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RESEARCH ARTICLE

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# Taurolithocholic acid but not tauroursodeoxycholic acid rescues phagocytosis activity of bone marrow-derived macrophages under inflammatory stress

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## Abstract

Spinal cord injury (SCI) causes cell death and consequently the breakdown of axons and myelin. The accumulation of myelin debris at the lesion site induces inflammation and blocks axonal regeneration. Hematogenous macrophages contribute to the removal of myelin debris. In this study, we asked how the inflammatory state of macrophages affects their ability to phagocytose myelin. Bone marrow-derived macrophages (BMDM) and Raw264.7 cells were stimulated with lipopolysaccharides (LPS) or interferon gamma (IFNγ), which induce inflammatory stress, and the endocytosis of myelin was examined. We found that activation of the TLR4-NFκB pathway reduced myelin uptake by BMDM, while IFNγ-Jak/STAT1 signaling did not. Since bile acids regulate lipid metabolism and in some cases reduce inflammation, our second objective was to investigate whether myelin clearance could be improved with taurolithocholic acid (TLCA), tauroursodeoxycholic acid or hyodeoxycholic acid. In BMDM only TLCA rescued myelin phagocytosis, when this activity was suppressed by LPS. Inhibition of protein kinase A blocked the effect of TLCA, while an agonist of the farnesoid X receptor did not rescue phagocytosis, implicating TGR5-PKA signaling in the effect of TLCA. To shed light on the mechanism, we measured whether TLCA affected the expression of CD36, triggering receptor on myeloid cells-2 (TREM2), and Gas6, which are known to be involved in phagocytosis and affected by inflammatory stimuli. Concomitant with an increase in expression of tumour necrosis factor alpha, LPS reduced expression of TREM2 and Gas6 in BMDM, and TLCA

**Abbreviations:** Akt, protein kinase B (after AKR mouse strain thymoma); BMDM, bone marrow-derived macrophages; CD36, cluster of differentiation 36; CNS, central nervous system; CR3, complement receptor 3; CREB, cAMP response element binding protein; DMEM, Dulbecco's modified Eagle medium; DMSO, demethyl sulfoxide; EDTA, ethylenediaminetetra-acetic acid; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FXR, farnesoid X receptor; Gas6, growth arrest specific protein 6; HDCA, hyodexoycholic acid; IFNγ, interferon-gamma; IHC, immunohistochemistry; IL-, interleukin-; IR, immunoreactivity; Jak, janus kinase; LPS, lipopolysaccharide; LXR, liver X receptor; MBP, myelin basic protein; MerTK, Mer tyrosin-protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MyD88, myeloid differentiation primary response 88; M-CSF, macrophage/monocyte-colony stimulating factor; NFκB, nuclear factor kappa B; PBS, phosphate buffered saline; PFA, paraformaldehyde; PI3K, phosphatodylinositol-3-kinase; PKA, protein kinase A; PXR, pregnane X receptor; RA, retinoic acid; RPMI, Roswell Park Memorial Institute culture medium; RPS29, ribosomal protein S29; RT, room temperature; RT-PCR, reverse-transcription polymerase chain reaction; RXR, retinoid X receptor; SCI, spinal cord injury; SSO, sulfo-N-succinimidyl oleate; STAT1, signal transducer and activator of transcription 1; TGFβ, transforming growth factor beta; TGM2, transglutaminase 2; TGR5, Takeda G-protein coupled receptor 5; TLCA, taurolithocholic acid; TLR4, toll-like receptor 4; TNFα, tumor necrosis factor alfa; TREM2, triggering receptor expressed on myeloid cells 2; Tris, tris(hydroxymethyl) aminomethane; TUDCA, tauroursodeoxycholic acid; VDR, vitamin D receptor.

Lorenzo Romero-Ramírez and Jörg Mey contributed equally

significantly diminished this downregulation. These findings suggest that activation of bile acid receptors may be used to improve myelin clearance in neuropathologies.

KEYWORDS
bile acid, Gpbr-1, inflammation, macrophage, myelin, phagocytosis, TGR5

## 1 | INTRODUCTION

Spinal cord injury (SCI) produces debris from dying cells and degenerating fiber tracts. This elicits a lasting inflammatory response (Greenhalgh & David, 2014; Kopper & Gensel, 2018). In addition, myelin remnants prevent neurite regeneration (Fournier & Strittmatter, 2001; McKerracher et al., 1994) and interfere with remyelination (Kotter et al., 2001; 2006). For the restauration of tissue homeostasis, the clearance of myelin debris by phagocytes is therefore important (Grajchen et al., 2018; Neumann et al., 2009).

In the case of a disrupted blood spinal cord barrier, which often occurs with traumatic SCI, hematogenous macrophages infiltrate the tissue, migrate towards the center of the lesion and phagocytose apoptotic cells (David & Kroner, 2011). This process is termed efferocytosis. Macrophages also clear myelin-derived proteins and lipids (Brück et al., 1995; Church et al., 2017). However, despite the recruitment of phagocytes, cellular debris causes a chronic neuroinflammation. Macrophages that accumulate lipids in their cytosol acquire a particular morphology. In the context of atherosclerosis, they were, therefore, dubbed "foam cells". This term was later transferred to macrophages in other contexts including neuropathologies (Grajchen et al., 2018; Guerrini & Gennaro, 2019). Two polarized phenotypes of macrophages are traditionally recognized as M1 (inflammatory) and M2 (alternative activation). After SCI, M1 macrophages are assumed to exacerbate secondary degeneration, while M2 macrophages promote a regenerative response (David & Kroner, 2011). In the M1/M2 paradigm, the proinflammatory differentiation is induced by transcription factors nuclear factor kappa B (NFκB) and signal transducer and activator of transcription (STAT) 1, while the alternative state is induced by interleukin (IL)-4, transforming growth factor beta (TGFβ) and nuclear receptors (David & Kroner, 2011; Freilich et al., 2013; Jablonski et al., 2015). Limited sets of marker genes, such as IL-1β for M1 and arginase-1 for M2, are frequently assumed to represent the polarized expression profiles of these reactive phenotypes (Ransohoff, 2016).

There seems to be an antagonistic regulation between efferocytosis and lipid metabolism on the one hand and inflammation on the other. Bacterial lipopolysaccharides (LPS) or tumour necrosis factor alpha (TNFα) inhibit efferocytosis (Feng et al., 2010; McPhillips et al., 2007; Michlewska et al., 2009). Myelin debris, too, was found to induce an inflammatory phenotype of macrophages, which resulted in a poor capacity to phagocytose apoptotic neutrophils (Wang et al., 2015a). In experiments with microglia, however, interferon gamma (IFNγ) treatment raised the proportion of cells that were active phagocytes, while IL-4 decreased it, and TNFα and TGFβ had no effect (Chan et al., 2001). IFNγ inhibited nonopsonized phagocytosis but increased opsonized phagocytosis by peritoneal macrophages (Wang et al., 2015b). In a recent study with zebrafish, the activation of TLR4/MyD88 (upstream of NFκB) was required for the degradation of myelin debris (Cunha et al., 2020). Thus, the issue of how different inflammatory stimuli affect myelin clearance remains complicated.

Exemplified by the foam cells, the intracellular breakdown and subsequent recycling of lipids is a necessary step of myelin clearance (Zhu et al., 2017). In the liver, important regulators of lipid homeostasis are the bile acids (Farr et al., 2020; Pols et al., 2011b). Their biological effects are mediated by two different receptor pathways, which are both present in macrophages (Chiang, 2013; Fiorucci et al., 2018). Secondary bile acids deoxycholic acid and lithocholic acid preferentially activate a G-protein coupled receptor, known as the Takeda G-protein coupled receptor-5 (TGR5). This receptor, via adenylyl cyclase causes production of cAMP, which entails many possible downstream signals including phosphorylation of CREB. Several primary and secondary bile acids also pass the cell membranes and activate the nuclear farnesoid X receptor (FXR; Fiorucci et al., 2018; Guo et al., 2016). Hyodeoxycholic acid (HDCA) and derivatives of it are TGR5 ligands and co-activators of the liver X receptors (LXR; De Marino et al., 2017).

In addition to their involvement in lipid metabolism, the TGR5- and FXR-mediated pathways inhibit activation of the inflammatory transcription factor NFκB (Pols et al., 2011a; Yanguas-Casás et al., 2014) and thereby are anti-inflammatory and cytoprotective (Dong et al., 2020; Fiorucci et al., 2018; Kim et al., 2018). For this reason, bile acids are being investigated in several neuropathologies (Ackerman & Gerhard, 2016; Elia et al., 2016; Grant & DeMorrow, 2020; Rosa et al., 2018).

To better understand the role of bile acids in the process of inflammation and myelin clearance after SCI we investigated the following hypotheses: (1) Activation of the inflammatory pathways triggered by LPS/TLR4/NFκB and IFNγ/Jak/STAT signaling reduces the myelin phagocytosis by bone marrow-derived macrophages (BMDM). (2) Bile acids interfere with these inflammatory responses and thereby promote myelin endocytosis in vitro. (3) If the second hypothesis were corroborated we wanted to test possible mechanisms involving phagocytosis receptors, such as counteracting TNFα-inhibition of Gas6 expression (Feng et al., 2010), upregulation of CD36 (Wu et al., 2021) and triggering receptor on myeloid cells-2 (TREM2) (Takahashi et al., 2005). The most frequently used TGR5 ligand in preclinical studies is tauroursodeoxycholic acid (TUDCA), derived from the bile of bears. This was tested by us, together with taurolithocholic acid (TLCA), which in preliminary experiments counteracted effects of TLR4 signaling more potently than TUDCA (unpublished data), and with HDCA, which has been successfully used in rodent models of metabolic disorders (De Marino et al., 2017).

