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### Diversity of microglia

*Their contribution to multiple sclerosis lesion formation*

van der Poel, M.

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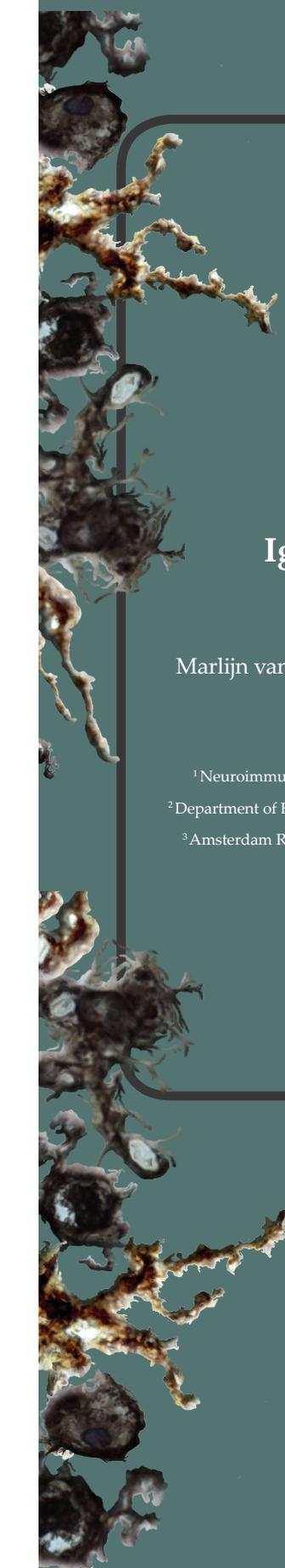
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## Chapter 7

### **IgG immune complexes break immune tolerance of human microglia**

Marlijn van der Poel<sup>1</sup>, Willianne Hoepel<sup>2,3</sup>, Jörg Hamann<sup>1,2\*</sup>, Inge Huitinga<sup>1\*</sup> and Jeroen den Dunnen<sup>2,3\*</sup>

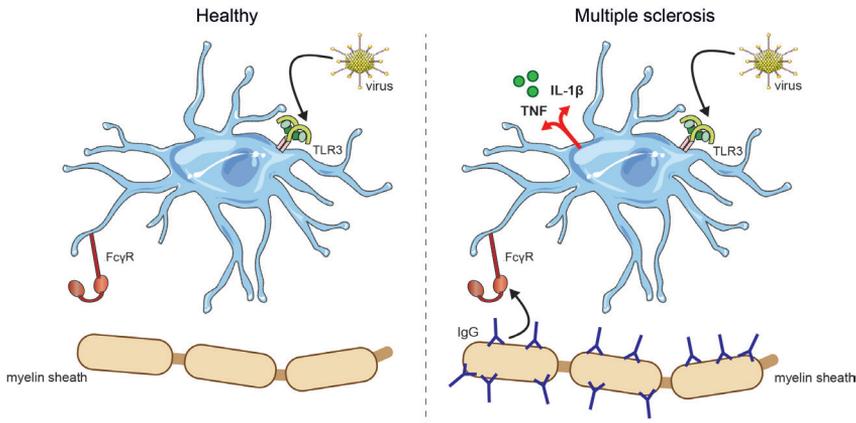
<sup>1</sup>Neuroimmunology Research Group, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands;

<sup>2</sup>Department of Experimental Immunology, Amsterdam University Medical Centers, Amsterdam, The Netherlands;

<sup>3</sup>Amsterdam Rheumatology and Immunology Center, Department of Rheumatology and Clinical Immunology, Amsterdam University Medical Centers, Amsterdam, The Netherlands.

\*Authors contributed equally to this work

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**Graphic abstract | IgG immune complexes break immune tolerance of primary human microglia.**

In healthy individuals, microglia are immune tolerant to viral stimuli. Here, we identified that myelin structures of the majority (8/11) of MS patients is bound by IgG. Moreover, we identified that the combination of these immune complexes with a viral stimulus breaks the immune tolerance of primary human microglia.

**Abstract**

Microglia are phagocytic cells involved in homeostasis of the brain and are key players in the pathogenesis of multiple sclerosis (MS). A hallmark of MS diagnosis is the presence of immunoglobulin G (IgG) antibodies, which appear as oligoclonal bands in the cerebrospinal fluid. Here, we demonstrate that myelin obtained post-mortem from 8 out of 11 MS brain donors is bound by IgG antibodies. Importantly, we show that IgG immune complexes strongly potentiate activation of primary human microglia by breaking their tolerance for microbial stimuli, such as LPS and Poly I:C, resulting in increased production of key pro-inflammatory cytokines, such as TNF and IL-1 $\beta$ . We identified Fc $\gamma$ RI and Fc $\gamma$ RIIa as the two main responsible IgG receptors for breaking of immune tolerance of microglia. Combined, these data indicate that IgG immune complexes potentiate inflammation by human microglia, which may play an important role in MS-associated inflammation and the formation of demyelinating lesions.

## Introduction

Microglia are phagocytic cells of the central nervous system (CNS) that play an important role in brain homeostasis but also have been implicated in neuroinflammatory diseases<sup>1</sup>. In the healthy CNS, microglia are kept in a homeostatic state by their microenvironment, while during inflammation they can be activated to secrete a wide range of cytokines and chemokines<sup>1,2</sup>.

Microglia are known as central players in multiple sclerosis (MS), since they are involved in demyelination and may trigger the adaptive immune response by interacting with infiltrating lymphocytes<sup>3</sup>. In active and mixed active/inactive MS lesions, microglia highly express HLA-DR and contain myelin<sup>4</sup>. We have previously shown that microglia in normal-appearing MS tissue are in a homeostatic state, identified by RNA-sequencing<sup>5</sup>. We further demonstrated that microglia isolated from post-mortem brain tissue do not respond to common TLR ligands, such as lipopolysaccharide (LPS)<sup>6,7</sup>. This immune tolerance of microglia to microbial stimuli may serve an important physiological purpose by maintaining homeostasis to prevent collateral neuronal damage caused by inflammation. Considering the contribution of pro-inflammatory microglia to axonal or myelin damage in MS<sup>8,9</sup>, there likely is an additional stimulus that converts microglial cells from immune tolerant into pro-inflammatory cells. Yet, the nature of this additional stimulus is still undefined for microglia in MS.

Oligoclonal bands (OCBs) detected in the cerebrospinal fluid (CSF) are a diagnostic marker for MS<sup>10,11</sup>. Antibodies isolated from MS serum or CSF have been identified to specifically target myelin lipids<sup>12</sup> or myelin proteins<sup>13</sup>, suggesting the existence of myelin-specific IgG autoantibodies. Interestingly, microglial cells are equipped with various Fc gamma receptors (FcγRs) that can recognize IgG<sup>6</sup>. Therefore, if anti-myelin IgG antibodies are indeed present in the CNS of MS patients, their binding to myelin and the subsequent formation of large insoluble IgG immune complexes (IgG-ICs) could promote immune activation of microglial cells and phagocytosis of myelin through these FcγRs.

