting.
13. Hemocytometer.
14. Trypan blue or equivalent viability stain.
15. Microscope (for phase contrast and brightfield imaging).
16. Fluorescence plate reader with filters for 485 nm and 535 nm excitation and emission, respectively (appropriate for FITC/fluorescein signal).

Related Products Available from Millipore

ECM644	*In Vitro* Vascular Permeability Assay (24-Well)
SCCE001	EndoGRO Human Umbilical Vein Endothelial Cells (HUVEC)
SCCME001-004	EndoGRO Media Products for Human Endothelial Cell Culture
IL038	Recombinant Human Interleukin-1β
GF023	Recombinant Human Tumor Necrosis Factor-α
SM-2002-C	0.05% Trypsin-EDTA in Hank's Balanced Salt Solution
BSS-1006-B	EmbryoMax® 1X Dulbecco's Phosphate Buffered Saline

Precautions

Refer to MSDS for further information.

Storage

Store kit materials at 2-8°C; use within 4 months f rom date of receipt. Do not freeze.

Assay Protocol

Cell Harvesting:

Perform the following steps in a sterile cell culture hood. Prepare the cells for the investigation as desired; the following procedure is only a suggested protocol for utilizing human umbilical vein endothelial cells (HUVEC) in monolayer formation. *It is recommended to use HUVEC for this assay not older than passage 5 after thaw of original frozen stock.*

1. Use cells that are at least 80% confluent.
2. Visually inspect cells before harvest, taking note of approximate cell number and morphology (e.g., cells should exhibit typical endothelial "cobblestone" morphology, not excessive rounding, elongation, piling, etc.).
3. Remove (aspirate) endothelial cell Growth Medium. Wash cells once with sterile PBS or HBSS (e.g., 5 mL for 75 cm^2 flask) (see **Materials Not Supplied**).
4. Add 5 mL cell detachment buffer (see **Materials Not Supplied**) and incubate at room temperature for 1-3 minutes.
5. Add 5 mL Growth Medium to inactivate cell detachment buffer, then gently pipet the cells off the flask and into a sterile centrifuge tube. Gently mix the cell suspension and remove a sample to count on a hemocytometer; dilute 1:1 with trypan blue to check viability during counting (see **Materials Not Supplied**).
6. Centrifuge cells to pellet (150 x g, 5 minutes).
7. Gently resuspend the pellet in Growth Medium to a concentration of 0.25 - 1 x 10^6 cells/mL.
8. Add additional compounds (cytokines, pharmacological agents, etc.) to the cell suspension if pretreatment of the endothelial cells is necessary.

Cell Seeding and Permeability Treatment:

Perform the following steps in a sterile hood.

Note: During all steps requiring removal or addition of liquid from the insert, be careful to not touch the insert membrane with pipette tips, etc., as this may damage membrane or monolayer integrity (i.e., some residual liquid should remain present on top of the membrane). For liquid removal, pipette tips should be rested above the apical assist feature at the bottom left of each insert to prevent contact with the membrane. Liquid addition should be performed at the insert wall to minimize monolayer disruption. Minimize the amount of time that the insert is empty of liquid to avoid drying of the cell monolayer (e.g., empty and refill inserts one at a time).

1. Hydrate each insert being utilized with 125 µL Growth Medium. Make sure solution completely covers the bottom of the insert.
2. Cover plate and incubate at room temperature for 15 minutes.
3. Prior to adding cells, remove 100 µL of the Growth Medium from the insert and discard.
4. Seed 100 µL of the cell suspension into the insert (e.g., 0.25 - 1 x 10^6 cells/mL = 0.25 - 1 x 10^5 cells/insert).
5. Carefully add 250 µL of Growth Medium to the receiver plate well through the basolateral access hole at the top right of each insert. *(Note: Ensure media completely covers the underside of the insert and that no bubbles are present. Air may get trapped at the interface.)*
6. Cover the plate and incubate the cells for 72 hours, or until a monolayer is formed (see **Monolayer Staining**), in a 37℃/ 5% CO_2 tissue culture incubator.
7. If a cell starvation step is not necessary, proceed to step 8. If a cell starvation step is necessary, replace the media in the insert (100 µL) and receiver plate well (250 µL) with

starvation media (e.g., endothelial cell Basal Medium + 0.5% FBS, see **Materials Not Supplied**). Continue incubation at 37℃ / 5% CO_2 for 6 to 24 hours.

