REVIEW ARTICLE

Microglia-induced Neurotoxicity: A Review of *in Vitro* Co-culture Models

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ABSTRACT

Microglia-induced neurotoxicity occurs when inflammation mediated by microglia causes loss of neuronal structures or functions in the central nervous system implicated in stroke, spinal cord injury, sepsis, neurodegenerative diseases and even psychiatric illnesses. Various co-culture in vitro microglia-induced neurotoxicity (MINT) models have been established to enable an in-depth study of this process and yet there is a dearth of information regarding usages, advantages and limitations of each of the components of this model. In this review, we examined 56 MINTs for the cells, stimuli, parameters, methods of neurotoxicity measurement and formats of co-culture used in their construction. We aim to provide foundational information, overall guideline and framework for the novice researcher to develop his/her own model and for the advancement of improved, novel and more representative MINT models.

Keywords: Microglia, Neurotoxicity, Coculture models, Neurites, Cell survival

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INTRODUCTION

Neuroinflammation is the brain's response to inflammatory challenge, initiated by the innate immune cells within the central nervous system (CNS) (1,2). Where in the peripheral circulation, innate immune cells consist of monocytes, tissue macrophages and neutrophils, microglia are the cells of the CNS (3–5). Microglia survey the brain's extracellular compartment by projecting and retracting filopodia to sample the environment for any changes in homeostasis (6,7). When exposed to insults such as lipopolysaccharide (LPS), found in the outer membrane of Gram-negative bacteria, or the beta-amyloid (Aβ) protein that accumulates in Alzheimer's disease (AD), microglia launch an inflammatory response to eliminate and manage infection, disease and traumatic insults and mediate repair of the CNS environment (7). Neuroinflammation is physiological and microglia are regarded as pro-homeostatic (7–9). However, during inflammation, nearby neurons, initially unaffected by the primary insult, may be damaged as a result of cytotoxic factors produced by microglia. Furthermore, the inflammatory process may persist into chronic inflammation that can disrupt neuronal functions, leading to neurotoxicity (10), defined as any adverse effect on the structure or function of the nervous system due to biological, chemical or physical agents (11).

Microglia-mediated neuroinflammation has been found to play an important role in the pathophysiology of neurodegenerative and psychiatric diseases (12–14). Neurodegeneration, or the progressive loss of structure or function of neurons, occurs in neurodegenerative diseases such as AD and Parkinson's disease (PD), is essentially a form of neurotoxicity (15). Both these groups of diseases impart a major burden on the health care system and the quality of life of its sufferers worldwide and in Malaysia, rising in numbers despite various studies and novel treatments applied (16,17), making it pertinent to find alternative approaches for the treatment of these diseases.

Alongside the search for direct neuroprotective therapy, neuroscientists are studying modulation of microglial inflammatory responses to ameliorate neurotoxicity (5). An important tool for studying microglia and their neurotoxic effects are *in vitro* models of microglia-induced neurotoxicity (MINT) (Table 1). Typically beginning with mono-cultures of microglia and mono-cultures of neurons, co-cultures are then

Table I : Types of microglia cells used in MINT models.

Microglia	Type/Name	Source/Organism		Age of Source Organism	References	Year
Primary cells	Whole brain	C57BL/6	Mouse	Postnatal (1-5 d.o.)	(47)	2020
					(36) (44) (45) (48)	2019
		Wistar	Rat	Postnatal (3-6 d.o.)	(49)	2008
		Fisher F344	Rat	Postnatal (1 d.o.)	(39)	2010
	Cortical	C57BL/6	Mouse	Postnatal (0-3 d.o.)	(38) (23) (42) (43) (46) (35)	2020 2019 2016
					(25)	2008
		Sprague-Dawley	Rat	Embryonic (d. 19-20)	(41)	2000
		(SD)			(40)	1997
				Postnatal (1-3 d.o.)	(34)	2020
					(37)(24)	2019
					(29)	2005
					(28)	2004
					(27)	2000
					(33)	1996
				Postnatal (3-5 d.o.)	(32)	2004
					(31)	1998
					(30)	1992
Cell lines	BV2	C57BL/6 (transformed)	Mouse		(74) (114) (115) (116)	2020
					(23) (24) (66) (70)(71) (72) (117) (113) (118)(119) (120)(121) (122)	2019
					(123) (124)	2018
					(69) (88)	2017
					(68)	2015
					(125)	2014
					(25) (126)	2008
	SIM-A9	C57BL/6 (Spontaneous)	Mouse		(67)	2018
	HAPI	Wistar (Spontaneous)	Rat		(127)	2019
					(52)	2018
	EOC 20	C3H/HeJ (Factor-dependent)	Mouse		(128)	2019
	THP-1	Acute monocytic leukemia cells	Human		(92) (93)	2019
	N9	CD-1 (Transformed)	Mouse		(129)	2019
	Microglial cell line	Unknown (Bioleaf. Shanghai, China)	Rat		(130)	2018

d.o. = days old, d. = day

established to evaluate the effects of substances on microglial response to neurons. Various *in vitro* MINT models have been developed. In this review, our objectives are to analyze common cell types, agents of activation, neurotoxic parameters and formats of culture used to develop MINT models, comparing and contrasting their properties, strengths and limitations between them. We have also extracted the specifics of each vital MINT model component as published in 56 research articles into four tables that will be useful for quick referencing and guideline. The components that were reviewed are depicted in Figure 1. Our mission is to inspire the young microglia-neuroscientist to develop novel and improved MINT models for advancement of knowledge and research into microglia-centred therapy for neuroinflammation-mediated neurotoxicity.