In the present study, we have taken into account that in the CNS of MS brain donors, microglial cells are likely to be exposed to IgG-ICs, which thereby provides an additional stimulus for microglia activation. We report that myelin obtained from post-mortem tissue of the majority of MS brain donors is indeed bound by IgG antibodies, while low or no IgG binding was observed in myelin of controls. Moreover, we demonstrate that exposure of human microglia to IgG-ICs breaks their immune tolerance for microbial stimuli such as Poly I:C or LPS, leading to high expression of pro-inflammatory cytokines and chemokines such as TNF, IL-1β, IL-8, and IL-12. Finally, we identified FcγRI and FcγRIIIa as the two main responsible IgG receptors for this effect.

## Materials and Methods

### *Human brain tissue*

Human brain tissue was provided by the Netherlands Brain Bank (NBB, Amsterdam, The Nether-

lands, <https://www.brainbank.nl>). Donors provided informed consent to perform autopsy and to use tissue, clinical and pathological information for research purposes, approved by the medical ethics committee of the VU medical center (Amsterdam, The Netherlands). The donor diagnosis was based on clinical and neuropathological information, which is provided in **Supplemental Table 1**. For microglia isolations, we used tissue from subsequent brain autopsies regardless of the diagnosis (**Supplemental Table 1**). For myelin isolation, we used tissue from other donors, 9 progressive MS, 1 relapsing-remitting and 1 progressive-relapsing MS brain donors and 11 non-neurological control donors were included (**Supplemental Table 1**). MS brain donors had an average disease duration of 29.5 years and average years until expanded disability status scale (EDSS) score 6 was 15 (**Table 1**). Control brain donors had an average age of 72 years, which is significant higher than the age of MS donors (63 years). The average post-mortem delay (PMD) for MS brain donors was also significant higher (8:37 hrs) as compared with control donors (6 hrs) (**Table 1**).

**Table 1 | Autopsy and clinical parameter statistics for donors used for myelin isolation**

Diagnosis	Age	Gender	PMD	MS type	Disease duration	Time to EDSS6
CON	72.4 ± 10.4	8 F 3 M	6:04 ± 1:24	-	-	-
MS	62.6 ± 11.1	6 F 5 M	8:37 ± 1:21	8 SP 1 RR 1 PP 1 PR	29.5 ± 14.9	15 ± 11.7
<i>p-value</i>	<i>p</i> <0.01	n.s.	<i>p</i> <0.001	-	-	-

Age in years; CON = non-neurological control; Disease duration in years; F = female; M = male; MS = multiple sclerosis; PMD = post-mortem delay in hrs:min; PP = primary progressive; PR = progressive-relapsing; RR = relapsing-remitting; SP = secondary progressive; Time to EDSS6 = time in years until patient reached expanded disability score 6.0. Data are represented as mean with SEM. Statistical testing: Gender = Fisher's exact test; Age and PMD = unpaired t-test; n.s. = not significant.

### *Myelin isolation*

Myelin was isolated from post-mortem normal-appearing white matter (NAWM) tissue of non-neurological control (n=11) and MS (n=11) donors, as previously described by our group. Briefly, after Percoll (GE Healthcare, Little Chalfont, UK) density centrifugation during the isolation protocol of microglia, the top-layer containing myelin was collected and purified by a sucrose gradient (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation, myelin was collected from the interface and washed in water to remove any remaining cells. Purified myelin was labelled with pHrodo dye (1:100; Invitrogen, Carlsbad, CA, USA). Myelin concentration was measured with the bicinchoninic acid protein assay kit (Pierce Thermo Scientific, Rockford, IL, USA). Detailed donor information and lesion loads for MS donors are provided in **Table 2** and **Supplemental Table 1**.

### Microglia isolation

Microglia were isolated from post-mortem subcortical white matter (WM) tissue of brain donors with different clinical background, as described previously<sup>5,14</sup>. Briefly, 6-8 gram of tissue was collected during autopsy and stored in Hibernate-A medium (Invitrogen) at 4°C. After tissue homogenization, Percoll density centrifugation was performed and the middle layer containing glial cells was collected, followed by magnetic activated cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) using CD11b beads (Miltenyi Biotec). CD11b-positive cells were collected in beads buffer (phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (BSA) and 2 mM EDTA) and viable cells were counted. Purity of microglia isolation was assessed by analysing CD45 (1:200, HI30; BD Bioscience, Franklin Lakes, NJ, USA), CD11b (1:200, ICRF44; eBioscience, Waltham, MA, USA), CD56 (1:100, HCD56; Biolegend, San Diego, CA, USA) and CD66b (1:100, G10F5; Biolegend) expression by flow cytometry.

**Table 2 | Lesion load of MS brain donors used for myelin isolation**

NBB donor	Diagnosis	Proportion active lesions <sup>16</sup>	Proportion mixed active-inactive lesions <sup>16</sup>	Lesion load in BRS <sup>16</sup>	Reactive lesion load in BRS <sup>16</sup>
2010-005	SP MS	0.54	0.104	7	1
2010-045	PR MS	0.07	0.07	1	0
2010-117	SP MS	0.25	0.705	26	2
2011-008	PP MS	0.61	0.35	10	2
2011-035	SP MS	0.37	0.6	9	2
2011-048	SP MS	0.14	0.18	7	0
2011-080	SP MS	0.15	0.68	15	4
2011-089	SP MS	0.73	0.135	11	3
2011-093	RR MS	0.091	0.454	0	0
2011-100	SP MS	0.21	0	3	0
2011-120	SP MS	0.52	0.2	3	2

BRS = brainstem; Lesion load = all WM lesions defined in standard location of brainstem; MS = multiple sclerosis; NBB = Netherlands Brain Bank; PP = primary progressive; Proportion active lesions = number of active lesions/all WM lesions; Proportion mixed active-inactive lesions = number of mixed active-inactive lesions/all WM lesions; PR = progressive-relapsing; RR = relapsing-remitting; Reactive lesion load = all reactive lesions defined in standard location of brainstem; SP = secondary progressive.

### Stimulation

Predestined wells in a 96-wells Maxisorp plate (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) were coated with 2 µg/ml IgG antibodies (Nanogam, Sanquin, Amsterdam, The Netherlands) for 1 hr at room temperature (RT), followed by blocking with PBS containing 10% fetal bovine serum (FBS) for 0.5 hr at 37°C. Directly after isolation, microglia (40,000-60,000 cells per well) were divided over wells coated with or without Nanogam, together with or without the TLR ligands LPS (100 ng/ml,

from E. coli 0111:B4, Sigma-Aldrich) or Poly I:C (20 µg/ml, Sigma-Aldrich), and cultured in medium (RPMI (Invitrogen) supplemented with 10% FBS and 1% penicillin-streptomycin) at 37°C in an incubator. After 3, 6, or 24 hrs in culture, microglia were lysed for RNA collection in 800 µl TRisure (Bioline, London, UK) and stored at -80°C. After 24 hrs, supernatant (200 µl) was collected for ELISA and stored at -20°C.

#### *FcγR blocking*

Isolated microglia were incubated with 20 µg/ml FcγR antibodies, anti-FcγRI (10.1, BD Biosciences), anti-FcγRIIa (IV.3, StemCell Technologies, Vancouver, Canada) or anti-FcγRIII (3G8, BD Biosciences), in culture medium for 30 min at 4°C, followed by adding culture medium resulting in a final antibody concentration of 5 µg/ml. FcγR-blocked microglia (50,000-60,000 cells per well) were divided over wells coated with or without Nanogam, together with or without Poly I:C. After 6 hrs, cells were lysed in 800 µl TRisure for RT-qPCR analysis.

