

Inflammatory stimuli promote lipid accumulation in myelin-phagocytosing macrophages: effects of LXR, PPARY and RXR agonists

Lorenzo Romero-Ramírez

lromeroramirez@sescam.jccm.es

Hospital Nacional de Paraplégicos/Instituto de Investigación Sanitaria de Castilla-la Mancha Toledo

Natalia Díaz-Rubio

Hospital Nacional de Paraplégicos/Instituto de Investigación Sanitaria de Castilla-la Mancha Toledo

Alejandro Arriero-Cabañero

Hospital Nacional de Paraplégicos/Instituto de Investigación Sanitaria de Castilla-la Mancha Toledo

Jörg Mey

Hospital Nacional de Paraplégicos/Instituto de Investigación Sanitaria de Castilla-la Mancha Toledo

Article

Keywords: CNS injuries, Inflammatory stimuli, Macrophages, Myelin, Nuclear receptors, Phagocytosis

Posted Date: October 23rd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11142135/v1>

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Additional Declarations: No competing interests reported.

Abstract

Central nervous system (CNS) injuries result in extensive cell death and release of myelin and other debris with detrimental effects on the regenerative process. Efficient clearance of myelin relies on phagocytosis and subsequent metabolism in resident microglia and infiltrating hematogenous macrophages. This process is impaired in CNS injuries.

We previously demonstrated that inflammatory stimuli, such as lipopolysaccharide (LPS) or interferon-gamma (IFN γ), inhibit myelin uptake in macrophages. Here, their impact on both myelin degradation and lipid droplet (LD) formation has been investigated.

When bone marrow-derived macrophages (BMDM) or Raw264.7 cells were treated with increasing concentrations of myelin, we found a correlating increase of BODIPY fluorescence within the cells, suggesting that lipids derived from myelin degradation are stored as neutral lipids in LD. Cells treated with inflammatory stimuli exhibited greater intracellular accumulation of myelin and higher BODIPY fluorescence, suggesting metabolic reprogramming under inflammatory conditions. To influence this process, we treated BMDM with nuclear receptor agonists involved in lipid metabolism. The treatment with T0901317 or bexarotene, agonists of liver X receptors and retinoid X receptors respectively, reduced LD formation in myelin-laden BMDM treated with proinflammatory stimuli. We consider these nuclear receptors as therapeutic targets in pathologies which involve impaired clearance of lipid debris.

Introduction

Traumatic spinal cord injury (SCI) results in immediate and extensive cell death, the extent of which is closely correlated with the severity of the initial insult. In addition to the primary mechanical damage, a cascade of secondary injury mechanisms is triggered, including excitotoxicity, oxidative stress, and a robust inflammatory response [1]. Among these, inflammation plays a central role in exacerbating tissue damage and impeding tissue regeneration [2].

A hallmark consequence of SCI is the widespread release of myelin debris derived from damaged axons. Efficient clearance of this myelin by phagocytic cells, such as resident microglia and infiltrating macrophages from the circulation, is essential for restoring tissue homeostasis and enabling axonal regeneration [3–5]. Myelin phagocytosis not only prevents the accumulation of cytotoxic debris but also influences the inflammatory profile and functional phenotype of macrophages [6].

Emerging evidence suggests that myelin uptake and degradation are intimately linked to lipid metabolism within macrophages [7]. Lipids derived from phagocytosed myelin are stored as neutral lipids (e.g. triglycerides and cholesteryl esters) in intracellular lipid droplets (LD), dynamic organelles involved in lipid homeostasis and immune regulation [8]. While LD formation is a normal consequence of myelin processing, excessive or dysregulated accumulation has been associated with impaired phagocytic capacity [9] and a shift toward a more pro-inflammatory state. After SCI, LD-loaded phagocytes behave as foam-like cells found in atherosclerotic plaques [10]. Inflammatory stimuli such

as lipopolysaccharide (LPS) or interferon-gamma (IFN γ) can further influence this process by altering both the efficiency of myelin clearance and the balance between lipid storage and metabolism [11, 12].

Understanding how inflammation modulates myelin phagocytosis and LD dynamics is critical to deciphering the cellular mechanisms underlying secondary injury in SCI [5]. This knowledge may inform new therapeutic strategies aimed at enhancing myelin clearance while minimizing detrimental inflammation [8, 13].

We showed previously that inflammatory stimuli, such as LPS or IFN γ , inhibit myelin uptake in macrophages [14, 15], and we found that pharmacological intervention with several nuclear receptor agonists increased myelin uptake in macrophages [16]. Here, we describe the effect of these inflammatory stimuli and agonists of nuclear receptors on myelin processing and metabolism.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), *Escherichia coli* lipopolysaccharides isotype 026: B6, penicillin/streptomycin mix (P/S) and 10xTrypsin-EDTA solution (T4174) were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) and GlutaMax™ were purchased from Gibco BRL (Gaithersburg, USA). Nuclear receptor agonists T0901317 (71810), bexarotene (B-2422) and rosiglitazone (R5773) were obtained from Cayman Chemical Company (Ann Arbor, USA), LC Laboratories (Woburn, USA) and LKT Laboratories (St Paul, USA) respectively. BODIPY 493/503 (D3922), Fluoromount G™ (00-4958-02), Fluoromyelin™ red (F34652), pHrodo Green STP Ester (P35369), murine recombinant macrophage-colony stimulating factor (M-CSF, Peprotech 315-02), murine recombinant IFN γ (Peprotech, 315-05), Sytox Dead Cell stain sample kit (S348629), microplate BCA protein assay kit, TRIzol reagent, RevertAid™ H Minus First Strand cDNA Synthesis Kit and primers for qPCR were purchased from ThermoFisher Scientific (Waltham, USA).

Cell culture

The murine macrophage cell line Raw264.7 (TIB-71, ATCC, Manassas, USA) was grown in DMEM supplemented with 5% FBS, GlutaMax™ and Penicillin/Streptomycin (P/S) in cell culture dishes. For cytometry experiments, cells were plated at a density of 300,000 cells/well in 12-well plates. For immunocytochemistry experiments, cells were plated at 100,000 cells/well in 24-well plates on glass coverslips treated with poly-L-Lysine (PLL, 50 μ g/mL). Cells were cultured in DMEM (2 g/L glucose) without phenol red supplemented with 1% FBS, GlutaMax™, and P/S.

Bone marrow-derived macrophages (BMDM) cultures were prepared from nine- to twelve-week-old C57BL/6J mice bred in the Animal Facility of Hospital Nacional de Paraplégicos (HNP) [16]. This procedure was reviewed and approved by the ethics committee for Animal Care of the Hospital Nacional

de Paraplégicos (228CEEA/2023), All methods were performed in accordance with national or international guidelines, including EU directive 2010/63/EU and ARRIVE guidelines. Mice were euthanized by gradual carbon dioxide displacement, followed by cervical dislocation to confirm death. After removing muscles and connective tissue, femur and tibia bones were superficially sterilized and collected in phosphate buffered saline (PBS). Under a sterile hood, the epiphyses were cut off and the bone marrow extracted by injections from both sides with 2 ml of DMEM using a 25G (femur) or 29G cannula (tibia). We collected, washed and resuspended extracts from one mouse in 1 ml of lysis buffer for erythrocytes containing 8.29 mg NH_4Cl , 1 mg KHCO_3 , 37 μg EDTA. After 2 min, lysis was stopped with 14 ml ice cold PBS. Cells were washed again and resuspended in medium, consisting of DMEM supplemented with 10% heat inactivated FBS and 1% P/S (10.000 u/ml) and 20 ng/ml of M-CSF. Cells were cultivated in non-treated culture dishes at a density of 10^7 cells/plate for seven days at 37°C, 5% CO_2 . For cytometry experiments and qPCR samples, cells were plated at a density of 500,000 cells/well in 12-well plates. For immunocytochemistry experiments, cells were plated at 150,000 cells/well in 24-well plates on glass coverslips treated with poly-L-lysine (50 $\mu\text{g}/\text{mL}$). Cells were cultured in DMEM (2 g/L glucose) without phenol red supplemented with 10% FBS, GlutaMax™, and P/S.

