Lipopolysaccharide activates microglia via neuraminidase 1 desialylation of Toll-like Receptor 4

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Abstract
Most cell surface receptors are sialylated, i.e. have sialic acid as the terminal residue of their sugar chains, but can be desialylated by sialidases, such as neuraminidase 1 (Neu1). Desialylation by Neu1 can activate immune cells, such as neutrophils, macrophages and monocytes. We investigated the role of Neu1 in activation of microglia using BV-2 cells (a murine microglial cell line) by cytokine ELISAs, enzyme activity assays, antibody/lectin binding and proximity labelling. We found that lipopolysaccharide (LPS) activation caused an increase in Neu1 protein on the cell surface, and an increase in surface sialidase activity that was prevented by Neu1 knockdown. Moreover, LPS induced interleukin 6 (IL-6) and MCP-1 release, which was reduced by Neu1 knockdown and increased by Neu1 over-expression. Neu1 knockdown also prevented the maintenance of IL-6 release by microglia after LPS was removed. Sialidase treatment of the cells was sufficient to induce IL-6 release, prevented by inhibiting toll-like receptor 4 (TLR4). Neu1 was found in close proximity to TLR4 on the surface of cells, and LPS induced desialylation of TLR4 on the cell surface, prevented by Neu1 knockdown. Sialic acid-binding immunoglobulin-like lectin E was found to bind to TLR4 via sialic acid residues and inhibit IL-6 release by BV-2 cells. We conclude that LPS causes Neu1 to translocate to the cell surface, where it desialylates TLR4, releasing inhibitory sialic acid-binding immunoglobulin-like lectin E, enhancing and maintaining inflammatory activation of the microglia. Thus, sialylation is a potent regulator of microglial activation, and Neu1 may be a target to reduce activation of microglia.

KEYWORDS
desialylation, microglia, neuraminidase 1, neuroinflammation, Toll-like receptor 4

1 | BACKGROUND

Microglia are brain-resident macrophages that mediate inflammation and phagocytosis in the central nervous system (CNS). They fight off infections by pathogens, and clear up dead neurons or debris, thus contributing to CNS health (Vilalta & Brown, 2018). However, chronic and sustained inflammation of the CNS has long been recognized as an important hallmark of neuropathology, including...
neurodegenerative diseases. Thus, microglia, as mediators of inflammation, are potential targets to treat diseases associated with CNS inflammation, including Alzheimer’s disease (Hickman, Izzy, Sen, Morsett, & Khoury, 2018).

One of the main receptors mediating inflammatory activation of microglia is Toll-like receptor 4 (TLR4). TLR4 is a N-glycosylated, 95 kDa large transmembrane protein that requires dimer formation for its activation. A well-characterized ligand of TLR4 is lipopolysaccharide (LPS) (Poltorak et al., 1998), which requires two co-receptors, MD2 and CD14, to activate TLR4 (Gioannini et al., 2004). Dimerization of TLR4 induces downstream activation of NF-κB signalling, which up-regulates hundreds of genes including those for pro-inflammatory cytokines, such as interleukin-6 (IL-6), and chemokines, such as monocyte chemoattractant protein 1 (MCP-1), resulting in inflammatory activation of immune cells such as microglia (Amith et al., 2010; Lehnardt et al., 2003). TLR4-mediated activation of microglia enables microglia to deal with infection and tissue damage, however, chronic TLR4 activation of microglia can also cause neurotoxicity and may contribute to neurodegenerative disease (Chakravarty & Herkenham, 2005; Lehnardt et al., 2003). Thus, it is important to understand how TLR4 is regulated in microglia.

In peripheral macrophages and dendritic cells, TLR4 is known to be regulated by neuraminidase 1 (Neu1). Neuraminidases (also known as sialidases) are enzymes that catalyse the cleavage of sialic acid residues from glycoproteins and glycolipids. Sialic acids are a family of negatively charged monosaccharide sugars with structures derivative of neuraminic acid, and normally are the terminal residue of the sugar chains of glycoproteins and glycolipids. Such sialic acid residues are important regulators of cell adhesion, protein folding and cell signalling (Schauer, 1985; Varki, 1997).

Neu1 is one of four mammalian neuraminidase isoenzymes and is predominately located in lysosomes. However, Neu1 is also found on the plasma membrane of differentiating monocytes and macrophages (Liang et al., 2006), where it was found to remove α-2,3-linked sialyl residues from TLR4 monomers inducing TLR4 dimerization and NF-κB signalling (Amith et al., 2010). Inflammatory activation of these cells induced the translocation of Neu1 to the plasma membrane surface, where it caused desialylation (i.e. the removal of sialyl residues) of TLR4 (Amith et al., 2010).

These findings suggest that the negatively charged sialyl residues of the TLR4 monomer cause steric or electrostatic hindrance of dimerization or ligand binding. However, more recent studies have shown the potential involvement of sialic acid recognizing immunoglobulin-like receptors (Siglecs). Chen et al. (2014) found that murine Siglec-E (and other Siglecs) was able to bind to TLR4, but this interaction was disrupted by Neu1 activity removing sialyl residues from TLR4 on the cell surface of dendritic cells. Thus, Siglec-E-deficient dendritic cells were more responsive to LPS as Siglec-E was inhibiting TLR4 function by binding to sialyl residues on TLR4 (Chen et al., 2014).