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

Adult male C57BL/6 mice were used for preparing BMDM. Myelin was extracted from brains of adult Wistar rats. All animals were bred at the animal facility of the *Hospital Nacional de Parapléjicos* (Toledo, Spain). Conforming to European directive 2010/63/EU, animal care and euthanasia were approved by the local Ethics Committee on Animal Welfare (163CEEA/2017).

### 2.2 | Reagents

Inflammatory pathways were induced with *Escherichia coli* LPS (isotype O26:B6; Sigma-Aldrich) at concentrations of 0.1, 1, 10 and 100 ng/ml and with mouse IFNγ (Peprotech) 10 and 20 ng/ml. TUDCA sodium salt was purchased from Calbiochem (580549), sodium TLCA from Sigma-Aldrich (T7515) and HDCA from Alfa Aesar (B20506). In preliminary experiments with Raw264.7 cells, using nitrite release as the inflammatory read out, we tested concentrations of 20–320 µM bile acids, dissolved in cell culture medium. Based on these results we chose 40 µM TLCA, 200 µM TUDCA and 160 µM HDCA in phagocytosis and gene expression assays. The activation of NFκB was inhibited with 2 µM Bay11-7082 (Sigma-Aldrich B5556). We blocked CD36 with 25 µM sulfo-N-succinimidyl oleate (SSO; Merck SML2148) and PKA with 2 µM H89 dihydrochloride (Sigma-Aldrich B1427). All-*trans* retinoic acid (RA; Sigma-Aldrich R2625) was used at 0.1 µM and GW4064 (Cayman 10006611-5) at 2 µM (Wu et al., 2021).

### 2.3 | Preparation of bone marrow-derived macrophages

Bone marrow-derived macrophages were prepared from femur and tibia bones of 6-month-old mice as described recently (Wu et al., 2021). After removing muscles and connective tissue, 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 Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum (FBS) using a 25G (femur) or 29G cannula (tibia). We collected, washed and resuspended extracts from one mouse in 1 ml lysis buffer containing 8.29 mg $NH_4Cl$, 1 mg $KHCO_3$, 37 µg ethylenediaminetetra-acetic acid. After 1 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, 1% GlutaMax™ and 1% penicillin/streptomycin (10,000 U/ml) and 20 ng/ml murine macrophage/monocyte-colony stimulating factor (M-CSF; Peprotech, 315-02). Cells were cultivated in nontreated culture dishes (d = 100 mm) at a density of 10 million cells/plate for 7 days at 37°C, 5% $CO_2$.

### 2.4 | Myelin isolation and labeling

Myelin was isolated from cerebral cortices of adult rat brains as described previously (Kotter et al., 2006; Wu et al., 2021). The procedure consisted in mechanical homogenization of brain tissue in ice-cold 0.3 M sucrose (T25 Digital Ultra-Turrax; 40 ml/brain), ultracentrifugation on 0.83 M sucrose (75,000*g*, 4°C, 30 min), suspension of the interface in 20 mM Tris/HCl buffer, washing (12,000*g*, 4°C, 15 min) and resuspension in 5–10 ml buffer. Protein concentration was determined (Bio-Rad protein assay) and the volume adjusted to 1 mg/ml protein. Aliquots of 0.5 ml were stored at −20°C until further processing.

For the analysis of phagocytosis with flow cytometry or a microplate reader, myelin was labeled with pHrodo Green STP Ester (Invitrogen, P35369). The dye was dissolved in dimethyl sulfoxide (DMSO) (500 µg/75 µl DMSO) and 1% added to myelin extract, which was previously washed and suspended in 0.5 ml of 0.1 M $NaHCO_3$ (pH 8.4). After incubation for 45 min at RT, the labeled myelin was spun down (8 min, 12,000*g*) and the pellet resuspended (1 mg/ml) in DMEM medium without phenol red with 1% FBS.

### 2.5 | Cell culture

The murine macrophage cell line Raw264.7 was cultivated in DMEM with 5% FBS, 1% GlutaMax™ and 1% penicillin/streptomycin (10,000 U/ml) at 37°C, 5% $CO_2$. For plating in multiwell dishes, cells were detached from tissue culture plastic with cold PBS. Raw264.7 cells were plated either in 12-well plates at 600,000 cells/well or in 96-well plates at 60,000 cells/well, depending on the assay.

Macrophage primary cultures were grown in DMEM/GutaMax™ and 10% FBS with the addition of 20 ng/ml M-CSF in 12-well plates at 700,000 cells/well or in 96-well plates at 70,000 cells/well. Phagocytosis assays were carried out in the absence of M-CSF.

### 2.6 | Phagocytosis assays

The effect of inflammation on endocytosis by Raw264.7 cells was first assessed using SkyBlue labeled latex particles with d = 0.1–0.3 µm (Spherotch, FP-0270-2; 1% wt/vol), which were suspended 1/1000 in DMEM/1% FBS. Cell cultures were put in six-well plates with DMEM/1% FBS (without phenol red) and incubated for 24 h. After adding LPS or IFNγ, the cells were cultivated for 14 h. Then, 150 µl of SkyeBlue bead suspension were given to the cells. Following another 8 h incubation at 37°C, 5% $CO_2$, cells were harvested and the internalization of fluorescent beads quantified with flow cytometry (Figure 1a).

To investigate myelin endocytosis, 12.5 µg myelin/ml was added instead of latex beads. Myelin exposure lasted also 8 h. With tests under inflammatory conditions, treatment with 2 µM Bay11-7082, 25 µM SSO, 0.1 µM all-*trans* RA, 2 µM GW4064, 40 µM

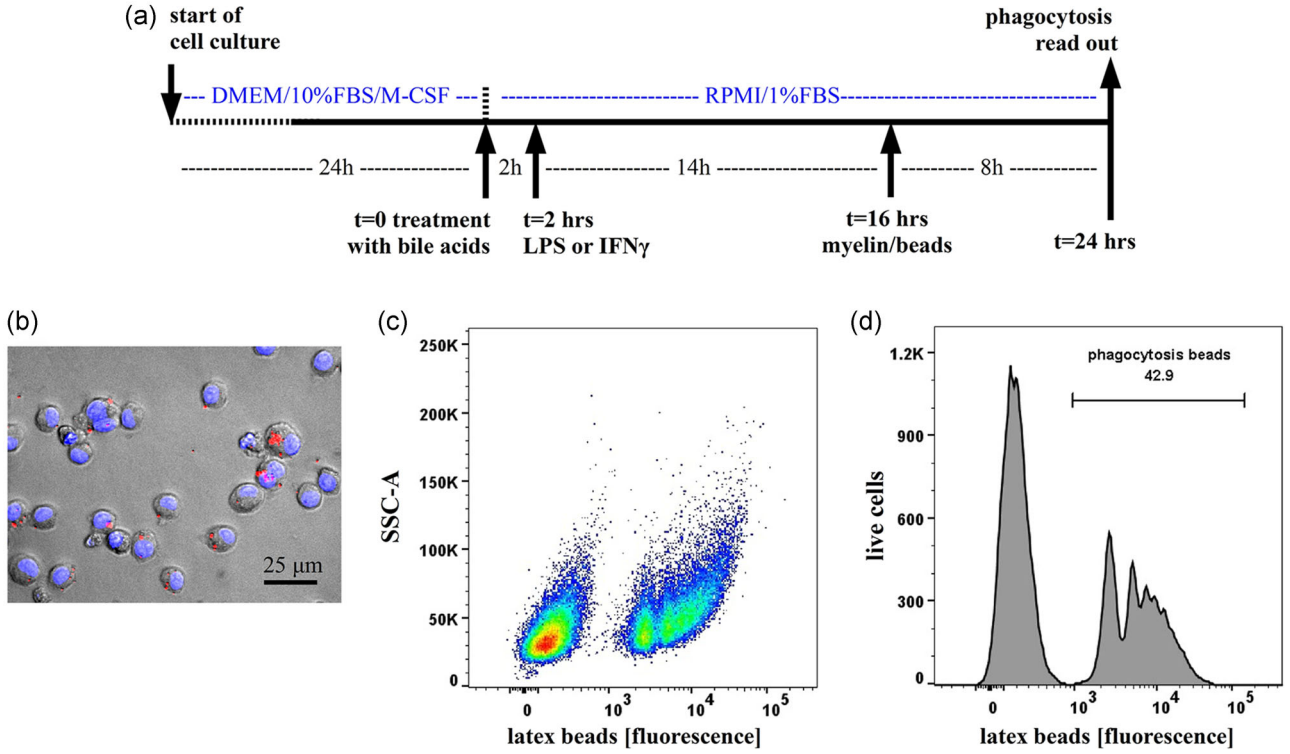


**FIGURE 1** Experimental set up for investigating phagocytosis. (a) Time line of in vitro phagocytosis experiments; cells were cultivated 24 h before bile acid treatment began at t0, inflammatory stimuli were applied 2 h later, and myelin exposure lasted 8 h. (b) Phase contrast image of Raw264.7 cells, superimposed with SkyBlue fluorescence of ingested latex beads (red) and Hoechst 33342 (blue). (c) Cytometry plot, side scatter vs fluorescence and (d) plotting the number of live cells versus SkyeBlue fluorescence illustrates distinct populations of cells without endocytosis, with uptake of one, two, or more latex beads. In this case 42.9% of the macrophages had ingested at least one fluorescent particle

TLCA, 200 µM TUDCA, or 160 µM HDCA began 2 h before LPS or IFNγ exposure, and myelin was added 14 h later (Figure 1a). When cells were investigated without inflammatory stimulation, treatment with RA or bile acids was added at t0. Assays were done in 96-well plates.

