Video Article

Development of an Insert Co-culture System of Two Cellular Types in the Absence of Cell-Cell Contact

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URL: https://www.jove.com/video/54356
DOI: doi:10.3791/54356

Keywords: Cellular Biology, Issue 113, Co-culture, insert, transwell, cell culture, secreted soluble factors, PC12, N9, lipopolysaccharide, cytokines, microglia, neurons, neuroinflammation

Date Published: 7/17/2016

Citation: Renaud, J., Martinoli, M.G. Development of an Insert Co-culture System of Two Cellular Types in the Absence of Cell-Cell Contact. *J. Vis. Exp.* (113), e54356, doi:10.3791/54356 (2016).

Abstract

The role of secreted soluble factors in the modification of cellular responses is a recurrent theme in the study of all tissues and systems. In an attempt to make straightforward the very complex relationships between the several cellular subtypes that compose multicellular organisms, *in vitro* techniques have been developed to help researchers acquire a detailed understanding of single cell populations. One of these techniques uses inserts with a permeable membrane allowing secreted soluble factors to diffuse. Thus, a population of cells grown in inserts can be co-cultured in a well or dish containing a different cell type for evaluating cellular changes following paracrine signaling in the absence of cell-cell contact. Such insert co-culture systems offer various advantages over other co-culture techniques, namely bidirectional signaling, conserved cell polarity and population-specific detection of cellular changes. In addition to being utilized in the field of inflammation, cancer, angiogenesis and differentiation, these co-culture systems are of prime importance in the study of the intricate relationships that exist between the different cellular subtypes present in the central nervous system, particularly in the context of neuroinflammation. This article offers general methodological guidelines in order to set up an experiment in order to evaluating cellular changes mediated by secreted soluble factors using an insert co-culture system. Moreover, a specific protocol to measure the neuroinflammatory effects of cytokines secreted by lipopolysaccharide-activated N9 microglia on neuronal PC12 cells will be detailed, offering a concrete understanding of insert co-culture methodology.

Video Link

The video component of this article can be found at https://www.jove.com/video/54356/

Introduction

The study of tissues, organs or systems *in vitro* is an attempt to simplify the very complex relationships existing between the several cellular subtypes that comprise multicellular organisms. Indeed, *in vitro* studies make it possible to acquire a detailed understanding of single cell populations. There are two major advantages of conducting *in vitro* experiments: 1) reduced cellular interactions, and 2) the ability to readily manipulate the cellular environment. Hence, these two advantages have allowed scientists to predict the behavior of specific cell types *in vivo*, leading to the ability to regulate outcomes of extrinsic influences in whole organisms. In that sense, *in vitro* cell culture often works as a bridge connecting basic and applied life sciences. Nonetheless, there are also several disadvantages of working *in vitro*, the most important one being that a certain reservation may dwell in the physiological relevance of observed phenotypes. Indeed, when a single cell type is grown in a vessel, the culture loses, to a various extent, its cell-cell connections with other cell types, its contribution to the humoral environment from the tissue and organism of origin, and the anchors within the tissue that enabled it to uphold a particular three-dimensional structure sometimes crucial for cell function.

The question of cell-cell relationships has been addressed by the development of mixed culture techniques. In this method, two or more cell populations are grown together in the same culture vessel. However, these mixed cultures bear important inconveniences. On one hand, some cell subtypes do not physically interact with one another in the tissue of origin and rely solely on paracrine communications sustained by secreted soluble factors and nearby receptors. This is the case for several inflammatory processes that depend on proximal cytokine signaling. In mixed cultures, physical interactions are unavoidable and make it impossible to study paracrine communications in the absence of cell-cell contacts that can yield altered results. On the other hand, achieving cell-specific interpretations from within a mixed population becomes unfeasible without the use of harsh separation techniques that could significantly affect results.

To solve these important issues, the use of conditioned media has been developed as a technique allowing for compartmentalized cultures and the study of paracrine signaling. This method requires the transfer of the supernatant of one cell type, thus named conditioned medium, to wells containing another population of cells. Yet, an important drawback is that short-lived molecules do not survive long enough in the conditioned medium to be transferred to the wells of the second population of cells. Even long-lived molecules will be greatly diluted over time due to diffusion. Furthermore, both cell populations only participate in unidirectional paracrine communication rather than in active bidirectional communication. This leads to the absence of feedback signaling that is vital in recreating accurate multicellular relationships as they exist *in vivo*.

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As a consequence and driven by the need to better simulate the original *in vivo* conditions in the *in vitro* cellular environment, several advances in cell culture techniques have been achieved over the years. One of the most significant advancements has been the use of permeable supports with microporous membranes for compartmentalizing cell cultures, used for the first time by Grobstein in 1953[1]. Such permeable supports have been tailored over the years to accommodate numerous cell types and to be used in several different applications. Nowadays, these supports exist as hollow inserts that are designed to rest in wells from a multiwell tissue culture plate or in circular dishes. In a co-culture system, the insert contains one cell type whereas the well or dish contains the other cellular population, allowing to study the contribution of two different populations of cells on their humoral environment (**Figure 1**). As a result, cellular polarity (basolateral vs apical secretion or signal reception) is preserved, thus conferring insert co-culture systems an important advantage over mixed cultures and conditioned medium techniques. Several types of membrane materials are available, the most common ones being polyester (PET), polycarbonate (PC) or collagen-coated polytetrafluoroethylene (PTFE), and they exist in different pore sizes ranging from 0.4 µm to 12.0 µm. These varieties of materials and pore sizes offer a spectrum of inserts exerting variable features relevant to optical properties, membrane thickness and cell adherence that make them practical at different levels for the following uses not limited to:
-studying cell differentiation, embryonic development, tumor metastasis and wound repair by chemotaxic assays through permeable membranes;
-evaluating drug penetration by assessing their transport through epithelial or endothelial monolayers cultured on permeable supports, and;
-performing cell co-cultures to analyze cell behavior modulations induced by secreted soluble factors in the absence of cell-cell contact.