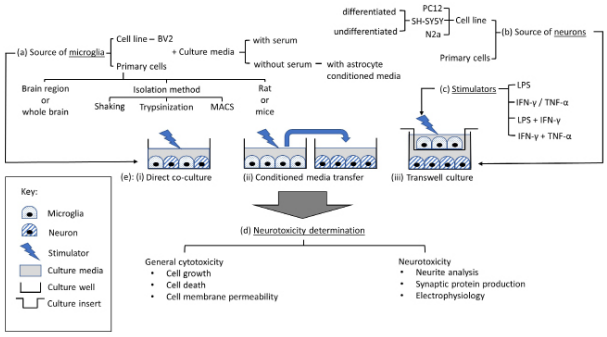

Fig. 1 : A summary of elements in a microglia-induced neurotoxicity (MINT) model: (a) Microglia (b) Neurons (c) Stimulator (d) Neurotoxicity determination (e) Format of co-culture.

1.0 Cells used in MINT models

1.1 Microglia

From a total of 56 research articles we reviewed, types of microglia used in the models are listed in Table I.

a. BV2 microglia cell line

Of the articles reviewed, 59% (33/56) used microglial cell lines and the most commonly used is the transformed microglial cell line, BV2 (25/33).

BV2 cells were developed by Italian scientists at the University of Perugia, Italy in 1990 by transfecting primary microglia cell culture from 7-day-old C57BL6 mice with the v-raf and v-myc oncogenes using a recombinant J2 retrovirus (18). The BV2 cells had extensions from their cell bodies, negative expression of astroglial and oligodendrocyte markers (glial fibrillary acid protein - GFAP and galactocerebroside - GC), were positive for macrophage antigen 1 and 2 (MAC1 and MAC2), nonspecific esterase (NSE) activity, absent peroxidase activity and with phagocytosis of Candida albicans. These are all features often expressed in primary microglia culture (18). As for their immunological responses, BV2 responded to LPS and Polyinosinic:polycytidylic acid (Poly I:C) by secreting the inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF), also equivalent to responses of primary microglia. Stimulation with interferon-γ (IFN-γ) induced tumoricidal activity similar to macrophages and application of both LPS and IFN-γ induced an enhanced tumoricidal effect, similar to peritoneal macrophages and primary microglia (18). In addition, BV2 showed electrophysiological characteristics of primary microglia. They have inward flow K+ channels and lack of outward flowing ones, dissimilar to macrophages (18). Since then, BV2 cells have been used widely in cell culture studies to represent microglia.

A decade into the new millennium, Henn et al. (2009) revisited BV2 cells using modern technologies in intracellular signalling pathways and transcriptome analysis. They found that BV2, upon LPS activation, up-regulated genes of cytokine productions, immune receptors, cell death and stress-related genes in a similar pattern to primary microglia, although less pronounced (19). BV2 also showed typical nitric oxide (NO) productions and functional response to IFN-γ and that they trigger nuclear translocation of NF-κB in astrocytes, similar to primary microglia. In addition, BV2 showed higher (41%) overlap in gene expression with microglia in vivo (19) while another study analysing hundreds of gene expression between primary microglia and in vivo models found 33-37% overlap (20). The genes surveyed in both of these studies included a variety of extracellular matrix-related genes. Hence, the range for comparisons here is very wide. When the focus is shifted to only inflammatory-related genes, for example at the subgroup of interferon-related genes, primary microglia did show a 95% overlap of gene expressions with in vivo intra-cerebroventricular LPS-injected model (20). When BV2 gene expression of cell cycling and DNA damage, cell adhesion, immune response and chemotaxis genes were studied, He et al. (2018) found that BV2 showed increased expression of these genes and low expression of cell adhesion genes, compared to primary microglia. However, they noted that most microglia-specific immune functions and pathways were retained except, for TGFβ signaling and chemotaxis towards C5a (21). When challenged with LPS, both BV2 and primary microglia do respond by up-regulating expressions of cytokines-related genes, but that BV2 secreted a larger amount, which could indicate different sensitivities between BV2 and primary microglia towards LPS (21). Butovsky et al. 2014 isolated microglia from adult mice and performed gene and miRNA profiling of over 1000 genes and cross-checked with Ly6C+ monocytes, which are usually recruited to the CNS during inflammation. From the results, they identified a group of genes and miRNA unique to only adult microglia. Surprisingly, when they assayed for their expression in primary microglia isolated from newborn mice, adolescent mice, N9 and BV2 cells, none of them expressed this unique panel. They found that primary microglia isolated from 21 days old to 2 months old mice expressed the

highest level of these signature adult microglia genes (22). Hence, this could be the best alternative for the representation of aged microglia, suitable for studies related to neurodegenerative diseases.

Nonetheless, when compared with primary newborn or postnatal microglia, BV2 is sufficient for the study of microglia-induced inflammation as they share various transcriptional and functional aspects of primary microglia, while any differences detected were mostly quantitative and not in the direction of the response itself. In addition, BV2 can multiply indefinitely making their culture much easier to maintain and cryopreserved for later studies. This ready availability makes BV2, as with other cell lines, cost-saving and its experimental data generation faster than primary microglia and in vivo studies (19). However, as mentioned, for studies into TGFβ signaling and chemotaxis towards C5a, primary microglia might be a more representative model (21).

b. Primary microglia

The remaining 41% of the articles reviewed (23/56) used primary microglia, while three studies (23–25) used both cell lines and primary microglia. Primary microglia have been regarded as the closest representation of in vivo phenomena and therefore is lauded to be the gold standard of in vitro models. Is this true?

