Video Article

Microglia as a Surrogate Biosensor to Determine Nanoparticle Neurotoxicity

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Abstract

Nanoparticles found in air pollutants can alter neurotransmitter profiles, increase neuroinflammation, and alter brain function. Therefore, the assay described here will aid in elucidating the role of microglia in neuroinflammation and neurodegenerative diseases. The use of microglia, resident immune cells of the brain, as a surrogate biosensor provides novel insight into how inflammatory responses mediate neuronal insults. Here, we utilize an immortalized murine microglial cell line, designated BV2, and describe a method for nanoparticle exposure using silver nanoparticles (AgNPs) as a standard. We describe how to expose microglia to nanoparticles, how to remove nanoparticles from supernatant, and how to use supernatant from activated microglia to determine toxicity, using hypothalamic cell survival as a measure. Following AgNP exposure, BV2 microglial activation was validated using a tumor necrosis factor alpha (TNF-α) enzyme linked immunosorbent assay (ELISA). The supernatant was filtered to remove the AgNP and to allow cytokines and other secreted factors to remain in the conditioned media. Hypothalamic cells were then exposed to supernatant from AgNP activated microglia and survival of neurons was determined using a resazurin-based fluorescent assay. This technique is useful for utilizing microglia as a surrogate biomarker of neuroinflammation and determining the effect of neuroinflammation on other cell types.

Video Link

The video component of this article can be found at https://www.jove.com/video/54662/

Introduction

Environmental pollutions, specifically those of the nanoparticle (NP) range (1 - 20 nm diameter), have been linked to obesity and other neurodegenerative diseases due to the ability to cross the blood brain barrier [1-3]. Elevated exposure to pollution may induce inflammation in the central nervous system including the hypothalamus [1]. One potential mechanism in which this occurs could be through nanoparticle induced activation of microglia (brain immune cells) [4]. Prior studies have used *in vivo* models to study the effects of NPs on brain health which are time-consuming, expensive, and do not directly answer the question of how NPs influence microglia. Microglia play a multifaceted role in the central nervous system, including maintenance of the brain microenvironment and communicating with surrounding neurons via the release of secreted factors and cytokines. Depending on the stimuli, microglia can be activated to an M1 pro-inflammatory or an M2 anti-inflammatory state. For example, M1 activated microglia release pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), while M2 activated microglia release anti-inflammatory cytokines including interleukin-4 (IL-4). To validate our surrogate *in vitro* biosensor for determining neurotoxicity of air pollutants, we measured microglial response to 20 nm silver nanoparticles (AgNPs). The goal of this article is to describe how an *in vitro* microglial cell line can be used as a surrogate biosensor marker for testing murine microglial response to NPs and how microglial activation affects hypothalamic cells. The long-term intended application of this validated model is to test effects of real-world pollutants on brain health and neurodegenerative disease. We provide a detailed description of an *in vitro* 96-well format assay for measuring microglial activation and hypothalamic cell survival following the exposure of microglial conditioned media.

Microglial activation was determined following AgNP exposure using a TNF-α enzyme linked immunosorbent assay (ELISA). To determine the effect of activated microglia on hypothalamic cells, the AgNPs were removed from microglial supernatant (conditioned media) using a filtration device. The filtration device retains cytokines while excluding the AgNPs based on size. Briefly, supernatant from microglia treated with or without AgNPs was collected, added to the filters, and centrifuged at 14,000 x g for 15 min. We were then able to determine the influence of microglial secreted cytokines on hypothalamic cell viability. Cell toxicity following exposure to conditioned media (containing cytokines) was determined via a resazurin-based assay as previously described [5,6]. Metabolically active cells reduce resazurin and produce a fluorescent signal proportional to the number of viable cells [7].

There are several advantages of using this technique over others (such as co-culture, trans-well setups, or *in vivo* experiments). Our model provides the ability to directly activate microglia and determine if secreted factors are toxic to neurons [8]. The current protocol uses immortalized BV2 microglia stimulated with 20 nm diameter nanoparticles, and immortalized murine hypothalamic cells (designated mHypo-A1/2) [9] for

determination of subsequent response. While this protocol has been optimized for these specific conditions, the methods can be altered to be used in other models of microglial-induced cell death, or with other cell types including primary microglia and neurons.