The purpose of this article is to describe general methodological guidelines to fulfill the third function stated above, that is to evaluate cellular changes mediated by secreted soluble factors in the absence of cell-cell contact using an insert co-culture system. Several different fields of research make use of insert co-culture systems in order to answer questions relevant to the effect of secreted soluble factors on populations of cells. Indeed, paracrine signaling that modulates cellular behavior at various levels is pertinent in all tissues and systems, which makes insert co-culture systems indispensible to ensure advances in these fields. Conversely, the use of inserts can confirm that signal transduction is by direct cell-cell contact and not by secreted factors. One of the most important uses of inserts is in inflammation studies[2-14] where the effect of secreted cytokines is evaluated in various cellular players of immunity. In particular, the study of inflammation in the central nervous system (CNS) has greatly profited from insert co-culture studies, which have allowed to better defining the distinct paracrine roles of neurons and microglia in driving neuroinflammation[15-21]. These systems were also devised to study the anti-inflammatory potential of molecules that relies on their ability to reduce or inhibit the secretion of pro-inflammatory factors[22-26]. Research pertaining to cancer[27-31], in particular the mechanisms underlying angiogenesis[32-34] and inflammation[35-42] in tumorigenesis, also benefits from insert co-culture systems. Moreover, soluble factors are of prime importance in the processes that drive differentiation and several studies have used inserts to answer questions in that particular field[43-50]. In the CNS, seeing as neural tissue has a very limited renewal potential, the study of neurotrophism and neuroprotection is fundamental and has been widely ensured by the use of stem cells in co-culture systems[51-56]. In addition, inserts are also utilized in as diverse fields as nephrology[57,58], endothelial interactions and angiogenesis[59-62], apoptosis signaling[63-65], inflammation in obesity and metabolic syndrome[22,23,66-67], inner ear hair cell protection[68,69], and even in fungus virulence[70,71] and parasitology[72,73].

This article offers general methodological guidelines in order to set up an experiment in view of evaluating cellular changes mediated by secreted soluble factors using an insert co-culture system. In particular, we will focus our attention on nerve cell co-cultures and their uses in studying neuroinflammatory process. Given the very vast spectrum of experiments that inserts make possible to pilot, it is unbearable to cover every aspect of this cell culture technique. As an example, a specific protocol to measure the effects of cytokines secreted by lipopolysaccharide (LPS)-activated N9 microglia on neuronal PC12 cells will be detailed, offering a concrete understanding of insert co-culture methodology.

Protocol

N.B.: Each of the following steps should be performed under sterile conditions in a laminar flow hood as required for mammalian cell culture. In addition, the general guidelines for optimal sterile cell cultivation apply, *e.g.*, discarding tips any time they may lead to cross-contamination, reducing the amount of time cells are exposed to the air when performing entire media changes, properly but gently stirring all cell suspensions to ensure their homogenous pipetting, etc. Moreover, inserts are a kind of plasticware that require special handling. First, whenever inserts are manipulated, avoid touching the fragile membrane, which tears easily and could therefore jeopardize the experiment. Also, it is not suitable to perform vacuum aspiration of the cell culture medium, as there is a risk of perforating the membrane or dissociating adherent cells. Next, inserts hang loosely in the multiwell tissue culture plate and, thus, caution must be employed when moving the plasticware or when pipetting to avoid dissociating adherent cells. In addition, when using inserts with large pore sizes, there is a possibility that the cell culture medium seeps through the membrane and, therefore, it is important to frequently monitor the level of liquid. Finally, note that the following protocol is designed for adherent cells and requires minor modifications in order to be suitable for suspension cells.

1. General guidelines for conducting insert co-culture experiments

1. Seeding cell type #1 in inserts
    1. Unwrap the inserts from their packaging.
    2. Place the inserts in an empty multiwell tissue culture plate of the proper dimension. To do so, grip the uppermost edge of the insert using tweezers.
    3. To improve the attachment and spreading of adherent cells, condition the inserts with cell culture medium prior to seeding. To do so, cover the entire surface of the membrane with cell culture medium using a micropipette.
    NOTE: Do this for as many inserts as required.
    4. Replace the lid on the plate and incubate for at least 1 hr or O/N under the same conditions (usually 37°C, 5-10% CO_2).
    5. When the inserts are conditioned, remove all of the cell culture medium using a micropipette. Discard the used medium.
    6. Seed cell type #1 in fresh cell culture medium in the same manner as in a multiwell plate. To do so, draw an appropriate volume of the cell suspension with a micropipette and dispense the liquid in the insert.
    NOTE: Prepare as many inserts as required.
    7. After seeding all the inserts, gently rock the plate left and right, then back and forth in order to distribute the cells evenly. Avoid making circular motions, as this will cause the cells to accumulate in the center of the inserts.

8. Place the lid on the plate and incubate as specified by as per cellular requirements (usually 37°C, 5-10% CO_2).

2. Seeding cell type #2 in multiwell tissue culture plates
    1. Seed cell type #2 in fresh cell culture medium according to Section 1.
    2. Prepare as many wells as required for the number of inserts. Rock the plate as in step 1.1.7) to ensure that the cells are evenly distributed in the wells.
    3. Place the lid on the plate and incubate under the same conditions (usually 37°C, 5-10% CO_2).

3. Refreshing medium in inserts
    1. Using a micropipette, remove part or all of the cell culture medium, in the inserts containing cell type #1. Discard the used medium.
    2. Draw an appropriate volume of fresh cell culture medium. Gently rest the tip on the inner wall of the insert and slowly dispense the cell culture medium.
    3. Place the lid on the plate and incubate as per step 1.1.4.

4. Refreshing medium in multiwell tissue culture plates
    1. Using a micropipette, remove part or all of the cell culture medium in the wells containing cell type #2. Discard the used medium.
    2. Draw an appropriate volume of fresh cell culture medium. Rest the tip on the inner wall of the well and slowly dispense the cell culture medium.
    3. Place the lid on the plate and incubate according to previously established cell culture protocols.