Myelin isolation and labeling

Myelin was isolated from cerebral cortices of adult rat brains as described previously [4, 14]. This procedure was reviewed and approved by the ethics committee for Animal Care of the Hospital Nacional de Paraplégicos (250OEBA/2025). All methods were performed in accordance with national or international guidelines, including EU directive 2010/63/EU and ARRIVE guidelines. Adult Wistar rats bred in the Animal Facility of HNP were euthanized by gradual carbon dioxide displacement, followed by cervical dislocation to confirm death. The brain tissue was mechanically homogenized in ice cold 0.3M sucrose, 40 ml/brain (T25 Ultra Turrax, IKA Labortechnik, Germany), ultracentrifugation on 0.83 M sucrose (75,000g, 4°C, 30 min), suspension of the interface in 20 mM Tris/HCl buffer (pH 7.2), washing (12,000g, 4°C, 15 min) and resuspension in 5–10 ml buffer. Protein concentration was determined with microplate BCA protein assay kit and aliquots of 0.25 ml were stored at – 20°C until further processing.

For the analysis of phagocytosis, myelin was labeled with Fluoromyelin™ red (F34652). The dye was dissolved in PBS and incubated with myelin (208 μg myelin/ μl dye) for 30 min at RT.

Myelin labeled with pHrodo Green STP Ester (P35369) was used to detect myelin in the lysosomes. The dye was dissolved in dimethyl sulfoxide (DMSO) at 8.9 mM and 1% added to myelin extract, which was previously resuspended in 0.1 M NaHCO_3 (pH 8.4) and incubated for 45 min at RT. Labeled myelin was spun down (5 min, 12,000g) and the pellet resuspended in DMEM medium without phenol red with 1% FBS for Raw264.7 cells and 10% FBS for BMDM.

Myelin degradation and metabolization assays by flow cytometry

Cells were plated in 12-well plates (Corning Incorporated, #3513). Next day, cells were treated with different concentrations of myelin (10, 25 or 50 µg/ml) labeled with Fluoromyelin™ or pHrodo. After 24 hours, the medium was replaced and LPS (10 ng/ml or 200 ng/ml) or IFNγ (10 or 50 ng/ml) was added for additional 24 or 48 hours. To detect lipid droplets, cells were incubated with BODIPY 493/503 (2 µM) in PBS for 15 minutes. They were washed twice with PBS and incubated with x1 trypsin, 10 mM EDTA in PBS for 5–10 minutes at 37°C, 5% CO₂. After trypsinization, cells were collected and spun down (3 min, 200g) in a microfuge. After centrifugation, cells were resuspended in 200µl of cytometry buffer (1 mM EDTA, 1% FBS in PBS) with Sytox blue (1:1000 dilution) to detect dead cells. Analysis was performed in a Cytoflex S cytometer (BD Biosciences) and data analysis was assessed using FlowJo 10.9 software (FlowJo, LLC-BD Biosciences).

Immunocytochemistry

Raw264.7 cells or BMDMs were plated at a density of 100,000 cells/well and 150,000 cells/well respectively on glass coverslips treated with PLL (50 µg/mL) in 24-well plates and were treated as described in the Cell Culture section. After the treatments, cells were fixed with 2% paraformaldehyde for 15 minutes at room temperature and washed three times with PBS. Coverslips were mounted on glass slides with Fluoromount G. Depending on the experiment, images were taken in a Leica microscope DMI6000B equipped with a Leica DFC 350 Fx camera or a Leica confocal microscope TCS SP5 and analyzed with ImageJ software.

Statistical analysis

GraphPad Prism software version 8.0 for Windows was used for statistical analysis and to create the graphs. Treatments were compared with student t-test, one-way or two-way ANOVA depending on the experiments, and the statistical significance between groups was determined using Sidak's or Tukey's post-hoc tests respectively. Data in graphs are presented as the mean ± SEM.

Results

Inflammatory stimuli increase FM-myelin accumulation in macrophages

First, we followed myelin uptake and processing by macrophages (Fig. 1 and Fig. 2). For this, myelin was purified, labeled with the fluorophore FluoroMyelin (FM-myelin), a fluorophore with high affinity for myelin [17], and cells were incubated with FM-Myelin for 24h. After that, non-phagocytosed FM-myelin was washed from the media and the median of fluorescence intensity (MFI) and the percentage of FM-positive cells were measured at 24h and 48h of incubation with flow cytometry (Fig. 1). As expected, MFI (Fig. 2A, 2E) and the percentage of FM-positive cells (Fig. 2C, 2G) were proportional to the concentration of myelin added to the cells. There was a reduction in both MFI (Fig. S1A, S1B) and percentage of FM-

positive cells (Fig. S2A, S2B) at 48h of incubation compared to 24h in both cell types. We interpret this loss of FM signal, which was significant (Fig. S1A-B, S2A-B), as a consequence of the intracellular processing of myelin.

To test whether an inflammatory response interfered with the processing of ingested myelin, the cell cultures were treated with LPS or IFN γ . Cytometry plots (Fig. 1A) and representative images (Fig. 1B) of treated cells suggested that FM-myelin accumulated within the cells. In the case of Raw264.7 cells, the treatment with LPS for 24h induced an increase in both MFI (Fig. 2A) and the number of FM-positive cells compared to controls (Fig. 2C). This effect persisted at 48 h treatment with LPS (Fig. 2B, 2D). IFN γ had only a small, though significant effect on MFI (Fig. 2B). In the case of BMDM, no changes were observed after 24 h of treatment (Fig. 2E, 2G), but both inflammatory stimuli increased cellular MFI and the percentage of myelin positive cells after 48 h (Fig. 2F, H). These results suggest that LPS interfered with intracellular breakdown of myelin in both cell types, though the effect took longer in BMDMs. In these cells, IFN γ also had the effect of maintaining higher levels of intracellular myelin.

LPS induces myelin accumulation in the acidic compartments of Raw264.7 cells

Myelin was labeled with pHrodo, a fluorophore that emits fluorescence at pH < 6, to study myelin processing in the lysosomes (Fig. 3A, 3B). As happened with FM-myelin labeling, the MFI of pHrodo (Fig. 4) increased as more myelin was added to the cells. At the lowest myelin concentration of 10 μ g/mL, most cells were already positive for pHrodo-myelin, such that the dose effect was not significant with respect to the percentage of positive cells (Fig. S3). Again, there was a reduction in pHrodo-myelin MFI between cells treated for 24h and 48h (Fig. S1C-D), but not in pHrodo-positive cells (Fig. S2C-D).

We also asked whether treatment with LPS and IFN γ affected the lysosomal myelin signal. Cytometry plots of selected treatments (Fig. 3A) and representative images (Fig. 3B) suggested myelin accumulation in the acidic compartments of pHrodo-myelin treated macrophages under inflammatory conditions. We found that LPS increased fluorescence intensity in acidic compartments of Raw264.7 cells, which became significant at a high load of 50 μ g/mL pHrodo-myelin (Fig. 4A, 4B). In contrast, in BMDM under these conditions, LPS reduced fluorescence intensity of pHrodo-myelin at 24h and had no effect at 48h (Fig. 4C, 4D). While both types of macrophages were influenced by LPS signaling, no effects were observed with IFN γ .