We have previously shown that activated microglia exhibit a neuraminidase activity on their cell surface (Nomura, Vilalta, Allendorf, Hornik, & Brown, 2017). However, it is currently unknown whether this neuraminidase activity originates from Neu1. Here, we provide evidence that LPS-activated microglia expose active Neu1 on their surface. We further show that desialylation of microglia by adding an exo-sialidase (a sialidase that cleaves terminal sialyl residues) affects pro-inflammatory cytokine release via TLR4. LPS-induced cytokine release is reduced by knockdown of endogenous Neu1 and increased by over-expression of Neu1. Moreover, we provide evidence that TLR4 and Neu1 interact at the cell surface, and that Neu1 is able to desialylate the glycan chains of TLR4 protein. We finally show that Siglec-E binds to TLR4 in microglia via recognition of sialyl residues and that lack of Siglec-E promotes LPS-dependent cytokine release. As surface Neu1 seems to be required for inflammatory signalling in microglia, it may be a novel target to reduce CNS inflammation.

2 | MATERIALS AND METHODS

2.1 | Materials

All reagents, chemicals and enzymes used were purchased from Sigma Aldrich unless indicated otherwise. All cell culture reagents were from Invitrogen. Protein A/G-coupled magnetic beads were from Thermo Fischer. Anti-FLAG M2 antibody (host: rabbit or mouse) and FITC-coupled peanut agglutinin were from Sigma Aldrich. TLR4 inhibitor Cli-095 was from InvivoGen. Lentiviral plasmids were purchased from Addgene. Biotin-tryramide was kindly provided by Dr Johanna Rees.

2.2 | Culture of cell lines and primary microglia

The BV-2 and HEK 293 cell lines (RRID:CVCL_0182, CVCL_0045, both from ECACC) (these cell lines are not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee) were maintained as previously described (Blasi, Barluzzi, Bocchini, Mazzolla, & Bistoni, 1990; Graham, Smiley, Russell, & Nairn, 1977). No further authentication was performed in the laboratory. Briefly, both cell lines were passaged every 3–4 days (maximum of 25 cell passages) and kept at 37°C in a 5% CO₂-humidified atmosphere. BV-2 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Twenty-four hours prior to applying cell treatments, medium was switched to DMEM with 0.5% FBS and antibiotics. HEK 293 cells were maintained in DMEM/F-12 medium supplemented with 10% FBS.

Primary glial cultures were prepared from post-natal days 3–5 Wistar rats (Charles River, RRID:RGD_2312511). Male and female adult rats were housed together to generate littermates. The animals were housed with access to food and water ad libitum in a colony room kept at 19–22°C and 40%–60% humidity, under a 12:12 hr light/dark cycle. After litter being born, both male and female pups were taken without assessment of weight. After sacrificing them by cervical dislocation, brain was dissected and brain cortex isolated as reported previously (Neher et al., 2011). Briefly, cortices were stripped of meninges, dissociated in trypsin-EDTA for 15 min at 37°C and digested tissue was triturated with a pipette. The cell suspension in
trypsin-EDTA was quenched by adding 10% FBS-containing DMEM supplemented with antibiotics (hereafter, termed glial medium). Cells were pelleted at 500 g for 7 min and re-suspended in glial medium. Cell suspension was sequentially passed through a 100 and 40 µm cell strainer and seeded in poly-L-lysine-coated T-75 flasks. Twenty-four hours post-seeding cellular debris was removed from flasks and medium was exchanged to fresh glial medium. Primary rat microglia were isolated 7–10 DIV by rigorous shaking of the flask. In total no more than 10 pups were required for this study. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1985). No institutional ethical approval was required.

2.3 | Cell treatments

Cells were treated for 18 hr with LPS from Salmonella enterica at 100 ng/ml, exo-sialidase from Vibrio cholerae at 80 µM/ml (or 2 hr at 200 µM/ml) or zymosan A from S. cerevisiae at 50 µg/ml. In some experiments, exo-sialidase was heat inactivated at 70°C for 10 min. The TLR4-specific inhibitor Cli-095 (Invivogen) was added at 5 µM for 3 hr prior to stimulation of cells. Polymyxin B was added at 100 U/ml 1 hr prior to LPS stimulation in primary glial cultures. To assay cytokine release induced by bacteria, we grew Escherichia coli until OD 0.6 (scatter at 630 nm) was reached and heat inactivated them at 70°C for 15 min to prevent bacterial proliferation in our BV-2 cultures. We treated BV-2 cells with inactivated E. coli at 1% v/v for 18 hr in antibiotic-free DMEM.

2.4 | Anti-Neu1 binding assay

Mechanically detached BV-2 microglia were washed in PBS with 1% BSA (staining buffer) and blocked for 15 min with 1 µg/10⁶ cells anti-FcR F(ab)² fragments (Jackson Immunoresearch) at room temperature. Cells were subsequently incubated with anti-Neu1 antibody (Aviva Biosystems, RRID:AB_1088413) or rabbit isotype control (SouthernBiotech, RRID:AB_2732899) at 10 µg/ml for 1 hr on ice. Cells were washed and incubated for 1 hr on ice with 488-coupled anti-rabbit detection antibody (Invitrogen). Mean fluorescence intensities of cells were evaluated by flow cytometry (BD Accuri C6).

2.5 | Neuraminidase activity assays

Cells were lysed in lysis buffer (PBS, 1% Triton X-100, protease inhibitors) on ice for 20 min and spun down at 20,000 g for 15 min. Lysates were added to a sodium acetate buffer (pH 4.5) or PBS (pH 7.2) containing the substrate methylumbelliferyl-N-acetylneuraminic acid at 15 µM. After 60 min incubation at 37°C, the reaction was stopped by adding glycine–NaOH buffer (pH 10.5) to each well and release of the fluorescent product methylumbelliferone was measured on a plate reader (365 excitation, 445 emission). Lysis buffer background was subtracted from sample signals and data presented as mean fluorescence intensities. Cell surface activity at neutral pH was performed as previously described (Nomura et al., 2017).