#### *RNA isolation*

RNA was isolated according to manufacturer's instructions (Bioline). Briefly, chloroform was added to TRisure samples and after centrifugation, aqueous phase was collected, mixed with ice-cold isopropanol and incubated with 1 µg glycogen (Roche, Basel, Switzerland) for 30 min at -20°C. Precipitated RNA was washed twice in ice-cold 75% ethanol and diluted in 10 µl deionized water.

#### *cDNA synthesis and RT-qPCR*

cDNA synthesis was performed according to manufacturer's instructions (Qiagen, Qiantitect Reverse Transcription kit). Purified RNA was incubated with gDNA Whipe-out buffer and subsequently incubated with QuantiTect Buffer, RT Primer Mix, and QuantiTect Reverse Transcriptase for 30 min at 42°C, followed by incubation at 95°C for 3 min.

Microglial gene expression levels of cytokines, chemokines and receptors were determined by RT-qPCR. Primer pairs were designed using the Integrated DNA Technologies website (eu.ifdna.com) and primer specificity was examined using cDNA derived from pooled brain tissue of MS and control donors. Optimal primers were selected based on dissociation curve and gene expression was normalized to the mean of housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and elongation factor-1 alpha (*EEF1A1*). Gene expression values were calculated using the  $2^{-\Delta\Delta CT}$  method. Primers used to determine gene expression are listed in **Supplemental Table 2**.

## ELISA

To assess the presence of IgG antibodies on myelin, 5 µg/µl myelin isolated from control WM or normal-appearing MS WM tissue were coated overnight on a 96-well high-affinity Maxisorp plate (Nunc). Plates were gently washed using PBS 0.05% Tween (PT) and blocked for 1 hr at 37°C with PT containing 1% BSA (PTB). After removing the block, plates were incubated for 1 hr at RT with polyclonal rabbit anti-mouse HRP (1:1,500; DAKO, Jena, Germany) to detect a-specific binding, and with goat anti-human IgG HRP (1:2,500; Jackson ImmunoResearch, Cambridge, UK) to detect IgG present on myelin.

Next, plates were gently washed and incubated with streptavidin Poly-HRP (1:10,000; Sanquin, Amsterdam, The Netherlands) for 30 min at RT. Again, plates were gently washed and developed using 1x TMB substrate solution (Invitrogen) and measured by 450nm (Biorad, Hercules, CA, USA).

Supernatants of stimulated microglia were analysed for TNF (eBioscience) and IL-1β (U-CyTech Bioscience, Utrecht, The Netherlands) protein expression, using indicated antibody pairs.

## Myelin phagocytosis

For phagocytosis experiments, microglia (100,000 cells per well) were stimulated with or without Poly I:C in a 96-wells Maxisorp plates (Nunc), coated with or without Nanogam and cultured at 37°C. After culturing for 20 hrs, dead cells and debris were washed away and adhered microglia were incubated for 24 hrs with pooled pH-rodo labelled myelin (10 µg/ml) from 7 control donors. Information for control donors is provided in **Supplemental Table 1**. After 24 hrs culturing with myelin, adhering microglia were de-attached with TrypLE Select (Thermo Fisher Scientific) incubated for 8 min at 37°C and transferred to a 96-wells plate. Uptake of pHrodo labelled myelin by microglia was measured with flow cytometry.

## Flow cytometry

Flow cytometry was performed to determine FcγRI, FcγRIIa, and FcγRIII protein expression on isolated WM control microglia. Furthermore, microglial CD45 and CD11b expression was determined for each donor we used in this study. Finally, we used flow cytometry to determine pHrodo labelled myelin uptake by microglia *in vitro*.

Microglia were incubated for 10 min with FcR Blocking Reagent (Miltenyi Biotec), followed by incubation with conjugated antibodies for 30 min on ice and measurement on BD Canto II (BD Biosciences). Viable cells were detected using viability dye eFluor 780 (1:1,500; eBioscience) and pHrodo conjugated myelin was detected in PE channel.

## Statistical analysis

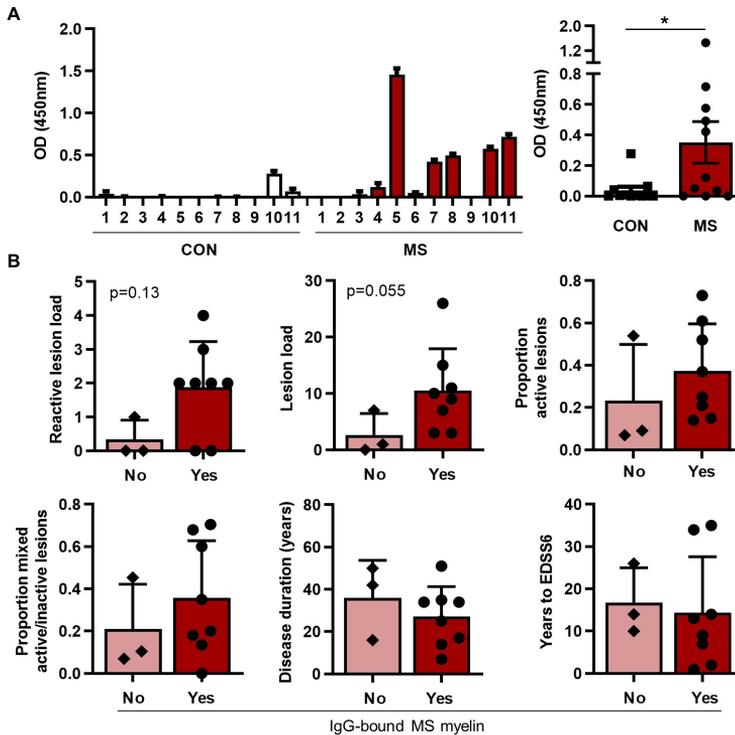
Statistical analysis was performed on data obtained from ELISA, RT-qPCR, and flow cytometry measurements. After testing for normality using Shapiro-Wilk normality test, parametric or non-parametric tests were performed to define *p*-values using GraphPad Prism software version 7.03 (GraphPad Inc., La Jolla, CA, USA). Statistical tests performed for each experiment are indicated in figure legends.

## Results

### *Myelin of MS donors is bound by IgG antibodies*

The presence of OCBs in the CSF is one of the key criteria for MS diagnosis<sup>10,11</sup>. It has previously been shown that antibodies derived from the serum or CSF of MS patients specifically target lipids or proteins of MS myelin<sup>12,13</sup>. Yet, the majority of these studies have only indirectly demonstrated myelin reactivity by isolating IgG from serum or CSF, and they have not directly assessed whether IgG is actually bound to myelin of MS patients. Here, we investigated whether myelin of MS brain donors is indeed bound by IgG through an ELISA-based setup, using post-mortem myelin isolated from NAWM of MS donors, while using non-neurological donors as controls. Importantly, we found that myelin from the majority of MS brain donors (8/11) is bound by IgG antibodies (**Figure 1A**), thereby corroborating the idea that anti-myelin antibodies are present in the CNS and indeed bind to myelin structures. In contrast, almost no IgG binding was observed to myelin that was isolated from non-neurological controls (**Figure 1A**), which is in line with the general absence of OCBs in the CSF of control individuals<sup>15</sup>. MS brain donors had a significantly lower age ( $62.6 \pm 11.1$  years) and higher PMD ( $8:37 \pm 1:21$  hours) as compared to control donors (age:  $72.4 \pm 10.4$  years; PMD:  $6:04 \pm 1:24$  hours) (**Table 1**), but when we matched age and PMD for control and MS brain donors, by removing in the control donor group the two eldest donors or two donors with lowest PMD, and in the MS donor group the two youngest donors or two donors with highest PMD, the number of donors with IgG-bound myelin was still higher in MS brain donors as compared to control brain donors (data not shown).