5. Transferring inserts containing cell type #1 to multiwell tissue culture plates containing cell type #2.
    NOTE: Perform this step when both cell types have reached the appropriate growth stage.
    1. Prior to transferring the inserts into wells, make any necessary medium changes, as previously described in steps 1.3) and 1.4).
        NOTE: At this point, it is important to dispense the appropriate volumes of media in both compartments as specified by the insert manufacturer's instructions.
    2. Using tweezers, grip the uppermost edge of an insert containing cell type #1 and gently place it in the appropriate well containing cell type #2.
    3. After transferring all inserts, check for the presence of air bubbles beneath the membrane of inserts.
        NOTE: Air bubbles prevent any exchange across the membrane of the insert and can jeopardize the entire experiment.
    4. If air bubbles are present, very gently lift the insert from the well using tweezers and plunge back into the cell culture medium. The bubbles will disappear. If they are still present, try gently dipping inserts back into the cell culture medium at an angle.
        NOTE: Do not knock or stir inserts to avoid dissociating adherent cells.
    5. After removing all the air bubbles and checking that the volumes of media in both compartments, place the lid on the plate and incubate.

6. Refreshing media in a co-culture system
    NOTE: Although the membrane readily allows media exchanges between insert and well, refreshing the medium is done in both compartments since the time required to reach equilibrium in the upper and lower compartments by diffusion alone can be quite long.
    1. Refreshing medium in inserts containing cell type #1 is done in the same way as in step 1.3).
    2. To refresh medium in wells containing cell type #2, gently slide the insert to the side to create a space wide enough to accommodate a pipette tip. Refresh the medium as in step 1.4).
    3. Check for the presence of air bubbles and verify the volumes as per steps 1.5.3) through 1.5.5).

2. Example: measuring the effects of cytokines secreted by LPS-activated N9 microglia on neuronal PC12 cells

NOTE: The following steps are designed for specific flask, well and dish sizes. However, the protocol can be customized for any plasticware dimensions. For media and composition see Materials Table.

1. Seeding and differentiating PC12 cells in multiwell tissue culture plates
    1. Warm routine PC12 cell culture medium, PC12 differentiation medium and trypsin-EDTA in a 37°C water bath.
    2. Use PC12 cells at 60-80% confluence from a 75 cm^2 flask.
    3. With a 15 mm Pasteur pipette, perform vacuum aspiration of the entire cell culture medium in the flask.
    4. Gently rinse the cell monolayer with 5 ml of sterile phosphate buffered saline and remove the liquid with a Pasteur pipette. Be careful not to dissociate cells at this step.
    5. Cover the cell monolayer with 3 ml of trypsin-EDTA and incubate for 2-3 min at 37°C.
    6. Ensure that all the cells are detached under a microscope. If very few cells are floating, incubate for a longer period for a maximum of 5 min.
    7. Add 10 ml of routine PC12 cell culture medium in order to inactivate the trypsin-EDTA.
    8. With a 10 ml pipette, gently triturate while making sure that most cells are dissociated from the bottom of the flask. Avoid creating air bubbles in the cell suspension.
    9. With the same 10 ml pipette, transfer the cell suspension in a 50 ml centrifugation tube.
    10. Centrifuge for 1 min at 3,200 x g
    11. Discard the supernatant with a Pasteur pipette while taking care not to disturb the pellet.
    12. Add 10 ml of routine PC12 cell culture medium with a 10 ml pipette.
        NOTE: This volume can be adjusted if the pellet is unusually small or big.
    13. Triturate with the same 10 ml pipette to homogenize the pellet. PC12 cells often clump together so at least 20 pipetting up and downare necessary.
        NOTE: While vigorous triturating is necessary, avoid creating air bubbles in the cell suspension.

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14. In a 1.5 ml tube, prepare an appropriate dilution of the cell suspension in trypan blue. Count the cells using a hemocytometer according to previously established protocols[74].
15. In a separate 50 ml tube, split the cell suspension with PC12 differentiation medium to obtain a diluted cell suspension appropriate for seeding wells from a 24-well plate (30,000 ¢/cm^2, 0.6 ml per well according to the manufacturer's protocol).
    NOTE: The 24-well plate must be previously coated with collagen as specified by previously established protocols[75].
16. Distribute 0.6 ml of the cell suspension per well using a micropipette.
17. When all the wells are seeded, rock the plate as in step 1.1.7).
18. To allow the proper differentiation of PC12 cells, incubate the 24-well plates for 7-9 days at 37°C in a 5% CO_2 humid atmosphere in PC12 differentiation medium[15,16] before performing co-culture experiments.
19. Perform medium changes every other day by removing half of the liquid and replacing it with an equal volume of fresh PC12 differentiation medium.