2.6 | siRNA-mediated RNAi

BV-2 cells at 70%–80% confluency were subjected to a lipid:siRNA mix containing 3% (v/v) Lipofectamine 3000 and 60 pmol of either TLR4- (target sequences: 1) 5’-GCAUAGGAGGUGCUCUUA, 2) 5’-GAGUGUCAGUUAACAUUA, 3) 5’-GGAUUGUAUCGCCUUCU, 4) 5’-UGAAGACCCUGAUCAGUG, smartpool siRNA, (Dharmacon), Neu1-(sense: 5’-GGAUAGUGGUUCUUAAUCUTT-3’, anti-sense: 5’-AGAUAGAACACAUUCCTG-3’), Siglec-E-targeting (sense: 5’-GAAUGACCAUCCGUCUCAATT-3’, anti-sense: 5’-UGAGACGCAUGGUACAUCUGG, Thermo Fischer) in serum-free OptiMEM. Transfection medium was removed after 3 hr incubation at 37°C and replaced by DMEM containing 10% FBS. Twenty-four hours after the transfection, BV-2 cells were re-seeded at appropriate density in low-serum DMEM. Primary rat microglia were transfected with magnetic particles (OZ Biosciences) using rat Neu1-targeting siRNA (sense: 5’-AGCAGGCUACUCAUCCUGATT, anti-sense: 5’-UCAGGG AUGAGUAGCGCGUGG, Thermo Fischer) as previously described (Carrillo-Jimenez et al., 2018). Treatments for cytokine/chemokine assays were applied 48 hr after transfection.

2.7 | cDNA synthesis and over-expression of Neu1 and TLR4-FLAG

The bicistronic lentiviral transfer plasmid pWPI with GFP as a reporter was purchased from Addgene. The Pme1 blunt end cloning site was modified by inserting the poly-linker 5’-AAACCTTCTATGGATCTA CTGATTTCCACTAGTCCATGTTT-3’ containing BamH1 and Spe1 restriction sites. Neu1 and TLR4 inserts were synthesized by PCR from whole mouse brain cDNA which was a kind gift from Dr Stefan Milde. PCR was performed using the Phusion High Fidelity PCR kit (NEB) according to the manufacturer’s protocol (Primers: Neu1-BamH1 fwd 5’-CTTCCATTGGATCCATGGTGGGGGCAGACCCGACC-3’, Neu1-Spe1 rev 5’-CATATGGGACTAGTTCCAAG CGTGGCGTAGAC GT-3’; TLR4-FLAG: TLR4-BamH1 fwd 5’-CTTCCATTGGATCCATGTTT-3’, TLR4-FLAG-Spe1 rev 5’-GAAACCCAGACT TGAGCCGATACAGTGGAGAGCCG-3’, Neu1-Spe1rev 5’-GAGACGACCTGAGGACTAGTTCCAAG CGTGGCGTAGAC GT-3’). Lentiviral particles were produced on HEK293T cells with envelope packaging vectors psPAX2 and pMD2.G as previously described (Balcaitis, Weinstein, Li, Chamberlain, & Möller, 2005). Viral supernatants of empty vector, Neu1- or TLR4-vector, transfected cells were taken off 48 hr post = transfection spun down and filtered through a 0.45 µm filter. They were added freshly to BV-2 cells in the presence of 8 µg/ml polybrene (Sigma) for 18 hr. Transduction as mean fluorescence intensities. Cell surface activity at neutral pH was performed as previously described (Nomura et al., 2017).
efficiency was monitored by GFP expression using flow cytometry. Cells with top 10% GFP expression were sorted by a Sony cell sorter.

2.8 | Surface biotinylation assay and western blotting

Neu1-over-expressing BV-2 cells were treated with vehicle or LPS for 18 hr. Cells were re-suspended in 1 mg/ml biotin 3-sulfo-N-hydroxysuccinimide ester sodium salt (Sigma) in PBS. After 30 min at 4°C under constant agitation, cells were washed in 200 mM glycine/PBS to quench the biotinylation reaction. Cells were subsequently lysed in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1x protease inhibitor mixture (Roche), 150 mM NaCl, 1% v/v Triton X-100 and 0.1 M sodium thiocyanate. Lysates were cleared of debris by centrifugation for 15 min and 72°C for 20 s. Expression of TLR4 was normalized to β-glucuronidase housekeeper gene expression (fwd 5’-TTCCATCCAGTTGCTTGG-3’, rev 5’-CTTCAT GTACT CCAGTGG-3’) were amplified by SYBR Green Master Mix (Sigma) according to the manufacturer’s protocol. Quantitative PCR and analysis were performed on a Rotor Gene Q cycler (Qiagen) with the following PCR cycle parameters: 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s.

2.11 | Proximity labelling using tyramide-biotin

Neu1-over-expressing cells were treated with LPS for 18 hr, mechanically detached and washed in PBS. Cells were incubated for 2 hr at 4°C under constant agitation with a TLR4 antibody (SantaCruz, RRID:AB_2240715) diluted 1:20 in blocking buffer (PBS supplemented with 5% BSA). Cells were washed 3 times with blocking buffer before adding HRP-coupled anti-rabbit antibody (Biorad) at 1:1,000 dilution. Cells were incubated with this secondary antibody for 1 hr at 4°C followed by three more washes in PBS. 80 µg/mL tyramide-biotin label) for 7 min at room temperature. The reaction was quenched by adding 100 U/ml catalase to the cells and left to incubate for another 5 min at 4°C. Cells were re-suspended in antibody strip buffer (50 mM Tris HCl pH 7.4 with fresh 0.03% H2O2 containing 80 µg/mL tyramide-biotin label) for 7 min at room temperature. Mean fluorescence of lectin-stained beads was assessed by flow cytometry in the FL-1 channel.