Protocol

1. Microglial Cell Culture Maintenance

1. Warm cell culture medium (Dulbecco's Modified Eagle Medium; DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/neomycin (PSN) to 37 °C.
2. Obtain frozen stock of BV2 microglial cells at passage 18 - 25 from storage at -80 °C. Rapidly thaw cells in 37 °C water bath.
3. Gently transfer cells to a 75 cm^2 vented flask containing 10 ml cell culture media.
4. Incubate flask in 5% CO_2 at 37 °C. Aspirate media after 24 hr and replace with fresh media. Grow cells until approximately 70 - 80% confluency.

2. Counting and Plating Cells

1. In a 37 °C water bath, warm DMEM and 1x-trypsin-EDTA solution.
2. Aspirate media from cells and add 500 µl of trypsin. Incubate at 37°C for 2 - 5 min.
3. Using a scraper, remove cells from the flask and suspend in 5 ml of DMEM. Pass cells through a 70 µm strainer into a 50 ml tube three times.
4. Count cells using a hemocytometer and seed 8,000 cells/well in a black walled clear bottom plate in a final volume of 200 µl DMEM supplemented with 10% FBS and 1% PSN. Incubate plate in 5% CO_2 at 37 °C for 24 hr.
5. Remove old DMEM and replace with 200 µl of warmed DMEM supplemented with 1% PSN to serum starve microglia. Incubate plate in 5% CO_2 at 37 °C for 24 hr.

3. Activating Microglia

1. In separate tubes, dilute AgNPs (0.01, 0.05 or 0.1 µg/ml) and vehicle/neutral control (sodium citrate, 0.04 mM) in serum-free DMEM supplemented with 1% PSN to final working concentrations.
2. Remove 100 µl of media from each well and replace with 100 µl of appropriate treatment compound.
3. Incubate plate in 5 % CO_2 at 37 °C for 24 hr.

4. Filtering Conditioned Media

1. Collect 200 µl of media supernatant from each well and transfer into a filter (molecular weight cutoff of 10 kDa) with collection tube (1.5 to 2 ml capacity).
2. Centrifuge at 14,000 x g at room temperature for 15 min.
3. Discard the flow-through (supernatant with particles) and place the filter upside-down into a new collection tube.
4. Centrifuge at 1,000 x g at room temperature for 2 min.
   NOTE: The resulting filtered media (containing secreted cytokines) is concentrated by six-fold.
5. Bring the volume of the concentrate to 400 µl with fresh DMEM supplemented with 10% FBS and 1% PSN and store on ice.
   NOTE: To ensure residual AgNPs are removed from filtered media, generate an AgNP concentration based on the characteristic peak at about 390 - 420 nm [10]. Briefly, prepare aliquots of unfiltered AgNPs (0, 0.01, 0.025, 0.05, 0.1, and 0.2 µg/ml as described in step 3.1) and filtered vehicle control (sodium citrate, 0.04 mM) in serum-free DMEM. Aliquot 50 µl of filtered samples and standards into a black walled clear bottom plate. Using a spectrophotometer, measure absorbance at 390 - 410 nm and compare readings to the AgNP concentration curve. If filtered samples have the same absorbance value as the vehicle control sample, it can be assumed residual AgNPs are removed.
6. Use half of the filtered media to determine TNF-α secretion following instructions from a commercially available kit.

5. Hypothalamic Cell Culture Maintenance

1. Warm DMEM cell culture medium supplemented with 10% FBS and 1% PSN to 37 °C.
2. Obtain frozen stock of hypothalamic (mHypo-A1/2) cells at passage 18 - 25 stored at 80 °C. Rapidly thaw cells in 37 °C water bath.
3. Gently transfer cells to a 75 cm^2 vented flask containing 10 ml cell culture media.
4. Incubate flask in 5% CO_2 at 37 °C. Aspirate media after 24 hr and replace with fresh media. Grow cells until approximately at 70 - 80% confluency.

6. Determining Hypothalamic Cell Toxicity

1. Warm DMEM and 1x-trypsin-EDTA solution in a 37 °C water bath.
2. Aspirate media from hypothalamic cells and add 500 µl of trypsin. Incubate at 37 °C for 2 - 5 min. Remove cells from flask using a scraper as described above in step 2.
3. Count cells using a hemocytometer and plate hypothalamic cells at 5,000 cells/well in a black walled clear bottom plate in a final volume of 200 µl DMEM supplemented with 10% FBS and 1% PSN and incubate overnight in 5% CO_2 at 37 °C.
4. Remove 100 µl of old media using a multichannel pipetter and add 100 µl of filtered concentrated conditioned media, to a final concentration of 1x.
5. Incubate plate in 5% CO_2 at 37 °C for 24 hr.