Since we did not detect IgG presence on myelin in all MS brain donors, we determined whether IgG-bound myelin is associated with particular disease characteristics. Interestingly, MS brain donors that showed IgG bound to myelin displayed a trend towards higher lesion load as compared to MS brain donors without IgG presence on myelin (**Figure 1B**). No difference was observed for reactive lesions (which are characterized by absence of demyelination based on PLP and accumulation of HLA-DR<sup>+</sup> microglia/macrophages, defined by immunohistochemistry<sup>16</sup>), disease duration, disease severity (defined as years until patient reached EDSS6), or proportions of active and mixed active/inactive WM lesions<sup>16</sup> (**Figure 1B**). Combined, these data demonstrate that, in contrast to control brain donors the myelin of the majority of MS brain donors is bound by IgG, which correlates with a trend towards higher lesion load.



**Figure 1 | Myelin of MS donors is bound by IgG antibodies.** **A**) Presence of IgG antibodies on myelin isolated from control WM (n=11) and MS normal-appearing WM tissue (n=11) was detected by ELISA, and visualized per donor or grouped for control and MS donors. Unpaired T-test, \* $p < 0.01$ . **B**) Reactive lesion load, total lesion load, proportion of active lesions (defined as number of active lesions divided by total number of WM lesions)<sup>16</sup>, proportion of mixed active/inactive lesions (defined as number of mixed active/inactive lesions divided by total number of WM lesions)<sup>16</sup>, disease duration in years, and disability (defined as years until patient reached expanded disability status scale (EDSS) score 6) were compared between MS donors without or with IgG-bound myelin. Lesion load was examined in a standard location in the brainstem of MS donors. Reactive lesions were characterized by absence of demyelination and accumulation of HLA-DR<sup>+</sup> microglia/ macrophages, defined by immunohistochemistry<sup>16</sup>, and lesion load is the total number of lesions determined. Mann-Whitney test. Bars show mean with SEM.

### *IgG-ICs break immune tolerance of human microglia for TLR ligands*

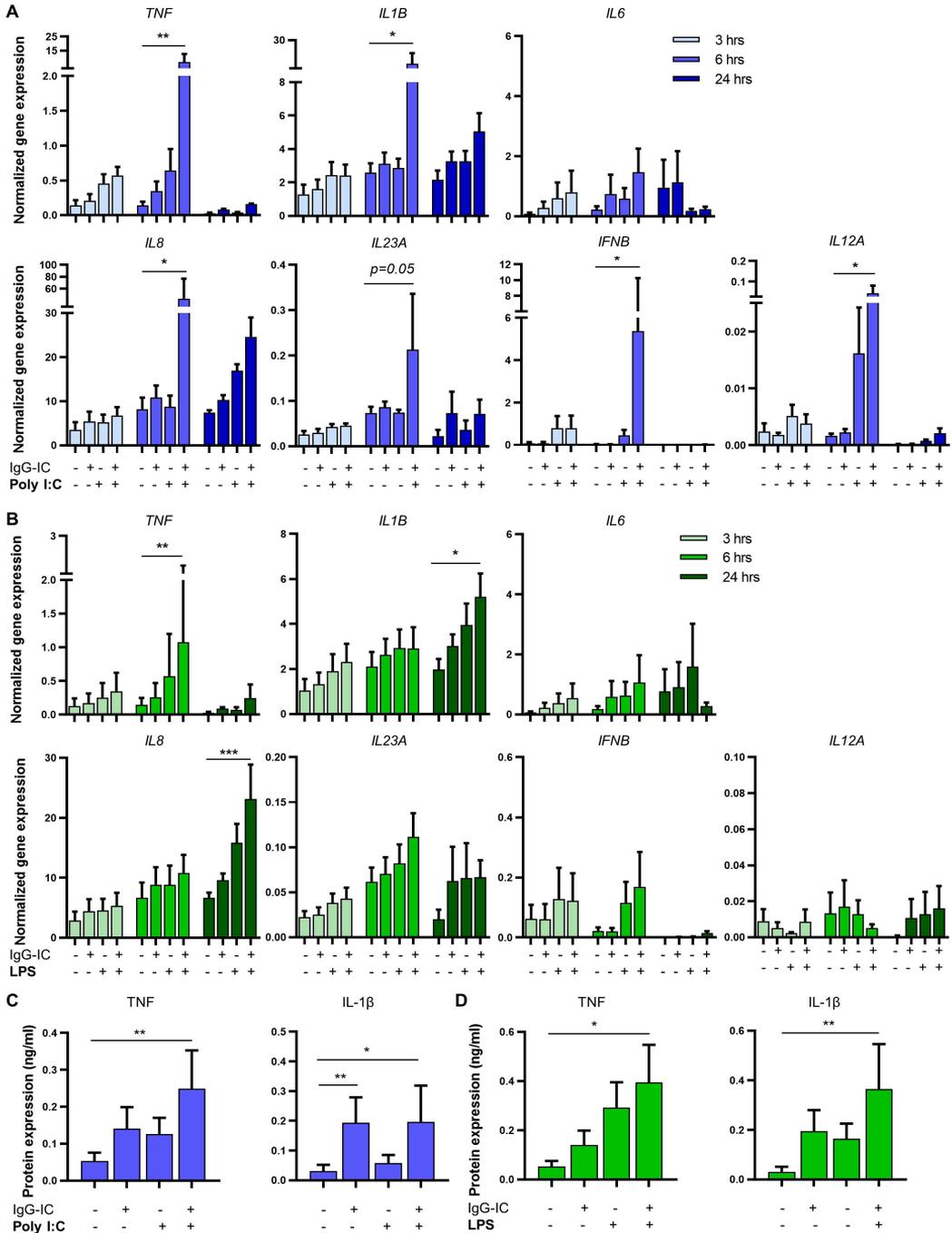
Previously we showed that human microglia are non-responsive to classic pro-inflammatory stimuli, which includes Toll-like receptor (TLR) ligands such as LPS<sup>7</sup>. In addition to testing TLR4 ligand LPS, which enabled the comparison to previous studies, we also used TLR3 ligand Poly I:C as a viral mimic, since it has been suggested that viruses can play a role in MS aetiology<sup>17,18</sup>. In addition, both TLR4 and TLR3 are well-known for recognizing non-microbial endogenous danger signals that become available during stress, damage, and cell death<sup>19</sup> that are also present under neuropathological conditions, including MS. Since OCBs are a hallmark of MS diagnosis, the presence of IgG antibodies that bind to myelin in MS brain donors may act as an additional danger signal, next to microbial/endogenous stimuli, to stimulate primary microglia. To test whether IgG antibodies have an effect on the immune activation profile of microglia, we isolated microglia from post-mortem WM