2.9 | Cytokine release assays

Primary microglia or BV-2 microglia were seeded at 20,000 cells/well (or 5,000 cells/well for MCP-1 release) in a 96-well plate and treated with a variety of stimuli for 18 hr. Culture supernatants were measured using an IL-6 or MCP-1 ELISA kit according to the manufacturer’s instructions (Rat/Mouse ELISA Max Deluxe, Biolegend). Absorption of wells was assessed by an Optima Plate Reader (BMG Technologies) and IL-6 concentration determined via linear regression from a standard curve.

2.10 | Quantitative PCR

RNA was extracted by Monarch Total RNA Extraction kit (N.E.B.) and cDNA synthesized by the SuperScript II First-Strand Synthesis kit (Thermo Fischer) according to the manufacturer’s instructions. Murine TLR4 (primer pair: fwd 5’-ATGAAAAAGCTCGTTAC-3’, rev 5’-CTCTCGGTTGAGTACAC-3’), Neu1 (fwd 5’-CTTCATCGCAGAC-3’), β-glucuronidase housekeeper gene expression (fwd 5’-GGTTAGAGTGCGTCCCACTCA-3’ or 5’-AAAGGGAATGCGCCTCACTCA-3’). The infrared dye was visualized using the IRDye 680 anti-mouse detection antibody (1:20,000, Li-Cor, RRID:AB_626632) were added at 1:200 overnight at 4°C before eluted fractions were loaded on a 4%–12% Bis-Tris NuPage gel and proteins were detected using the Li-Cor system.

2.12 | Detection of TLR4 desialylation

TLR4-FLAG construct was transduced via lentivirus into BV-2 microglia. TLR4-FLAG expressing or control cells were subjected to LPS (100 ng/ml, 24 hr) or sialidase (200 µU/ml) treatments and subsequently lysed. Lysates from TLR4-FLAG expressing or control cells were pre-cleared for 1 hr with protein A/G magnetic beads and anti-FLAG antibody (Sigma, RRID:AB_2240715) was added over night at 4°C. FLAG epitope was pulled down over 4 hr with protein A/G magnetic beads. Beads were stained with FITC-labelled peanut agglutinin for 20 min at room temperature. Mean fluorescence of lectin-stained beads was assessed by flow cytometry in the FL-1 channel.
incubated with protein G agarose (Thermo Fischer) overnight at 4°C. Elution and western blotting was performed as described in Surface Biotinylation. Detection of FLAG was performed by anti-FLAG antibody (Sigma, RRID:AB_796202).

2.14 | Study design & sharing of materials

The study was not pre-registered. No randomization or blinding was performed. Custom-made materials will be shared upon reasonable request.

2.15 | Statistical analysis

Analysis of data was performed using Graphpad Prism (Vers. 6.0) and data shown represented as a mean of at least n = 3 independent experiments ± standard error of mean (SEM). Normality of acquired data was tested by Shapiro–Wilk test. No test for outliers was performed. Statistical significance was assessed by ANOVA followed by Tukey’s or Sidak’s post hoc test or by t tests where indicated. p-values of p ≤ .05 are considered significant.

3 | RESULTS

3.1 | LPS activation of BV-2 microglia results in increased presence and activity of Neu1 on the cell surface

We have previously reported that LPS-activated BV-2 and primary rat microglia show an increased sialidase activity on their cell surface (Allendorf, Puigdellivol, & Brown, 2020; Nomura et al., 2017), so we investigated here whether this activity was caused by Neu1. To do this, we firstly tested whether siRNA-mediated knockdown of Neu1 affected the surface sialidase activity of BV-2 microglia. We found that knockdown of Neu1 had no effect on the background sialidase activity of the cells, but inhibited the increase in surface activity induced by LPS (non-targeting siRNA + vehicle: 19,474 ± 911, +LPS: 29,261 ± 648 mean fluorescence intensity (MFI) ± SEM; Neu1-targeting siRNA + vehicle: 18,578 ± 510, +LPS: 22,685 ± 713 MFI ± SEM, Figure 1a). This suggests that there are sialidases other than Neu1 (such as Neu3) present on the surface of non-activated microglia, but LPS induced Neu1 activity on the surface, and most of the LPS-induced sialidase activity is due to Neu1. In contrast to LPS, zymosan, a ligand for TLR2, did not induce a significant increase in surface neuraminidase activity (17,811 ± 1,074 MFI ± SEM).

To test whether Neu1 protein is present on the surface of the cells, we added an anti-Neu1 antibody to live BV-2 cells, and found significant binding measured by flow cytometry, and this binding was increased by LPS activation of the cells (anti-Neu1 + vehicle: 72,017 ± 4,946 MFI ± SEM, anti-Neu1 + LPS: 130,350 ± 13,517 MFI ± SEM), Figure 1b). The binding of an isotype control antibody was much lower and this binding did not change with LPS (isotype + vehicle: 27,968 ± 7,950 MFI ± SEM, isotype + LPS: 35,201 ± 7,505 MFI ± SEM), Figure 1b). Thus, Neu1 protein is present on the surface of BV-2 microglia, and its level increases after treatment with LPS.