6. Add 22 µl of resazurin reagent and incubate plate for 20 min in 5% CO_2 at 37 °C.
7. Using a multimode spectrophotometer, record fluorescence (560 nm EX/590 nm EM) to measure cell viability. Report results as relative fluorescence units (RFU).

Representative Results

We show that microglia function as a surrogate biosensor for brain response to nanoparticles using the protocol above. Our results include measurement of the toxic effects of microglial activation on downstream neuronal cell death. **Figure 1** demonstrates a workflow of the protocol to activate microglia and determine if secreted cytokines reduce viability of hypothalamic neurons. TNF-α secretion was significantly increased following AgNP exposure (**Figure 2**). When hypothalamic neurons are exposed to conditioned media from AgNP-activated microglia, cell viability is significantly reduced (**Figure 3**). **Figure 4** outlines the potential mechanism in which AgNPs activate microglia and contribute to hypothalamic cell death.

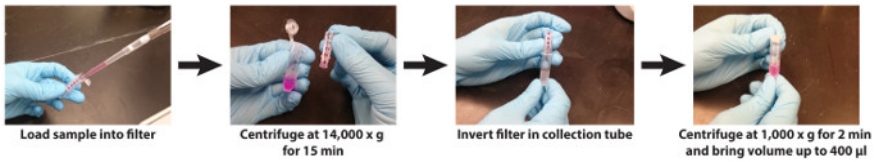

Figure 1. Workflow for AgNP Filtration Method. Supernatant from activated microglia is added to filtration devices and centrifuged to remove AgNPs from media. Please click here to view a larger version of this figure.

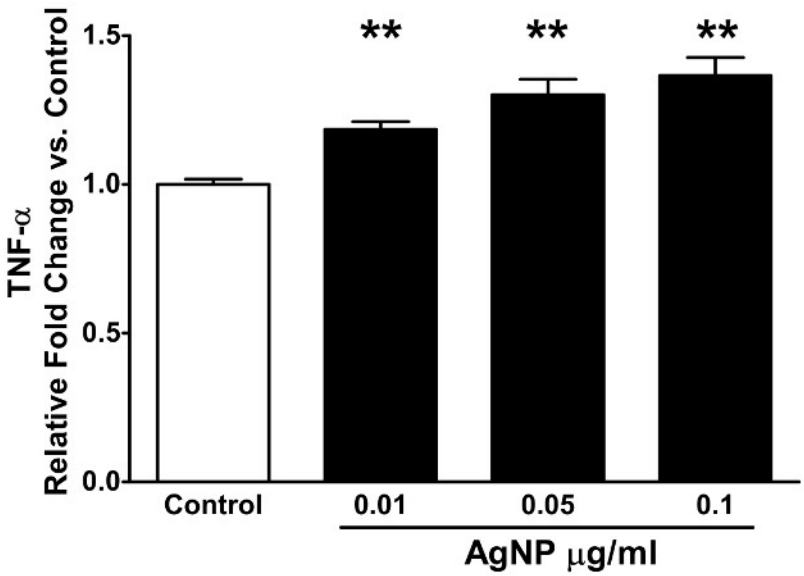

Figure 2. TNF-α Secretion Is Increased Following AgNP Exposure. Microglial BV2 cells were treated with vehicle control or AgNPs for 2 hr. TNF-α secretion was significantly increased following exposure to AgNPs. Data are presented as mean ± SEM. **p < 0.001 *vs.* control as assessed by Student's unpaired *t*-test. Please click here to view a larger version of this figure.