2. Seeding N9 microglia in the inserts and treating with LPS
    1. One day before performing the co-culture experiments, pre-treat PTFE 0.4 µm-pore inserts with routine N9 cell culture medium to optimize cell adherence, following steps 1.1.1) through 1.1.4)
    2. Warm routine N9 cell culture medium and trypsin-EDTA in a 37°C water bath.
    3. Meanwhile, weigh approximately 10 mg of LPS in a 1.5 ml tube for later use.
       NOTE: Since LPS is a potent pro-inflammatory endotoxin and requires particular safety precautions, glasses, gloves, and a particulate respirator are strongly recommended.
    4. Use N9 cells at 80-90% confluence from a 75 cm^2 flask.
    5. Follow steps 2.1.3) to 2.1.14). Always use routine N9 cell culture medium instead of routine PC12 cell culture medium. Also note that N9 microglia do not clump together as much as PC12 cells do, so fewer pipetting up and down may be necessary at step 2.1.13).
    6. In a separate 50 ml tube, split the cell suspension with N9 cell culture medium to obtain a diluted cell suspension appropriate for seeding inserts designed to hold in 24-well plates (60,000 ¢/cm^2, 0.05 ml per insert according to the manufacturer's protocol, for membrane surface area see manufacturer information).
       NOTE: These inserts are already coated with collagen by the manufacturer and are optimized for cell adhesion.
    7. Distribute 0.05 ml of the cell suspension per insert using a micropipette.
    8. When all inserts are seeded, rock the plate as in step 1.1.7).
    9. Perform serial dilutions of LPS using N9 treatment medium to obtain 4 µg/ml, 2 µg/ml and 1 µg/ml working solutions.
    10. Pipette 0.05 ml of the 4 µg/ml working solution in one the set of inserts in order to yield a final dilution of 2 µg/ml.
    11. Repeat step 2.2.10) for the other two working solutions in different sets inserts, yielding final dilutions of 1 µg/ml and 0.5 µg/ml respectively.
    12. Incubate the plates containing the inserts for 24 hr at 37°C in a 5% CO_2 humid atmosphere to allow N9 microglia activation with LPS before performing the co-culture experiments.

3. Co-cultivating neuronal PC12 cells with N9 microglia
    1. At 7-9 days of differentiating PC12 cells, activate the N9 microglia after 24 hr of incubation with LPS by warm N9 treatment medium and PC12 treatment medium in a 37°C water bath.
    2. Perform a total medium change for N9 microglia as in step 1.3), replacing the entire used medium by 0.1 ml of N9 treatment medium. Do this for every insert.This is necessary to remove all traces of LPS, leaving only activated N9 microglia in the inserts.
    3. Perform a total medium change for neuronal PC12 cells as in step 1.4). Transfer the inserts into the 24-well plates as in step 1.5).Incubate the N9-PC12 co-cultures for 24 hr or 48 hr at 37°C in a 5% CO_2 humid atmosphere.
    4. After 24 hr or 48 hr, harvest the supernatant and/or the cells for cytotoxicity, enzyme-linked immunosorbent assay (ELISA), Western blot, or other assays.

Representative Results

The use of insert co-culture systems is particularly pertinent in the study of neuroinflammatory processes that showcase paracrine relationships between different cellular players of the CNS. Immunity in the CNS is accomplished mainly by resident cells called microglia that monitor their environment in their resting ramified state (**Figure 2A**) and are capable of sensing disturbances that could trouble the very precious homeostasis necessary for proper neuronal function[76-78]. Microglial activation, characterized by the adoption of an amoeboid shape (**Figure 2B**) and the multiplication of the cell population termed microgliosis (**Figure 3**), followed by the release of pro-inflammatory mediators, such as cytokines, constitutes the chief aspect of neuroinflammation[79]. Cytokine release serves the noble purpose of protecting neurons against harmful assaults and is closely monitored. However, when neuroinflammation escapes this tight control, it adopts a destructive nature and may seriously injure the CNS. Inasmuch as neural tissues have a very limited renewal potential, the CNS is all the more susceptible to such auto-destructive inflammatory responses. Studying the crosstalk, that exists between neurons and microglia is imperative in elucidating underlying mechanisms of neuroinflammation. Unlike mixed cultures, insert co-culture systems enable the investigator to identify which cell population is generating the toxic effects and which one is being affected.

Here, PC12 cells differentiated for 7-9 days with nerve growth factor were co-cultured with LPS-activated N9 microglia with the goal of quantifyingthe noxious effects of inflammation-derived soluble factors on neurons. To do so, neuronal PC12 cells were cultivated in 24-well plates while N9 microglia were seeded in inserts. N9 microglia were treated for 24 hr with LPS, a very potent pro-inflammatory endotoxin comprised in the outer membranes of gram-negative bacteria. LPS is known to activate toll-like receptor 4 thus eliciting a robust inflammatory response in a wide variety of immune effector cells, including the immortalized murine cell line N9 microglia[80,81]. The following representative results show that N9 microglia activated with LPS have a tendency to increase their population (**Figure 3**) and to secrete soluble pro-inflammatory cytokines, such as interleukin-6 (IL-6), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), that readily cross the membrane of the co-culture inserts and cause cytotoxic damage to neuronal PC12 cells growing in the lower compartment.

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The foremost concern before transferring the LPS-activated N9 microglia to the wells containing neuronal PC12 cells consists in confirming that the PC12 are properly differentiated. Seven-day differentiated PC12 cells should exhibit obvious neuronal phenotypes such as a flatter cell body, which projects several long neurites sometimes themselves displaying varicosities (**Figure 4**). Once this checkpoint is performed, N9 microglia inserts containing fresh medium devoid of LPS can be transferred into the wells containing differentiated PC12. After an incubation period of 24 hr or 48 h, the supernatant in the lower compartment is harvested and a cytotoxicity assay, based on lactate dehydrogenase release[15,16], as well as an ELISA[15], to measure cytokines are performed. It is important to harvest the supernatant in the lower compartment in order to measure the cytokines that have indeed crossed the permeable membrane and that may activate receptors on the surface of PC12 cells. Results demonstrate that LPS-activated N9 microglia from the upper compartment generate a dose- and time-dependent cytotoxic effect on differentiated neuronal PC12 cells set in the lower compartment (**Figure 5**). The 0.5 µg/mL LPS condition is however not significantly cytotoxic at 24 hr or 48 h. Levels of cytotoxicity were found to reach almost 100% in the 2 µg/ml LPS 48 hr condition. Moreover, results show that the concentration of pro-inflammatory cytokines IL-6, IFN-γ and TNF-α in the supernatant increases concurrently with observed cytotoxicity. Specifically, IL-6 concentrations are significantly increased after 24 hr only for the 2 µg/ml condition, whereas 1 µg/ml of LPS also yields significantly raised levels of the cytokine after 48 hr (**Figure 6**). Microglia secrete IFN-γ that reaches the lower compartment in a significantly increased manner when they are treated with 1 µg/ml and 2 µg/ml, and are incubated with differentiated neuronal PC12 cells for either 24 hr or 48 hr (**Figure 7**). The 0.5 µg/ml LPS condition once again does not increase IFN-γ levels significantly. Finally, TNF-α concentrations in the lower compartment were quantified by ELISA and were found to be the most augmented by LPS activation of microglia, reaching levels almost seven-fold more important than the control condition (**Figure 8**). In particular, both 24 hr and 48 hr incubation periods caused important increases in TNF-α levels in the supernatant. However, the 1 µg/ml LPS condition yielded a significant rise of TNF-α levels only after 48 h. As a whole, these results show that secreted pro-inflammatory cytokines are at least partly responsible for the cytotoxic effects observed in differentiated neuronal PC12 cells following a 24 hr or 48 hr incubation period with microglia activated by different concentrations of LPS.