In conclusion, LPS affected lysosomal myelin processing in Raw264.7 cells and in BMDMs, but both cell types reacted differently.

Inflammatory stimuli increase BODIPY staining in myelin-laden macrophages

When macrophages phagocytose lipids, free fatty acids (FFA) and cholesterol are accumulated as neutral lipids (triglycerides and cholesteryl esters) in LD. BODIPY 493/503 staining is commonly used to detect LD in cell cultures (Fig. 5A and 5B). Here, we used BODIPY staining to study this after myelin phagocytosis and to investigate the effects of inflammatory stimuli by flow cytometry (Fig. 5A). As expected, the MFI (Fig. 6A and 6D) and the percentage of BODIPY-positive cells (Fig. S4A and S4C) increased as more myelin was added to the cells. In RAW264.7 cells, the amount of LD (BODIPY MFI: Fig. S1E) and the percentage of BODIPY-positive cells (Fig. S2E) decreased between 24h and 48h, which did not occur in BMDM (Fig. 1F and 2F).

The effect of inflammatory stimuli on LD accumulation was in line with FM-myelin and pHrodo measurements: BODIPY staining rose significantly when myelin-loaded RAW264.7 cells were treated with LPS or IFN γ for 24h (Fig. 6A) and 48h (Fig. 6B). This was confirmed by determining the percentage of BODIPY-positive Raw264.7 cells (Fig. S4A, B). Again, more time was required to affect BMDM (Fig. 6C, D). The combination of myelin with either LPS or IFN γ significantly increased BODIPY MFI in the macrophages when treatment lasted 48h (Fig. 6D) but not 24h (Fig. 6C). The percentage of BODIPY-positive cells was not affected significantly (Fig. S4C, D). Thus, inflammatory stimuli caused that more FFA and cholesterol accumulated in LDs in myelin-laden macrophages.

In conclusion, inflammatory stimuli may slow down myelin processing and induce accumulation of myelin products (e.g. FFA and cholesterol) as triglycerides and cholesteryl esters in LD. These myelin-laden macrophages under inflammatory stress resemble foam cells of atherosclerotic plaques.

Lipid accumulation, driven by myelin uptake and inflammatory stimuli, elevates SSC and FSC in macrophages

Since both myelin phagocytosis and inflammatory stimuli induced lipid accumulation, we evaluated their effect on two cellular parameters using flow cytometry: side-scatter (SSC), which reflects internal complexity (such as nuclear shape, granules or vesicles) and forward-scatter (FSC), which correlates with cell size.

As observed with FM and BODIPY staining, SSC increased as more myelin was added to the cells (Fig. 7A and S5A). In the case of RAW264.7 cells loaded with myelin, the treatments with inflammatory stimuli for 24h (Fig. S5A) or 48h (Fig. S5B) significantly increased SSC compared to control. As with FM and BODIPY, this effect became significant with BMDM at 48h of treatment (Fig. 7B), but not at 24h (Fig. 7A). Myelin ingestion also caused an increase in cell size of BMDM, as indicated by FSC cytometry data (Fig. 7C). In addition, LPS and IFN γ further increased the FSC when treatment lasted 48h (Fig. 7D). In the cell line, there was no strong increase of FSC with myelin (Fig. S5C), however, the effect of the inflammatory stimuli at 48h was similar to BMDM (Fig. S5D).

In RAW264.7 cells loaded with myelin, the treatment with LPS, but not IFN γ , increased FSC compared to control at 24h (Fig. S5E). Again, in the case of BMDM, both inflammatory stimuli had this effect on FSC after 48h, but not yet after 24h of treatment (Fig. S5G, H). In conclusion, the cellular remodeling induced by myelin uptake and inflammatory stimuli is governed by the magnitude of lipid accumulation.

Agonist of LXR and RXR reduced lipid accumulation in myelin-laden macrophages under inflammatory stress

Since some agonists of nuclear receptors increased myelin uptake in macrophages [16], we studied their effect on the processing of phagocytosed myelin in BMDM. None of the agonists altered total FM-myelin staining (Fig. 8A) or percentage of FM-positive cells (Fig. S6A) with or without inflammatory stimuli. However, bexarotene, an agonist of RXR, reduced BODIPY MFI in BMDM treated with myelin and IFN γ (Fig. 8B). In addition, T0901317, an agonist of LXR, reduced BODIPY staining in cells treated with myelin together with LPS or IFN γ (Fig. 8B). In contrast, rosiglitazone, an agonist of PPAR γ did not have any effect. The nuclear receptor agonists did not significantly reduce the percentage of BODIPY-positive cells that were treated with myelin and inflammatory stimuli (Fig. S6B).

Interestingly, the agonists that reduced BODIPY staining lowered significantly SSC (Fig. 8C), but not FSC (Fig. 8D), linking cell complexity (SSC) with the accumulation of LD (BODIPY).

Discussion

CNS injuries cause cell death and the release of cellular debris that compromise tissue regeneration. The phagocytosis and degradation of the debris in the spinal cord is challenging due to the abundance of myelin with a high content of lipids [18]. Myelin debris has many detrimental effects for regeneration as cytotoxic, proinflammatory and inhibitor of axonal regeneration [3, 4, 19, 20]. The presence of lipid-laden macrophages in the injured spinal cord has been described [6]. These foam-like cells are similar to the macrophages of atherosclerotic plaques and may last in the spinal cord for long time with detrimental effects for regeneration [6, 8]. Since the inflammatory environment at the injury site reprograms the metabolism of macrophages [6, 12], we studied the effects of inflammatory stimuli on myelin phagocytosis [14, 15]. We previously showed that NF κ B and JAK/STAT pathways were involved in the inhibition of myelin uptake in macrophages [14, 15].

In the present study we have investigated the effect of inflammatory stimuli on myelin degradation and metabolism. We evaluated myelin endocytosis (FluoroMyelin), internalization of myelin in lysosomes (pHrodo-myelin) and accumulation of LD (BODIPY) with two related measures: fluorescence levels of cells in cytometry and the percentage of positively labeled cells using thresholds based on non-myelin controls.

Inflammatory stimuli interfered with intracellular processing of ingested myelin

Our results demonstrate that FluoroMyelin is a robust marker for myelin endocytosis, as both MFI and the percentage of FM-positive cells increased in a concentration-dependent manner across both cell types. Using this approach, we observed that the MFI and the percentage of FM-positive cells decreased between 24 and 48h after myelin removal from the medium, indicating that the internalized myelin was metabolized during this time.

Myelin was labeled with pHrodo, a fluorophore that emits fluorescence at pH < 6, to study myelin processing in the lysosomes. As with FM-myelin, pHrodo MFI and the percentage of positive cells increased with the concentration of pHrodo-myelin added, and pHrodo MFI was reduced at 48h compared to 24h of incubation. LPS not only induced FM-myelin accumulation in RAW264.7 cells, but also pHrodo-myelin after 24h of treatment, suggesting myelin accumulation in the lysosomes. In the case of BMDM, LPS had the opposite effect. Future experiments may reveal how these results are influenced by the pH of the lysosomes, the number of lysosomes and the amount of myelin in them in the different cell types.

Pro-inflammatory stimuli increase BODIPY fluorescence in myelin-laden BMDM

Finally, the cells were labeled with BODIPY 493/503, a fluorophore that labels neutral lipids, to study LD formation. As with the other fluorophores, MFI and the percentage of BODIPY-positive cells increased with the concentration of myelin added to the cells. The observed reduction in FM and pHrodo-myelin during the 24h-48h after cells had endocytosed the myelin indicate that the cells were able to metabolize the ingested myelin. That cholesterol and free fatty acids obtained from myelin degradation in the lysosomes are partially redirected to the synthesis of neutral lipids (triglycerides and cholesteryl esters) that are stored in LD. Our results suggest that the magnitude of this process is dependent on the amount of phagocytosed myelin, exogenous signals (such as pro-inflammatory stimuli) and the cellular metabolic demand [21].