Primary microglia studies have disadvantages that may produce erroneous results or faulty interpretations. For example, primary microglia isolation has been conducted from various ages and types of rats and mice, from various anatomic locations of the brain or even whole brain isolation and with various isolation techniques from mixed glial culture. Hence the term "primary microglia" itself is not accurate as they remain in mixed glia population for a minimum of two weeks in vitro, during which they actively engulf neuronal cell debris from the traumatic isolation procedures and thus are somewhat modified (19). Additionally, degenerative diseases occur in the adult or elderly. Primary microglia are isolated from newborn or postnatal rat/mice pups. How far they emulate responses of adult/elderly microglia is unknown. Furthermore, it is well established that microglia phenotype and density vary widely between the cortex, cerebellum and other anatomic locations of the brain (26). Cultures derived from whole brain homogenates disregard these differences. Hence, primary microglia may not be as representative as previously thought (19).

To isolate the microglia from brain tissue, among the 23 studies we have reviewed, 17 used shaking (24,25,27–41), four used mild trypsinization (23,42–44), two used magnetic associated cell sorting (MACS) (45,46) and remaining three used fluorescence associated cell sorting (FACS) (47), adhesion to polyethyleneimine (PEI) flasks (48) and Percoll gradient technique (49), respectively.
Lin et al. (2017) isolated microglia from newborn Sprague-Dawley (SD) rat brain cortices and isolated microglia using 2 techniques: 1) mild trypsinization and 2) shaking. The first involves incubation with 0.25% trypsin-EDTA which will detach astrocytes and other cells, leaving microglia attached to the bottom of the flask. For the shaking method, cultures are placed on an orbital shaker at 220 rpm for 1 hour. Here, the detached cells are the microglia. They found that cells from both techniques stained Iba1 positive and that cells purified using mild trypsinization are ramified, similar to "resting" microglia in vivo, while from shaking were heterogeneous, a mixture of amoeboid, ramified and other morphologies, which indicated some degree of activation. The yield was 20% more with isolation using mild trypsinization. Purity, determined via flow cytometry by labelling CD11b and CD45, was 10% more with cells isolated using mild trypsinisation (93-94% vs. 83-88%). mRNA expressions of genes related to activation states and CD68 labelling showed that cells isolated from shaking are more activated. Although the baseline cytokine levels were higher in cells isolated using shaking, the direction of response toward LPS and IFN-γ was similar in both cells. Phagocytosis of E. coli occurs more in cells isolated with shaking. All this indicated that the isolation technique significantly affects yield, purity and degree of cell activation in vitro, though they respond similarly to an inflammatory stimulus. The only difference is to what degree or how strongly they responded, in other words, quantitative differences, not in their pattern or direction of response (50).

The effect of isolation techniques on mice pup primary microglia seems to be reversed. He et al. (2018) compared the effects of three isolation techniques: 1) shaking, 2) mild trypsinization and 3) CD11b MACS on microglia RNA transcriptions (21). Even though they were all isolated from postnatal day 1-3 C57BL/6 pups, all from the same anatomic region – the cortices, all cultured as mixed glia for 2 weeks, the subsequent different isolation techniques impacted on the final state of the cells. Mild trypsinization produced morphologically amoeboid microglia indicating a more activated state, in contradiction to Sprague Dawley rat pups' primary microglia isolated by Lin et al. (50), indicating that inter-species differences are significant. Shaking and CD11b MACS produced ramified microglia. LPS, Aβ and age-activated mildly trypsinized microglia also showed higher expression of 1180 genes, compared to shaking and CD11b MACS cells. Nonetheless, the amount and percentage of Iba1 positive cells were similar amongst the three techniques (about 95%) (21).

The issue about inter-species differences in microglia response was further investigated by Lam et al. (2017) in their extensive study of rat and mice primary microglia gene expression, protein production and blocked K+ channel responses after stimulation with a pro-inflammatory stimulus (IFN-γ with TNF-α), an alternative activation stimulus (IL-4) and an acquired

deactivation stimulus (IL-10) (51). Primary microglia from both species changed morphology and migratory capacity similarly in all the treatments. The blockage of certain K+ channels also affected the migration of cells from both species in the same manner. The differences were in responses between the species are in their gene expression and protein production. They differ in degree of gene expression, the direction of expression and the absence/presence of certain genes such as CD206, ARG1. Even resting levels and response of certain markers such as Iba1, CD11b, CD68 differed between the species (51). Hence it is advisable, in our opinion, to conduct a thorough literature review of specific similarity and differing responses of primary microglia between rat and mice primary culture studies, before generalizing a specific response from one species onto another.

Another issue with cultured microglia, be it from cell lines or primary cells, is the addition of serum into the culture media used. So far, we have identified two popular media used in past years, Roswell Park Memorial Institute-1640 (RPMI-1640) and Dulbecco Modified Eagle Media (DMEM), sometimes supplemented with Ham's F12 medium (DMEM/F12), with the majority choosing the latter.

Commercial media used contains serum to maintain the viability of the microglia in vitro. When serum is intentionally removed, microglia isolated from postnatal rat brains died within days (68). To circumvent this limitation, Bohlen et al. (2017) replaced the serum with astrocytes concentrated conditioned medium (CM) and discovered that microglia required signals and trophic factors from astrocytes to survive. The idea is that in vivo, microglia receives various trophic signals from neurons, astrocytes and other cells in the brain. Emulating those signals in vitro may create more representative microglia, rather than with serum. The serum contains blood microelements that in reality never come in contact with microglia due to the blood-brain-barrier. They found that astrocyte-derived factors primarily CSF-1/IL-34, TGF-β2 and cholesterol prevented ex vivo death of primary microglia. When serum was added to the cultures previously devoid of serum, microglia showed increased phagocytic capacity, indicating that the cells may not be as phagocytic as were thought and that elements in serum may have induced microglia activation (68, 69). However, microglia could very well respond differently if neuronal CM or oligodendrocyte CM are also added to the culture. It could be that microglia may be very phagocytic but that this cannot be shown in in vitro culture with merely astrocytic CM as its supplement. Microglia in vivo clearly does not just associate with astrocyte, but also with other glia, neurons and the cerebrospinal fluid (70). In fact, it is now established that in certain diseases, the blood-brain-barrier does allow the entry of a limited number of T-lymphocytes (71). Furthermore, in studies where astrocytic CM or CSF-1/IL-34, TGF-β2 and cholesterol were used in place of serum, there was a loss of expression of some microglial-specific genes including Tmem119 and Sall1 (72,73). Hence, in our opinion, effects of elements from neurons, other glia, extracellular milieu and lymphocytes must also be ascertained and added to the culture media, if specific diseased situations are to be emulated, in a serum-free in vitro environment.