8. Carefully remove the media from the insert and the receiver plate well (the inserts may be lifted out while emptying the well) so as not to touch or disturb the monolayer.
9. Transfer the inserts to a new Receiver Tray.
10. Add the vascular permeability factor to the inserts being tested (see **Materials Not Supplied**) in 100 µL of media (endothelial cell Basal Medium, Growth Medium, or other as appropriate for controls). Add 250 µL of the solution to the receiver plate wells.
11. Cover the plate and incubate the cells for an appropriate duration for the treatment of interest (e.g., 2 to 24 hours) in a 37℃ / 5% CO_2 tissue culture incubator.

FITC-Dextran Permeability Testing:

1. Following completion of permeability treatment, transfer the inserts to a new Receiver Tray.
2. Prepare a working solution of FITC-Dextran in media (Basal Medium, Growth Medium or other as appropriate for controls). A FITC-Dextran dilution of 1:40 is suggested, although the final dilution may need to be optimized by the end user. Prepare a sufficient volume of solution to add 75 µL per insert. Protect the FITC-Dextran solution from light at all times.
3. Add 250 µL of media (Basal Medium, Growth Medium or other as appropriate for controls) to each well of the Receiver Tray for each insert being analyzed.
4. Carefully remove the media from the insert so as not to touch or disturb the cell monolayer.
5. Add 75 µL of FITC-Dextran working solution (see step 2) to each insert. Incubate the plate, protected from light, for 20 minutes at room temperature. (This permeation time is suggested, although the duration may need to be optimized by the end user.)
6. Stop permeation by removing the inserts from the wells. Thoroughly mix the media in the wells of the Receiver Tray (now containing FITC-Dextran that crossed the monolayer). Inserts may be temporarily held in a previous Receiver Tray that has been emptied of liquid.
7. Remove 100 µL of the media from each well of the Receiver Tray, and transfer to wells of the black 96-well opaque plate provided for fluorescence measurement.
8. Read the plate using a fluorescence plate reader with filters appropriate for 485 nm and 535 nm excitation and emission, respectively (or similar FITC/fluorescein-compatible wavelengths).

Monolayer Staining:

1. After completion of FITC-Dextran permeability testing, the endothelial monolayer may be stained for brightfield imaging of monolayer integrity. This may also be performed on a spare cell-seeded insert following the 72 hour monolayer formation time to check for monolayer confluency prior to starvation or permeability treatment.
   (NOTE: Stained inserts should not be used for FITC-Dextran permeability testing or further cell culture; sample numbers should be planned to account for discarding of inserts stained prior to permeability testing. Sterile filtration of Cell Stain and performance of all staining steps in a sterile hood may be desirable if other inserts in the tray assembly will be returned to cell culture.)
2. With an insert in an empty receiver plate well (no liquid in well), carefully remove the media from the insert so as not to touch or disturb the cell monolayer.
3. Add 50 µL of Cell Stain to the insert, cover the plate and incubate at room temperature for 20 minutes.
4. Carefully remove the Cell Stain to hazardous waste (see MSDS).
5. Carefully rinse the insert twice with PBS or HBSS, filling the insert and receiver plate well with 100 µL and 250 µL of buffer, respectively, for each rinse. The insert may be left in the second rinse during microscopic (brightfield) imaging.