Although pro-inflammatory stimuli alone have been reported to induce LD formation in BMDMs [11, 23], we did not detect an increase of both BODIPY MFI or the percentage of BODIPY-positive cells following treatment with LPS or IFN γ in either cell type. Several factors may account for these discrepancies. First, variations in fetal bovine serum (FBS) batches across studies could play a critical role, as free fatty acids present in FBS are required for pro-inflammatory stimuli-induced LD biogenesis in macrophages [12, 23]. In addition, some groups added exogenous free fatty acids to the media to induce LD formation in macrophages under inflammatory conditions [24]. Second, different from our single-stimulus approach, other groups have combined LPS and IFN γ to synergistically drive LD formation [22].

The combination of both myelin phagocytosis and pro-inflammatory stimuli raised the BODIPY labeling in both cell types much more when than cells were treated only with myelin, suggesting an increase in LD formation. While BMDM required 48h of pro-inflammatory stimulation to exhibit BODIPY MFI, RAW264.7 cells achieved this change within only 24h. The mechanism involved in pro-inflammatory stimuli-induced LD formation might be due to inhibition of lipolysis and mitochondrial β -oxidation. [12, 25].

While both MFI and the percentage of BODIPY-positive cells did not change from 24h to 48h of incubation in BMDM, both were reduced in RAW264.7 cells. Even considering that the threshold was somewhat arbitrary, it is clear that most RAW264.7 cells did not accumulate LD. Even with 50 µg/mL myelin and LPS, about 87% of the cells were BODIPY-negative at 24h. As dynamic organelles, LD content undergo content regulation by cellular metabolic demand [21]. In the case of RAW264.7 cells, these lipid stores might serve both as a source of energy and building blocks for lipid synthesis to sustain their high proliferation rate, unlike the response of differentiated BMDM. These results underscore the limitations of RAW264.7 cells for modeling LD formation or foam cell development and validate BMDM as a more physiologically relevant model.

LXR and RXR agonists reduced BODIPY labeling in BMDM under inflammatory conditions

We showed previously that the treatment with agonists of nuclear receptors increased myelin uptake [16]. Following these results, their effects on myelin processing and metabolism have now been studied. Bexarotene, an agonist of RXR, reduced BODIPY MFI in BMDM treated with myelin and IFN γ . The treatment with T0901317, an agonist of LXR, reduced both LPS or IFN γ -induced BODIPY MFI in BMDMs. On the contrary, rosiglitazone, an agonist of PPAR γ , did not have any effect. Interestingly, there is a correlation between the effects of the agonists of nuclear receptors on BODIPY MFI and SSC, which confirmed the important role of LD formation for SSC measurement in cytometry. The signaling of both LXR and RXR are interconnected as LXR form obligate heterodimers with RXR required for LXR binding to LXRE (LXR recognition sequence), a specific DNA recognition motif localized in the promoters of target genes [26]. Both T0901317 [27] and bexarotene [28, 29] have shown effects in lipid metabolism and as anti-inflammatory drugs. Both agonists have been reported to increase cholesterol efflux [30, 31] and triglyceride synthesis [32, 33]. Our results indicate that their effect on lipid recycling also reduces the accumulation of LD, as measured with BODIPY fluorescence in myelin-laden BMDM, when these were treated with IFN γ . When cells were exposed to LPS, only T0901317 had this effect.

In conclusion, we have established an *in vitro* model to study foam cell formation and test potential therapeutic targets. In addition, we have shown the important role of the pro-inflammatory stimuli on lipid metabolism with direct implications on foam cell formation after SCI.

Declarations

Competing interests

The authors declare no competing interests.

Funding

Experiments were paid for by Grant PID2021-128446NBI00 funded by the Ministerio de Ciencia, Innovación y Universidades, MICIU/AE/<https://doi.org/10.13039/50110 00110 33> and ERDF7EU to J.M. and L.R.R. A.A.C. received a doctoral scholarship grant from the Wings for Life Spinal Cord Research Foundation (WFL-ES-01/24, grant 259).

Author Contribution

Conceptualization: L.R.R.; Methodology: L.R.R. Formal analysis and investigation: L.R.R., N.D.R and A.A.C; Writing original draft preparation: L.R.R.; Writing, review and editing: L.R.R. and J.M.; Funding acquisition: L.R.R. and J.M.; Resources: L.R.R. and J.M.; Supervision: L.R.R. and J.M. All the authors read and approved the manuscript.

Acknowledgement

Dr. Virginia Vila del Sol and Ángela Marquina of the cytometry core facility of the HNP provided valuable technical help, perform the evaluation of the samples by flow cytometry and obtained the data. The assistance of Irene García-Moreno Galán with cytometry experiments is greatly appreciated. Dr. Jose-Ángel Rodríguez Alfaro and Dr. Javier Mazarío Torrijos of the microscopy core facility of the HNP provided valuable technical help with image acquisition.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

