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Thymoquinone from nutraceutical black cumin oil activates Neu4 sialidase in live macrophage, dendritic, and normal and type I sialidosis human fibroblast cells via GPCR Gαi proteins and matrix metalloproteinase-9

Trisha M. Finlay · Preethi Jayanth · Schammim Ray Amith · Alanna Gilmour · Christina Guzzo · Katrina Gee · Rudi Beyaert · Myron R. Szewczuk

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Abstract Anti-inflammatory activities of thymoquinone (TQ) have been demonstrated in *in vitro* and *in vivo* studies. However, the precise mechanism(s) of TQ in these anti-inflammatory activities is not well understood. Using a newly developed assay to detect sialidase activity in live macrophage cells (*Glycoconj J* doi:10.1007/s10719-009-9239-8), here we show that TQ has no inhibitory effect on endotoxin lipopolysaccharide (LPS) induced sialidase activity in live BMC-2 macrophage cells. In contrast, the parent black seed oil (BSO) and another constituent of BSO *para*-cymene (p-CY) completely block LPS induced sialidase activity. All of these compounds had no effect on cell viability. On the other hand, TQ induces a vigorous sialidase activity in live BMC-2 macrophage cells in a dose dependent manner as well in live DC-2.4 dendritic cells, HEK-TLR4/MD2, HEK293, SP1 mammary adenocarcinoma cells, human WT and 1140F01 and WG0544 type I sialidosis fibroblast cells. Tamiflu (oseltamivir phosphate) inhibits TQ-induced sialidase activity in live BMC-2 cells with an IC_{50} of 0.0194 μM compared to an IC_{50} of 19.1 μM for neuraminidase inhibitor DANA (2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid). Anti-Neu1, -2 and -3 antibodies have no inhibition of TQ-induced sialidase activity in live BMC-2 and human THP-1 macrophage cells but anti-Neu4 antibodies completely block this activity. There is a vigorous sialidase activity associated with TQ treated live primary bone marrow (BM) macrophage cells derived from WT and hypomorphic cathepsin A mice with a secondary Neu1

T. M. Finlay · P. Jayanth · S. R. Amith · A. Gilmour · C. Guzzo · K. Gee · M. R. Szewczuk (✉)
Department of Microbiology & Immunology,
Queen's University,
Kingston, ON K7L3N6, Canada
e-mail: szewczuk@queensu.ca

T. M. Finlay
e-mail: tmfinlay@ucalgary.ca

P. Jayanth
e-mail: 6pj1@queensu.ca

S. R. Amith
e-mail: ray.amith@ucd.ie

A. Gilmour
e-mail: 5amg@queensu.ca

C. Guzzo
e-mail: 3cg8@queensu.ca

K. Gee
e-mail: kgee@queensu.ca

R. Beyaert
Department for Molecular Biomedical Research, VIB,
Unit for Molecular Signal Transduction in Inflammation,
Technologiepark 927,
B-9052 Zwijnaarde, Belgium
e-mail: Rudi.Beyaert@dmbr.vib-UGent.be

R. Beyaert
Department of Biomedical Molecular Biology, Ghent University,
Ghent, Belgium

Present Address:
T. M. Finlay
Hotchkiss Brain Institute, University of Calgary,
Calgary, AL T2N 4N1, Canada

Present Address:
S. R. Amith
Conway Institute, University College Dublin,
Belfield,
Dublin 4, Ireland