We used two independent assays for evaluation with either flow cytometry or a microplate reader. In flow cytometry, uptake was measured on a FACS Canto machine (BD Biosciences) and analyzed with FlowJo 10.6.2 software. The viability marker 7-amino-actinomycin D (1 µg/ml; BD Biosciences) was added 10 min before analysis. Phagocytosis was expressed as a percentage of live cells that took up latex beads or myelin (Figure 1b–d).

For myelin phagocytosis assays with a microplate reader, cells were incubated with 12.5 µg/ml pHrodo-labeled myelin at 37°C for 8 h. To quantify myelin uptake, we washed cells with PBS to eliminate pHrodo fluorescence in the cell culture medium. Cells were incubated with 5 µg/ml Hoechst 33342 nuclear staining for 2 min. Quantitative assessment was performed with a fluorescence plate reader (Infinite M1000 Pro, Tecan) using the pHrodo and Hoechst 33342 dye excitation and emission maxima of approximately 505 nm, 525 nm and 381 nm, 497 nm respectively. A phagocytosis index for each treatment was calculated as the absorbance ratio of pHrodo versus Hoechst 33342. Data were checked with MTT assays of cell viability.

## 2.7 | Immunocytochemistry (IHC) and fluorescence microscopy

The validity of myelin-pHrodo labeling for quantitative phagocytosis assays was checked with IHC. Cells were washed with PBS, fixed in 4% paraformaldehyde/PBS for 15 min, washed, blocked and permeabilized for 1 h in 10% normal goat serum, and 0.1% Triton X-100 at RT. Cultures were incubated with a primary antibody against myelin basic protein (rat anti MBP; Abcam, ab7349, 1/2000; overnight at 4°C), Alexa594-labeled secondary antibody (goat anti-rat, 1/500, Invitrogen, A11007; 2 h at RT), and Hoechst 33342 nuclear staining (5 µg/ml in PBS; 5 min at RT). Photos were taken with Leica epifluorescence or confocal microscopes at ×40 magnification using a digital camera (ImageJ-win64 software; Figure 2a–f).

## 2.8 | Quantification of gene expression

Ribonucleic acid was isolated with TRIzol, according to the manufacturer's instructions, then treated with DNase I and reverse transcribed with Superscript III reverse transcriptase and oligo (dT) primers as described previously (Wu et al., 2021). Real-time quantification of gene expression was performed using a SYBR Green RT-PCR assay. Each 15 µl SYBR green reaction mixture consisted of 1 µl

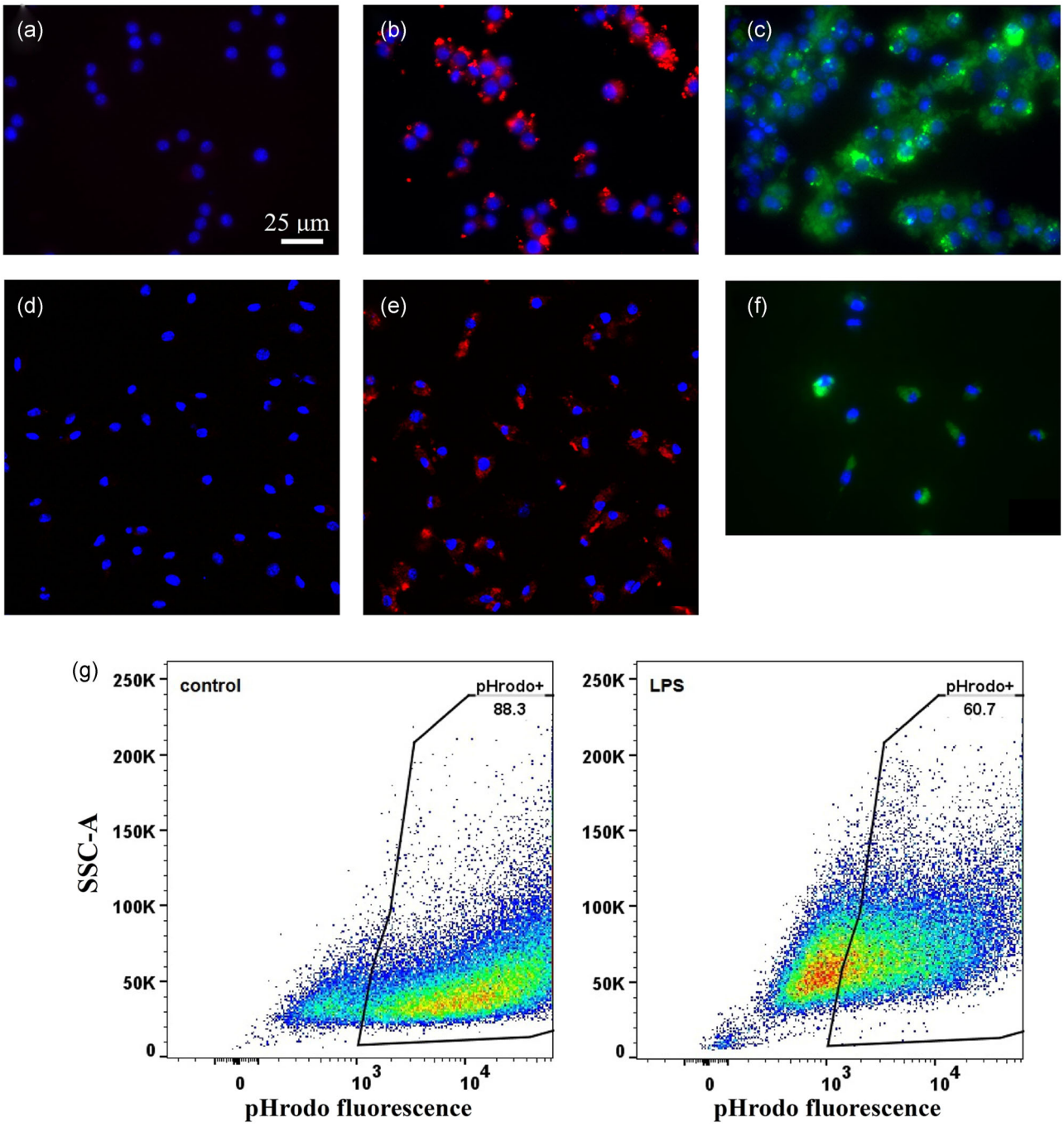


**FIGURE 2** Phagocytosis of myelin by Raw264.7 cells and BMDM. (a, b) MBP immunostaining (red) of Raw264.7 cells in the absence (a) and after incubation with myelin (b). (c) Myelin was labeled with the pH-dependent dye pHrodo (green) and added to the cell culture. (d, e) MBP IR (red) of BMDM in the absence (d) and after incubation with myelin (e). (f) BMDM incubated with pHrodo-labeled myelin. Cell nuclei were stained with Hoechst 33342 (blue) in (a–f); all photos were taken with the same magnification, scale bar in (a). (g) Cytometry plots of Raw264.7 cells after incubation with pHrodo-labeled myelin with and without exposure to LPS; the gating window that includes pHrodo-positive cells (based on experiments without myelin) shows 88.3% of the cells with myelin under control condition and 60.7% cells in the presence of 1 ng/ml LPS. BMDM, bone marrow-derived macrophage; LPS, lipopolysaccharide

complementary DNA, 7.5 µl SYBR Green PCR-mix (2×), 0.75 µl forward and reverse primer (10 pM) and 4.75 µl distilled water. PCR was performed with 5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 60 s at 60°C and a separate dissociation step. Specificity of the PCR product was confirmed by checking the melting curves. All samples were run in triplicates, and the level of expression of each gene was compared with the expression of the ribosomal gene RPS29. Amplification, detection of specific gene products and quantitative analysis were performed using an ABI 7900HT sequence detection system (Applied Biosystems). Three or four independent cell culture