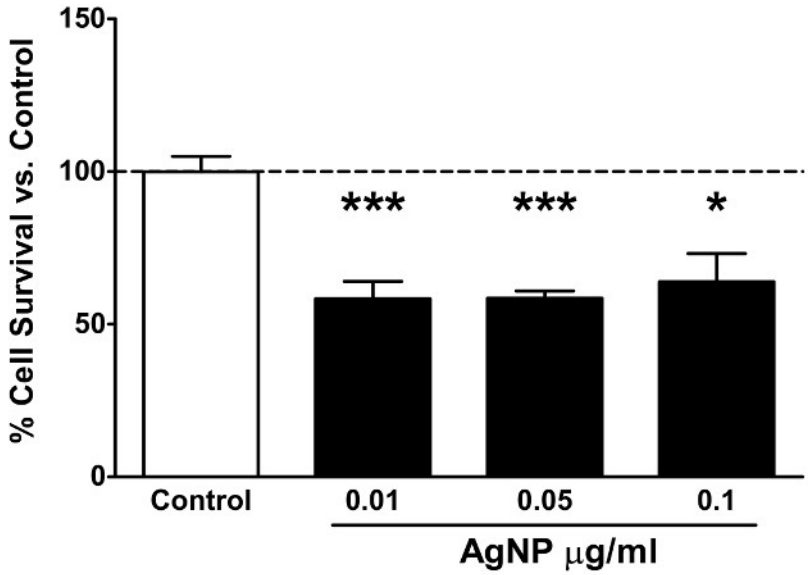

Figure 3. Hypothalamic Cell Viability Is Reduced Following Microglial Conditioned Media Exposure. Hypothalamic cells show increased cell death following exposure to conditioned media from microglia stimulated with AgNP. Data are presented as mean ± SEM.*p < 0.05 *vs.* control as assessed by Student's unpaired *t*-test. Please click here to view a larger version of this figure.

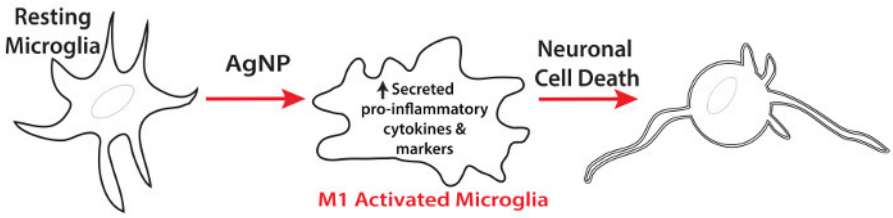

Figure 4. Potential Pathway of AgNP Induced Microglial Activation and Influence on Hypothalamic Neurotoxicity. Following exposure to silver nanoparticles, microglia are activated to an M1 pro-inflammatory state, characterized by increased secretion and expression of pro-inflammatory cytokines and makers. The release of the pro-inflammatory cytokines and makers contributes to surrounding neuronal cell death. Please click here to view a larger version of this figure.

Discussion

Recent studies support that environmental exposure contributes to obesity and other neurodegenerative diseases [11,12]. However, techniques used in previous studies are time consuming and expensive. Economic considerations, physiologically relevant delivery systems, ethical issues with extensive use of *in vivo* animal models, and difficulty translating findings into meaningful health advisories are a few of the major challenges that have impeded advancements in studying NP-induced neurotoxicity [13]. Therefore, use of an *in vitro* microglial surrogate biosensor has become an essential tool for environmental exposure to neurotoxins [14]. In the methods described above, we provide a direct way to determine how nanoparticles such as AgNPs activate microglia and induce neurotoxicity. It should be noted that because the cells are not fixed or lysed, other downstream applications including gene expression and Western blots could be performed on the microglia or neurons. In addition to performing a TNF-α ELISA, the supernatant can be used to analyze an array of cytokines and secreted factors from the stimulated microglia.

Microglial activation can be a result of many different factors including environmental exposures, infection, ischemia, and injury resulting in morphological and physiological changes. Chronic M1 microglial activation is associated with neurodegenerative diseases such as obesity, Alzheimer's disease, and Parkinson disease [5,15-19]. Further, the secretion of pro-inflammatory cytokines from microglia, including TNF-α, contributes to the onset of surrounding neuronal cell death [5,15,20]. Other markers that can be investigated after microglial activation are changes in reactive oxygen species (ROS) and nitrogen species [17]. Because pro-inflammatory cytokines such as TNF-α are regulated by the transcription factor nuclear factor kappa B (NFκB) [21], other downstream targets within this pathway can be profiled using this protocol. Additionally, microglial activation (acute or chronic) is stimuli dependent. Therefore, the timing and stimuli should be taken into consideration when performing the assays. The main limitation to this assay is that it is an *in vitro* study. As such, results should be cautiously extrapolated, and overinterpretation of results avoided, until biological significance has been verified *in vivo*. However, utilizing an *in vitro* test such as the microglial model described here, as a pre-screening method is a crucial first step to understanding toxicity of nanoparticles. Additionally, the specific particle used in testing should be considered carefully when interpreting results. Our protocol utilizes manufactured single metal AgNPs to serve as a surrogate for environmental pollutant generated nanoparticles. These particles were chosen not to mirror real-world environmental pollutants, but as standardized control particles to validate the methodology. Environmental pollutants represent multiple species of NPs, therefore particle morphology (size, shape, charge) and type of NP (silver, gold, titanium, carbon) should be important aspects of experimental design. Without taking such considerations into account, results may not be physiologically relevant, and thus difficult to interpret.