deficiency (NeuI KD), but not from Neu4 knockout (Neu4 KO) mice. Pertussis toxin (PTX), a specific inhibitor of Gαi proteins of G-protein coupled receptor (GPCR) and the broad range inhibitors of matrix metalloproteinase (MMP) galardin and piperazine applied to live BMC-2, THP-1 and primary BM macrophage cells completely block TQ-induced sialidase activity. These same inhibitory effects are not observed with the GM1 ganglioside specific cholera toxin subunit B (CTXB) as well as with CTX, tyrosine kinase inhibitor K252a, and the broad range GPCR inhibitor suramin. The specific inhibitor of MMP-9, anti-MMP-9 antibody and anti-Neu4 antibody, but not the specific inhibitor of MMP-3 completely block TQ-induced sialidase activity in live THP-1 cells, which express Neu4 and MMP-9 on the cell surface. Neu4 sialidase activity in cell lysates from TQ-treated live THP-1 cells desialylates natural gangliosides and mucin substrates. RT-PCR and western blot analyses reveal no correlation between mRNA and protein values for Neu3 and Neu4 in human monocytic THP-1 cells, suggesting for the first time a varied post-transcriptional mechanism for these two mammalian sialidases independent of TQ activation. Our findings establish an unprecedented activation of Neu4 sialidase on the cell surface by thymoquinone, which is derived from the nutraceutical black cumin oil. The potentiation of GPCR-signaling by TQ via membrane targeting of Gαi subunit proteins and matrix metalloproteinase-9 activation may be involved in the activation process of Neu4 sialidase on the cell surface.

Keywords Thymoquinone · Para-cymene · Black seed oil · Cell signaling · Receptor activation · TOLL-like receptor · Neu3 sialidase · Neu4 sialidase · Cellular sialidase · GPCR · Matrix metalloproteinase

Abbreviations

TQ	thymoquinone
p-Cy	para-cymene
Oseltamivir phosphate	Tamiflu
DANA	2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid
LPS	lipopolysaccride
IC_{50}	50% inhibition concentration
BM	bone marrow
M-CSF	monocyte colony-stimulating factor
4-MUNANA	2′-(4-methylumbelliferyl)-α-*N*-acetylneuraminic acid
PVDF	polyvinylidene fluoride
DMSO	dimethyl sulfoxide
HEK293	human embryonic kidney 293 cells
Neu1 KD	hypomorphic cathepsin A mice with the secondary ~90% reduction of the Neu1 activity
Neu4 KO	Neu4 knockout
GPCR	G-protein coupled receptor
CTX	cholera toxin
CTXB	cholera toxin subunit B
MMP	matrix metalloproteinase
MMP-9i	specifc inhibitor of MMP-9
MMP-3i	specifc inhibitor of MMP-3
PMA	4-beta-phorbol 12-myristate 13-acetate

Introduction

The black seeds of the *Nigella sativa* plant are primarily used for the extraction of the oils that are used in traditional medicine. Of the many constituents of the black cumin oil (BSO) eluted by high pressure liquid chromatography, thymoquinone (TQ) has been shown to be one of the most active in many anti-inflammatory effects including treatment and prevention of disease [1] and in multiple animal study models of allergic lung inflammation [2–4], LPS stimulated basophil cells [5], and in rat models of adjuvant-induced arthritis [6]. TQ is part of a chemical structural group known as benzoquinones. Other benzoquinones such as aziridinyl benzoquinone are used as anti-neoplastic agents [7], and TQ is believed to share similar properties to the benzoquinones already in use as therapeutic drugs. Although the medicinal properties of TQ have been extensively studied, the precise molecular mechanism(s) of anti-inflammatory effects of TQ is not well understood.

To gain an insight into the anti-inflammatory properties of TQ, we used a newly developed assay to detect membrane sialidase activity that is initiated by ligand binding to TOLL-like (TLR) receptors in live primary bone marrow (BM) macrophage cells and macrophage and dendritic cell lines [8, 9]. Neu1 sialidase and not Neu2,-3 and -4 was found to form a complex with TLR-2,-3 and-4 receptors, and was also expressed on the cell surface of primary macrophage cells and macrophage cell lines [8]. Activation of Neu1 is induced by TLR ligands binding to their respective receptors. In the present study, we show that TQ has no inhibitory effect on endotoxin lipopolysaccharide (LPS) induced sialidase activity in live BMC-2 macrophage cells. Instead, TQ induced a vigorous sialidase activity in macrophage cells, dendritic cells, TLR-deficient HEK293 cells, human WT and type I sialidosis fibroblast cells as well as in primary bone marrow (BM) macrophage cells derived from WT and Neu1-deficient mice, but not from Neu4 knockout (Neu4KO) mice. The neuraminidase inhibitor Tamiflu (oseltamivir phosphate) as well as neutralizing antibodies against Neu4 and MMP-9 completely blocks this activity. The findings in this report suggest that TQ is a novel agonist of Neu4 sialidase activity in live cells.