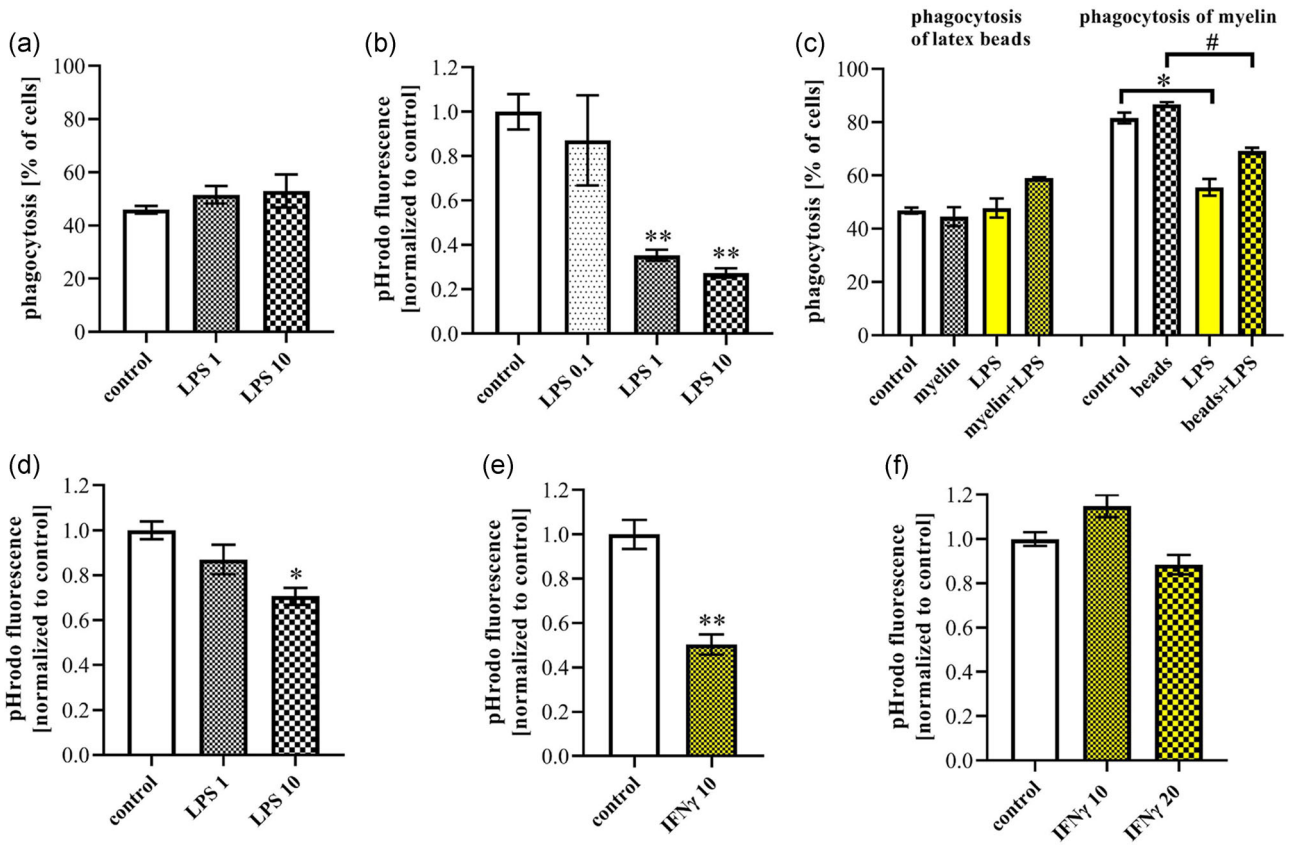


**FIGURE 3** Effect of LPS and IFNγ on the phagocytosis activity of Raw264.7 cells and BMDM. (a) Proportion of Raw264.7 macrophages (% of live cells, counted with cytometry) with endocytosis of latex beads when incubated without and with 1 or 10 ng/ml LPS. (b) Phagocytosis of pHrodo-labeled myelin by Raw264.7 cells when incubated without and with 0.1, 1, or 10 ng/ml LPS. (c) There was no interference between ingestion of latex beads (bars on the left side) and pHrodo-labeled myelin (bars on the right side); plotted are numbers of cells counted as positive for phagocytosis (mean ± *SEM*; $n = 3$ independent experiments with every condition; *, $^{\#}p < 0.05$, only effects of LPS on myelin uptake were significant, irrespective of the presence of latex beads). (d) Level of myelin phagocytosis by BMDM under control condition, when incubated with 1 ng/ml or with 10 ng/ml LPS. (e) Myelin phagocytosis by Raw264.7 cells incubated without and with 10 ng/ml IFNγ. (f) Myelin phagocytosis by BMDM incubated without or with 10 or 20 ng/ml IFNγ. Data show means ± *SD*; ANOVA, post hoc Dunnett's test (a–c, e) or *t* test (d), in comparison with the control condition: $^{*}p < 0.05$, $^{**}p < 0.01$. ANOVA, analysis of variance; BMDM, bone marrow-derived macrophage; IFNγ, interferon gamma; LPS, lipopolysaccharide

preparations were analyzed in all experiments. PCR efficiency was verified by dilution series (1, 1/3, 1/9, 1/27, 1/81, and 1/243) and relative messenger RNA (mRNA) levels were calculated using the comparative ΔCt method with normalization to the housekeeping gene. Gene identifiers, primer sequences, product sizes and melting temperatures are listed in Table 1.

## 2.9 | Statistical analysis

In the graphs data are presented as mean values ± *SEM*. Effects of different treatment conditions were analyzed with one-factor analysis of variance using GraphPad Prism v5 software. In case of significant effects, treatment conditions were compared with post hoc Sidak's multiple comparisons or Dunnett's tests, depending on the question. In some cases, when only two data sets were compared we a used two-tailed *t* tests considering $p < 0.05$ as statistically significant.

# 3 | RESULTS

## 3.1 | The effect of inflammatory stimuli on the phagocytosis of latex beads and myelin by macrophages

Our first objective was to study whether clearance of myelin is affected by the inflammatory state of macrophages. For this we established phagocytosis assays with the Raw264.7 macrophage cell line and with primary cultures of murine BMDM. Cells were incubated with fluorescent latex beads and subsequently analyzed with flow cytometry. Within 8 h, the particles were ingested by the cells (Figure 1b). Flow cytometry unequivocally differentiated between cells with and without phagocytosis and allowed us to quantify whether one or more beads had been taken up (Figure 1c,d). For measuring myelin phagocytosis, we prepared myelin from brains of adult rats and labeled it with pHrodo. This dye

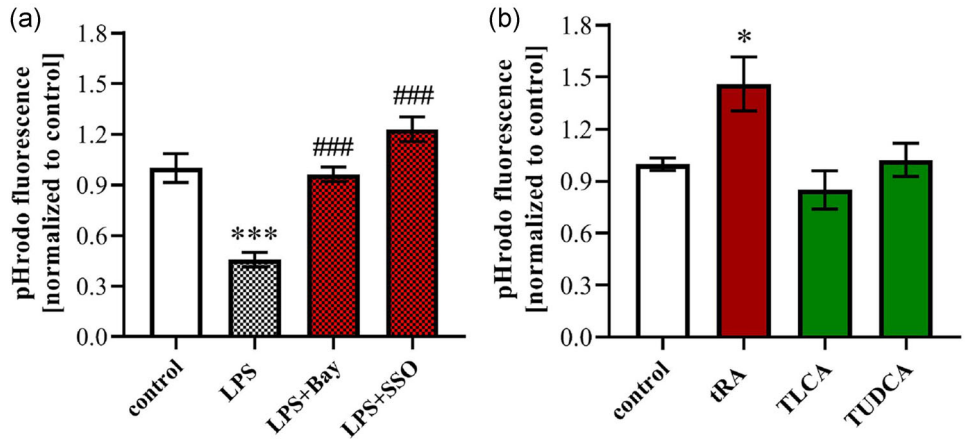


**FIGURE 4** Effect of anti-inflammatory treatment on phagocytosis. (a) Inhibitors of NFκB (Bay11-7082) and of CD36 (sulfo-N-succidimidyl oleate, SSO) restored phagocytosis activity of Raw264.7 cells in the presence of 1 ng/ml LPS; ANOVA, $F(3, 42) = 59.8$, $p < 0.0001$; post hoc Tukey test, versus control: $^{***}p < 0.001$, versus LPS: $^{###}p < 0.001$. (b) In the absence of LPS, all-*trans* RA (tRA) increased phagocytosis activity, while bile acids TLCA and TUDCA had no effect; ANOVA, $F(3, 30) = 4.5$, $p = 0.01$; post hoc Dunnett's test versus control $^{*}p < 0.05$. ANOVA, analysis of variance; NFκB, nuclear factor kappa B; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid

shows pH-dependent fluorescence and thereby indicates the inclusion of myelin within lysosomes.

Cellular uptake of myelin was also visualized with an antibody against MBP. The Raw264.7 cells do not express this myelin-associated protein, but after incubation with myelin they showed intracellular immune reactivity of MBP (Figure 2a,b). Microscopical inspection of pHrodo fluorescence localized this dye inside of the cells as well (Figure 2c). The same methods demonstrated myelin phagocytosis in BMDM (Figure 2d–f). Fluorescent labeling of cells could then be assessed with flow cytometry (Figure 2g) or by measuring pHrodo fluorescence with a microplate reader. The example experiment with cytometry shown in Figure 2g illustrates that the number of cells counted in the myelin/pHrodo-positive FACS gate decreased when cells were exposed to LPS (from 88% of live cells in the absence of LPS to 61% with 0.1 ng/ml LPS in the medium).