Example Data

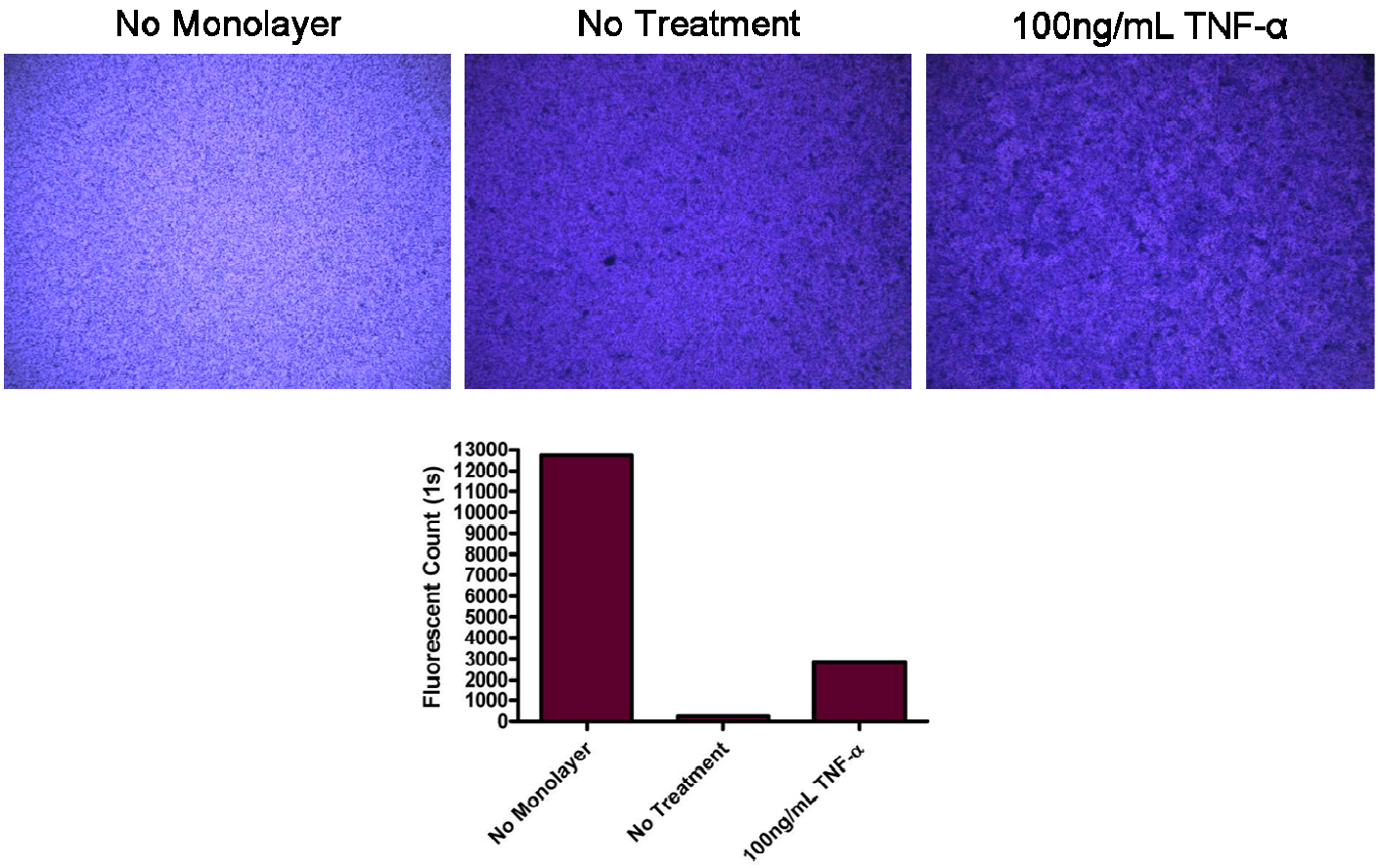


Figure 1. Example staining and permeability analysis of HUVEC monolayers.