Materials and methods

Reagents Thymoquinone (TQ, 99% pure, Aldrich, St. Louis, MO, USA) was reconstituted in 1× Tris buffered saline (TBS) containing 57% dimethyl sulfoxide (DMSO, Bio Shop Canada Inc., Burlington, ON, Canada). Black seed oil (BSO) (Minyak Bijirin, Hitam Baraka, Expolanka Commodities, Sri Lanka) derived from *Nigella sativa* and *para*-cymene (p-Cy, 99% pure, Sigma-Aldrich, St. Louis, MO, USA) were used at the indicated optimal dosage. TLR4 ligand lipopolysaccharide (LPS, 3 μg/mL, from *Serratia marcescens* and purified by phenol extraction; Sigma, St. Louis, MO), and TLR2 ligand, killed *Mycobacterium butyricum* (5 μg/ml, DIFCO) were used at indicated optimal dosage.

The sialidase substrate, 2′-(4-methylumbelliferyl)-α-*N*-acetylneuraminic acid (98% pure, 4-MUNANA, Biosynth International Inc., Itasca, IL, USA) was used at optimal concentration of 0.318 mM for the live cell sialidase assay as described previously [8]. Neuraminidase natural substrates, total ganglioside extract (brain, porcine ammonium salt; Avanti Polar Lipids, Inc., Alabaster, Alabama) and mucin from bovine submaxillary glands (Type I-S, Sigma) were used at final 500 μg/mL for the sialic acid (NANA) assay kit (BioVision Research Products, Mountain View, CA).

Tamiflu (99% pure oseltamivir phosphate, Hoffmann-La Roche Ltd., Mississauga, Ontario, Lot # BS00060168) was used at indicated concentrations as well as DANA (2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, Sigma).

Cell lines BMC-2 and BMA macrophage cells [10] and DC2.4 dendritic cells [11] were obtained from Dr Ken L. Rock, University of Massachusetts Medical School, Worcester, MA. The HEK293 parental cell line was a gift from Dr Leda Raptis of the Department of Microbiology & Immunology, Queen's University, Kingston, Ontario. Stable HEK-TLR cells were obtained by calcium phosphate transfection of a pCDNA3 expession vector for a specific chimeric TLR with an in frame C-terminal YFP and selection in 0.8 μg/ml G418. The HEK-TLR4/MD2 cell line was generated by additional co-transfection of an expression plasmid for human MD2. All cells were grown at 37°C in 5% CO_2 in culture media containing DMEM (Gibco, Rockville, MD) supplemented with 5% horse serum (Gibco) and 3% fetal calf serum (FCS) (HyClone, Logan, Utah, USA).

1140F01 and WG0544 cells are Neu1 deficient skin fibroblast cells that were isolated from patients with lysosomal storage disorder type I sialidosis and immortalized [12]. These cells were provided by Dr. Volkan Seyrantepe in Dr. Alexey Pshezhetsky's laboratory, Department of Pediatrics and Biochemistry, Montreal University, Service de Genetique, Ste-Justine Hospital, 3175 Cote-Ste-Catherine, H3T1C5, Montreal, QC, Canada. Cells were grown in 1× DMEM medium (Gibco) containing 5% horse serum (Gibco) and 3% fetal calf serum (HyClone) and passaged with 1× Trypsin-EDTA. SP1 cells are a non-metastatic infiltrating mouse mammary adenocarcinoma cell line provided by Dr. Bruce Elliott, Pathology and Molecular Medicine, Queen's University, Kingston, Ontario, Canada [13]. Cells were grown in DMEM (Gibco) medium containing 5–10% fetal calf serum (HyClone) at 37°C in 5% carbon dioxide.