To test whether inflammatory signals influence phagocytosis, cells were stimulated with LPS, which activates TLR4/NFκB signaling, or IFNγ, which activates the Jak/STAT1 pathway. Concentrations of 0.1–10 ng/ml LPS did not influence the uptake of latex beads by the macrophage cell line (Figure 3a). The same concentrations of LPS did, however, significantly decrease the phagocytosis of myelin (Figures 2g and 3b). Combinations of SkyeBlue beads and myelin revealed no mutual interference. Additional myelin exposure did not alter the endcytosis of latex beads, and endocytosis of beads did not affect the simultaneous ingestion of myelin nor the negative effect of LPS on this function (Figure 3c).

Since the clearance of myelin is biologically more relevant, we did not further study the ingestion of latex beads. Stimulation with LPS also reduced the phagocyosis activity of BMDM primary cultures, although higher concentrations of LPS (10 ng/ml) were needed to exert a significant effect on these macrophages (Figure 3d).

When stimulated with 10 ng/ml IFNγ, the myelin ingestion by Raw264.7 cells decreased strongly (Figure 3e). However, in the case of BMDM, the cytokine did not have this effect, and even 20 ng/ml IFNγ did not significantly reduce myelin uptake (higher concentrations compromised cell viability). These results may indicate that activation of NFκB signaling affected phagocytosis by macrophages but that STAT1 signaling did not.

To support this conclusion, we inhibited the transcriptional activity of NFκB with 2 μM Bay11-7082 simultaneously with exposure to LPS. This restored myelin phagocytosis completely (Figure 4a). Alternatively, we inhibited CD36, which is associated with inflammation but also serves as a scavenger receptor. The CD36 inhibitor SSO abolished the effect of LPS.

## 3.2 | Taurolithocholic acid rescues phagocytosis activity of BMDM

Our second objective was to find out whether bile acids that were shown to be anti-inflammatory in macrophages (Pols et al., 2011a) can also improve myelin clearance. In the absence of inflammation, TLCA and TUDCA had no measurable effects on phagocytosis (Figure 4b). As a positive control, we added 0.1 μM all-*trans* RA. This significantly increased myelin endocytosis, corroborating a recent publication (Wu et al., 2021).

In accordance with our hypothesis, the reduction of myelin phagocytosis by Raw264.7 cells in the presence of LPS was partially prevented by bile acids. Flow cytometry demonstrated that LPS reduced the proportion of myelin/pHrodo positive cells from 88% to 66%, which increased to 83% when also treated with 40 μM TLCA (Figure 5a). Experiments measuring pHrodo fluorescence with a microplate reader confirmed this and also showed that 200 μM TUDCA and 160 μM HDCA rescued the function to a similar degree

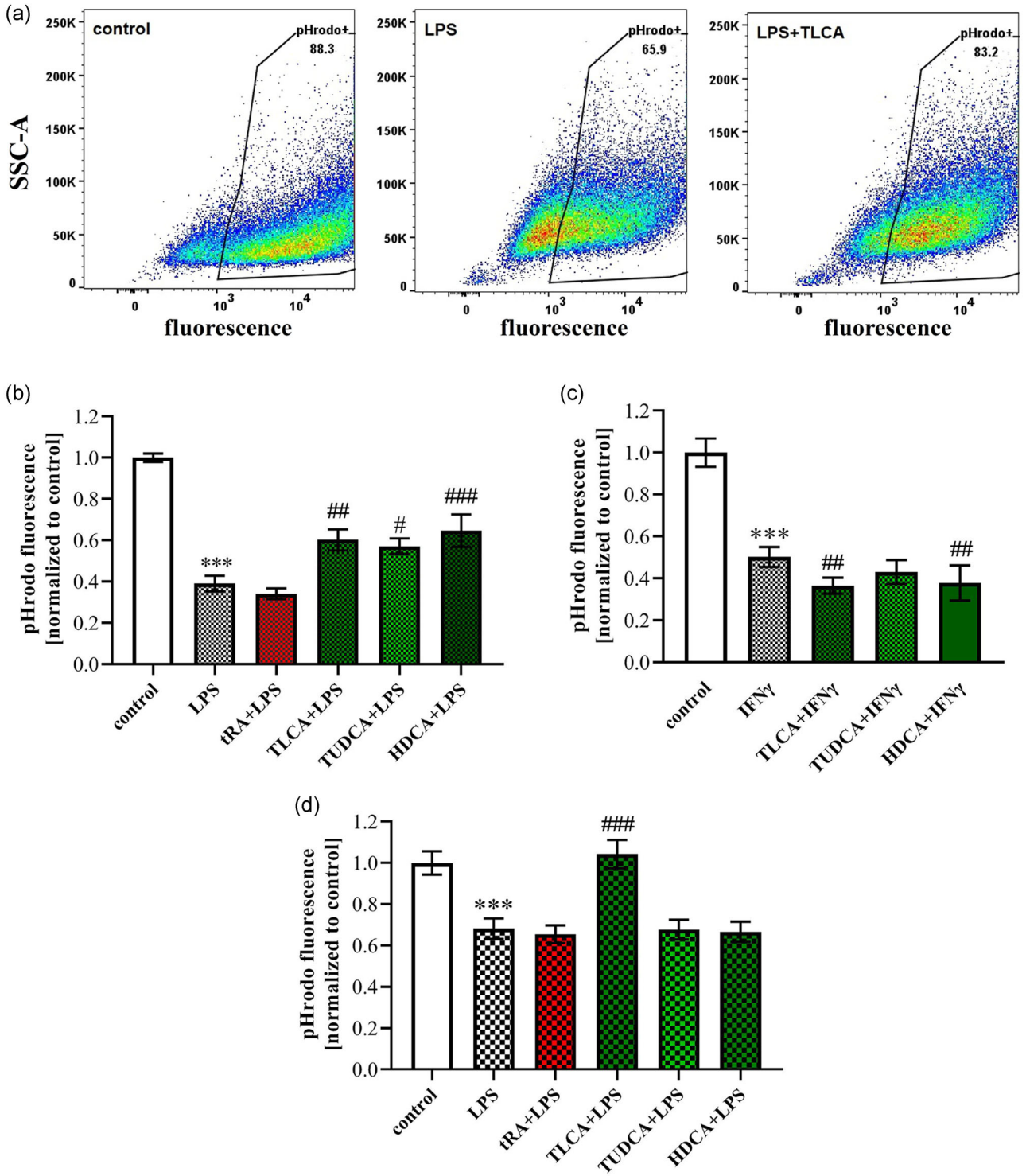


**FIGURE 5** (See caption on next page)

(Figure 5b). Surprisingly, treatment with 0.1 μM all-*trans* RA, which is known to reduce the expression of inflammatory cytokines (Dheen et al., 2005; Kampmann et al., 2008; van Neerven et al., 2010a, 2010b) did not have this effect. In the cell line, IFNγ also reduced myelin uptake. This was not prevented by any of the bile acids (Figure 5c).

The same experiments were repeated with BMDM. We confirmed the principal finding with TLCA and RA but, as before for the response to IFNγ, there were relevant differences, in this case with respect to the other TLR5 ligands. Lipopolysaccharides decreased myelin uptake to 65% compared to the control condition, RA had no effect on this, while TLCA completely rescued the phagocytosis (Figure 5d). In contrast, TUDCA and HDCA did not affect this activity at all. Since all these ligands in the concentrations used here have been shown to activate TGR5 signaling, different signal transduction may be at work regarding the effect on myelin endocytosis. Specifically, TLCA is known to also activate the nuclear receptor FXR. Thus, we attempted to clarify whether FXR activation is involved in myelin phagocytosis. The FXR agonist GW4604 did not increase phagocytosis in the presence or absence of LPS, while, on the other hand, an inhibitor of PKA blocked the effect of TLCA (Figure 6). The result implicates the involvement of TGR5 in our experiments because this receptor exerts its effects by activating PKA.

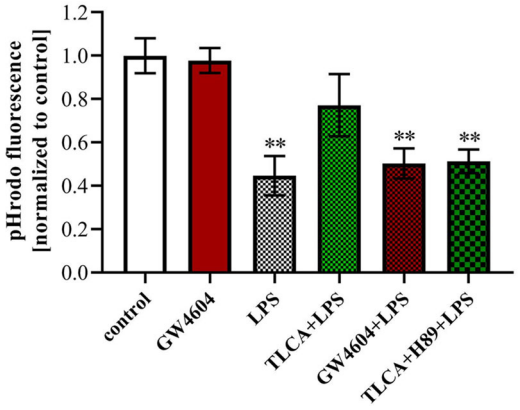


**FIGURE 6** Activation of FXR did not rescue myelin phagocytosis in the presence of LPS. Quantification of pHrodo/myelin uptake by Raw264.7 cells using a microplate reader; conditions were without additional treatment (control), 2 μM FXR agonist GW4604, 1 ng/ml LPS, LPS + 40 μM TLCA, LPS + 2 μM GW4604, LPS + 40 μM TLCA + 2 μM PKA inhibitor H89. One-factor ANOVA with $F(5, 33) = 8.5$, $p < 0.0001$ showed a significant effect of treatment. The reduction of myelin clearance by LPS was abolished by TLCA but not by GW4604 nor by TLCA when PKA activation was blocked; post hoc Sidak's multiple comparisons test: $^{**}p < 0.01$, $^{***}p < 0.001$ versus control; $^{\#}p < 0.05$ versus LPS. ANOVA, analysis of variance; LPS, lipopolysaccharide; TLCA, taurolithocholic acid

## 3.3 | TLCA, TUDCA, and HDCA have differential effects on the expression of genes involved in inflammation and phagocytosis

Previous studies demonstrated that TGR5 activation by bile acids interferes with NFκB transcription factors (Pols et al., 2011a; Yanguas-Casás et al., 2017). Since the negative effect of LPS on phagocytosis could be abolished using an inhibitor of NFκB, we considered the hypothesis that this anti-inflammatory activity of bile acids is responsible for releaving the inhibition of phagocytosis by LPS. The expression of two crucial inflammatory cytokines, IL-1β and TNFα was measured with quantitative RT-PCR. As expected, both genes were upregulated when cells were exposed to LPS (Figure 7). The addition of bile acids could significantly reduce this response.