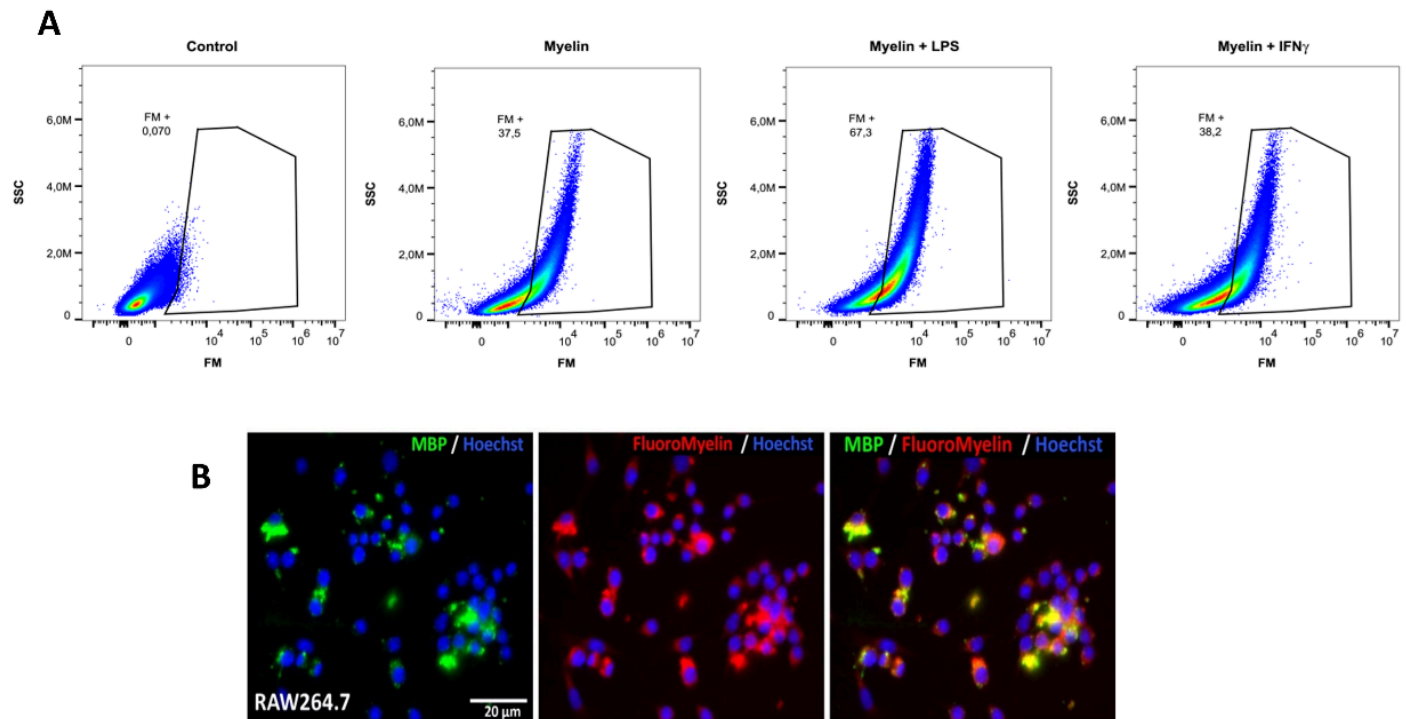


Figure 1

RAW264.7 cells accumulate FluoroMyelin-labeled myelin (FM-myelin) under pro-inflammatory conditions. (A) Cytometry plots of RAW264.7 cells of untreated cells (Control) or after incubation with FM-myelin for 24h followed by medium replacement with and without inflammatory stimuli (LPS or IFN γ) for additional 24h. The gating window that includes FM positive cells (based on experiments without myelin, Control) shows 37.5% of the cells with myelin and 67.3% and 38.2% cells in the presence of 10 ng/ml LPS or IFN γ respectively. (B) Representative images of RAW264.7 incubated with FM-myelin (red). Cells were immunostained for MBP (green) and cell nuclei were stained with Hoechst 33342 (blue). Scale bar 20 μ m. FM-myelin evaluation with cytometry is validated, with LPS but not IFN γ the myelin remains in the cells longer in RAW264.7 cells.

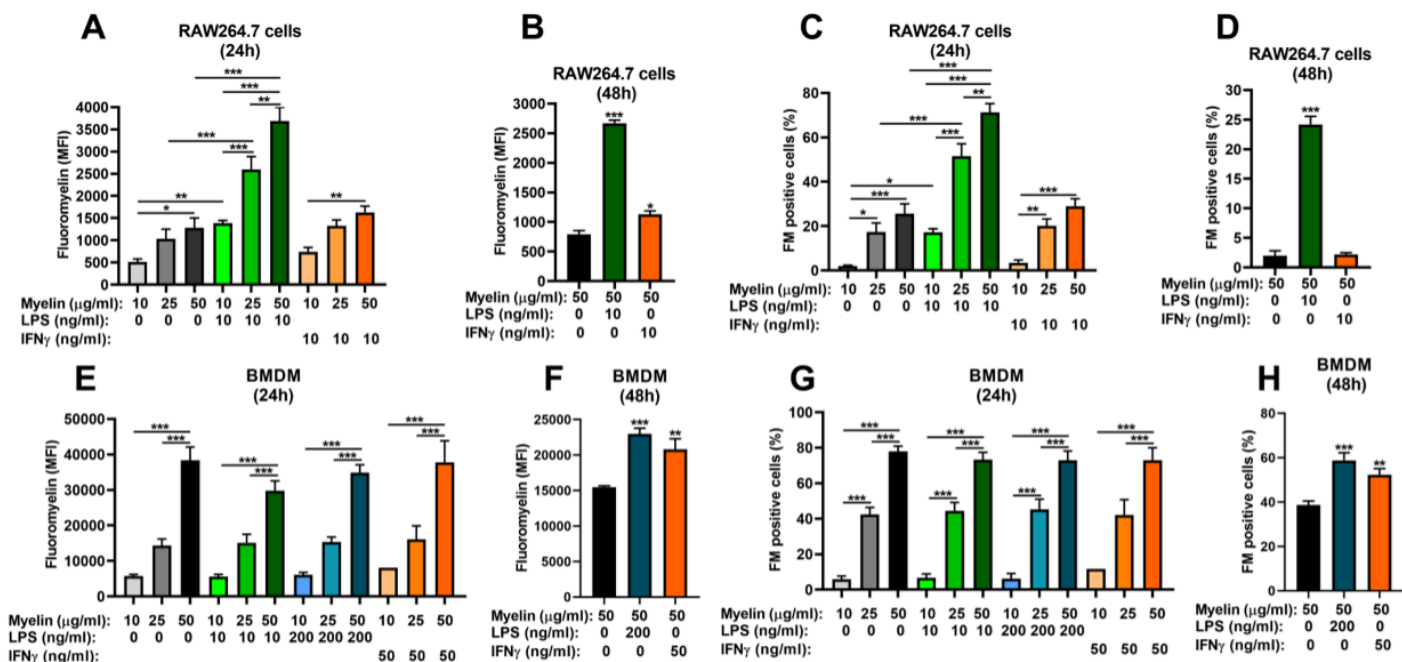


Figure 2

Macrophages accumulate myelin under pro-inflammatory conditions. RAW264.7 (A-D) or BMDM (E-F) were incubated with FM-myelin at different concentrations (10, 25 or 50 μg/mL) for 24h. After washing non-phagocytosed myelin, cells were treated with LPS (10 or 200 ng/mL) or IFNγ (10 or 50 ng/mL) for additional 24h (A,C) or 48h (B,D). After this treatment, the FM fluorescence intensity was detected by flow cytometry. Panels (A,B,E,F) show median fluorescence intensity (MFI) per cell and (C,D,G,H) the percentage of FM positive cells. Results represent means ± SEM of n³ independent experiments. Two-way (A,C,E,G) or one-way (B,D,F,H) ANOVA revealed significant differences in: (A) $F(2,27)=35.21$ ($p<0.001$) for myelin concentration, $F(2,27)=59.70$ for pro-inflammatory treatments ($p<0.001$) and $F(4,27)=4.88$ ($p<0.01$) for interaction; (B) $F(2,4)=237.6$ ($p<0.001$); (C) $F(2,27)=74.92$ ($p<0.001$) for myelin concentrations, $F(2,27)=76.45$ ($p<0.001$) for pro-inflammatory treatments and $F(4,27)=6.08$ ($p<0.01$) for interaction; (D) $F(2,6)=173.0$ ($p<0.001$); (E) $F(2,59)=82.29$ ($p<0.001$) for myelin concentrations; (F) $F(2,12)=15.64$ ($p<0.001$); (G) $F(2,59)=112.3$ ($p<0.001$) for myelin concentrations; and (H) $F(2,12)=13.48$ ($p<0.001$). Tukey's (A,C,E,G) or Sidak's (B,D,F,H) post-hoc test indicated statistical significance between treatments $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