HUVEC at passage 4 were seeded at 50,000 cells per insert and cultured for 72 hours in growth medium ("No Monolayer" negative control cultured in growth medium only). Following this culture period, monolayers underwent "No Treatment" (growth medium only) or treatment with 100ng/mL TNF-α in growth medium for 23 hours. Monolayer staining and FITC-Dextran permeability testing were performed as described in the **Assay Protocol**. Stained cells were brightfield-imaged on an inverted microscope at 5X objective magnification. Fluorescence intensities were quantified (one second counting time) on a VICTOR^2 1420 Multilabel Counter using Wallac software (PerkinElmer Life Sciences). The "No Monolayer" sample demonstrated high permeability in the absence of an occlusive endothelial cell monolayer. The "No Treatment" sample exhibited a visually confluent monolayer, as supported by the finding of low FITC-Dextran permeability (a "positive control" for monolayer integrity). Disruption of monolayer integrity was observable both visually and by quantification of increased plate well solution fluorescence following TNF-α treatment.

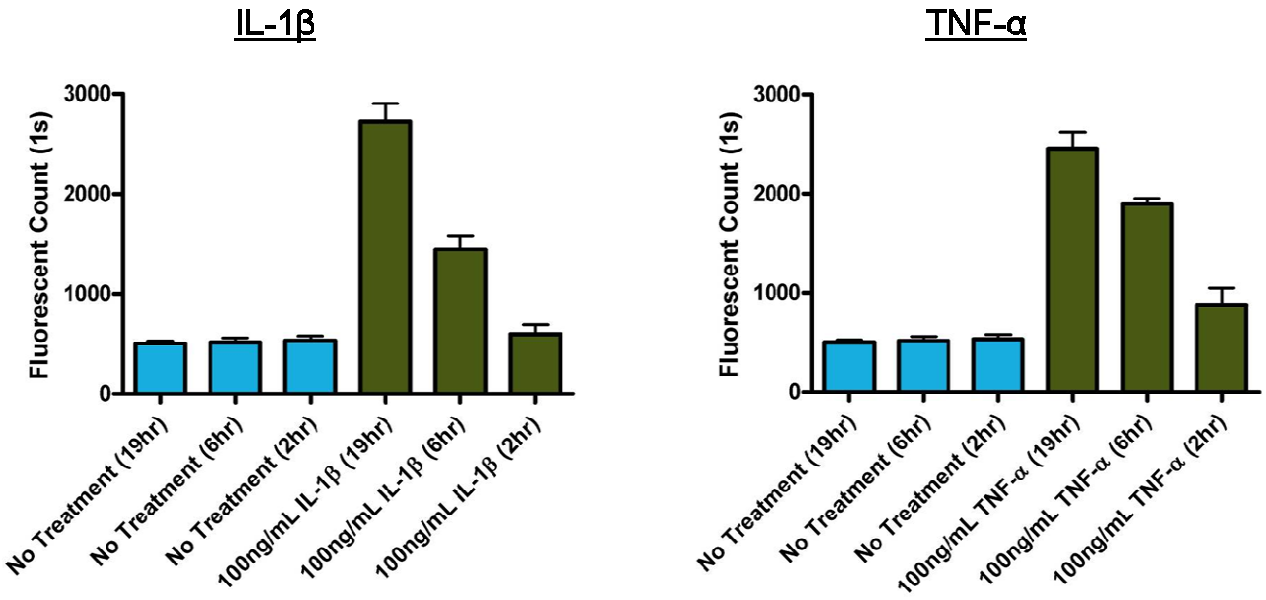


Figure 2. Time-courses for stimulation of vascular permeability by IL-1β and TNF-α.

HUVEC at passage 1 were seeded at 50,000 cells per insert and cultured for 72 hours in growth medium. Following this culture period, monolayers underwent "No Treatment" (growth medium only) or treatment with 100ng/mL IL-1β or TNF-α in growth medium for a range of durations. FITC-Dextran permeability testing was performed as described in Figure 1. Triplicate samples demonstrated low permeability for non-treated samples, and time-dependent increases in permeability following IL-1β or TNF-α treatment. Bars are mean + SEM.