In recent experiments we provided evidence that retinoids affect phagocytosis by increasing the expression of transglutaminase-2 (TGM2) and of the scavenger receptor CD36 (Wu et al., 2021). Using bile acids on Raw264.7 cells, the enzyme TGM2 was not significantly affected by treatment (Figure 8a). Expression of CD36 significantly increased under inflammatory conditions and increased further when the cells were treated with HDCA, while TLCA and TUDCA had no significant effect (Figure 8b). Similarly, significantly more transcripts of the complement receptor CR3 were found with HDCA treatment and not with TLCA and TUDCA (Figure 8c). Another potential mechanism implicates inhibition of the growth arrest specific gene-6 (Gas6) by TNFα. The Gas6 protein augments phagocytosis of apoptotic cells by binding to phosphatidyl serine (Feng et al., 2010). In Raw264.7 cells we did not see a decrease of Gas6 expression under inflammatory conditions nor a significant effect of bile acids (Figure 8d). While these changes do not correlate with effects on phagocytosis, we found a highly significant

**FIGURE 5** Ligands of TGR5 reduced the inhibitory effect of LPS on phagocytosis. (a) Flow cytometry plots of Raw264.7 cells under the control condition, in the presence of 1 ng/ml LPS and LPS plus 40 μM TLCA illustrate how LPS reduced myelin uptake which recovered in the presence of the bile acid. (b) Quantification of pHrodo-myelin uptake by Raw264.7 cells using a microplate reader; conditions were without LPS, 1 ng/ml LPS, LPS + 0.1 μM RA, LPS + 200 μM TUDCA, LPS + 40 μM TLCA, LPS + 160 μM HDCA. One-factor ANOVA with $F(5, 78) = 27.5$, $p < 0.0001$ showed significant treatment effects; post hoc Sidak's multiple comparisons test: $^{***}p < 0.001$ LPS versus control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ LPS + bile acids versus LPS. (c) Quantification of myelin uptake by Raw264.7 cells, same conditions as in (b) but with 10 ng/ml IFNγ as the inflammatory stimulus. The reduction of phagocytosis by IFNγ was highly significant but bile acids had no rescue effect: ANOVA $F(4, 40) = 171$, $p < 0.0001$; post hoc Sidak's multiple comparisons test: $^{***}p < 0.001$ IFNγ versus control, $^{\#\#}p < 0.01$ IFNγ + bile acids versus IFNγ. (d) Quantification of myelin uptake by BMDM as in (b), using 10 ng/ml LPS to induce an inflammatory phenotype. LPS-dependent reduction and reversal with TLCA were highly significant: ANOVA $F(5, 58) = 11.0$, $p < 0.0001$; post hoc Sidak's multiple comparisons test: $^{***}p < 0.001$ LPS versus control, $^{\#\#\#}p < 0.001$ LPS + TLCA versus LPS. ANOVA, analysis of variance; HDCA, hyodeoxycholic acid; IFNγ, interferon gamma; NFκB, nuclear factor kappa B; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid

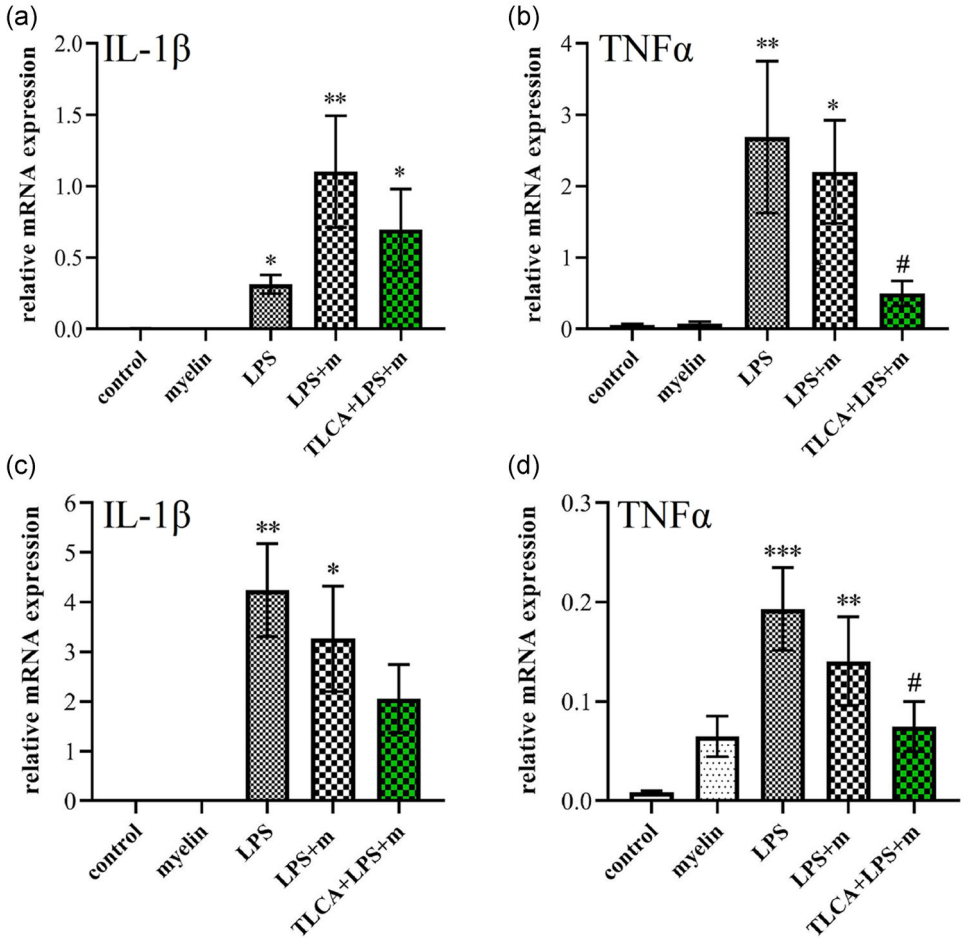


**FIGURE 7** Effects of bile acids on LPS-induced inflamatory genes. Following exposure to myelin, LPS and bile acids, mRNA was extracted from cell cultures, and gene expression measured with quantitative RT-PCR. (a, b) Expression of IL-1β and TNFα by Raw264.7 cells exposed to myelin, 1 ng/ml LPS, 40 μM TLCA or a combination of these as indicated. (c, d) Expression of IL-1β and TNFα by BMDM exposed to myelin, 10 ng/ml LPS and the same concentrations of bile acids as for the cell line. Data were evaluated with ANOVA [a: $F(4, 35) = 3.8$, $p < 0.05$; b: $F(4, 35) = 5.0$, $p < 0.01$; c: $F(4, 35) = 7.3$, $p < 0.001$; d: $F(4, 37) = 6.2$, $p < 0.001$] and post hoc Sidak's multiple comparisons test; $^{*}p < 0.05$, $^{**}p < 0.001$ indicate induction by the inflammatory stimulus and $^{\#}p < 0.05$ effects of TLCA. In both cell types the bile acid significantly inhibited the LPS-induced upregulation of TNFα but not of IL-1β. ANOVA, analysis of variance; BMDM, bone marrow-derived macrophage; HDCA, hyodeoxycholic acid; IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; mRNA, messenger RNA; NFκB, nuclear factor kappa B; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid

downregulation by LPS of the TREM2. Its expression was partly rescued by TLCA and HDCA, though these changes fell just short of being significant (Figure 8e).

In BMDM the only bile acid that rescued myelin phagocytosis in the presence of LPS was TLCA. Therefore, we then tested whether TLCA influenced the expression of CD36, TREM2, and Gas6 in the primary cultures. Our previous study implicated CD36 in the effect of RA on myelin phagocytosis (Wu et al., 2021), but this was not the case with TLCA (Figure 9a). The expression of Gas6 was measured in BMDM because the evidence regarding the clearance of apoptotic cells seemed compelling (Feng et al., 2010). In contrast to the cell line, we found a highly significant reduction with LPS, and TLCA was able to recover mRNA levels at least partially (Figure 9b). The same pattern was observed for TREM2 (Figure 9c), indicating that these two proteins may play a role in the effect of bile acids on phagocytosis under inflammatory stress.

## 4 | DISCUSSION

We investigated whether bile acids affect myelin clearance by macrophages under inflammatory conditions. The results suggest two conclusions: (1) Activation of the LPS-TLR4/NFκB pathway reduces myelin phagocytosis by BMDM in vitro, while IFNγ-Jak/STAT1 signaling does not. (2) Taurolithocholic acid rescues myelin phagocytosis in BMDM when this activity is suppressed by LPS.