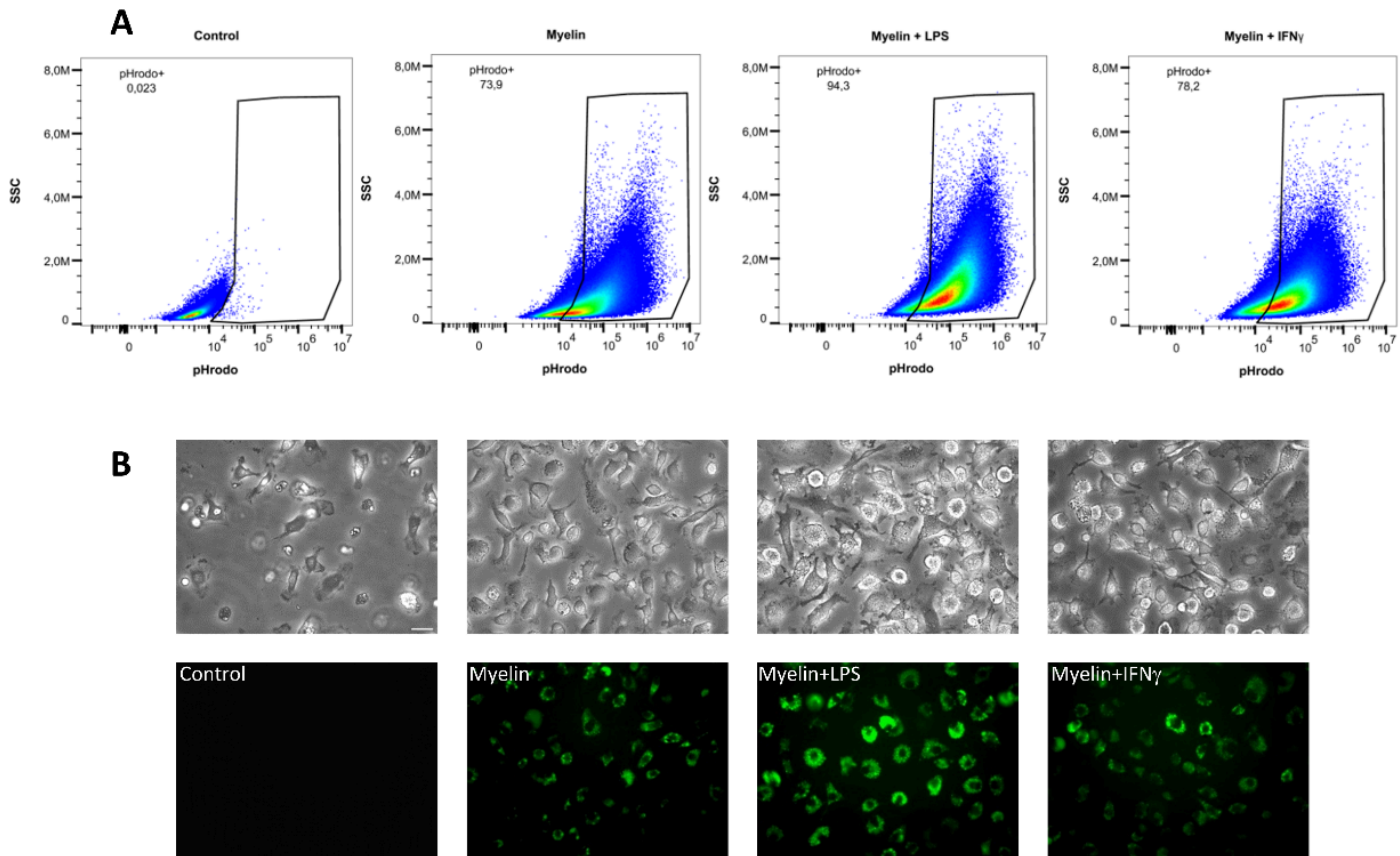


Figure 3

LPS induces myelin accumulation in the acidic compartments of RAW264.7 cells. (A) Cytometry plots of RAW264.7 cells of untreated cells (Control) or after incubation with pHrodo labeled myelin (pHrodo-Myelin) for 24h and with and without exposure to inflammatory stimuli (LPS or IFN γ) for additional 24h. The gating window that includes pHrodo positive cells (based on experiments without myelin, Control) shows 73.9% of the cells with myelin and 94.3% and 78.2% cells in the presence of 10 ng/ml LPS or IFN γ . (B) BMDMs incubated with pHrodo-Myelin (green) for 24h and with and without exposure to inflammatory stimuli (LPS or IFN γ) for additional 24h. Scale bar 20 μ m.

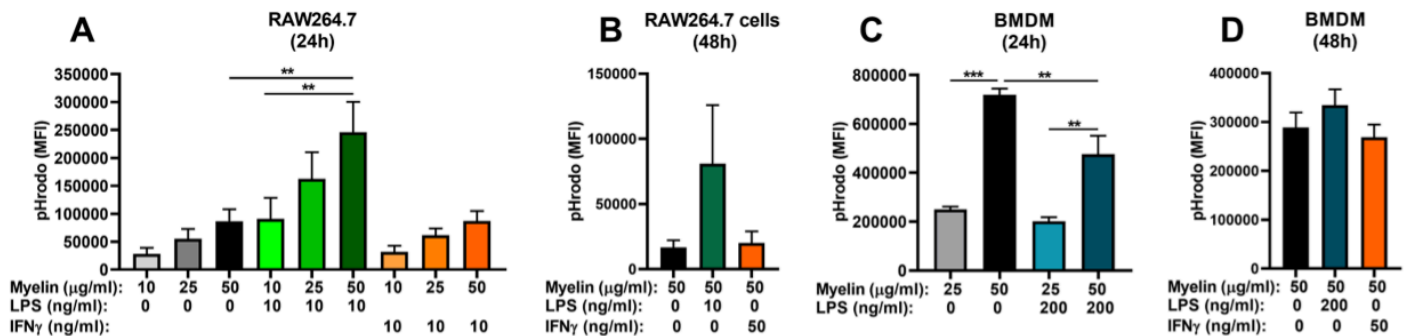


Figure 4

Myelin fluorescence in lysosomes correlates with extracellular myelin concentration and is influenced by LPS. RAW264.7 (A,B) or BMDM (C,D) were incubated with pHrodo-myelin at different concentrations (10, 25 or 50 $\mu\text{g/mL}$) for 24h. After washing non-phagocytosed myelin, cells were treated with LPS (10 or 200 ng/ml) or IFN γ (10 or 50 ng/mL) for additional 24h (A,C) or (48h B,D) . After this time, the median fluorescence intensity (MFI) was determined by flow cytometry. Results represent MFI means $\pm$ SEM of n^3 experiments. Two-way ANOVA revealed significant treatment effect in (A) $F(2,24)=5.71$, $p<0.01$ for myelin concentrations and $F(2,24)=11.42$ ($p<0.001$) for pro-inflammatory treatments and (C) $F(1,8)=80.81$ ($p<0.001$) for myelin concentrations; $F(1,8)=12.46$ ($p<0.01$) for pro-inflammatory treatments; and $F(1,8)=5.52$ ($p<0.05$) for interaction. Tukey's post-hoc test indicated statistical significance between treatments. $**p<0.01$, $***p<0.001$.