We used surface biotinylation to confirm that Neu1 was on the BV-2 cell surface after LPS activation. Incubation of live cells with the water-soluble sulfo-NHS-biotin compound results in biotinylation of cell surface proteins (but not proteins inside the cell). Streptavidin pull down of these biotinylated proteins, followed by western blot for Neu1, revealed the presence of a band corresponding to the size of Neu1 (52 kDa), and this band was more intense in the LPS-treated condition (Figure 1ci upper panel, quantified in Figure 1cii: 2.1 ± 0.4-fold increase in Neu1 signal). However, LPS treatment of BV-2 cells did not change the total (surface and inside) expression levels of Neu1 (Figure 1ci lower panel), suggesting that LPS causes translocation of Neu1 from the inside to the surface of the cells.

3.2 | Sialidase treatment of BV-2 microglia induces IL-6 cytokine release mediated by TLR4

The above results indicate that LPS causes Neu1 to translocate to cell surface resulting in an increased sialidase activity there. To test what effect an increased surface sialidase activity has on microglia, we treated BV-2 microglia with sialidase from V. cholerae, which is commonly used to effectively desialylate cells (Nomura et al., 2017). Surprisingly, we found that sialidase treatment dramatically induced the release of IL-6 cytokine measured by ELISA in the culture medium (536 ± 49 pg/ml secreted IL-6 ± SEM, Figure 2a). This release could be blocked by (a) heat inactivating the enzyme and by (b) pre-treatment with the TLR4 inhibitor Cli-095 (41.3 ± 1.9 and 12.6 ± 11.4 pg/ml secreted IL-6 ± SEM). The finding that heat inactivation of the sialidase prevented IL-6 release, but this heat treatment had no effect on the activity of LPS (untreated LPS: 224 ± 26 pg/ml, heated LPS: 194 ± 30 pg/ml secreted IL-6 ± SEM), Figure 2a), indicates that the sialidase effect is not mediated by LPS contamination. However, the finding that pre-treatment with the TLR4 inhibitor Cli-095 prevented sialidase-induced IL-6 release suggests that the sialidase induces IL-6 release via activating TLR4. Importantly, we found that sialidase treatment of primary rat microglia (rather than BV-2 microglia) induced a substantial release of IL-6, which was blocked by Cli-095, but not by LPS-sequestering polymyxin B (Figure S1a).

To test whether a reduction in TLR4 protein, as opposed to TLR4 activity, affected the sialidase-induced IL-6 release, we knocked down TLR4 in BV-2 by siRNA and found that this significantly reduced TLR4 mRNA expression (83 ± 5% reduction in mRNA ± SEM, Figure 2b) and the sialidase-induced IL-6 release (non-targeting siRNA: 269 ± 47 pg/ml, TLR-4 targeting siRNA: 90 ± 26 pg/ml secreted IL-6 ± SEM, Figure 2c). LPS was used as a positive control to validate effective TLR4 knockdown or Cli-095 inhibition of TLR4 (non-targeting siRNA: 249 ± 29 pg/ml, TLR-4 targeting siRNA: 93 ± 24 pg/ml secreted IL-6 ± SEM, Figure 2a,c). We conclude that sialidase activity can activate TLR4 signalling in BV-2 microglia.
3.3 Knockdown of Neu1 in BV-2 microglia prevents LPS-induced IL-6 & MCP-1 release

Next, we tested whether alterations in the endogenous levels of Neu1 could affect TLR4-mediated inflammatory activation of microglia by measuring the release of IL-6 and MCP-1. We used a Neu1 siRNA knockdown that substantially decreased the endogenous sialidase activity of BV-2 lysates (si-Neu1: 4,274 ± 1,139, si-NT: 526 ± 171 mean fluorescence units ± SEM, Figure S1b). Interestingly, knockdown of Neu1 significantly reduced LPS-induced IL-6 release by the BV-2 microglia (si-NT + LPS: 351 ± 130 pg/ml, si-Neu1 + LPS: 99 ± 36 pg/ml secreted IL-6 ± SEM, Figure 3b). We confirmed this finding in primary rat microglia (Figure S1c). Moreover, we found similar trends when applying heat-inactivated E. coli rather than LPS to BV-2 microglia (Figure S1c). The TLR2 agonist zymosan induced IL-6 release that was not affected by knockdown of Neu1 (si-NT + zymosan: 145 ± 16 pg/ml secreted IL-6 ± SEM, Figure 3b), indicating some specificity to TLR4 signalling, rather than a general effect of Neu1 on microglial activation. We also tested whether the release of the chemokine MCP-1 was affected by Neu1 knockdown. Indeed, we observed a significant reduction in baseline and LPS-induced MCP-1 release (si-NT: 857.7 ± 204.6 pg/ml, si-Neu1: 94.52 ± 54.81 pg/ml, si-NT + LPS: 3,244.0 ± 211.4 pg/ml, si-Neu1 + LPS: 1708.0 ± 268.7 pg/ml secreted MCP-1 ± SEM, Figure S1c).

The above results indicate that Neu1 may play a role in the inflammatory response to LPS, but as LPS induced desialylation of the microglia via Neu1, we wondered whether Neu1 might maintain activation of the microglia in the absence of LPS. We therefore tested whether Neu1 knockdown affected the IL-6 release by BV-2 microglia treated with LPS for 18 hr, then washed to remove LPS and cultured for a further 24 or 48 hr in new media. We found that BV-2 microglia secrete significant amount of IL-6 cytokine 24 and 48 hr after media replacement (19.6 ± 5.5 and 22.3 ± 5.7 pg/ml).