The activation of bile acid receptors may therefore be explored for therapeutic strategies to improve myelin clearance in pathologies such as SCI, where this is a limiting factor. Results indicate, however, that caution should be used not to generalize to conditions that do not involve TLR4 signaling, to bile acids other than TLCA or to the endocytosis of different substrates such as apoptotic cells. Unfortunately, TLCA itself does not lend itself for clinical applications because it causes liver cholestasis (Denk et al., 2010) and the growth of cholangiocarcinoma cells

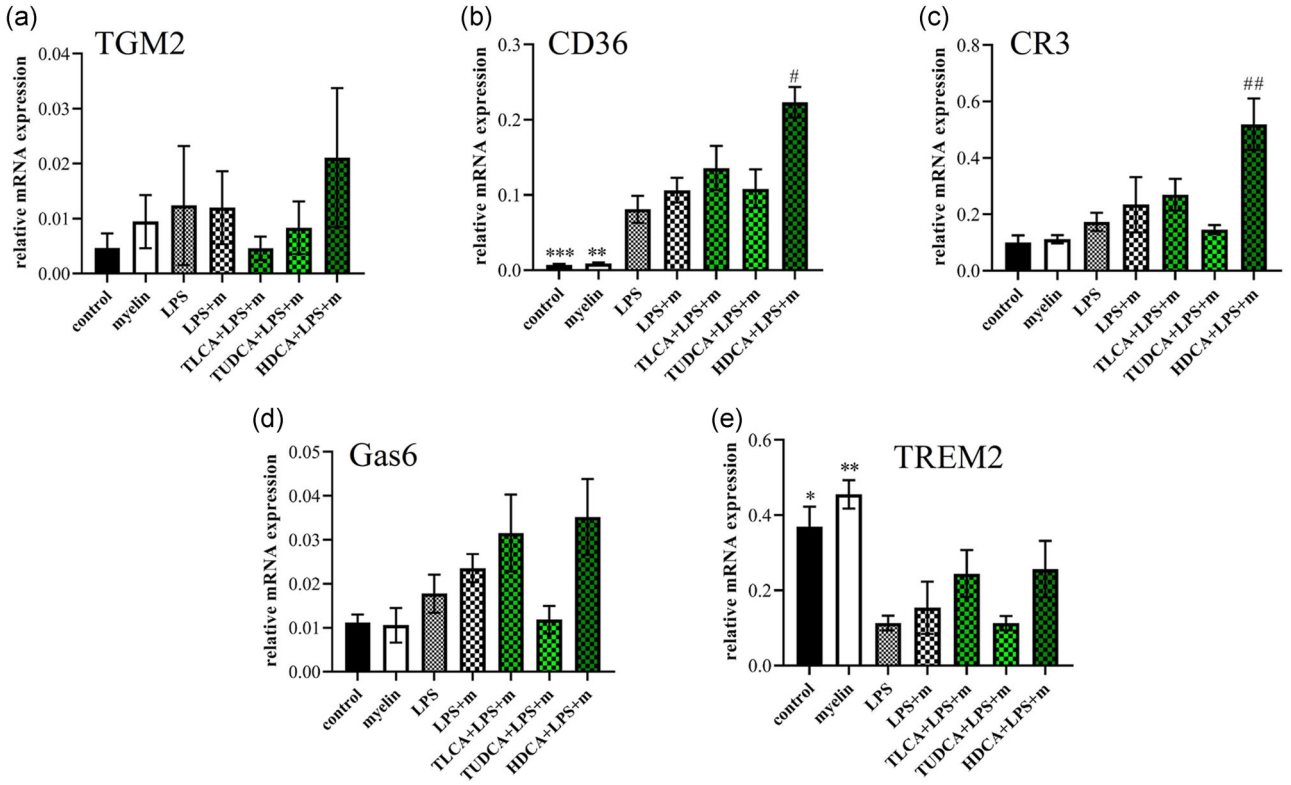


**FIGURE 8** Effects of bile acids and inflammation on expression of phagocytosis related genes in Raw264.7 cells. Expression of (a) TGM2, (b) CD36, (c) CR3, (d) Gas6, and (e) TREM2 by Raw264.7 cells exposed to myelin, 1 ng/ml LPS, 40 μM TLCA, 200 μM TUDCA, 160 μM HDCA or combinations as indicated in the graphs. Data were evaluated with ANOVA and post hoc Dunnett's test using LPS + myelin for comparison; *, ** are used to indicate proinflammatory activity and #, ## to indicate effects of bile acids; TGM2: $F(6,35) = 0.6$, n.s.; CD36: $F(6, 39) = 19$, $p < 0.0001$; CR3: $F(6, 39) = 7.3$, $p < 0.0001$; Gas6: $F(6, 49) = 2.9$, $p < 0.05$; n.s.; TREM2: $F(6, 38) = 6.5$, $p < 0.0001$. ANOVA, analysis of variance; HDCA, hyodeoxycholic acid; TGM2, triggering receptor on myeloid cells-2; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid

(Amonyingcharoen et al., 2015). Neither TUDCA nor HDCA occur naturally in the CNS of rats (Tripodi et al., 2012). The concentration of HDCA in rat bile is 0.01 mM and in rat serum 3.6 μM. TUDCA was detected at 0.5 μM in rat serum (Hagio et al., 2009; Suzuki et al., 2013). Our data raise several questions that suggest future experiments.

## 4.1 | Are nuclear receptors involved in the bile acid effects?

While the influence of TLCA on endocytosis in the presence of LPS seems to involve TGR5/PKA- and not FXR signaling, it remains unclear why the other TGR5 ligands did not have a similar effect on myelin ingestion by BMDM. All three bile acids have been shown to raise cAMP levels (Cipriani et al., 2011; De Marino et al., 2017; Yanguas-Casás et al., 2017). Since the applied concentrations of TUDCA and HDCA reduced inflammatory reactions (our data), it is unlikely that higher concentrations of TUDCA or HDCA would have been effective in supporting phagocytosis. The most likely alternative pathways by which bile acids affect cell physiology is the activation of nuclear receptors (De Marino et al., 2017; Ding et al., 2015; Wang et al., 1999). For instance, HDCA suppressed cell proliferation of intestinal epithelial cells through FXR-PI3K/AKT signaling without involving TGR5 activation (Song et al., 2019). The FXR is expressed in macrophages and is involved in the regulation of autoimmunity (Hucke et al., 2016). However, a compelling study with FXR deficient mice provides evidence to the contrary (Albrecht et al., 2017): Activation of FXR with GW4064 did not affect the expression of more than 80 genes involved in inflammatory immune responses nor the release of TNFα and NO by astrocytes and microglia. When testing the effect of GW4064 on myelin phagocytosis, we found no rescue effect in the presence of LPS (Figure 6) and therefore discard the implication of FXR in this context. It can be relevant, however, that related nuclear receptors PXR, VDR and LXR are activated by some bile acids (Chiang, 2013; De Marino et al., 2017). Of these, the VDR and LXR form heterodimers with RXR (van Neerven et al., 2008), and direct activation of RXR with bexarotene improved myelin endocytosis by BMDM (Wu et al., 2021). Mechanisms involving activation of these receptors by bile acids should therefore be considered.

## 4.2 | Can NFκB inhibition by TLCA explain the effects on phagocytosis?

Our pharmacological results do not support a dualistic model that contrasts a phagocytic versus an inflammatory phenotype of