Insert co-culture systems of LPS-activated N9 microglia and PC12 cells have been shown to be particularly useful in the study of neuroinflammation and in elaborating strategies to counteract it. Our group recently showed that LPS-activated N9 microglia grown in cell culture inserts exhibit increased transcription of pro-inflammatory cytokines, which in turn induce apoptosis of nerve growth factor-differentiated PC12 cells by crossing the micropores of the insert[15,16]. In the same paradigm, pre-treatment of the microglial population with the polyphenolic compound resveratrol (0.1 µM, 3 hr) prevented the transcription of pro-inflammatory cytokines and thus impeded apoptosis of differentiated neuronal PC12 cells by blocking caspase-3 activation and, thereafter, DNA cleavage. Another group has also demonstrated the neuroprotective effects of vitamin E in a N9-PC12 co-culture[26]. In addition to N9-PC12 co-cultures, different immortalized cell lines or primary cultures have also been employed in a context of neuroinflammation. For example, the murine microglia BV2 cell line was co-cultured with human neuroblastoma SH-SY5Y cells to show the anti-apoptotic effect of the polyphenolic luteolin apparently mediated by its anti-inflammatory potential[17]. As well, BV2 microglia activated by injured PC12 cells were shown to exert anti-apoptotic effects in mesenchymal stem cells[18]. Reciprocal signaling was demonstrated to be important in the anti-apoptotic mechanisms underlying primary microglia neuroprotection conferred upon primary cerebellar granule neurons[19]. Furthermore, primary hippocampal neurons were co-cultivated with primary microglia activated by secreted amyloid precursor protein in order to evaluate the release of glutamate by cysteine exchange and the subsequent weakening of synaptic function[20]. Finally, one group also showed the crosstalk that exists between primary hippocampal neurons and primary microglia pertaining to fractalkine signaling, a chemokine constitutively expressed by neurons in the CNS whose receptor is found on the surface of microglia[21].

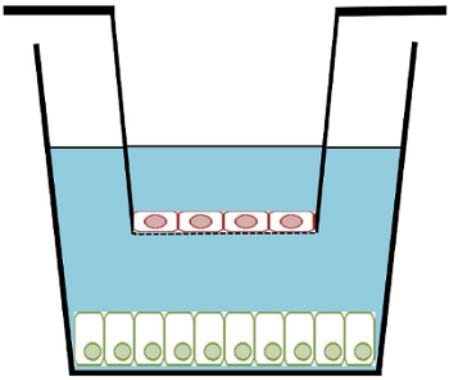

Figure 1. Schematic representation of an insert co-culture system. The upper compartment is composed of the insert which holds one cell type and its apical humoral environment. The lower compartment consists of a well or dish containing the second cell type. The insert rests on the edges of the multiwell tissue culture plate or dish. The insert is designed to lie just above the bottom of the well so as not to touch the population of cells growing below. The medium in the lower compartment is in contact with both the basal surface of the first cell type and the apical surface of the second cell type. Please click here to view a larger version of this figure.

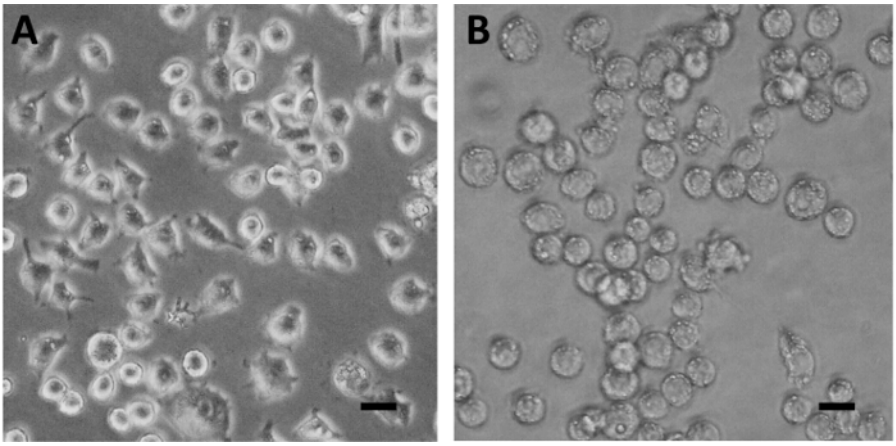

Figure 2. Microphotographs of N9 microglia. (**A**) Untreated resting N9 microglia in inserts exhibit a ramified cellular morphology allowing them to actively monitor their environment. (**B**) N9 microglia in inserts treated with lipopolysaccharide (LPS) for 24 hr display an amoeboid shape typical of the activated phenotype. This microphotograph was taken just before transferring the insert containing these activated N9 microglia to the wells containing differentiated PC12 cells as illustrated in **Figure 4**. Scale bar = 25 µm. Please click here to view a larger version of this figure.