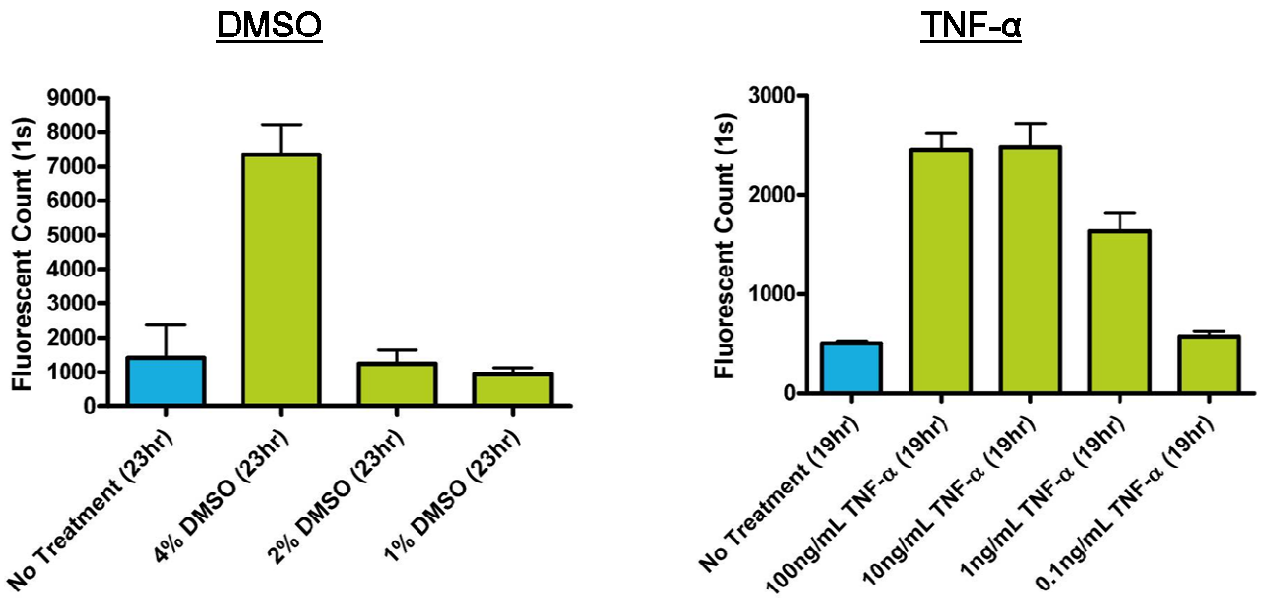


Figure 3. Dose responses to DMSO and TNF-α in the vascular permeability assay.

HUVEC were seeded at 50,000 cells per insert (passage 4 for DMSO, passage 1 for TNF-α) and cultured for 72 hours in growth medium. Following this culture period, monolayers underwent "No Treatment" (growth medium only) or treatment with DMSO or TNF-α in growth medium at a range of concentrations for 23 (DMSO) or 19 (TNF-α) hours. FITC-Dextran permeability testing was performed as described in Figure 1. Triplicate samples demonstrated that DMSO, a common solvent for treatment compounds, did not detrimentally affect monolayer integrity until a concentration of 4% DMSO in growth medium. Low permeability was observed for non-treated samples, with dose-dependent increases in permeability with increasing TNF-α concentration (reaching a plateau at 10-100ng/mL TNF-α). Bars are mean + SEM.