1.2 Neurons

From the 56 research articles we reviewed, types of neuronal cells used in the models are listed in Table II. Twenty six of the studies reviewed used only neuronal cell lines, 25 only primary neuronal cells and 5 used both primary neurons and neuronal cell lines. One study used primary neurons from two regions of the brain, the cortex and hippocampus (46), while another used both differentiated and undifferentiated cell line (52).

a. Neuronal cell lines

Some common neuronal cell lines used in MINT models are PC12, SH-SY5Y and N2a, either differentiated or undifferentiated. Generally, undifferentiated ones are more sensitive to neurotoxic chemicals and tend to be affected at a lower dose to its differentiated counterpart (53). Undifferentiated ones are used to represent developing neurons, for example during embryonic development before they terminally differentiate into conducting neurons, hence they are used to model developmental diseases, whereas differentiated neurons are used to model mature neurons. Neuronal cell lines are advantageous in that they can be propagated, frozen down, revived and re-cultured indefinitely until they are to be used. Then, all that is required is the addition of a differentiating agent, most common being retinoic acid (RA) or serum-deprivation and nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF). RA functions to halt cell division, bringing cells into the G0 phase of the cell cycle, while growth factors are to encourage the growth of neurons and expression of neuronal markers such as include βIII-tubulin, synaptophysin, microtubule-associated protein-2 (MAP2), neuron-specific enolase (NSE), synaptic associated protein-97 (SAP-97) and neuronal-specific nuclear protein NeuN (54).

PC12 originated from the rat's adrenal phaeochromocytoma in 1976. They produce and store dopamine, sometimes noradrenaline (55). As they possess several properties of dopaminergic neurons (56), they are used to represent dopaminergic cells of Parkinson's disease (PD). They can be differentiated into terminal neurons using NGF (54). SH-SY5Y cells are neuroblastoma cells, originally taken from bone tumors, a clone of the SK-N-SH cell line isolated in the 1970s. It is also catecholaminergic, not specifically dopaminergic but does express tyrosine hydroxylase (TH) and also used to model PD, neurons or neurodegenerative diseases (57). Once differentiated, it can express neuronal markers such as β-III tubulin in neurite outgrowths (58). N2a,

Table II. Types of neurons used in MINT models.

Neurons	Type/Name	Source/Organism		Age of Source Organism	References	Year
Primary cells	Cortical	C57BL/6	Mice	Embryonic (d.15-20)	(47)	2020
					(36) (43) (44) (46) (48)	2019
					(35)	2016
					(25)	2008
		SD	Rat	Embryonic (d.17-20)	(23) (72)	2019
					(125)	2014
					(32)	2004
					(33)	1996
				Postnatal (1 d.o.)	(37) (127)	2019
		Wistar	Rat	Postnatal (1 d.o.)	(88)	2017
		Kunming	Mice	Postnatal (1 d.o.)	(113)	2019
	Hippocampal	C57BL/6	Mice	Embryonic (d.16-18)	(38)	2020
					(46)	2019
		SD	Rat	Embryonic (d. 18-19)	(40)	1997
					(27) (41)	2000
			Rat	Postnatal (1 d.o.)	(130)	2018
	Mesencephalic	SD	Rat	Embryonic (d.14)	(24)	2019
					(29)	2005
		Fisher F344	Rat	Embryonic (d.14)	(39)	2010
	Cerebellar	C57BL/6	Mice	Postnatal (7 d.o.)	(42)	2019
		SD	Rat	Postnatal (2-4 d.o)	(31)	1998
				Postnatal (8 d.o.)	(30)	1992
		Wistar	Rat	Postnatal (3-6 d.o.)	(49)	2008
	Spinal Cord	SD	Rat	Embryonic (d.13-14)	(28)	2004
Cell lines	PC12	NEDH (Phaeochromocytoma – Adrenal gland)	Rat		(121)	2019
					(69)	2017
					(68)	2015
	PC12 (diff.)				(72)	2019
					(67)	2018
	SH-SY5Y	Homo sapiens (Neuroblastoma – Bone marrow metastases)	Human		(74) (115)	2020
					(43) (129) (92) (93) (120) (122)	2019
					(52) (123) (124)	2018
	SH-SY5Y (diff.)				(34)	2020
					(128)	2019
					(52)	2018
	N2a	Neuroblastoma	Mouse		(66) (119)	2019
					(25)	2008
	N2a (diff.)				(45)	2019
	MN9D	Dopaminergic (Neuroblastoma hybrid)	Mouse		(24) (70) (71)	2019
	HT-22	Hippocampal (Transformed)	Mouse		(114)	2020
					(23) (117)	2019
	SK-N-SH	Neuroblastoma	Human		(116)	2020
	B35	Neuroblastoma	Rat		(126)	2008
	CATH.a (diff.)	C57BL/6 x DBA/2 (Transformed)	Mouse		(118)	2019

d.o. = days old, d. = day, diff. = differentiated

also known as Neuro-2a cells are also neuroblastoma-derived cells, that can be differentiated into neurons with serum-deprivation, forskolin or RA (59).