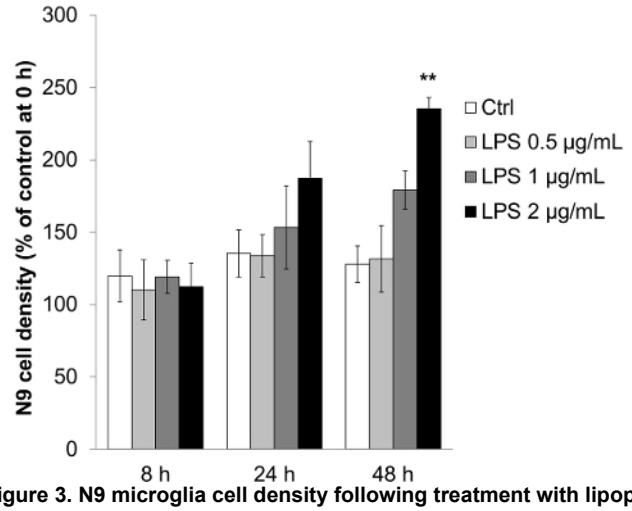

Figure 3. N9 microglia cell density following treatment with lipopolysaccharide (LPS). The effect of treating N9 microglia with different concentrations of LPS for different time spans was assessed by estimating the number of cells using a hemocytometer[69]. Significant differences between groups were ascertained by one-way analysis of variance, followed by Tukey's post-hoc analysis with the GraphPad Instat program, version 3.06 for Windows (San Diego, CA; www.graphpad.com). All data, analyzed at the 95% confidence interval, are expressed as means ± standard error of the mean from 3 independent experiments in which 10 wells were considered. Asterisks indicate statistical differences between the treatment and control condition (**p < 0.01). Please click here to view a larger version of this figure.

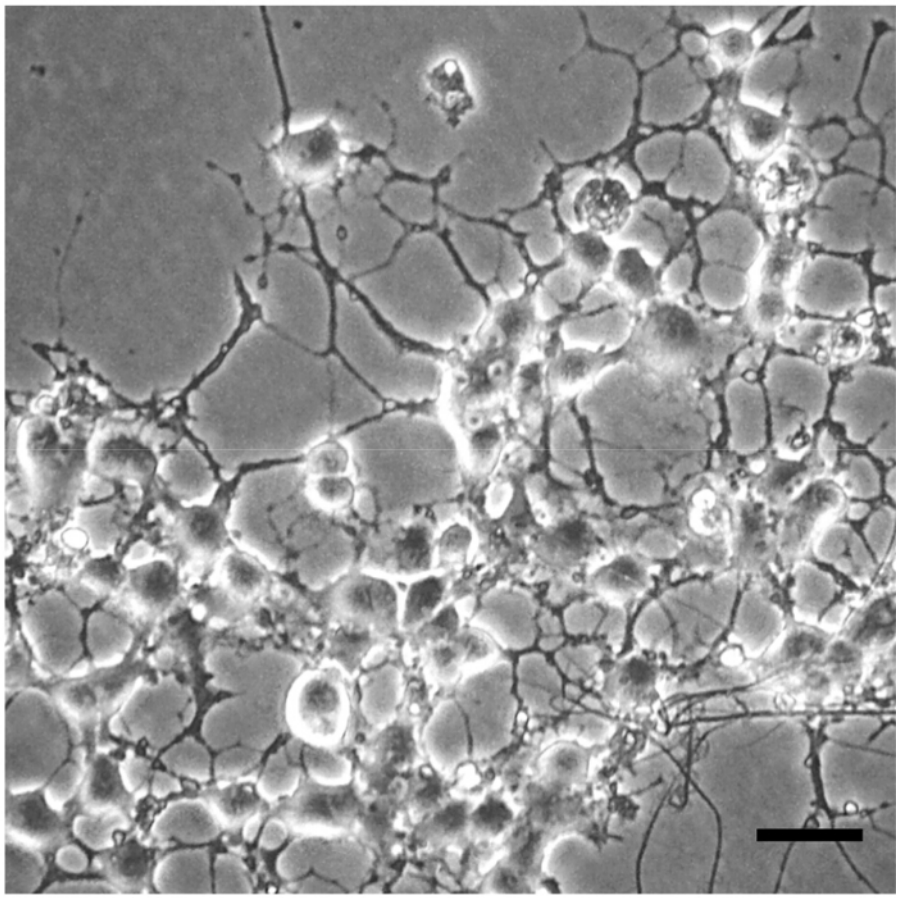

Figure 4. Microphotographs of differentiated neuronal PC12 cells. Seven-day nerve growth factor-differentiated neuronal PC12 cells show obvious neuronal phenotypes such as long neurites and varicosities. This microphotograph was taken just before transferring the inserts containing activated N9 microglia as pictured in **Figure 2**. Scale bar = 25 µm. Please click here to view a larger version of this figure.

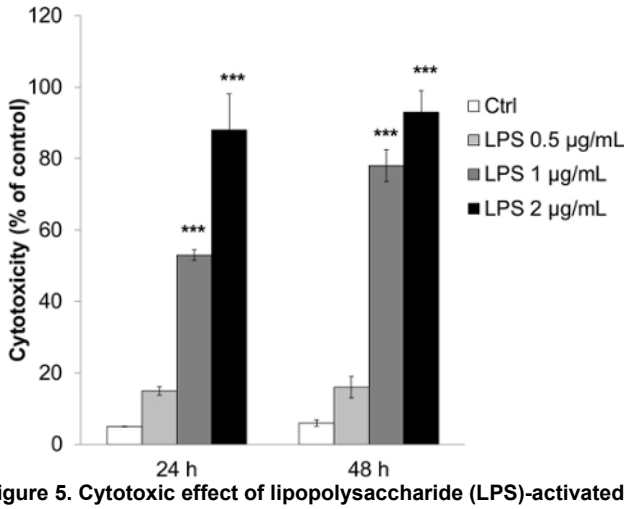

Figure 5. Cytotoxic effect of lipopolysaccharide (LPS)-activated microglia on differentiated neuronal PC12 cells. N9 microglia were activated with different concentrations of LPS for 24 h, then transferred to wells containing differentiated neuronal PC12 cells for 24 hr or 48 hr. Cytotoxicity was evaluated using a lactate dehydrogenase release assay performed on the supernatant of the lower compartment. Significant differences between groups were ascertained by one-way analysis of variance, followed by Tukey's post-hoc analysis with the GraphPad Instat program, version 3.06 for Windows (San Diego, CA; www.graphpad.com). All data, analyzed at the 95% confidence interval, are expressed as means ± standard error of the mean from 3 independent experiments in which 6 wells were considered. Asterisks indicate statistical differences between the treatment and control condition (***$p < 0.001$). Please click here to view a larger version of this figure.