Mouse models Wild-type (WT) and Neu4 KO (Neu4 knockout) [14] were obtained from Dr. Alexey Pshezhetsky's laboratory. The Neu1 KD (Neu1 deficient and cathepsin A deficient) mice have a hypomorphic cathepsin A phenotype with a secondary ~90% reduction of the Neu1 activity [15].

Primary mouse bone marrow macrophage cells Bone marrow (BM) cells were flushed from femurs and tibias of mice with sterile Tris-buffered saline (TBS) solution. The cell suspension was centrifuged for 3 min at 900 rpm, and the cell pellet resuspended in red cell lysis buffer for 5 min. The remaining cells were washed once with sterile TBS, and then resuspended in RPMI conditioned medium supplemented with 10% FCS and 20% (v/v) of L929 cell supernatant as a source of monocyte colony-stimulating factor (M-CSF) according to Alatery and Basta [16] and 1× L-glutamine-penicillin-streptomycin (Sigma-Aldrich Canada Ltd., Oakville, Ontario) in sterile solution. The primary BM macrophages were grown on 12 mm circular glass slides in RPMI conditioned medium for 7 days to 8 days in a humified incubator at 37°C and 5% CO_2. By day 7 these primary macrophage cells are more than 95% positive for macrophage marker F4/80 molecule as detected by flow cytometry [16].

Sialidase activity in viable cells Primary BM macrophages and TLR-expressing cells were grown on 12 mm circular glass slides in conditioned medium as described above. After removing medium, 0.318 mM 4-MUNANA substrate [2′-(4-methylumbelliferyl)-α-*N*-acetylneuraminic acid] (Biosynth International Inc., Itasca, IL, USA) in Tris buffered saline pH 7.4 was added to each well alone (control), with predetermined dose of TLR specific ligand (LPS, 3–5 μg/mL), or in combination of TLR ligand and 200–500 μM Tamiflu as previously described [8, 17]. The substrate is hydrolyzed by sialidase to give free 4-methylumbelliferone, which has a fluorescence emission at 450 nm (blue color) following an excitation at 365 nm. Fluorescent images were taken after 1 min to 2 min using epi-fluorescent microscopy (40× objective).

Free sialic acid assay Human monocytic THP-1 cells (1×10^6 cells) were treated for 5 min with 250 μg/mL TQ in

57% DMSO and Tris buffer pH 7.4 or left untreated as controls. The cells were washed once, pelleted and lysed in lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 0.2 mg/ml leupeptin, and 1 mM phenylmethanesulfonyl fluoride (PMSF)). To 25 μL of cell lysates was added 25 μL of 500 μg/mL mucin or total ganglioside extract for 30 min and the free sialic acid released was measured according to protocol of the sialic acid (NANA) assay kit (BioVision Research Products, Mountain View, CA, USA). The kit utilizes an enzyme coupled reaction in which the free sialic acid is oxidized resulting in the development of the Oxi-Red probe to give fluorescence (ex/em 535/587) using a Varioskan spectrophotometric microplate reader (Thermo Electron Corp., Finland). The sialic acid concentration of the test samples are calculated from a standard curve after subtracting background from control and the sample volume used in the assay.

Antibodies Rabbit anti-human Neu1 IgG, mouse anti-human Neu2 IgG and rabbit polyclonal anti-MMP9, were all acquired from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Other antibodies were mouse anti-human Neu3 IgG (Medical & Biological Laboratories Co., Ltd., Japan) and rabbit anti-human Neu4 polyclonal antibody (ProteinTech Group Inc., Chicago, IL, USA). AlexaFluor-labeled secondary antibodies included goat anti-rabbit AlexaFluor 488 (Molecular Probes, Eugene, OR, USA), goat anti-rabbit AlexaFluor 594 (Molecular Probes), goat anti-mouse 594 (Invitrogen, Corp.) were used at predetermined optimal concentrations in these studies. Horse radish peroxidase-labeled goat anti-rabbit antibodies were obtained from Santa Cruz Biotechnology.