Neuronal cells are highly heterogenous, displaying various membrane channels, receptors and producing and responding to a variety of neurotransmitters. For example, in studying PD, ideally, dopaminergic neurons are used (57). Some studies demonstrate the dopaminergic properties of their neurons, while others do not. Likewise, channels like VGCCs and NMDA receptor density differences, between cerebellar granule neurons and N2a have shown significant differences in sensitivity to marine toxic compounds

(60). Hence, including membrane channels, receptors and neurotransmitter characterization of neuronal cells used prior to experimentation is advisable for a more informed interpretation and application of results for future studies.

b. Primary neurons

Besides utilising neuronal cell lines, primary neurons can be cultured from rodents (61). However, their limitations are that they do not undergo mitosis, hence they cannot be expanded in vitro (62). The limited number of cells prevents the usage of primary cells for high-throughput assays. Primary neurons taken from whole brains are also highly heterogeneous, making interpretation of results difficult (61).

2.0 Stimulators for microglial activation

Stimulators used for microglia activation in the studies reviewed are listed in Table III. The most common stimulant used in MINT models is lipopolysaccharide (LPS), present in the outer membrane of Gram-negative bacteria, which contains Lipid A, the endotoxin that initiates innate immune response (63). LPS acts via binding to toll-like receptor 4 (TLR4) present on microglia which then activates intracellular proteins for gene transcriptions of inflammatory molecules (64). Therefore, LPS is often used to represent a bacterial infection, an exogenous stimulator, a threat from the outside. However, in many sterile diseases such as stroke and neurodegenerative diseases, microglia are activated by substances released from cell damage and other endogenous stimuli such as pro-inflammatory cytokines IFN-γ and TNF-α (65). Hence, these have also been used in various MINT models.

2.1 Exogenous activators

LPS has been reliable and consistent in activating microglia to a neurotoxic state in many MINT models. Conditioned media (CM) from LPS-activated BV2 microglia reduced cell viability of SH-SY5Y cells, N2a and primary cortical neurons (43,66). Similarly, CM from LPS-treated SIMA9 cells caused reduced viability of differentiated PC12 neurons (67) and LPS-treated BV2 CM reduced viability of undifferentiated PC12 (68,69). In dopaminergic neurons, CM from LPS-stimulated BV2 reduced viability and TH-positive MN9D cells (70). Besides cell lines, LPS-induced primary microglial cells reduced viability and induced cytotoxicity via apoptosis of MN9D cells (71). In trans-well cultures, LPS-stimulated BV2 cells caused reduced viability and neuritic staining density and length in primary cortical neurons derived from embryonic Sprague-Dawley rats and C57BL6 mice (23,72). Also in trans-well cultures, LPS-activated BV2 and LPS-activated primary cortical neurons caused apoptosis and suppressed cell proliferation in MN9D and primary mesencephalic neurons (24). In a primary mesencephalic neuron-microglia mixed culture, LPS addition reduced length of TH-positive neurons and cell survival as determined with immunostaining (73).

Twenty six publications in this review used LPS alone as the stimulator. Thirteen used 1μg/ml, while 6 used a lower concentration of 100 ng/ml. Among these, three studies were in contradiction. Liu et al. (74) reported no direct toxicity of LPS on SHSY-5Y cells for LPS concentrations of 0.1 to 100μg/ml but neglected to report any toxicity on BV2 cells. They used 10μg/ml LPS for stimulation of BV2, similar to Flavin et al. (41). However, Kaewmool et al. (75) tested 0.06-8μg/ml LPS on BV2 cells and discovered that BV2 cell viability decreased significantly at 8μg/ml of LPS. Therefore, probably unknown to Liu et al. (74) and Flavin et al. (41), the concentration they used, 10μg/ml, could have imparted significant cytotoxicity to BV2 cells, contaminating the conditioned medium collected with dead BV2 intracellular components, which may exaggerate results of their experiments. In our own experience, we have used 1μg/ml of LPS for activating the microglia and our results have not revealed any cytotoxic effect of this concentration on the microglia based on viability assays (76–79).

2.2 Endogenous activators

Microglia are activated not only by exogenous threats but also by endogenous threats such as cell stress, damage and death (65). They activate by responding to cytoplasmic substances released from damaged/dead cells and to cytokines produced by other cells. An important cytokine for macrophage and microglia activation is IFN-γ.

IFN-γ is upregulated in the dorsal horn of the spinal cord during peripheral nerve injury (80), in aged brains, following traumatic brain injury, at early stages of neurodegenerative diseases including AD, PD and vascular dementia (81). Microglia exposed to these high levels of IFN-γ are then activated into a reactive form, producing other proinflammatory cytokines (82,83). Therefore, besides LPS, IFN-γ is also used as a stimulator of microglia activation in neuroinflammatory studies. Where LPS represents a specific exogenous threat; namely infection, IFN-γ represents a more generic and endogenous response.

Likewise, tumor necrosis factor-α (TNF-α) is also an endogenous activator of microglia. TNF-α is present in the healthy CNS as a regulator of synaptic plasticity, learning, memory and other functions of the CNS. In diseased conditions, such as neurodegenerative diseases, ischemia and trauma to the brain, TNF-α is found in higher levels and its main secretor are astrocytes and microglia (84). The secreted TNF-α then acts onto the microglia to stimulate them to an activated inflammatory state, releasing high levels of glutamate and causing glutamate excitotoxicity in the neurons (85,86). TNF-α is also involved in the nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) signaling pathway that perpetuates neuroinflammation in the astrocytes and microglia (87). TNF-α is both activated by NFκB and activates NFκB.