tissue, regardless of donor clinical diagnosis, and immediately stimulated them with either plate-bound IgG-ICs, TLR ligands Poly I:C or LPS, or a combination. Purity of microglia isolations was defined based on CD45 expression, and negative selection for CD15 (marker for granulocytes). The cell samples we collected contained on average 94-98% microglial cells (**Supplementary Figure 2B**). Confirming previous results<sup>7</sup>, individual stimulation with Poly I:C or LPS induced very little transcription of pro-inflammatory cytokine and chemokine genes, such as *TNF*, *IL1B*, *IL6*, *IL23A*, *IFNB*, *IL12A*, and *IL8* (encoding the proteins TNF, IL-1 $\beta$ , IL-6, IL-23p19, IFN- $\beta$ , IL-12 p35, and IL-8, respectively) (**Figure 2A** and **B**). Similarly, individual stimulation with IgG-ICs also hardly induced any cytokine or chemokine production (**Figure 2A** and **B**). However, strikingly, the combination of Poly I:C and IgG-ICs strongly and synergistically amplified transcription of the pro-inflammatory cytokines *TNF*, *IL1B*, *IL23A*, *IFNB*, *IL12A*, and the chemokine *IL8*, while the production of *IL6* was hardly increased (**Figure 2A**). A similar response was observed upon co-stimulation with LPS and IgG-IC, although in general the effect was less pronounced and/or the amplification appeared to occur at a later time point (**Figure 2B**). To verify that the transcription data correlated with protein secretion, we determined cytokine levels in the supernatant of cultured human microglia, which showed a similar pattern for TNF and IL-1 $\beta$  after co-stimulation (**Figure 2C-D**).

In addition to pro-inflammatory cytokines, we studied the gene expression of additional chemokines (*CCL2*, *CXCL9*), microglia activation markers (*ITGAX*, *SPP1*), and T-cell costimulatory molecules (*CD40*, *CD86*, *CD274*) after co-stimulation (**Supplementary Figure 1**). We observed a trend towards increased expression of the chemokine *CCL2* and a significantly higher expression of T cell-interacting receptor *CD274* upon IgG-IC co-stimulation. Combined, these data indicate that while microglia are tolerogenic to stimulation with individual TLR ligands, co-stimulation with IgG-ICs breaks this tolerance by strongly promoting the production of various pro-inflammatory genes.

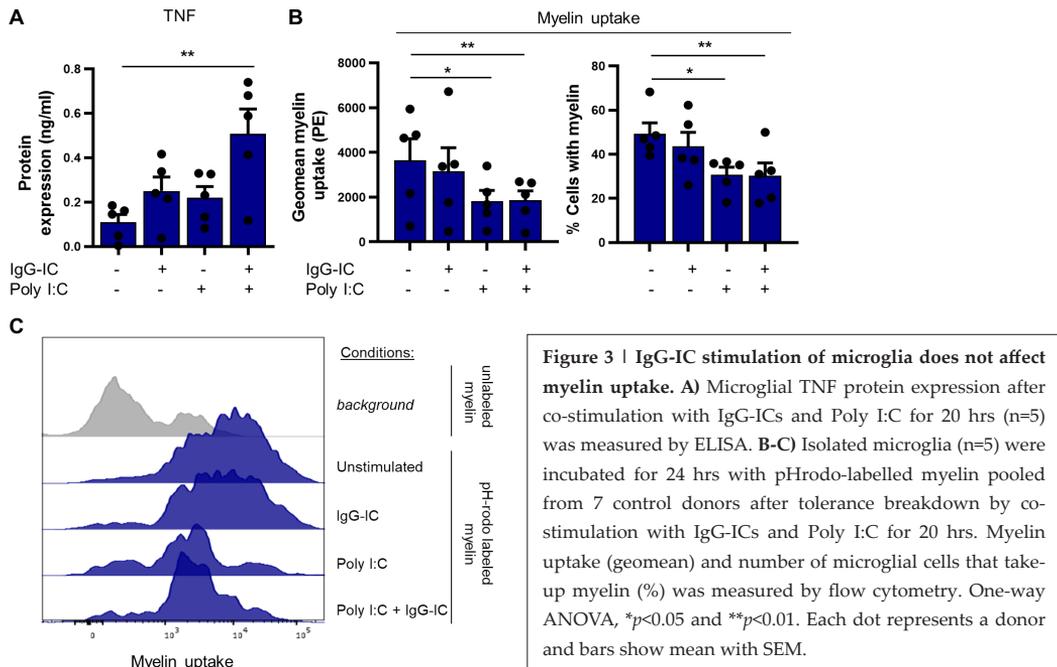
#### *IgG-IC stimulation of microglia does not affect myelin uptake*

Cross-talk between Fc $\gamma$ R and TLRs has recently been established to be important to potentiate pro-inflammatory cytokine production by myeloid immune cells<sup>20</sup>, but it is less clear whether Fc $\gamma$ R-TLR cross-talk also affects other important immune functions, such as phagocytosis. Therefore, we next set out to investigate whether Fc $\gamma$ R-TLR cross-talk also has an impact on the general phagocytic capacity of microglia, determined by uptake of non-opsonized control myelin by microglia. In MS, microglia play a central role in demyelination by taking up myelin<sup>4</sup>, which may be affected by prior Fc $\gamma$ R-activation. Therefore, we stimulated microglia with IgG-ICs, Poly I:C, or a combination, and subsequently measured uptake of pHrodo-labelled myelin, isolated from control donors, in lysosomes by flow cytometry (gating strategy is visualized in **Supplementary Figure 2A**). We verified that the microglia that were used for the phagocytosis experiments indeed showed IgG-IC-induced immune activation by measuring increased TNF protein production (**Figure 3A**). However, while stimulation with Poly I:C reduced the uptake of control myelin by microglia, stimulation with plate-bound IgG-ICs had no effect on phagocytosis, neither alone nor in combination with Poly I:C (**Figure 3B-C**). Please note that the decreased uptake upon Poly I:C stimulation may partly be related to cell viability, since the number of viable cells was lower after Poly I:C stimulation



**Figure 2 | IgG-ICs break the tolerance of human microglia for TLR ligands.** Microglia were isolated from WM tissue, regardless of donor clinical diagnosis, and stimulated as indicated with IgG-IC and the TLR ligands Poly I:C (n=4) (A) or LPS (n=5) (B). Gene expression was determined for the pro-inflammatory cytokines/chemokines *TNF*, *IL1B*, *IL6*, *IL8*, *IL23A*, *IFNB*, and *IL12A* by RT-qPCR. Expression of pro-inflammatory cytokines *TNF* and *IL1-β* was confirmed at protein level after 24 hrs, for Poly I:C stimulation (C) and LPS stimulation (D) by ELISA. Two-way ANOVA or one-way ANOVA, \*p<0.05, \*\*p<0.01, and \*\*\*p<0.001. Bars show mean with SEM.