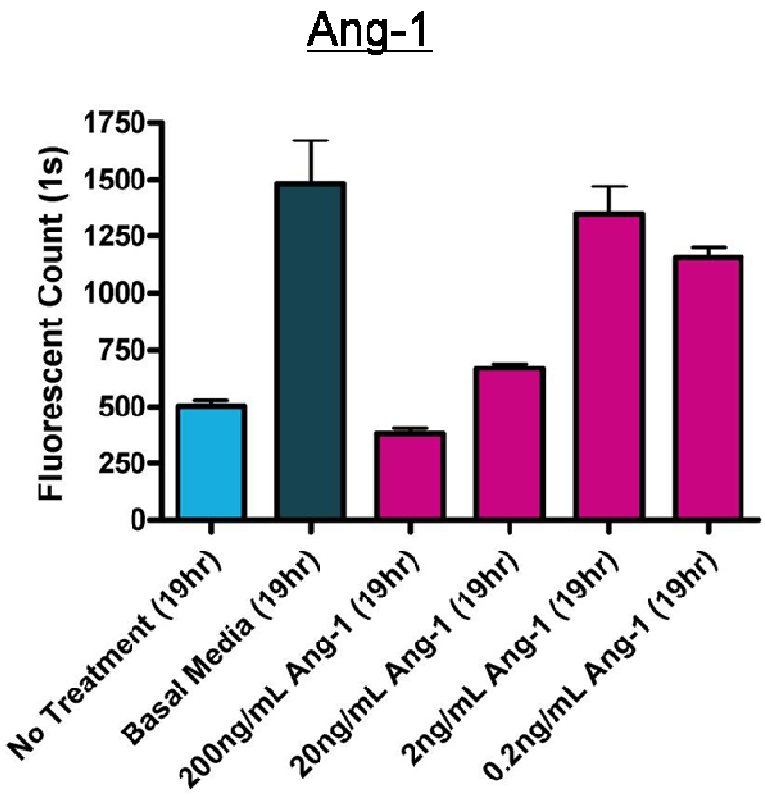


Figure 4. Dose responsive inhibition of vascular permeability by Ang-1.

HUVEC at passage 1 were seeded at 50,000 cells per insert and cultured for 72 hours in growth medium. Following this culture period, monolayers either underwent "No Treament" (growth medium only), were exposed to basal medium (known to increase permeability relative to growth medium, due to a lack of essential supplements), or were treated with Ang-1 in basal medium at a range of concentrations for 19 hours. FITC-Dextran permeability testing was performed as described in Figure 1. Triplicate samples demonstrated that Ang-1 can inhibit basal medium-induced permeability (down to "No Treatment"-levels) in a dose-dependent manner. Bars are mean + SEM.

Troubleshooting

Problem	Possible Solutions
Incomplete/non-confluent endothelial cell monolayer	• Use endothelial cells at lower passage number or from new thaw/stock of cells. • Allow a longer culture duration for monolayer formation. • Plate cells at a higher initial seeding density. • Growth media or supplemental components may be old/degraded – use freshly-made growth media. • Avoid mechanical disruption or excessive fluid shear that may compromise insert membrane attachment or monolayer integrity. • During monolayer formation, treat cells with known endothelial survival factors, e.g., Ang-1, VEGF, etc.
Low fluorescent signal with known activator (e.g., TNF-α or IL-1β)	• Increase FITC-Dextran permeation time. • Use a higher concentration (lower dilution) of FITC-Dextran for permeability testing. • Decrease initial endothelial cell seeding density. • Ensure that FITC-Dextran stock or plate well solutions are protected from light.
High fluorescent signal in control wells	• Check monolayer and insert integrity (see Possible Solutions for "incomplete/non-confluent endothelial cell monolayer"). • Decrease FITC-Dextran permeation time. • Use a lower concentration (higher dilution) of FITC-Dextran for permeability testing.
No/minimal effect by treatment with test permeability factor	• Perform permeability treatment with range of treatment concentrations (dose response) and/or durations to detect transient responses. • Efficacy of permeability treatments may vary with cell type, so treatment conditions may need to be modified. • The media composition used for monolayer formation and permeability factor treatment may influence permeability effects, and may need to be adjusted to detect effects of different factors.

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