**FIGURE 1** Active neuraminidase 1 (Neu1) enzyme is found on the cell surface of BV-2 microglia after lipopolysaccharide (LPS) treatment. (a) (i) Neuraminidase activity of BV-2 microglia treated with: LPS (100 ng/ml) or zymosan (100 µg/ml) or vehicle, and either: Neu1-targeting (si-Neu1) or non-targeting (si-NT) siRNA. Data presented as mean fluorescent intensities ± SEM of activity assays from at least three independent cell culture preparations. Statistical analysis was performed by one-way ANOVA followed by Tukey’s post hoc. ****p < .0001, ##p < .001. (ii) Western blot of protein lysate from si-NT or si-Neu1 treated BV-2. Upper panel shows Neu1 signal and lower panel shows β-actin signal as loading control. (b) Binding of anti-Neu1 or isotype-control antibody to LPS-treated or untreated BV-2 microglia as measured by flow cytometry. Left: Green fluorescence intensity distribution of a representative experiment. Right: Quantification of data presented as mean fluorescence intensities ± SEM from binding assays of three independent cell culture preparations. Statistics: one-way ANOVA with Tukey’s post hoc test. **p < .01. (c) Anti-Neu1 antibody probed Western blot of surface biotinylation assay. Untreated or LPS-treated (100 ng/ml, 18 hr) Neu1-over-expressing microglial cells were subjected to sulfo-NHS-biotin or vehicle. The resulting lysates (i)-lower panel) and their respective streptavidin pull-down fractions (ii)-top panel) were analysed for Neu1 presence. Blot representative of pull downs from three independent cell culture preparations. (ii): Quantification of streptavidin pull-down bands probed against Neu1. Mean signal intensities ± SEM of pull downs from three independent cell culture preparations. Statistics: one-way ANOVA with Tukey’s post hoc test. *p < .05 versus vehicle + NHS-Biotin.
ml secreted IL6 ± SEM, respectively); however, this was completely prevented by Neu1 knockdown (1.7 ± 1.7 and 0.9 ± 0.9 pg/ml secreted IL6 ± SEM, respectively; Figure 3d). Thus, Neu1 appears to be required to maintain inflammatory activation of microglia after LPS exposure.

3.4 | Over-expression of Neu1 in BV-2 microglia increases LPS-induced IL-6 & MCP-1 release

To further investigate the role of endogenous Neu1, we over-expressed Neu1 in BV-2 cells by lentiviral transduction. Highly expressing cells were sorted by GFP expression as the construct expressed both GFP and Neu1. A vector-expressing GFP, but not Neu1, was used as control. In Figure 4a we show that over-expression of Neu1 resulted in a substantial increase in cell lysate neuraminidase activity measured at pH 4.5 (empty vector: 5,403 ± 1,129, Neu1 vector: 44,092 ± 7,974 mean fluorescence intensities ± SEM). As shown in Figure 4b over-expression of Neu1 did not induce IL-6 release from BV-2 cells, but it substantially increased the IL-6 release in response to LPS (empty vector + LPS: 193 ± 49 pg/ml, Neu1 vector + LPS: 410 ± 18 pg/ml secreted IL-6 ± SEM), but not in response to zymosan (empty vector + zymosan: 234 ± 45 pg/ml Neu1 vector + zymosan: 262 ± 61 pg/ml secreted IL-6 ± SEM). We also observed a significantly increased release of chemokine MCP-1 in the Neu1-over-expressing line upon LPS stimulation (empty vector + LPS: 967 ± 78 pg/ml, Neu1 vector + LPS: 1,435 ± 143 pg/ml MCP-1 ± SEM, Figure 4c). These results for Neu1 over-expression are complimentary to the Neu1 knockdown results (Figure 3) and indicate that Neu1 somehow amplifies the microglial response to LPS.

3.5 | Neu1 and TLR4 proteins are found in proximity on the cell surface of LPS-activated microglia

We have established that Neu1 protein is found on the cell surface of LPS-activated microglia and that Neu1 knockdown modulates TLR4 signalling. We therefore investigated whether these two proteins could interact at the cell surface. We used a proximity labelling assay that allows selective biotinylation of proteins surrounding an antibody-tagged target protein on the cell surface (radius approximately 100 nm) (Rees et al., 2015). TLR4 was used as the antibody-labelled target with an HRP-coupled anti-rabbit antibody used as a secondary antibody detecting TLR4 binding sites. We included controls with only secondary antibody added or samples with no tyramide-biotin label added. As shown in Figure 5 (top panel) the biotinylation reaction and

![Figure 2](image-url) - Sialidase treatment induces release of the pro-inflammatory cytokine interleukin 6 (IL-6) mediated by toll-like receptor 4 (TLR4). (a) IL-6 cytokine release was measured in BV-2 culture supernatants 18 hr after indicated treatments. Data presented as mean ± SEM of at least three independent cell culture preparations. Statistics: one-way ANOVA with Tukey’s post hoc test. ****p < .0001. (b) TLR4 mRNA levels as measured by qPCR 48 hr post-knockdown. Expression of three independent knockdowns was normalized to the si-NT condition ± SEM Statistics: Student’s t test, ***p < .001. (c) IL-6 cytokine release of non-targeting (NT) or TLR4-targeting siRNA transfected microglia. Data presented as mean ± SEM of at least three independent cell culture preparations. Statistics: one-way ANOVA with Tukey’s post hoc test, **p < .01 versus si-NT + vehicle, ##p < .01 versus si-NT + sialidase
the subsequent pull down were successful in detecting the majority of streptavidin signal in the primary and secondary conditions. As little signal is seen in the secondary-only condition, we can conclude that TLR4 binding of the primary antibody was specific. As for the anti-Neu1 blot (Figure 5, bottom panel), we only detected a 52 kDa signal (corresponding to the size of Neu1) in the primary and secondary antibody condition and not in our controls. These results indicate that Neu1 can be found in close proximity to our target protein TLR4 on LPS-activated microglia. Cells were not fixed or permeabilized and the tyramide-biotin label is not cell permeable. We therefore may further conclude that this interaction is occurring on the cell surface of microglial cells.