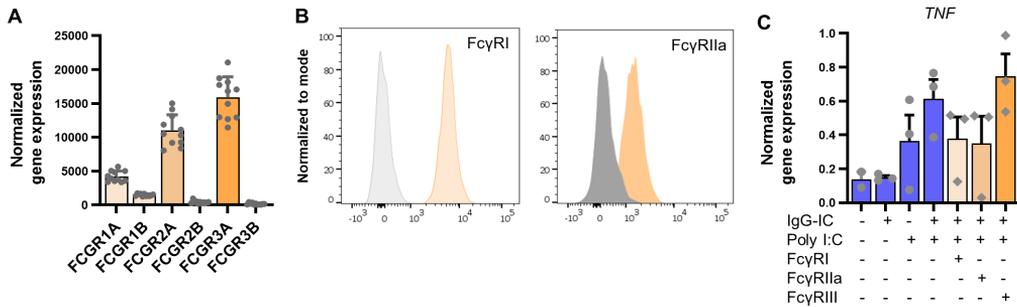
(Supplementary Figure 2A). In conclusion, these data indicate that (co-)stimulation of human microglia with IgG-ICs does not affect uptake of control myelin.



### Breaking of tolerance by IgG-ICs is dependent on FcγRI and FcγRIIa

Next, we set out to investigate which receptor on human microglia is responsible for IgG-IC-induced tolerance breakdown. The main receptors for IgG on human myeloid immune cells belong to the family of FcγRs. As shown in **Figure 4A** (and previously by our group<sup>6</sup>), human microglia highly expressed FcγRIa (encoded by *FCGR1A*), FcγRIIa (*FCGR2A*), and FcγRIIIa (*FCGR3A*). Subsequently, we confirmed expression of FcγRsI and IIa on protein level using flow cytometry (**Figure 4B**; gating strategy in **Supplementary Figure 2B**).

To determine whether FcγRs are responsible for the synergistic inflammatory response upon co-stimulation with IgG-ICs, we blocked different FcγRs that are expressed by microglia with specific antibodies during (co-)stimulation and assessed pro-inflammatory cytokine gene expression. Blocking of FcγRI and FcγRIIa completely blocked IgG-IC-induced TNF expression, while blocking of FcγRIII had no effect (**Figure 4C**). Combined, these data indicate that the binding of IgG is essential for breaking of immunological tolerance, which is mediated by FcγRI and FcγRIIa.



**Figure 4 | Breaking of tolerance by IgG-ICs is dependent on Fc $\gamma$ RI and Fc $\gamma$ RIIa.** **A**) Microglia isolated from WM tissue of control donors (n=11) expressed Fc $\gamma$ R genes *FCGR1A*, *FCGR2A*, *FCGR3A*; data is extracted from RNA-sequencing dataset<sup>5</sup>. **B**) Protein expression of Fc $\gamma$ RI and Fc $\gamma$ RIIa by microglia isolated from control WM tissue (n=4) was determined by flow cytometry. **C**) Microglial Fc $\gamma$ RI, Fc $\gamma$ RIIa, or Fc $\gamma$ RIII were blocked with specific antibodies, and gene expression of pro-inflammatory cytokine TNF was determined by RT-qPCR after 6 hrs tolerance breakdown by IgG-ICs and Poly I:C (n=3). As negative control, microglia were stimulated with either IgG-IC or Poly I:C. Each dot represents a donor and bars show mean with SEM.

## Discussion

Human microglia are generally non-responsive to microbial stimuli, thereby maintaining brain homeostasis and preventing neuronal damage by inflammation. Yet, considered their contribution to axonal or myelin damage in MS, there likely is an additional stimulus that converts tolerogenic microglia into pro-inflammatory cells. Here, we provide evidence that IgG-ICs may act as such an additional stimulus, which breaks microglial immune tolerance for microbial stimuli, leading to increased expression of key pro-inflammatory cytokines such as TNF and IL-1 $\beta$ . We identified Fc $\gamma$ RI and Fc $\gamma$ RIIa as the two responsible IgG receptors for this effect. Moreover, we identified that the majority of the MS brain donors displays IgG-bound myelin, which strengthens the concept of anti-myelin antibody immune complexes as a relevant secondary stimulus that promotes inflammation in MS brain tissue.

In this study, we identified that myelin in 8 out of 11 MS brain donors is bound by IgG antibodies, while myelin of most control brain donors showed very little IgG binding. Although this could to some extent be caused by non-specific binding, the observed IgG binding most likely reflects the presence of antigen-specific interactions of IgG antibodies with myelin lipids or/and proteins in MS donors<sup>12,13</sup>. Interestingly, a number of studies have indeed shown a worse disease course for MS patients with OCBs present in the CSF as compared to MS patients without OCBs<sup>11,21,22</sup>, thereby corroborating the idea that clonal IgG antibodies play an important role in MS disease progression.

Our data suggest that IgG antibodies contribute to MS-associated inflammation by breaking the tolerance of microglia to microbial stimuli. In this study, we used plate-bound IgG as a standardized approach to simulate IgG opsonization. Previously, we and others have extensively compared

plate-bound IgG immune complexes to IgG-opsonized bacteria<sup>23</sup>, viruses<sup>24</sup>, beads<sup>24</sup>, fibrinogen bound antibodies<sup>25</sup> or heat-aggregated IgG immune complexes<sup>26</sup> and have shown that they all elicit a very similar response by myeloid immune cells. While these data indicate that plate-bound IgG closely mimics other IgG immune complexes, in future studies it would be valuable to further validate these findings by testing IgG-opsonized MS myelin. While microglia are generally non-responsive to stimulation with individual TLR ligands, co-stimulation with IgG-ICs strongly potentiated the expression of various pro-inflammatory genes. These include key pro-inflammatory cytokines and chemokines, such as TNF, IL-1 $\beta$ , IL-8, IL-12, IL-23, and type I IFNs, several of which have been implicated in MS pathogenesis. For example, IL-23 is produced in active MS lesions and is elevated in serum and CSF of relapsing-remitting MS patients<sup>27,28</sup>. In addition, many of the up-regulated cytokines and chemokines are involved in the activation of CD8<sup>+</sup> T cells, which also play an important role in MS pathology, since they are found in higher numbers in active MS lesions<sup>29,30</sup>. However, surprisingly, IgG-ICs also strongly amplified the production of IFN- $\beta$ , which is one of the most commonly used therapies to treat RRMS by reducing the number of active lesions<sup>31,32</sup>. Yet, since the majority of the amplified genes strongly promote inflammation, the net response induced by IgG-ICs will most likely promote pathology by activating the local tissue and through recruitment of additional immune cells.

For the stimulation of primary human microglia we selected two classical TLR ligands, LPS (TLR4) and Poly I:C (TLR3). While Fc $\gamma$ R stimulation amplified the response to both ligands, the effect was most pronounced for Poly I:C. Since TLR4 signals through both MyD88 and TRIF, and TLR3 only uses TRIF, these data may indicate that Fc $\gamma$ R signaling particularly enables or amplifies the TRIF pathway in human microglia. In addition to recognizing microbial structures, TLR3 and TLR4 also recognize various non-microbial danger signals<sup>19</sup>, indicating that Fc $\gamma$ R-TLR cross-talk may not only occur during infection, but also during cell damage and death, as observed under neuropathological conditions including MS. Since Fc receptors have been shown to engage in cross-talk with various different receptor families present on myeloid cells, including RIG-I-like receptors, NOD-like receptors and C-type lectin receptors<sup>23,26,33</sup>, cross-talk with other receptors is also likely to occur in microglia.