Flow cytometry of cell surface Neu4 and MMP-9 in live cells Cells were grown in 25-cm^2 flasks at 90% confluence. For cell surface staining, live cells in serum free cold phosphate-buffered saline (PBS) were stained for Neu4 (53 kDa) with rabbit polyclonal anti-Neu4 (ProteinTech Group, Inc., Chicago, USA), for 15–20 min at 4°C, washed and followed with Alexa Fluor488 goat anti-rabbit IgG. After washing with cold PBS buffer, the cells were prepared for flow cytometry analysis. Forty thousand cells were acquired on a Beckman Coulter (Miami, FL) Epics XL-MCL flow cytometer and analyzed with Expo32 ADC software (Beckman Coulter). For overlay histograms, control cells treated with only Alexa488 conjugated goat anti-rabbit IgG are represented by the unfilled histogram with the dashed line. Auto-fluorescence of untreated cells is depicted by gray-filled histogram. Cells treated with anti-Neu1 antibody together with Alexa488 secondary antibody are depicted by the unfilled histogram with the black line. The mean fluorescence for each histogram is indicated for 80% gated cells.

Immunocytochemistry for Neu4 expression Cells were grown for 48 h on 12 mm circular glass slides in DMEM medium containing 5% fetal calf serum (for BMC-2 macrophage cells) or RPMI medium containing 10% fetal calf serum, 20% M-CSF and 1× L-glutamine-penicillin-streptomycin (Sigma-Aldrich Canada Ltd., Oakville, Ontario) (for primary BM macrophage cells) at 37°C in 5% carbon dioxide. After removal of medium, cells were fixed with 4% paraformaldehyde for 30 min and left non-permeabilized. Cells were treated with 1 μg/mL of rabbit anti-human Neu4 polyclonal antibody (ProteinTech Group, Inc., Chicago, USA) for 60 min at 37°C. Cells were then washed and stained with secondary antibodies labeled with AlexaFluor 594 for 60 min at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective).

Western blots and immunoprecipitation Human monocytic THP-1 cells are left cultured in media or in media containing 100 μg/mL TQ for 5 min. Cells (1×10^7 cells) are pelleted and lysed in lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 0.2 mg/ml leupeptin, 1% β-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride (PMSF)). Cell lysate proteins are resolved by 8% gel electrophoresis (SDS-PAGE). The initial blots are stripped and further probed for β-actin with monoclonal mouse anti-β-actin (Sigma-Aldrich Canada Ltd., Oakville, Ontario). Cell lysates are immunopreciptated with 2 μg of mouse monoclonal anti-Neu3 antibodies for 18 h. Following immunoprecipitation, complexes are isolated using protein A magnetic beads, washed 3× in buffer (10 mM Tris, pH 8, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100 and 0.2 mM sodium orthovanadate) and resolved by 8% gel electrophoresis (SDS-PAGE). Proteins are transferred to polyvinylidene fluoride (PVDF) transfer membrane blot. The blot is probed for either Neu3 (48 kDa) with anti-Neu3 (MBL Medical and Biological Laboratories Co., Ltd. Japan) or Neu4 (53 kDa) with anti-Neu4 (Protein Tech Group, Inc., Chicago, USA) followed by HRP conjugated secondary IgG antibodies or Clean-Blot IP Detection Reagent for IP/Western blots (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL) and Western Lightning Chemiluminescence Reagent Plus. The chemiluminescence reaction was analyzed with x-ray film. Sample concentration for gel loading was determined by Bradford reagent.