From our search of the literature, we found only two studies using IFN-γ alone to stimulate microglia (45,88). Seven studies out of the 56 MINT models we reviewed used a combination of IFN-γ with LPS.

Using IFN-γ alone, O'Farrell et al. (88) reported reported that CM from IFN-γ stimulated BV2 microglia reduced the number of primary neurites, neuritic length and branches of primary cortical Wistar rat pup neurons. Additionally, Sholl analysis revealed a significant reduction in the quantity of branches nearer to the soma of the neuron at distances of 10-30 μm (88). In the study by Chen et al. 2019, IFN-γ-activated primary microglia from C57BL6 pups reduced the viability of differentiated N2a neurons (45).

2.3 **Combining exogenous and endogenous activators**

Several studies combined LPS and IFN-γ (LPS+IFN-γ). Peng et al. (2016) transferred CM of LPS+IFN-γ-activated primary microglia onto primary cortical neuron culture. The CM reduced the activity of mitochondria evidenced by reduced neuronal basal respiration and spare capacity, similar to reports of studies on multiple sclerosis patients' brains (35). Reinhardt et al. (2019) repeated a similar study they performed in 2012 (89), co-culturing motor neurons differentiated from mice embryonic stem cells (ESCs) with LPS+IFN-γ-activated BV2 cells. The result was a significant reduction in total neurite length (90). In studies on human cells, THP-1, a cell line from human monocytic leukemic cells with comparable microglia-like responses (91), was activated with LPS+IFN-γ. Its CM was found to significantly reduce the viability of SH-SY5Y cells (92,93).

Interest in exploring microglia activation with a combination of both LPS and an endogenous activator is due to an emerging theory on the pathogenesis of neurodegeneration which hypothesizes that LPS is involved in the loss of neurons in neurodegenerative diseases (94). In healthy human systemic blood circulation, LPS is found in very low concentrations (95). However, increased levels were found in AD and amyotrophic lateral sclerosis (ALS) (96). LPS potentiates Aβ and tau protein aggregation in AD (97). In PD, the gut has increased permeability to LPS, resulting in a change of its microbiota (98). An increase in the population of LPS-producing bacterial species in the gut worsens the disease in patients and mouse models (99). A detailed review of this theory was written by Brown et al. (94) and this is an interesting avenue to pursue in this area.

2.4 Combination of two endogenous activators

In comparing the effect of LPS and a combination of two different endogenous activators, IFN-γ and TNF-α on primary rat microglia culture, Lively et al. (2018) reported that both stimuli activated microglia into a pro-inflammatory state but LPS stimulation induces higher level NO production, higher and broader range of pro-inflammatory genes and proteins expression. Morphology was also slightly different with LPS inducing amoeboid morphology while IFN-γ/TNF-α induced microglia into rounded cells, in chains with small processes (100). Investigations on the effects of this combination on neurotoxicity parameters and other combinations of endogenous activators on MINT should be pursued to increase our understanding.

3.0 Neurotoxicity determination

In the publications that we have reviewed, methods for determining neurotoxicity can be categorised into two groups: (1) general cytotoxicity and (2) neurotoxicity. General cytotoxicity methods evaluate the cell growth, cell death, cell membrane permeability, mitochondrial activity and synthesis of macromolecules, while evaluations of neurotoxicity are neuron-specific such as assays for neurotransmitter synthesizing enzymes, neuronal receptors, morphological endpoints such as neurite analysis, synaptic protein synthesis and functional assays such as electrophysiology (101,102). According to Bal-Price et al. (2010), the ideal structure of a neurotoxicity study would be to first determine cell viability/death, followed by tests of generic cell function such as energy metabolism and finally ending in tests of specific/niche cell function such as neurotransmission and axonal transport (103). Due to the complexity of the CNS with its various types and functions of cells, it is recommended that a variety of endpoints is conducted in any one study to have a more holistic understanding of a particular phenomenon or disease. Furthermore, in vitro MINT models are not considered a replacement of in vivo models, as cognitive and behavioural effects cannot be assessed in vitro, but as a supplement to animal models and for refinement of animal experiments (101–103).

Most of the studies reviewed used the MTT assay to determine viability (Table IV). MTT abbreviates 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a metabolic dye that turns into a purple formazan crystal once reduced by mitochondrial enzymes (104). It is an easy, cheap and simple method to measure metabolically active cells (105). As it uses a plate reader, this colorimetric assay is amenable for high throughput screening. Similar assays in this group are MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) and XTT (sodium 3 -[1-(phenylaminocarbonyl)-3,4-tetrazolium)-bis-(4-methoxy-6-nitro) benzene sulphonic acid hydrate) which has the advantage of producing soluble coloured products, omitting the second step used in MTT assays (104).

Other, more specific, methods were also used to determine types of cell death, growth, metabolic activity and protein-synthesizing capacity before and after toxic insult. For example, Annexin V and propidium iodide (PI) assay was used to differentiate the population

of cell death due to apoptosis or necrosis in several MINT models (36)(24)(71)(74). Annexin V binds to phosphatidylserine which is normally present on the inner surface of cell membranes but becomes exposed to the outer environment during apoptosis. PI on the other hand only stains the nucleus when the cell membrane is damaged in necrosis. With fluorescent labelling of Annexin V, populations of cells that stain with either can then be enumerated using flow cytometry (106). There is a possibility of false positives using PI to stain genetic material as they have been found to also stain cytosolic RNA in late apoptotic cells. Therefore studies that do not use RNAse to remove them might be exposed to an estimate of 40% false positivity (107). Other methods used to determine apoptosis were determining cleaved caspase 3 activity (36)(72) and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling), assay (108).

Table III : Microglia stimulus and co-culture formats in MINT models.