Critical steps of this protocol are performing proper aseptic technique, using appropriate doses of stimuli, and using the correct filtration device. Considerations for troubleshooting when planning studies to evaluate microglial activation and neuronal cell death include determining appropriate concentrations and incubation time of the stimuli, cell densities, and incubation time for reagents used in the assay. To optimize this assay dose response and time course studies should be performed. Additionally, it is essential to choose the appropriate supernatant filtration device to ensure separation of nanoparticles from secreted cytokines. The filters used in this protocol are able to separate nanoparticles and proteins based on size [22,23]. Since AgNPs have a molecular weight of 0.107 kDa and the pro-inflammatory cytokine TNF-α is 17 kDa [24,25], using a filter that has a 10 kDa molecular weight cutoff therefore allows factors or proteins less than 10 kDa (including AgNPs) to be excluded from the conditioned media. To validate that AgNPs were completely removed from the conditioned media, absorbance measurements of the media were taken after filtration and compared to an AgNP standard curve. We can therefore conclude that the cytotoxic effects of the conditioned media was due to cytokines/proteins such as TNF-α released from activated microglia, and not due to residual AgNPs [5].

The microglial model described here can be used to provide valuable insight into the role of microglia-induced cell death after nanoparticle exposure, and validates the effectiveness of utilizing microglia as a surrogate biosensor in determining nanoparticle toxicity [5]. Modifications of this protocol can be used to fit other models of microglial-induced cell death including models of neurodegenerative disease, utilization of other cell types (primary, transfected, immortalized), or various alternate stimuli (fatty acids, amyloid-beta) [5,26,27]. Although literature supports that BV2 microglia are well characterized and commonly used, it should be noted that slight differences in cytokine profiles or activation response may be observed if using primary microglia or other immortalized cell lines. Therefore, the end user can adapt this model to fully characterize the cytokine profile released by NP-activated microglia using either ELISAs or arrays. Future applications of this technique include utilizing primary cell cultures and testing other environmental nanoparticles including those generated from manufacturing, internal combustion engines, aircraft turbine engines, and coal based power generation plants [28-30].

Disclosures

The authors have no conflicts to disclose.