We show that tolerance breakdown by Fc $\gamma$ R-TLR cross-talk does not alter the general phagocytic capacity of microglia, as determined by the uptake of non-opsonized control myelin. Yet, Fc $\gamma$ Rs are very likely to be involved in the enhanced uptake of IgG-opsonized particles such as IgG-bound myelin<sup>34,35</sup>. Previous studies indicate that the two different Fc $\gamma$ R effector functions (cytokine production and phagocytosis) are controlled by distinct signaling pathways<sup>20,26,36</sup>. Nevertheless, when microglia recognize IgG-bound MS myelin, the two Fc $\gamma$ R effector functions will likely be activated simultaneously, leading to both phagocytosis and cytokine production. Indeed, we have previously shown that myelin of MS brain donors is phagocytosed more efficiently by microglia than control myelin<sup>37</sup>. Notably, in the present study, we used the same control and MS myelin as in the study by Hendrickx and colleagues<sup>37</sup> to determine the presence of bound IgGs. Therefore, retrospectively, our current finding that MS myelin is IgG-bound, while control myelin is not, may indicate that the increased uptake of MS myelin in the Hendrickx study was related to IgG opsonization of the myelin, resulting in increased uptake via Fc $\gamma$ Rs.

Since immune-activated microglia contribute to MS pathology by secreting pro-inflammatory mediators and phagocytosing myelin, it would be of high interest to study if microglial tolerance breakdown also occurs *in situ* in MS brain tissue and could trigger MS lesion initiation, for instance by identifying presence of viral genes together with myelin bound IgG-ICs in MS tissue. Activated microglia can appear in normal-appearing MS tissue as clusters<sup>38</sup>, and future studies should focus on the presence of the two activating stimuli near these microglial clusters, since they have been suggested to be the first stage of lesion formation and microglial immune activation might start here.

The molecular mechanisms underlying microglial activation are poorly understood. Our data clearly demonstrates that immune activation upon co-stimulation with IgG-ICs is regulated at the level of gene transcription. Interestingly, IgG-IC induced microglial tolerance breakdown is reminiscent of previous studies on other tolerogenic immune cells, where immune complexes of (auto)antibodies have been shown to break the tolerance of intestinal dendritic cells and synovial 'M2' macrophages<sup>33,39</sup>. In this regard, microglia seem to more closely mimic synovial macrophages, which also show amplification of gene transcription<sup>33</sup>, than intestinal dendritic cells, in which IgG or IgA co-stimulation specifically amplifies gene translation<sup>39</sup>. The cytokine profile of microglia cells upon IgG co-stimulation partially overlaps with that of the other myeloid immune cells, as illustrated by increased production of TNF, IL-1 $\beta$ , and IL-23, but also shows microglia-specific responses, such as the strongly increased production of IFN- $\beta$ , which (in contrast) is strongly suppressed in human macrophages, monocytes, dendritic cells, and Langerhans cells<sup>24</sup>. This supports the concept that Fc receptor activation by IgG antibodies contributes to the generation of tissue-specific immunity<sup>26</sup>.

In addition to modulating gene transcription and gene translation, IgG-ICs has also been shown to activate caspase-1, which is required for the production of IL-1 $\beta$  by cleaving pro-IL-1 $\beta$  into its functional form<sup>23</sup>. Interestingly, we observed an increased expression of IL-1 $\beta$  protein (but not mRNA) upon individual stimulation with IgG-ICs, which may suggest that IgG can also activate caspase-1 in human microglia. The signalling molecules involved in IgG-induced gene transcription and/or caspase-1 activation in human microglia will be an important topic for future investigations.

Taken together, these data indicate that the tolerogenic phenotype of primary human microglia is converted into a pro-inflammatory phenotype upon co-stimulation with IgG immune complexes. While this could serve a physiological purpose during viral infections of the CNS, the presence of IgG antibodies on myelin of the majority of MS donors suggests that this inflammatory response is activated undesirably in MS patients, thereby promoting chronic inflammation. Interfering with this mechanism may provide new tools to attenuate inflammation and pathology in patients suffering from MS.