Stimulus	References	Culture Formats	References
LPS	(23) (25) (27) (28)(41) (42) (43) (49) (52)(117) (129) (108) (115) (116) (119) (121) (124) (126)(125) (128)	Direct Co-culture	(27) (28) (29) (32) (39) (42) (44) (45) (46) (47) (126)
IFN-γ	(45) (88)3-dioxygenase (IDO	Trans-well	(23) (24) (30) (31) (32) (33) (34) (66) (72) (113) (124) (125) (128) (130)
LPS + IFN-γ	(31) (35) (46) (90) (92) (93) (123)	Conditioned Media/ Supernatant Transfer	(23) (25) (29) (32) (34) (35) (36) (37) (38) (40) (41) (43) (48) (49) (52) (67) (68) (69) (70) (71) (74) (117) (129) (88) (92) (93) (113) (114) (115) (116) (118) (121) (122) (123) (127)
Others	LPS + IFN-γ + TNF-α + IL-1α + IL6: (30)		
	LPS + Aβ: (120) (122)		
	LPS + Hypoxia: (40)		
	LPS + $MnCl_2$: (39)		
	IFN-γ + Aβ: (33) (32)		
	$CoCl_2$: (34)		
	KU-60019 (36)		
	TCDPP: (38)		
	Aβ42: (44)		
	Alpha-synuclein: (48)		
	CM from OGD-N2a: (66)		
	Necrotic-CM: (117)		
	MPP+: (128)		
	OGD: (47) (127)		
	$LaCl_3$: (113)		
	CdTe/ZnS QDs (114)		
	LPA: (118)		
	DAC: (121)		
	High glucose and Oxygen-glucose deprivation: (130)		
	ALS/DC IgG: (28)		
	Thrombin: (29)		
	Myelin: (49)		

LPS = lipopolysaccharide, IFN-γ – Interferon gamma, TNF-α = Tumour Necrosis Factor Alpha, IL-1α = Interleukin-1 Alpha, IL-6 = Interleukin-6, MCSF = Macrophage colony stimulating factor, Aβ = beta amyloid, CoCl2 = cobalt chloride, KU-60019 = ataxia-telangiectasia mutated (ATM)-specific inhibitor, TCDPP = Tris(1,3-dichloroisopropyl) phosphate, Aβ42 = beta amyloid peptide 42, OGD = oxygen-glucose deprivation, CM = conditioned media, LaCl3 = lanthanum chloride, CdTe/ZnS QDs = cadmium telluride/ zinc sulfide quantum dots, LPA = lysophosphatidic acid, DAC = docetaxel, adriamycin, and cyclophosphamide, MPP+ = active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine (MPTP).

As for neuron-specific neurotoxicity measurements, neurite analysis via morphological assessment of immunocytochemical staining is the most common method undertaken (36)(72)(88)(90). Although one study did measure neurites using only bright field microscopy (23), in our opinion, lack of contrast between culture plate and neurite outgrowth does not allow for a confident assessment of the neurites. Immunostaining of microtubular components specific to mature neurons such as beta-tubulin III (Tuj-1) and MAP2 will increase contract and reduce noise for more accurate determination. Neurite analysis involves measurement of neurite number, length, branches and Sholl analysis (38,101,102,109). Although these are widely used and accepted method of determining neurotoxicity (110), in our opinion, functional aspects of neuronal activity must also be as regularly and widely tested. For example, the production of synaptic proteins, synaptic and electrical activity of the neurons must also be determined. Inclusion of these evaluations in addition to neurite analysis will give a more comprehensive understanding (103).

4.0 Co-culture formats

The formats for co-culture of microglia and neurons in MINT models reviewed are: (1) direct cell-to-cell culture (DC), (2) CM/supernatant transfer (CMT) and (3) trans-well culture (TC). Of the three, CMT is the most popular. All three methods are relatively easy and simple to conduct, do not require exorbitant cost or specialized skills or materials except for TC which requires the purchase of cell culture inserts. The choice of the format depends on which aspects of cell interactions one wishes to investigate. Direct cell-to-cell culture involves culturing each cell population separately prior to seeding one on top of the other for the examination of the cell to cell interactions. In this way, an activator can be added to each culture first to examine any direct toxic effect on the individual cells, before seeding one on top of the other. For example, LPS can be added to microglia monoculture and neuronal monoculture to determine individual direct toxicity prior to seeding activated microglia onto the neurons. The advantage of DC is that both bi-directional cell-to-cell and secreted factors interactions can be observed cumulatively. In this view, it is the most in vivo-like of the three formats. If one would like to dissect the interactions further to focus only on uni-directional effects of secreted factors however, CMT is the best format. CMT involves activating microglia monoculture with an activator, then collecting its culture media, centrifuged to remove cellular debris and transferring the supernatant obtained to neuronal culture. The possible limitation of this process is the loss of vital factors produced by the activated microglia which may be degraded or depleted over time, as in most studies reviewed, the CM collected is stored and cryopreserved before its final usage. This can lead to false negatives or reduced degree of effects produced (111).

If one is interested in bi-directional secreted factors interactions, TC is the best format. It separates microglia from neurons using a semi-permeable porous membrane which, depending on the pore size selected, allows secreted factors from both cells to affect each other but does not allow physical contact between the cells. The physical distance between the cells is uniform and therefore can be standardized. Cell culture inserts come in various sizes for various number of wells format, therefore are suitable for limited cell population such as primary microglia/neuronal culture or unlimited ones, like cell lines. Furthermore, after the interaction, each cell population can be analysed separately simply by removing the insert from the wells (112).