RNA isolation and reverse transcriptase PCR Total RNA was extracted from THP-1 cell pellets using TRI Reagent RNA Isolation Reagent (Sigma-Aldrich Canada Ltd., Oakville, Ontario L6H 6J8). RNA measurement was performed using the ND-1000 (NanoDrop Technologies, Wilmington, DE 19810, USA) spectrophotometer. Human brain total RNA was purchased as a positive control for Neu3 and Neu4 expression at a concentration of 1 μg/μL

(Clontech Laboratories, Inc., Mountain View, CA). RNA (2 μg) was reverse transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase Enzyme (Invitrogen). Equal aliquots of cDNA (2 μL) were used for Neu3, Neu4, and β-actin amplification. Specific primers for Neu3 and Neu4 were used as previously described [18] and the sequences for β-actin primers follows: 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′ and 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′ with 3 uL 5X Taq Polymerase Master Mix (New England Biolabs, Pickering, Ontario, L1W 3W9) containing dNTPs, $MgCl_2$, KCl, and stabilizers. β-actin served as the housekeeping gene for THP-1 and total human brain mRNA expression. PCR cycles were performed on the Px2 Thermal Cycler (Thermo Electron Corporation). The following cycling parameters were used: 45 s at 95°C, 1 min at 55°C, 45 s at 72°C for a total of 40 cycles. The amplified products were resolved by electrophoresis on 1.2% agarose gels and visualized by UV detection of ethidium bromide intercalation on the Alpha Innotech HD2 Imager (Alpha Innotech, Cell Biosciences, Inc., Santa Clara, CA).

Cell viability HEK-TLR4 cells were grown overnight in 24 well plates on 12 mm circular glass cover slides in DMEM medium containing 5% fetal bovine serum and 0.8 μg/ml G418 at 37°C in 5% carbon dioxide. After removal of medium, 50 μl of 200 nM Tamiflu was added to the cells for 1 h at 37°C. Tamiflu was removed and cells were left untreated (control) or treated with 100 μl of 500 μM or 1 mM hydrogen peroxide (H_2O_2), or 100 μl of 500 μg/ml, 250 μg/ml or 100 μg/ml TQ for 1 h at 37°C. Stain mixture was prepared using 1 μl of Acridine orange and 1 μl of ethidium bromide solution (Sigma-Aldrich) in 998 μl of sterile 1× TBS. To stain and mount cells, 1 μl of stain mixture was used with 4 μl of DAKO mounting medium. This stain combination caused nonviable cells to appear red and viable cells to appear green. Stained cells images were taken using epi-fluorescent microscopy (40× objective).

Results

Thymoquinone (TQ) activates membrane associated sialidase activity in live BMC-2 macrophage cells

Recently, TOLL-like receptor (TLR) ligands binding to their respective receptors have been shown to induce cell surface Neu1 sialidase activity in live macrophage cells [8]. This sialidase activity is revealed in the periphery surrounding the cells using a fluorogenic sialidase specific substrate, 4-MUNANA [2′-(4-methylyumbelliferyl)-α-*N*-acetylneuraminic acid], which fluoresces at 450 nm and caused by the emission of 4-methylumbelliferone. Since TQ has been reported to have anti-inflammatory properties [2, 3], we hypothesized that TQ would inhibit LPS-induced sialidase activity in live macrophage cells. In Fig. 1a, TQ did not inhibit LPS-induced sialidase activity in live BMC-2 macrophages, but unexpectedly induced sialidase activity at concentrations as low as 33 ng/mL (Fig. 2a). This TQ-induced sialidase activity was only associated with live cell treatment (Fig. 2b); TQ in the presence of substrate alone had no effect and neither was the solvent dimethyl sulfoxide (DMSO) in solution with Tris buffered saline (Fig. 1c). A positive control sialidase (*Clostridium perfringens*), which has a specific activity of 1 U per 1 mmol of *N*-acetylneuraminic acid per minute was also added separately to the 4-MUNANA substrate.

Since TQ is derived from the black seed oil (BSO), we also asked whether BSO and its other component *para*-cymene (*p*-Cy) are able to inhibit the sialidase activity associated with LPS treated live macrophage cells. Both BSO and *p*-Cy inhibited the LPS-induced sialidase activation in live BMC-2 macrophage cells (Fig. 1a) in a dose dependent manner (Fig. 1b). Surprisingly, BSO and *p*-Cy had no inhibitory effect on TQ-induced sialidase activity in these live BMC-2 cells. In addition, TQ, BSO and p-Cy at these concentrations had no effect on the cell viability of BMC-2 cells (Fig. 2d).