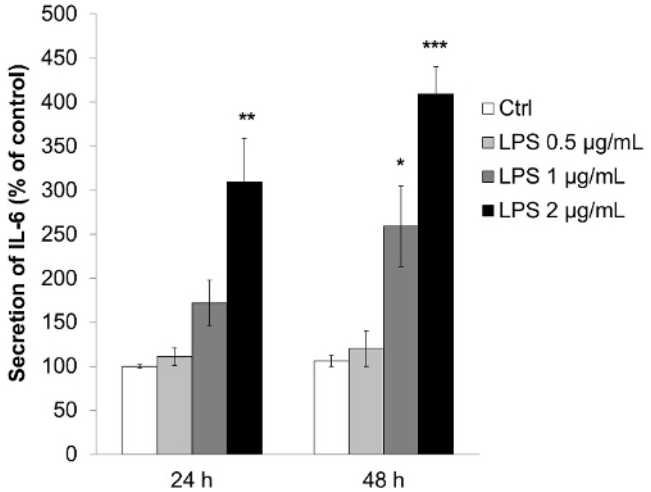

Figure 6. Concentration of interleukin-6 (IL-6) in the supernatant following N9 activation by lipopolysaccharide (LPS). N9 microglia were activated with different concentrations of LPS for 24 h, and then transferred to wells containing differentiated neuronal PC12 cells for 24 hr or 48 hr. IL-6 concentrations in the supernatant of the lower compartment were assessed using an ELISA. Significant differences between groups were ascertained by one-way analysis of variance, followed by Tukey's post-hoc analysis with the GraphPad Instat program, version 3.06 for Windows (San Diego, CA; www.graphpad.com). All data, analyzed at the 95% confidence interval, are expressed as means ± standard error of the mean from 3 independent experiments in which 6 wells were considered. Asterisks indicate statistical differences between the treatment and control condition (***p < 0.001, **p < 0.01 and *p < 0.05). Please click here to view a larger version of this figure.

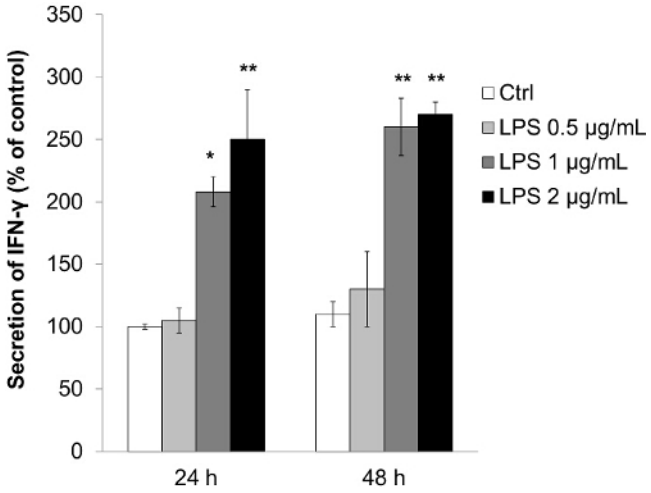

Figure 7. Concentration of interferon gamma (IFN-γ)in the supernatant following N9 activation by lipopolysaccharide (LPS). N9 microglia were activated with different concentrations of LPS for 24 h, and then transferred to wells containing differentiated neuronal PC12 cells for 24 hr or 48 hr. IFN-γ concentrations in the supernatant of the lower compartment were assessed using an ELISA. Significant differences between groups were ascertained by one-way analysis of variance, followed by Tukey's post-hoc analysis with the GraphPad Instat program, version 3.06 for Windows (San Diego, CA; www.graphpad.com). All data, analyzed at the 95% confidence interval, are expressed as means ± standard error of the mean from 3 independent experiments in which 6 wells were considered. Asterisks indicate statistical differences between the treatment and control condition (**p < 0.01 and *p < 0.05). Please click here to view a larger version of this figure.

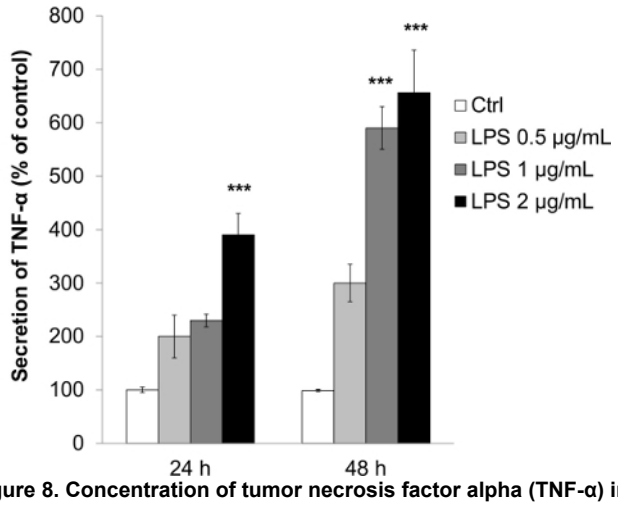

Figure 8. Concentration of tumor necrosis factor alpha (TNF-α) in the supernatant following N9 activation by lipopolysaccharide (LPS). N9 microglia were activated with different concentrations of LPS for 24 h, and then transferred to wells containing differentiated neuronal PC12 cells for 24 hr or 48 h. TNF-α concentrations in the supernatant of the lower compartment were assessed using an ELISA. Significant differences between groups were ascertained by one-way analysis of variance, followed by Tukey's post-hoc analysis with the GraphPad Instat program, version 3.06 for Windows (San Diego, CA; www.graphpad.com). All data, analyzed at the 95% confidence interval, are expressed as means ± standard error of the mean from 3 independent experiments in which 6 wells were considered. Asterisks indicate statistical differences between the treatment and control condition (***$p < 0.001$). Please click here to view a larger version of this figure.