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## Supplemental Material

**Supplemental Table 1** | Characteristics of brain donors

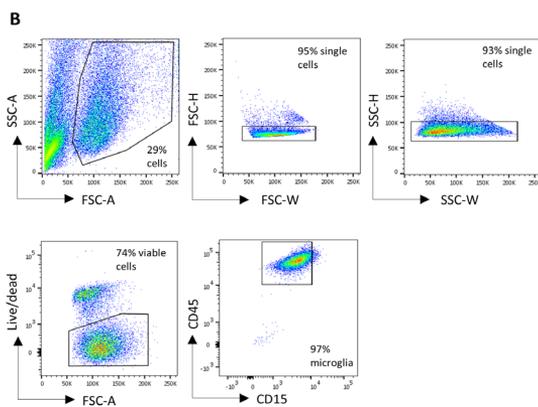
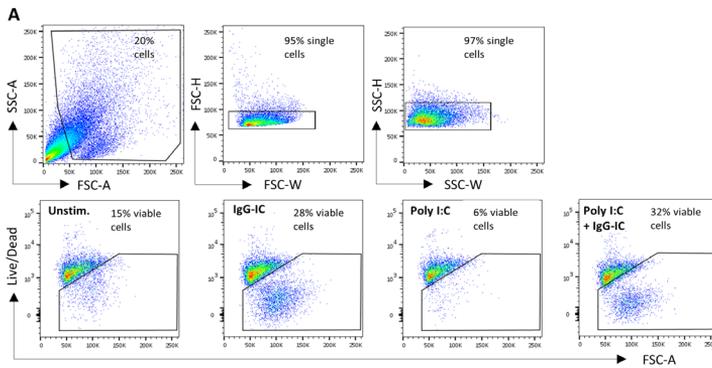
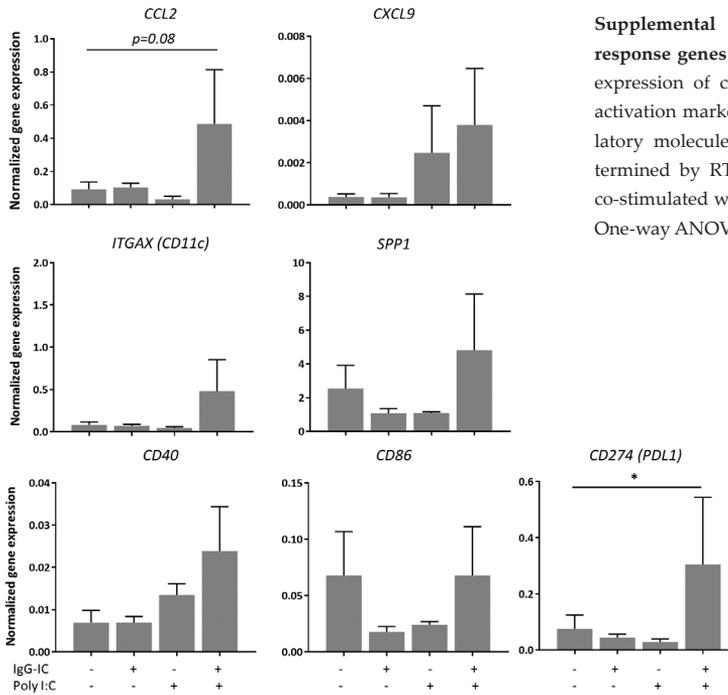
NBB donor	Diagn.	Age	Gender	PMD	Cause of death	Disease duration	Time to EDSS6	Experiment
2010-062	CON	94	F	5:50	Cachexia	-	-	ELISA on myelin
2010-068	CON	85	M	8:35	Cardiac arrest	-	-	ELISA on myelin
2010-070	CON	60	F	7:30	Infected pneumectomy cavity	-	-	ELISA on myelin
2011-021	CON	85	F	7:05	Terminal renal insufficiency	-	-	ELISA on myelin
2011-039	CON	91	F	4:15	Heart infarction	-	-	ELISA on myelin
2011-046	CON	89	F	4:45	Euthanasia	-	-	ELISA on myelin
2011-049	CON	83	F	4:40	Ileus with pancreatic cancer	-	-	ELISA on myelin
2011-091	CON	76	M	6:45	Lung cancer	-	-	ELISA on myelin
2012-048	CON	81	M	6:40	Euthanasia	-	-	ELISA on myelin
2012-049	CON	70	F	7:35	Cachexia by endstage pancreas carcinoma	-	-	ELISA on myelin
2012-052	CON	64	F	5:40	Palliative sedation	-	-	ELISA on myelin
2010-005	SP MS	68	F	10:40	Euthanasia	42	26	ELISA on myelin
2010-045	PR MS	84	F	7:35	Euthanasia	50	10	ELISA on myelin
2010-117	SP MS	60	F	10:40	Euthanasia	7	1	ELISA on myelin
2011-008	PP MS	54	M	8:15	Euthanasia	14	9	ELISA on myelin
2011-035	SP MS	50	F	7:35	Euthanasia	17	7	ELISA on myelin
2011-048	SP MS	53	M	10:00	Euthanasia	25	13	ELISA on myelin
2011-080	SP MS	56	F	8:25	Respiratory insufficiency by pneumonia	34	2	ELISA on myelin
2011-089	SP MS	64	M	7:30	Euthanasia	35	35	ELISA on myelin
2011-093	RR MS	56	M	10:10	Suicide by medication	16	14	ELISA on myelin
2011-100	SP MS	71	F	7:05	Cachexia with slowly progressive MS and metastatic breast cancer	34	14	ELISA on myelin
2011-120	SP MS	73	M	8:45	Urosepsis	51	34	ELISA on myelin
2013-016	CON	83	M	5:15	Myocardial infarction and palliative sedation	-	-	Co-stimulation
2018-112	CON	95	F	4:20	Ileus caused by tumor	-	-	Co-stimulation
2018-123	CON	82	F	6:20	Cachexia and pulmonary hypertension	-	-	Co-stimulation
2018-130	LB	66	M	8:00	Euthanasia	-	-	Co-stimulation
2018-139	PD	74	F	8:10	Euthanasia	-	-	Co-stimulation
2019-019	PD	87	F	6:20	Ceased oral intake and medication	-	-	Co-stimulation + myelin uptake
2019-021	ALS	71	M	5:55	ALS	-	-	Co-stimulation + myelin uptake
2019-023	AD	85	F	7:20	Prerenal insufficiency, heart failure	-	-	Co-stimulation + myelin uptake

NBB donor	Diagn.	Age	Gender	PMD	Cause of death	Disease duration	Time to EDSS6	Experiment
2019-026	PD	55	M	5:05	Euthanasia	-	-	Co-stimulation + myelin uptake / FcγR block
2019-035	Autism	60	M	6:15	Euthanasia	-	-	Co-stimulation + myelin uptake
2019-036	CON	85	F	6:50	Metastatic long cancer	-	-	Co-stimulation + FcγR block
2019-039	Dep.	61	F	4:45	Euthanasia	-	-	Co-stimulation + FcγR block

AD = Alzheimer's disease; Age in years; ALS = amyotrophic lateral sclerosis; CON = non-neurological control; Dep. = depression; Diagn. = diagnosis; Disease duration in years; F = female; LB = Lewy body dementia; M = male; MS = multiple sclerosis; NBB = Netherlands Brain Bank; PD = Parkinson's disease; PMD = post-mortem delay in hours:minutes; PP = primary progressive; PR = progressive-relapsing, RR = relapsing-remitting; SP = secondary progressive; Time to EDSS6 = time in years until patient reached expanded disability score 6.0.

**Supplemental Table 2** | Primers used for RT-qPCR on cultured human microglia

Gene symbol	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>CCL2</i>	CAGAAGTGGGTTTCAGGATTCC	ATTCTTGGGTTGTGGAGTGAG
<i>CD274 (PDL1)</i>	CAGGGCATTCCAGAAAGATG	GCTACCATACTCTACCA-CATATAGG
<i>CD40</i>	GGCTTCTTCTCCAATGTGTCA	TAAAGACCAGCACCAAGAGGAT
<i>CD86</i>	AACACAATGGAGAGGGAAGAG	ACTTTTGTGCGATGAAGATGTC
<i>CXCL9</i>	CCCTTCTCTCGAGAAAATTG	TGCTTTTCTTTTGGCTGACC
<i>EEF1A1</i>	AAGCTGGAAGATGGCCCTAAA	AAGCGACCCAAAGGTGGAT
<i>GAPDH</i>	TGACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGA
<i>IFNB</i>	GAAGGCCAAGGAGTACAGTC	GCTAGGAGATCTTCAGTTTCCG
<i>IL1B</i>	GGCAGGGAACCAGCATCT	CCGACCACCACTACAGCAA
<i>IL6</i>	CAGCCACTCACCTTTCAGAA	TGCCTCTTGTCTGCTTTCACA
<i>IL8</i>	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTTC
<i>IL12A</i>	TGCTGGCAGTTATTGATGAGC	GCCCGAATTCTGAAAGCATGA
<i>IL23A</i>	CGTCTCCTTCTCCGTTCAAA	CTCAGGGTTGCTGCTCCAT
<i>ITGAX (CD11C)</i>	TGGAGGACTTTGATGCTCTG	TCCTGTGCCATCTCCAATTC
<i>SPP1</i>	AGGCTGATTCTGGAAGTTCTG	CATGGCTTTCGTTGGACTTAC
<i>TNF</i>	GGCGTGGAGCTGAGAGATA	CAGCCTTGGCCCTGAAGA



**Supplemental Figure 2 | Flow cytometry gating strategy for human microglia.** **A)** Representative dot plots showing gating strategy for Figure 3B, based on cell gating by sideward scatter (SSC) and forward scatter (FSC), single cell gating by FSC and SSC height/width duplet exclusion and gating on viable microglia cells by live/dead marker. Viable cells are shown for each condition. Unstim=unstimulated condition. **B)** Representative dot plots showing gating strategy for Figure 4B, based on cell gating by sideward scatter (SSC) and forward scatter (FSC), single cell gating by FSC and SSC height/width duplet exclusion, gating on viable microglial cells by live/dead marker. After negative selection for CD15, percentage of CD45<sup>+</sup> cells is shown.