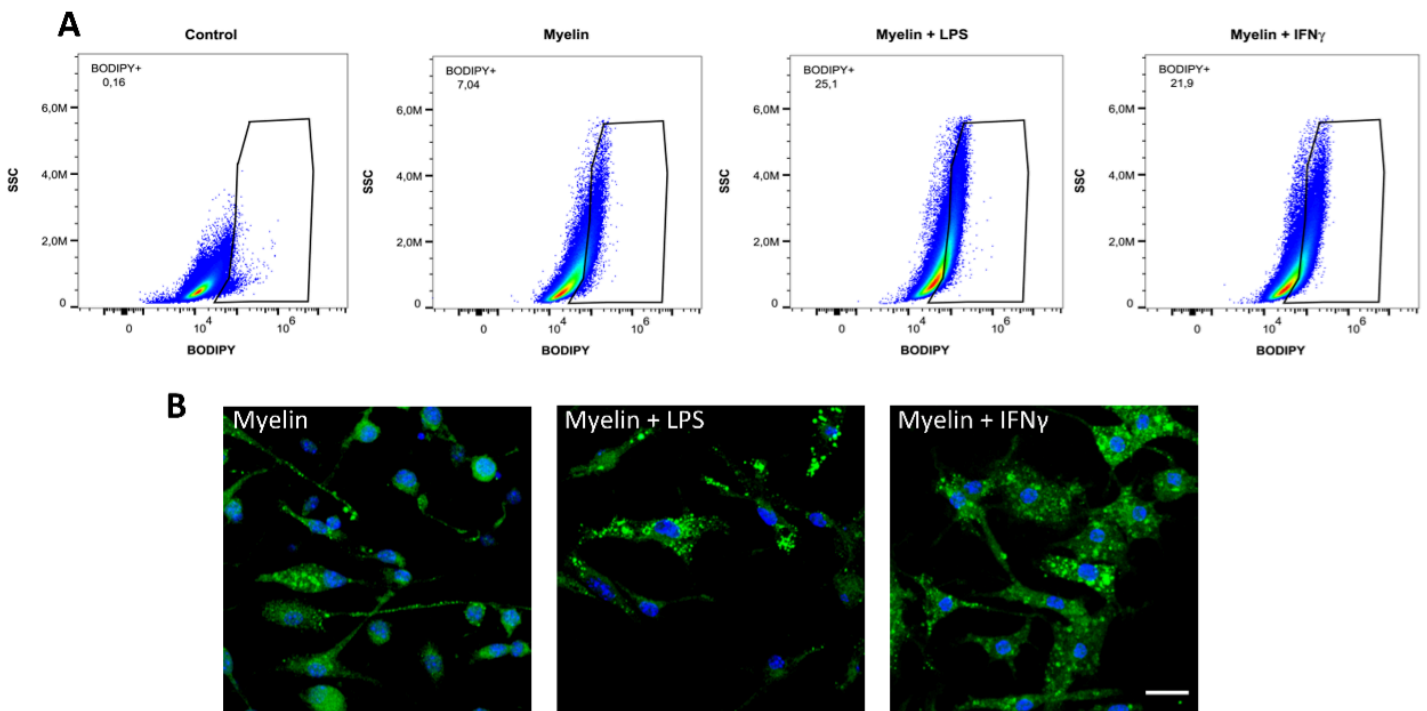


Figure 5

RAW264.7 cells increase BODIPY staining under pro-inflammatory conditions.(A) Cytometry plots of RAW264.7 cells of untreated cells (Control) or after incubation with myelin for 24h and with and without exposure to inflammatory stimuli (LPS or IFN γ) for additional 24h. After the treatments, cells were incubated with BODIPY 493/503 dye. The gating window that includes BODIPY positive cells (based on cells not labeled with BODIPY) shows 0.16% of cells not treated with myelin, 7.04% of the cells treated with myelin and 25.1% and 21.9% cells in the presence of 10 ng/ml LPS or IFN γ respectively. (B) BMDM labeled with BODIPY 493/503 (green). Cells were incubated with myelin for 24h and with or without exposure to inflammatory stimuli (LPS or IFN γ) for additional 48h. Cell nuclei were labeled with Hoechst 33342 (blue). All photos were taken with the same magnification. Scale bar 20 μm .

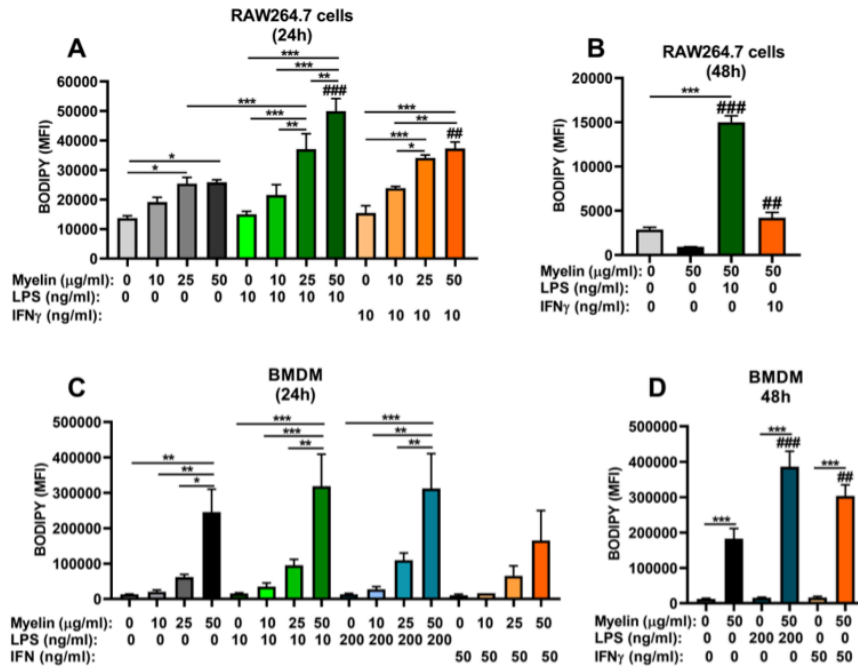


Figure 6

BODIPY staining correlates with extracellular myelin concentration and is enhanced by LPS and IFN γ . RAW264.7 (A,B) or BMDM (C,D) were incubated with myelin at different concentrations (10, 25 or 50 $\mu\text{g/mL}$) for 24h. After washing non-phagocytosed myelin, cells were treated with LPS (10 or 200 ng/mL) or IFN γ (10 or 50 ng/mL) for additional 24h (A,C) or 48h (B,D). After this time, cells were incubated with BODIPY 493/503 dye, and the median fluorescence intensity (MFI) was determined by flow cytometry. Results represent the BODIPY MFI means $\pm$ SEM of n^3 experiments. Two-way ANOVA revealed significant treatment effect in (A) $F(3,36)=48.14$ ($p<0.001$) for myelin concentrations; $F(2,36)=15.17$, $p<0.001$ for pro-inflammatory treatments; and $F(6,36)=4.37$, $p<0.01$ for interaction; (C) $F(3,83)=22.16$, $p<0.001$ for myelin concentrations; and (D) $F(1,18)=180.8$, $p<0.001$ for myelin concentrations; $F(2,18)=8.63$, $p<0.01$ for pro-inflammatory treatments; and $F(2,18)=8.04$, $p<0.01$ for interaction. One-way ANOVA revealed significant treatment effect in (B) $F(3,8)=158.8$, $p<0.001$. Tukey's (A,C) or Sidak's (B,D) post-hoc test indicated statistical significance between controls and treatments. $*p < 0.05$, $**p<0.01$, $***p < 0.001$, between 50 $\mu\text{g/mL}$ of myelin and treatments (#).