TQ induces sialidase activity in live DC-2.4 dendritic cells, HEK-TLR4/MD2, HEK293, SP1 mammary adenocarcinoma cells and human WT and sialidosis type I fibroblast cells

We also tested whether TQ-induced sialidase activity could be observed in non-macrophage cells. The data clearly show this to be the case. TQ induced sialidase activity in live DC2.4 dendritic cells, HEK-TLR4/MD2 cells, HEK293, and SP1 mammary adenocarcinoma cells (Fig. 3a). The results from TQ-induced sialidase activity in TLR-deficient HEK293 cells suggest that TOLL-like receptors may not be the target receptors for TQ.

It is noteworthy that Neu1 deficiency is a human autosomal recessive inborn error of metabolism disease in patients with sialidosis or mucolipidosis I. Patients with sialidosis have a build-up of sialylated glycoconjugates in tissues and urine causing neuropathic or non-neuropathic symptoms depending on the onset of the disease. Studies on over-expression of Neu4 in sialidosis fibroblast cells show a clearing of the storage material accumulated in the lysosome in these disorders [19]. Here, we asked whether or not TQ treatment of live human 1140F01 and WG0544 type I sialidosis fibroblast cells would reveal similar sialidase activity. The data in Fig. 3a clearly indicate this to be the case. These latter data show that