3.6 | LPS or sialidase treatment of BV-2 microglia reduces sialylation of TLR4

One potential explanation for the effects of Neu1 knockdown (or over-expression) on IL-6 signalling is that Neu1 is directly targeting the TLR4 receptor as reported in macrophages and dendritic cells (Amith et al., 2010). As TLR4 is N-glycosylated and Neu1 removes sialyl residues from N-glycans, we tested whether LPS-induced Neu1 activity on the cell surface will remove sialic acid residues from TLR4 protein. We firstly tagged TLR4 with a C-terminal FLAG tag and expressed it in BV-2 microglia using lentiviral transduction. We then captured TLR4-FLAG from sialidase-treated or LPS-stimulated microglia via FLAG pull down on beads and tested the binding of
peanut agglutinin (PNA). PNA binds the penultimate sugars of desialylated glycans (galactose followed by N-acetylglucosamine), and its binding is blocked by sialylation, and so PNA binding is used to measure desialylation (Lotan, Skutelsky, Danan, & Sharon, 1975). Flow cytometry experiments showed that PNA bound to TLR4 from BV-2 cells, and that sialidase treatment of the cells increased PNA binding to TLR4 (Figure 6a), indicating desialylation of TLR4 (PNA to TLR4: 1,071 ± 118, PNA to TLR4 + sialidase: 1841 ± 259 mean green fluorescence ± SEM). Moreover, to show the specificity of the staining we used galactose to block the binding of PNA to the beads. Pre-incubation of PNA with 200 mM galactose was sufficient to block PNA binding to bead-captured TLR4 (vehicle: 1,006 ± 215, vehicle + galactose: 604 ± 144, sialidase: 2,348 ± 242, sialidase + galactose: 739 ± 127 mean green fluorescence ± SEM, Figure 6b). LPS treatment of BV-2 microglia resulted in desialylation of TLR4 as shown by increased PNA binding (vehicle: 923 ± 176, LPS: 1848 ± 320 mean green fluorescence ± SEM, Figure 6c). Moreover, knockdown of Neu1 prevented the LPS-induced desialylation of TLR4 (si-Neu1 vehicle: 1,001 ± 197, si-Neu1 LPS: 922 ± 302 mean green fluorescence ± SEM, Figure 6b). Thus, LPS induces desialylation of TLR4 via Neu1.

3.7 | Siglec-E recognizes sialyl residues on TLR4 and negatively regulates TLR4-dependent cytokine release

Desialylation of receptors can regulate activity either directly or via reduced binding of sialic acid-binding proteins (Siglecs), and there is an existing literature on the interaction of Siglec-E and TLR4 in dendritic cells (Chen et al., 2014). We tested whether Siglec-E can bind TLR4 by adding Siglec-E ectodomain to TLR4-FLAG-expressing BV-2 that were pre-treated with sialidase or vehicle. The subsequent pull downs for Siglec-E were probed for FLAG. We indeed found a
FLAG band (corresponding to the size of TLR4) in the Siglec-E pull down, which was reduced in the sialidase-treated condition (58 ± 8% reduction in signal ± SEM, Figure 7a). Thus, Siglec-E appears to bind to TLR4 on microglia, and this binding is reduced by desialylation of the cells.

If LPS-induced desialylation of TLR4 amplifies TLR4 activation via reduced binding to Siglec-E, then we might expect reduced expression of Siglec-E to also amplify TLR4 activation. To test this, we knocked down Siglec-E in BV-2 microglia and found that this significantly increased LPS-induced IL-6 release (non-targeting siRNA: 365 ± 101 pg/ml, Siglec-E targeting siRNA: 648 ± 65 pg/ml secreted IL-6 ± SEM, Figure 7b). This increase in TLR4 response was relatively specific as the response to TLR2-agonist zymosan was not significantly affected by Siglec-E knockdown (non-targeting siRNA: 107 ± 33 pg/ml, Siglec-E–targeting siRNA: 38 ± 13 pg/ml (secreted IL-6 ± SEM)). Thus, Siglec-E inhibits TLR4-mediated inflammation in microglia, and this may be via sialic acid-mediated binding to TLR4.

4 | DISCUSSION

We have previously shown a surface sialidase activity on BV-2 cells and on primary microglia after treatment with the TLR4 ligand LPS (Nomura et al., 2017, Allendorf et al., 2020). However, it was unknown which of the four mammalian sialidase isoforms was responsible for this activity. Here, we show that Neu1 knockdown substantially reduces the LPS-induced sialidase activity on the surface of BV-2 microglia. LPS did not increase whole-cell Neu1 expression at protein or mRNA level (Figure S1a), thus LPS appears to cause translocation of Neu1 onto the cell surface, as occurs with monocytes and neutrophils (Amith et al., 2010; Feng et al., 2011). Overall, this data indicate that Neu1 is responsible for the LPS-induced sialidase activity on the surface of microglia. However, as Neu1 knockdown prevented the LPS-induced surface sialidase activity, but not the LPS-independent surface sialidase activity (Figure 1a), the latter activity may be as a result of another neuraminidase, such as Neu3. Neu3 is known to be constitutively present on the cell surface, but with a different substrate specificity from Neu1 (Miyagi & Yamaguchi, 2012).