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References

1. Block, M. L., & Calderon-Garciduenas, L. Air pollution: mechanisms of neuroinflammation and CNS disease. *Trends Neurosci.* **32**, 506-516 (2009).
2. Brochu, P., Bouchard, M., & Haddad, S. Physiological daily inhalation rates for health risk assessment in overweight/obese children, adults, and elderly. *Risk Anal.* **34**, 567-582 (2014).
3. Jerrett, M. *et al.* Traffic-related air pollution and obesity formation in children: a longitudinal, multilevel analysis. *Environ Health*. **13**, 49 (2014).
4. Kraft, A. D., & Harry, G. J. Features of microglia and neuroinflammation relevant to environmental exposure and neurotoxicity. *Int J Environ Res Public Health.* **8**, 2980-3018 (2011).
5. Duffy, C. M. *et al.* Role of orexin A signaling in dietary palmitic acid-activated microglial cells. *Neurosci Lett*. **606**, 140-144 (2015).
6. Butterick, T. A. *et al.* Use of a caspase multiplexing assay to determine apoptosis in a hypothalamic cell model. *J Vis Exp*. (2014).
7. Xiao, J. *et al.* Monitoring of cell viability and proliferation in hydrogel-encapsulated system by resazurin assay. *Appl Biochem Biotechnol*. **162**, 1996-2007 (2010).
8. Blasi, E., Barluzzi, R., Bocchini, V., Mazzolla, R., & Bistoni, F. Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. *J Neuroimmunol*. **27**, 229-237 (1990).
9. Belsham, D. D. *et al.* Ciliary neurotrophic factor recruitment of glucagon-like peptide-1 mediates neurogenesis, allowing immortalization of adult murine hypothalamic neurons. *FASEB J*. **23**, 4256-4265 (2009).
10. Paramelle, D. *et al.* A rapid method to estimate the concentration of citrate capped silver nanoparticles from UV-visible light spectra. *Analyst*. **139**, 4855-4861 (2014).
11. Wei, Y. *et al.* Chronic exposure to air pollution particles increases the risk of obesity and metabolic syndrome: findings from a natural experiment in Beijing. *FASEB J*. (2016).
12. Levesque, S., Surace, M. J., McDonald, J., & Block, M. L. Air pollution & the brain: Subchronic diesel exhaust exposure causes neuroinflammation and elevates early markers of neurodegenerative disease. *J Neuroinflammation.* **8**, 105 (2011).
13. Block, M. L. *et al.* The outdoor air pollution and brain health workshop. *Neurotoxicology*. **33**, 972-984 (2012).
14. Carson, M. J., Crane, J., & Xie, A. X. Modeling CNS microglia: the quest to identify predictive models. *Drug Discov Today Dis Models*. **5**, 19-25 (2008).
15. Valdearcos, M. *et al.* Microglia dictate the impact of saturated fat consumption on hypothalamic inflammation and neuronal function. *Cell Rep* . **9**, 2124-2138 (2014).
16. Perry, V. H., & Holmes, C. Microglial priming in neurodegenerative disease. *Nat Rev Neurol.* **10**, 217-224 (2014).
17. Block, M. L., & Hong, J. S. Chronic microglial activation and progressive dopaminergic neurotoxicity. *Biochem Soc Trans*. **35**, 1127-1132 (2007).
18. Vincenti, J. E. *et al.* Defining the Microglia Response during the Time Course of Chronic Neurodegeneration. *J Virol*. **90**, 3003-3017 (2015).
19. Grabert, K. *et al.* Microglial brain region-dependent diversity and selective regional sensitivities to aging. *Nat Neurosci*. **19**, 504-516 (2016).
20. Lull, M. E., & Block, M. L. Microglial activation and chronic neurodegeneration. *Neurotherapeutics*. **7**, 354-365 (2010).
21. Oeckinghaus, A., Hayden, M. S., & Ghosh, S. Crosstalk in NF-kappaB signaling pathways. *Nat Immunol*. **12**, 695-708 (2011).

22. Gifford, J. C. *et al.* Thiol-modified gold nanoparticles for the inhibition of Mycobacterium smegmatis. *Chem Commun (Camb)*. **50**, 15860-15863 (2014).
23. Colella, M., Lobasso, S., Babudri, F., & Corcelli, A. Palmitic acid is associated with halorhodopsin as a free fatty acid. Radiolabeling of halorhodopsin with 3H-palmitic acid and chemical analysis of the reaction products of purified halorhodopsin with thiols and NaBH4. *Biochim Biophys Acta.* **1370**, 273-279 (1998).
24. Sherry, B., Jue, D. M., Zentella, A., & Cerami, A. Characterization of high molecular weight glycosylated forms of murine tumor necrosis factor. *Biochem Biophys Res Commun.* **173**, 1072-1078 (1990).
25. National Center for Biotechnology Information. *PubChem Compound Database*. <https://pubchem.ncbi.nlm.nih.gov/compound/104755 > (2004).
26. Koenigsknecht-Talboo, J., & Landreth, G. E. Microglial phagocytosis induced by fibrillar beta-amyloid and IgGs are differentially regulated by proinflammatory cytokines. *J Neurosci*. **25**, 8240-8249 (2005).
27. McCarthy, R. C. *et al.* Characterization of a novel adult murine immortalized microglial cell line and its activation by amyloid-beta. *J Neuroinflammation*. **13**, 21 (2016).
28. Schauer, J. J. *et al.* Source apportionment of airborne particulate matter using organic compounds as tracers. *Atmos Environ*. **30**, 3837-3855 (1996).
29. Kleeman, M. J. *et al.* Source apportionment of fine (PM1.8) and ultrafine (PM0.1) airborne particulate matter during a severe winter pollution episode. *Environ Sci Technol*. **43**, 272-279 (2009).
30. Borm, P. J. *et al.* The potential risks of nanomaterials: a review carried out for ECETOC. *Part Fibre Toxicol*. **3**, 11 (2006).