From our review, many of the studies published, utilized only one format to evaluate MINT. Four studies used two formats (23,29,34,113) and only one study used all three formats (32). Unfortunately, the former four studies did not compare and investigate the differences of results between the two formats used. They merely use both formats to strengthen the confidence in the neurotoxicity observed. The studies also did not take advantage of the fact that a TC format allows bidirectional communications between the cells. Hence, the effect of neurons on the microglia could have also been studied. Instead, the studies focused only on the effects on the neurons. In Yan et al. (2019), lanthanum chloride ($LaCl_3$)-activated microglia in a TC showed higher levels of apoptosis and lower levels of necrosis compared to CMT (113). This shows that even though both formats reduced the viability of neurons by apoptosis and necrosis, the level of each type of cell death differs, possibly due to degradation of some factors in activated-microglial CM, either due to processing (centrifugation) or storage process (cryopreservation and thawing). The quality of proteins in the CM may be affected by possible denaturation due to the freezing and thawing process. Zhou et al. (2019) evaluated different parameters of neurotoxicity on each of their model. In their CMT, they evaluated the viability of the neurons, while in TC, they measured neurite outgrowth. They also used different cells for each of the formats: BV2 and hippocampal neuronal cell line, HT-22 for CMT and BV2 and cortical neurons for TC. MTT assay showed reduced viability in the CMT study and bright field microscopy showed reduced integrity of neurite outgrowths. They did not evaluate the fate of the microglia after the co-cultures (23). Merlo et al. (2020) showed a similar reduction of neuronal viability in both TC and CMT models of chemical hypoxia-activated microglia (34). As for the one study that employed all three formats, intriguingly, they found contradictory effects on the neurons. Li M. et al. (2004) studied the effect of IFN-γ and fibrillar beta amyloid (fAβ) on microglia-induced neurotoxicity of primary cortical neurons. Neurotoxicity was detected in DC but not in TC and CMT (32). Hence, they eliminated the neurotoxic mechanism of secreted factors.

Table IV : Methods of neurotoxicity assessment in MINT models

Method of Neurotoxicity Assesment			
General Cytotoxcity		Neurotoxicity	
Assay type	**References**	**Assay type**	**References**
MTT/MTS assay – viability	(23) (34) (43) (47) (66) (67) (68) (69) (70) (71) (74) (129) (92) (93) (115) (116) (121) (124) (125) (128) (130)	Neurite analysis - Immunostaining with anti-Beta III tubulin /Tuj-1/MAP2/NeuN	(36) (38) (42) (72) (88) (90) (127)
Hoechst 33342/Annexin V +/- PI – apoptosis/necrosis	(24) (36) (49) (71) (74) (113) (116) (122) (130)	Neurite analysis (Without immunostaining)	(23) (39) (40) (41)
CCK-8 - viability	(46) (117) (114) (120) (122) (123) (127)	Synaptic protein expression - Synapsin 1 & Homer 1	(36)
LDH - viability	(31) (46) (66) (71)	Neuron morphology - Phase contrast microscopy	(31) (123)
Caspase 3/7 or Cleaved caspase 3 - apoptosis	(36) (49) (52) (72)	Neuron nuclear morphology - Bright field microscopy	(23)
Immunocytochemistry (ICC) to neuronal markers – viability	(24) (25) (27) (28) (29) (32) (39) (42) (126)	Neuronal protein expression - Western blot:	(24) (46) (70) (74) (117) (128)
Flow cytometer (LIVE/DEAD stain) - viability	(45)	Neuronal gene expression - PCR	(24) (74) (128)
Calcein AM - viability	(128)	Neuronal inflammatory signalling pathway - ICC to NF-κB p65	(74)
CellTiter 96® Assay - viability	(119)	Neuronal cytokines production - ELISA	(24)
Alamar Blue - viability	(108)	Neuronal mitochondrial permeability transition pore (mPTP) opening assay	(24)
Fluorescein diacetate (FDA) staining - viability	(33)	Neuronal dopamine uptake	(29) (39)
Trypan Blue - viability	(30)	Neuronal cell surface receptors analysis – flow cytometry	(44)
Acridine orange - viability	(40) (41)	Neuronal mitochondrial membrane Potential	(24) (52)
Ethidium bromide – viability	(40) (41)	Neuronal intracellular reactive oxygen species (ROS) - dihydroethidium (DHE) and hydrogen peroxide assay	(24)
Apoptag kit - apoptosis	(40)		
TUNEL assay - apoptosis	(108) (41)		
DAPI staining for nuclear morphology - apoptosis	(42)		
Propidium iodide (PI) - cell cycle analysis	(24)		
Monodansylcadaverine fluorescence - autophagy	(52)		
Oxygen Consumption rate – metabolic state	(35)		

Neurotoxicity of the specific combination of IFN-γ+fAβ-stimulated microglia therefore most likely occur to due to specific cell contact, cell proximity or close paracrine mechanism.

CONCLUSION

In conclusion, the many in vitro MINT models thus far has enriched our knowledge on many possible impacts of microglial activation on the health of neurons, from cell viability to types of cell death, to impacts on neurite outgrowths, neuronal protein, gene expressions and possible intracellular signalling pathways for these events. However, the bulk of the studies is heavily skewed towards the screening of various anti-inflammatory or anti-neurotoxic therapeutics. The breadth of the potential of MINT models in investigating neuron-specific functions such as its electrophysiology, axonal transports and synaptic ability has not been explored as widely. Cell types used have also been well-characterized for routine usage in MINT models. However, generalizations between rat and mouse studies, between and within primary microglia and cell lines, should be made with caution and if possible, characterizations of the cells used should be done prior to the experiments. There is also a lack of studies comparing the different formats of co-culture and feedback signals of neurotoxic neurons to microglia. Hence, although it seems like a lot has been done with MINT models, the potential to explore a lot more is tremendous in the pursuit of understanding the complete picture of microglia-induced neurotoxicity.

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