In the lysosome, Neu1 is typically found to be in complex with protective protein cathepsin A (PPCA) and β-galactosidase (β-Gal), where it degrades glycoproteins and glycolipids (Portier, Michaud, Tranchemontagne, & Thauvette, 1990). Liang et al. (2006) have suggested that pro-inflammatory stimulation or maturation of monocytes induces the translocation of lysosomal Neu1 to the cell surface. It remains unclear, however, if PPCA and/or β-Gal are transported together with Neu1 from the lysosome to the surface, and how Neu1 is tethered to the plasma membrane. Previously it has been suggested that Neu1 is a luminal protein, however, a recent report suggests it has two putative transmembrane domains (Maurice et al., 2016). Moreover, it is currently unknown how Neu1 is able to be active at the cell surface as its pH optimum is pH 4.5–5. Possibly proteins bound to Neu1 at the surface (or loss of protein binding) alter its pH sensitivity. We have evidence that Neu1 might be active at neutral pH as lysates from Neu1 over-expressing BV-2 microglia show a significantly higher sialidase activity at pH 7 (Figure S1b) compared with mock-transfected microglia.

Neu1 regulates inflammatory signalling in innate immune cells of the periphery (Amith et al., 2010). It remains unclear, however, to what extent Neu1 is important to microglial inflammatory signalling. As Neu1 is not commercially available we used an exo-sialidase from V. cholerae to model desialylation of the microglial cell surface. Surprisingly, we found that desialylation by this enzyme caused a substantial release of the pro-inflammatory cytokine IL-6 via TLR4 activation. We cannot rule out that this preparation of V. cholerae contained LPS. However, heat treatment of LPS did not affect its ability to induce IL-6 release from BV-2, whereas heat treatment of the sialidase prevented it from inducing IL-6 (Figure 2a). Furthermore, LPS-sequestering agent polymyxin B treatment suppressed the LPS induced, but not the sialidase-induced IL-6 release in primary rat
microglia. We, therefore, conclude that desialylation may activate microglia via desialylation of TLR4. Amith et al. (2010) found similarly that desialylation of macrophages by endogenous Neu1 or an exogenous bacterial sialidase induced TLR4 dimerization and NF-κB activation (Amith et al., 2010).

Knock-down and over-expression experiments revealed a role of Neu1 in modulating cytokine and chemokine release. We moreover found evidence that Neu1 and TLR4 interact on the cell surface of BV-2 microglia where Neu1 desialylated TLR4. There are a variety of potential mechanisms by which the sialylation state of TLR4 may modulate TLR4 function: (a) sialic acid residues may hinder TLR4 dimerization by electrostatic repulsion, (b) sialic acid-binding proteins (Siglecs) may sequester TLR4 monomers, and desialylation may release Siglec-promoting TLR4 dimerization, (c) sialylation and/or Siglec binding may affect turnover or internalization of TLR4, i.e. desialylation promotes retention of TLR4 on the cell surface (as shown by Wu, Ren, & Chen, 2016) or (d) desialylation may unmask underlying galactose residues that are recognized by oligomeric galectins, e.g. galectin-3, inducing TLR4 cross-linking (Burguillos et al., 2015; Nomura et al., 2017).

We investigated the possible involvement of Siglec receptors as Siglecs have been found to bind to TLRs (Chen et al., 2014). We focused on the murine Siglec-E as it was found to be a negative regulator of the oxidative burst in microglia (Claude, Linnartz-Gerlach, Kudin, Kunz, & Neumann, 2013) and of TLR4 activation in dendritic cells (Chen et al., 2014). Moreover, Siglec-E has been found to be required for endocytosis of TLR4 in murine dendritic cells (Wu et al., 2016). Note, however, that an independent study on murine dendritic cells did not find Siglec-E to negatively regulate TLR4 (Nagala et al., 2018). Our experiments indicate that Siglec-E
ectodomain was able to bind to microglial TLR4 via sialic acid residues. Moreover, the IL-6 cytokine response to LPS (but not to zymosan) was stronger in the Siglec-E knockdown, which indicates that Siglec-E negatively regulates TLR4 signalling. It might do this by promoting TLR4 endocytosis (as reported by Wu et al. in dendritic cells) or by preventing TLR4 dimerization by steric hindrance.

A limitation to this study is that we mainly focused on using the BV-2 microglial cell line to study the effects of Neu1 expression and activity on TLR4-mediated cytokine/chemokine release. We complemented this with key experiments on primary rat microglia. However, both BV-2 and primary microglia have been challenged as appropriate models of in vivo microglia. Therefore, it would be of interest to test whether the in vivo inflammatory response to intracerebral injection of LPS is reduced by co-injection with a neuraminidase inhibitor or in mice with reduced Neu1 expression or activity. Other limitations include that we only investigated the role of the Neu1-TLR4 pathway with LPS as stimulus and IL-6 and MCP-1 as inflammatory response. It would be interesting to repeat these experiments with other stimuli and other measures of microglial activation.

In summary, we provide evidence that LPS induces Neu1 translocation to the surface of BV-2 microglia, where it desialylates TLR4, reducing Siglec-E binding and amplifying inflammatory activation of BV-2 microglia. Thus, cell surface Neu1 is a potential drug target to reduce neuroinflammation.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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