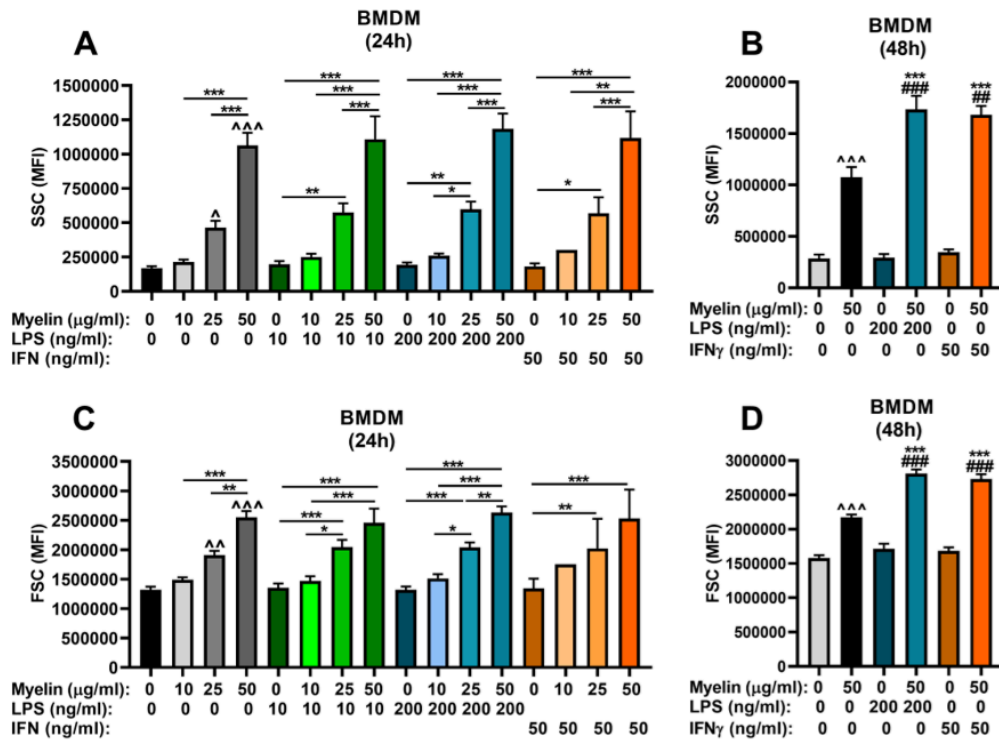


Figure 7

Effect of myelin and pro-inflammatory stimuli on side-scatter (SSC) and forward scatter (FSC) in BMDM. Cells were incubated with myelin at different concentrations (10, 25 or 50 $\mu\text{g/mL}$) for 24h. After washing non-phagocytosed myelin, cells were treated with LPS (10 or 200 ng/mL) or IFN γ (50 ng/mL) for additional 24h (A,C) or 48h (B,D). After this time, SSC (A,B) and FSC (C,D) was determined by flow cytometry. Results represent means $\pm$ SEM of n^3 independent experiments. Two-way ANOVA revealed significant effect in (A) $F(3,83)=91.52$, $p<0.001$ for myelin concentrations; (B) $F(1,12)=333.1$, $p<0.001$ for myelin concentration, $F(2,12)=11.61$ $p<0.01$ for pro-inflammatory treatments and $F(2,12)=9.56$ $p<0.01$ in interaction; (C) $F(3,83)=73.55$ $p<0.001$ for myelin concentrations; and (D) $F(1,12)=367.1$ $p<0.001$ for myelin treatment, $F(2,12)=25.50$ for pro-inflammatory treatments and $F(2,12)=11.31$ $p<0.01$ in interaction. Tukey's post-hoc test indicated statistical significance between treatments $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, between control and treatments (^) and between 50 $\mu\text{g/mL}$ of myelin and treatments (#).

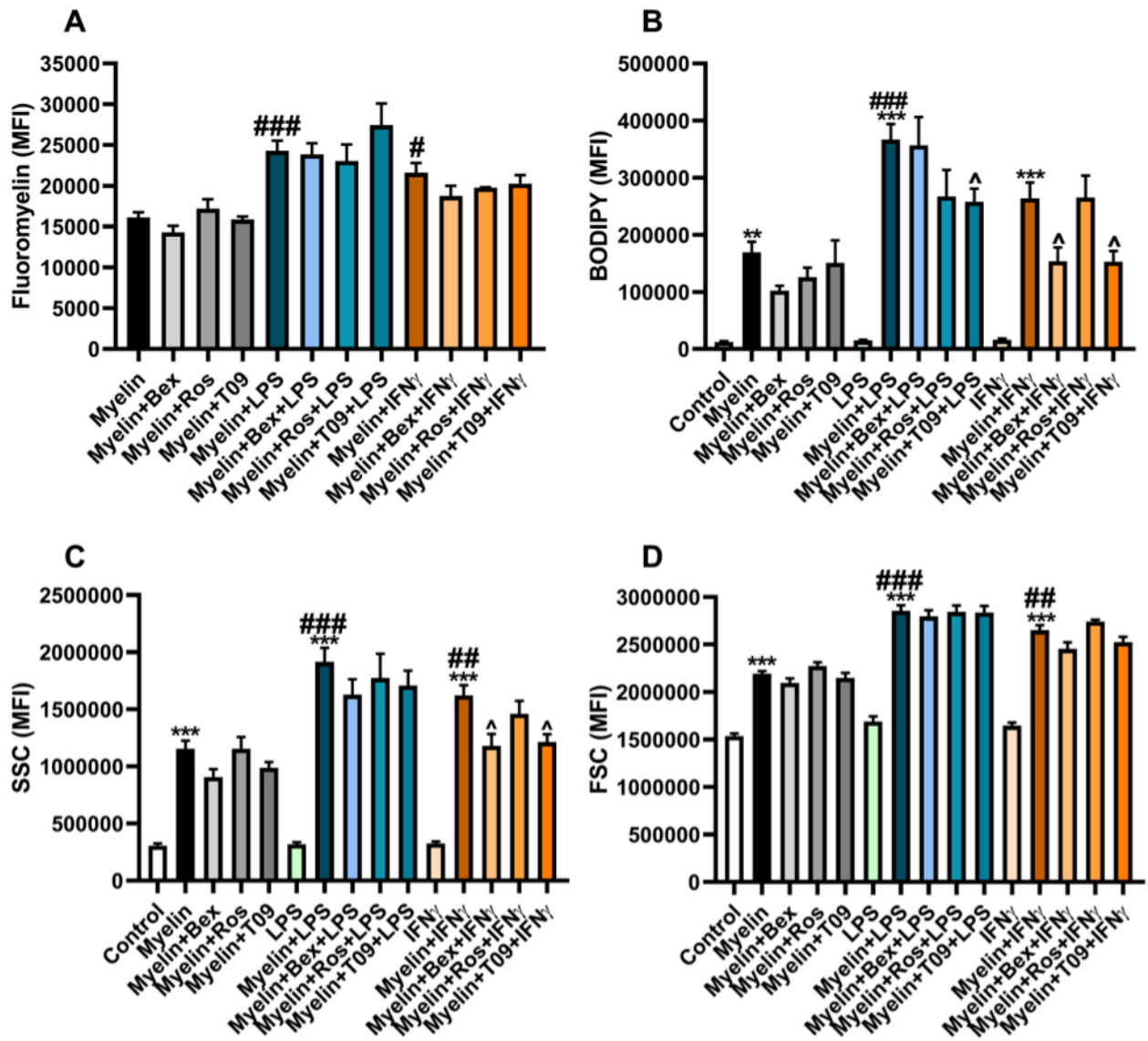


Figure 8

Agonists of nuclear receptors reduced lipid accumulation in BMDM under pro-inflammatory conditions.

BMDM were incubated with myelin (50 μ g/mL) for 24h. After washing non-phagocytosed myelin, cells were pretreated with 0.5 μ M bexarotene (Bex), 10 μ M rosiglitazone (Ros) or 10 μ M T091317 (T09) for 1h. Subsequently, 200 ng/mL LPS or 50 ng/mL IFN γ was added and incubated for 48h. Cells were analyzed with flow cytometry. (A) Effect of treatments on intracellular myelin accumulation (FM-labeled myelin MFI), (B) lipid droplet formation (BODIPY MFI), (C) on cell complexity (side scatter, SSC) and (D) forward scatter (FSC). Results represent the MFI (A,B) or arbitrary units of scattered light (C,D) means $\pm$ SEM of n^35 experiments. One-way ANOVA revealed significant treatment effect in (A) $F(11,57)=8,37$, $p<0.001$; (B) $F(14,73)=22.77$, $p<0.001$; (C) $F(14,73)=33.39$, $p<0.001$; and (D) $F(14,73)=69,12$ $p<0.001$; Sidak's post-hoc test indicated statistical significance between control and treatments. * $p < 0.05$, ** $p<0.01$, *** $p < 0.001$, between myelin and treatments (#), and between myelin+LPS or myelin+IFN γ and treatments (^).

Supplementary Files

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