Discussion

The most critical step of any insert co-culture system experiment actually dwells in choosing the proper insert to use. Pore size and membrane material must be taken into thorough account, without forgetting to consider the type of cells that will be seeded and the purpose of the experiment. For example, chemotaxic assays may use the same type of membrane than cell co-cultures to analyze cell behavior modulations induced by secreted soluble factors in the absence of cell-cell contact. However, both types of experiments require different pore sizes: larger ones for the former, to allow cell migration, and smaller ones for the latter, to preclude cell migration and cell-cell contact. For the evaluation of cellular changes mediated by secreted soluble factors in the absence of cell-cell contact, pore sizes of 0.4 µm or 3 µm are usually appropriate as they allow for large molecules, such as proteins, to cross but prevent most cells from doing so. Membrane material largely depends on the cell type and the techniques to be used at the end of the cell culture protocol. Briefly, PET membranes offer good cell visibility as they are clear but are not collagen coated, which can be a problem for adherent cells that required for the support to be treated. They also have the best chemical resistance to fixatives that, alongside their good optical properties, makes them ideal for histological studies. On the other hand, PC membranes offer poor cell visibility as they are translucent and are not collagen coated. However, they possess the highest density of pores and the most diversified availability of pore sizes. Finally, PTFE membranes are clear when wet but only allow visualization of cell outlines in the microscope. As a major advantage, they are collagen coated, which also makes them a little bit thicker. It is important to note that coating PET or PC membrane inserts with collagen can obstruct the pores and impede the passage of soluble factors. In any case, most insert manufacturers offer comprehensive guides to assist researchers in selecting the proper insert. In our specific paradigm, the choices of plating density, media, serum concentration, days of differentiation, days of LPS activation, concentrations of LPS, and coating of flasks for PC12 cells have been optimized over years of working with these co-culture systems[15,16]. Noteworthy, the plating density of N9 microglia in the inserts was chosen at 60,000 ¢/cm^2 in order to obtain 40-50% confluence at the moment of commencing the co-culture and, thus, to give them space to multiply upon their activation by LPS. Other conditions when optimized may also yield satisfactory results, such as substituting collagen by L-lysine in the flasks intended for native PC12 maintenance.

In order to increase the sustainability of the results, several different controls can be performed. When trying to prove that soluble factors are responsible for the observed effects, mixed cultures manifesting direct cell-cell contact and conditioned medium experiments should be conducted in parallel. Moreover, making use of antibodies to neutralize a specific secreted factor will help in identifying the molecule responsible for the paracrine effect that is perceived. When possible, block a receptor or a portion of its signaling pathway to pinpoint the exact identity of the receptor being activated. If a molecule is used to activate one cell population prior to the co-culture experiment with a second cell type, such as in the example described previously, it is important to take into consideration the presence of the molecule in the system. As a first option, the medium should be entirely changed before the co-culture experiment in order to ensure that the molecule is absent from the system altogether. As a second option, a condition should be added where the molecule alone is incubated with the second cell type in order to assess its effect in the absence of the first cell type. If there is no effect of the molecule on the second cell type, there will be no need to change the medium before the co-culture experiment. Likewise, if both cell types are not grown in the same cell culture medium, similar precautions must be taken to make sure that the differences in medium composition do not affect one or the other cell population. Although both media will be separated by the insert membrane at first, diffusion is bound to ensure that they will mix over longer incubation periods. Moreover, several problems can arise from aberrant disruption of soluble factor-receptor relationships. Among several causes of error, the excessive use of enzymatic dissociation and the presence of important concentrations of serum can interfere with cell surface receptors. Other causes of error in insert co-culture systems include, but are not limited to 1) improper cell culture techniques, such as removing the totality of the cell culture medium in too many wells at the same time causing cells to dry, 2) a monolayer that is too confluent in the insert, responsible for sealing off the insert membrane and preventing apically-secreted molecules to reach the lower compartment, and 3) misadjusted cell confluence, which causes an over- or under-expression of soluble factors leading to physiologically irrelevant results or absence of any effect.

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While only one scenario of insert co-culture was presented here, there are numerous ways to make use of this very versatile technique. Here, one cell type was pre-treated with a molecule responsible for inducing the secretion of a soluble factor that, upon transferring the insert to the well, affects the behavior of the second cell type. It is also possible to incubate both cell types together in a co-culture system before separating them to detect the increased vulnerability or resistance of one or both cell populations to an ulterior treatment. However, when considering its most rudimentary form, this technique can make use of two populations of cells incubated together without any treatment, with the goal to assess basal paracrine reciprocal signaling.

The most important limitation of this technique is that very short-lived molecular entities such as reactive oxygen species do not survive the distance that separates the cell population growing in the upper compartment and the one in the lower compartment[82]. The latest development in co-culture techniques has attempted to answer this problem by using a microfabricated culture substrate able to maintain two cell populations in microscale proximity[83]. In addition to bearing all of the advantages of insert co-culture systems, such as population-specific detection of changes and bidirectional signaling, this microfabricated screen was also demonstrated to make possible the detection of short-range interactions that were not before detectable due to the decay of soluble factors over distances. This cell culture platform is the latest innovation in cell co-culture and promises to help unravel signaling mechanisms between cells that were overlooked before.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was funded by a Natural Sciences and Engineering Research Council (NSERC) Canada grant to MGM. JR is a NSERC-Vanier student fellow.

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