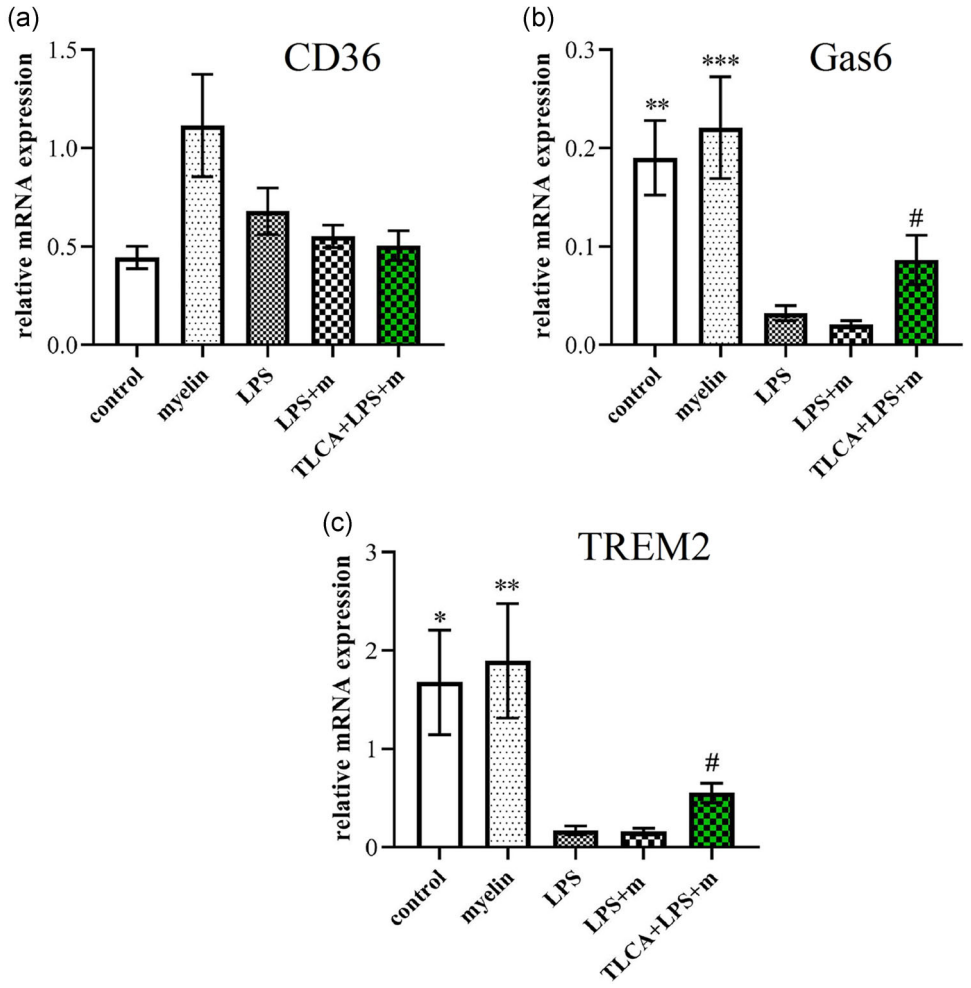


**FIGURE 9** Effects of bile acids and inflammation on expression of phagocytosis related genes in macrophage primary cultures. Expression of (a) CD36, (b) Gas6, and (c) TREM2 by BMDM cells exposed to myelin, 10 ng/ml LPS, LPS + myelin and LPS + myelin + 40 μM TLCA. Data were evaluated with ANOVA and post hoc Dunnett's test using LPS + myelin for comparison; *, **, *** are used to indicate proinflammatory activity, # the effect of bile acid treatment; CD36: $F(4, 45) = 3.8$, $p < 0.01$, post hoc tests n.s.; Gas6: $F(4, 45) = 8.6$, $p < 0.0001$; TREM2: $F(4, 44) = 5.8$, $p < 0.001$. ANOVA, analysis of variance; BMDM, bone marrow-derived macrophage; LPS, lipopolysaccharide; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid

macrophages. RA is known to inhibit expression of inflammatory genes including TNFα (Austenaa et al., 2008; Dheen et al., 2005) but did not rescue myelin endocytosis in the presence of LPS. Interferon-γ, which induces inflammatory responses via STAT1, strongly affected phagocytosis by Raw264.7 cells but did not interfere with myelin uptake by BMDM (Figure 3). Traditionally, two modes of differentiation of macrophages are distinguished, referred to as M1 (pro-inflammatory) and M2 (alternative activation; Freilich et al., 2013), and phagocytosis activity is associated with the M2 polarization (Wang et al., 2015b). Since cAMP induces M2 associated genes (Ghosh et al., 2016), we expected the TGR5 agonists to promote this activation in our cells. Both bile acid signaling mechanisms, via TGR5 and via FXR, converge on the inhibition of the NFκB responsive element (Fiorucci et al., 2018) which is inhibited by the nuclear corepressor-1 (downstream of FXR) and affected by phospho-CREB (downstream of TGR5-cAMP; Zhang et al., 2019). In addition, TGR5 can influence macrophage migration without involving NFκB (Perino et al., 2014).

## 4.3 | How important are bile acid effects on Gas6, TREM2, CR3, and CD36?

Activation of TLR4 with subsequent NFκB-induced production of TNFα inhibited the phagocytosis of neutrophils by macrophages (Michlewska et al., 2009). One mechanism for this is the suppression of Gas6 by TNFα. The former binds to phosphatidylserine of apoptotic cells, activates MerTK in the phagocyte and thereby initiates endocytosis (Wu et al., 2015). Blocking TNFα with a neutralizing antibody or adding exogenous Gas6 restored efferocytosis in LPS treated macrophages (Feng et al., 2010). Since phosphatidylserine makes up about 4% of myelin dry weight (Blewett, 2010),

**TABLE 1** Gene indentifiers and primer sequences used in quantitative RT-PCR

| Gene | Gene ID (NCBI reference sequence) | Primer sequences | Product Tm [°C] | Product size [bp] |
|---|---|---|---|---|
| RPS29 | NM_ 009093.2 | sense: GCCGCGTCTGCTCCAA | 60 | 54 |
| | | antisense: ACATGTTCAGCCCGTATTTGC | | |
| CR3 | NM_008401 | sense: GACCCTGGCCGCTCACGTATC | 62 | 127 |
| | | antisense: TCCACGCAGTCCGGTAAAATT | | |
| CD36 | NM_001159557 | sense: GGAGCCATCTTTGAGCCTTCA | 61 | 217 |
| | | antisense: GAACCAAACTGAGGAATGGATCT | | |
| Gas6 | NM_019521.2 | sense: GAACTTGCCAGGCTCCTACTCT | 61 | 124 |
| | | antisense: GGAGTTGACACAGGTCTGCTCA | | |
| IL1β | NM_008361 | sense: CTGGTGTGACGTTCCCATTA | 57 | 74 |
| | | antisense: CCGACAGCACGAGGCTTT | | |
| TNFα | NM_013693.3 | sense: AGGCTGTCGCTACATCACTG | 60 | 75 |
| | | antisense: CTCTCAATGACCCGTAGGGC | | |
| TGM2 | NM_009373 | sense: GACAATGTGGAGGAGGGATCT | 60 | 120 |
| | | antisense: CTCTAGGCTGAGACGGTACAG | | |
| TREM2 | NM_001272078 | sense: CTGGAACCGTCACCATCACTC | 62 | 183 |
| | | antisense: CGAAACTCGATGACTCCTCGG | | |

*Note*: See list for abbreviations, all gene sequences are from mouse.
Abbreviations: IL, interleukin; TGM2, transglutaminase-2; TNF, tumour necrosis factor.

secretion of Gas6 may also be relevant for myelin clearance. Regarding the influence of bile acids, our data with BMDM support this model while those with Raw264.7 cells do not. In the cell line, neither a reduction of Gas6 mRNA with LPS treatment nor its upregulation by bile acids was observed. Experiments using murine macrophages with deficient MerTK signaling showed that their phagocytic deficiency (and thus the potential mechanism via Gas6) was restricted to apoptotic cells and was independent of Fc receptor-mediated phagocytosis or ingestion of other particles (Scott et al., 2001). On the other hand, Gas6 expression was blocked by LPS in BMDM, as expected, and we observed a significant increase with TLCA (Figure 9b).

Another potential candidate to mediate the effect on myelin clearance is TREM2. This transmembrane immune receptor, which is expressed in microglia (Ulland & Colonna, 2018) and macrophages (Turnbull et al., 2006), is important for phagocytosis and lipid metabolism. TREM2 deficient microglia cells fail to degrade myelin-derived cholesterol and accumulate cholesteryl ester similar to foam cells in atherosclerosis (Nugent et al., 2020). Since this is generally associated with a proinflammatory response, the rescue effect of TLCA on TREM2 expression makes it a possible regulator of damage-associated activation of microglia (Ulland & Colonna, 2018).

HDCA, which partly rescued phagocytosis in Raw264.7 cells, may elicit this effect via upregulation of CR3 and/or the scavenger receptor CD36 (Figure 8b,c). This transmembrane glycoprotein, which is involved in efferocytosis (Fadok et al., 1998), was recently shown to be required for the uptake of myelin debris by phagocytes. Pharmacological inhibition of CD36 reduced myelin clearance in microglia and macrophage cultures and in an animal model of multiple sclerosis (Grajchen et al., 2020). The interpretation that the effect of HDCA involves CD36 is supported by our PCR data. The observation that the CD36 inhibitor SSO restored phagocytosis (Figure 4a) may be explained by the fact that SSO also inhibits NFκB activation and TNFα expression (Dhungana et al., 2017). In contrast, in an animal study of SCI, knockdown of CD36 was associated with smaller lesion area and improved locomotor recovery (Zhu et al., 2017). CD36 deficient mice also showed less ER stress and apoptosis after SCI (Myers et al., 2014). Thus, the role of this receptor in myelin clearance remains to be clarified.

### 4.4 | Do bile acids affect the metabolic breakdown of myelin?

The lysosomal degradation of lipids is an essential component of myelin clearance. After SCI, a persistent accumulation of myelin debris is accompanied by the presence of so-called foamy macrophages, which lose their ability to phagocytose apoptotic cells (Wang et al., 2015a). Since we consider that improving myelin clearance will be beneficial, it is noteworthy that bile acids play an important role in the regulation of lysosomal degradation. An antagonistic interaction between CREB and FXR mediated gene activation regulates autophagic degradation of lipids (Seok et al., 2014), and the coordinated activity of these two bile acid pathways seems to be important for

postprandial lipid metabolism in the liver (Farr et al., 2020). This may also be relevant for myelin clearance in CNS pathologies. We hypothesize that bile acids may not only improve the uptake of myelin by phagocytes but also its metabolic digestion. The results of the present study suggest that this line of investigation should be pursued.

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## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

## AUTHOR CONTRIBUTIONS

**Jörg Mey and Lorenzo Romero-Ramírez**: designed and supervised the study. **Siyu Wu**: performed the experiments and analyzed the data. **Lorenzo Romero-Ramírez**: performed some preliminary experiments. **Jörg Mey**: participated in data analysis and wrote the manuscript. All authors participated in proofreading.

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