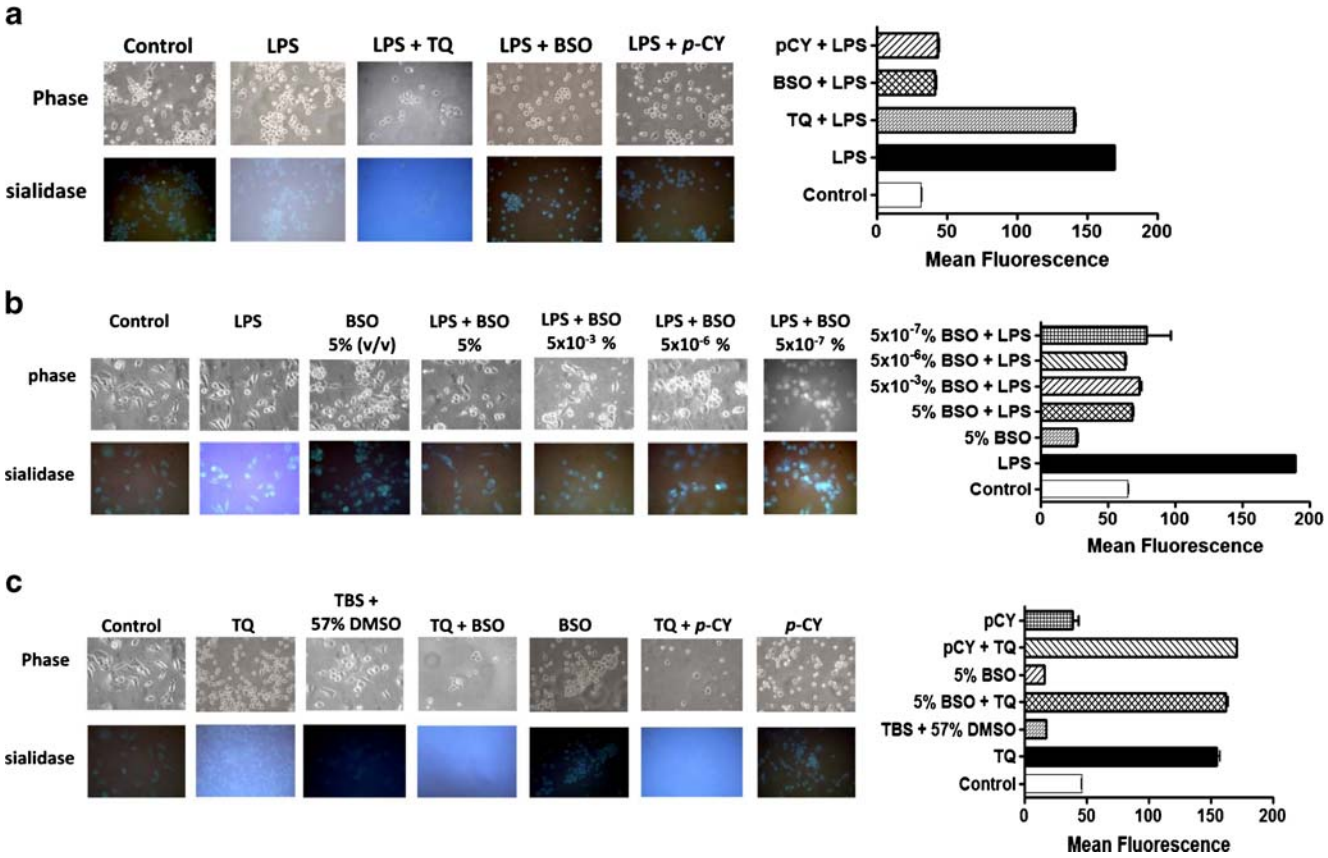


Fig. 1 **a** Inhibitory effect of black seed oil (BSO) and para-cymene (*p*-Cy) on LPS induces sialidase activity in live BMC-2 macrophage cells. BMC-2 cells were allowed to adhere on 12 mm circular glass slides in media containing 5% horse and 3% fetal calf sera for 24 h in a humified incubator at 37°C and 5% CO_2. After removing media, 0.318 mM 4-MUNANA (4-MU) substrate (2′-(4-methlyumbelliferyl)-α-*N*-acetylneuraminic acid) in Tris buffered saline pH 7.4 was added to cells alone (Control), with TLR ligand (3 μg/ml LPS phenol extracted from *Serratia marcescens*), or with ligand in combination with 250 μg/mL TQ, 5% BSO or 5% p-Cy. The substrate is hydrolyzed by sialidase enzymes to give free 4-methylumbelliferone, which has a fluorescence emission at 450 nm (*blue color*) following excitation at 365 nm. Fluorescent images were taken at 2 min after adding substrate using epi-fluorescent microscopy (40× objective). The mean fluorescence surrounding the cells for $n \geq 50$ replicates in each of the images was measured using Image J Software. The data are a representation of one out of three independent experiments showing similar results. **b** BSO inhibits sialidase activity associated with LPS treated live BMC-2 cells. Cells were allowed to adhere on 12 mm circular glass slides and assayed as described in (**a**) above. The data are a representation of one out of three independent experiments showing similar results. **c** BSO and p-Cy do not inhibit sialidase activity associated with TQ treated live BMC-2 cells. Cells were allowed to adhere on 12 mm circular glass slides and assayed as described in (**a**) above. The data are a representation of one out of three independent experiments showing similar results

Neu1 sialidase activity is not associated with TQ treatment of live cells.

Effect of neuraminidase inhibitors on TQ induced sialidase activity in live macrophage cells

Next, we tested whether neuraminidase inhibitors would inhibit the sialidase activity associated with TQ treatment of live macrophage cells. Surprisingly, this TQ-induced sialidase activity was completely blocked by the neuraminidase inhibitor Tamiflu (pure oseltamivir phosphate) in a dose dependent range of 0.25–250 μg/mL (Fig. 4a). In addition, the neuraminidase inhibitor DANA (2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid) had a limited inhibition of TQ-induced sialidase activity in live BMC-2 cells. Other studies using recombinant soluble human sialidases have shown that DANA inhibited Neu1 (IC_{50} of 168 μM), Neu2 (IC_{50} of 45.5 μM) and Neu4 (IC_{50} of 73.1 μM) [20]. To further elucidate the inhibitory capacity of Tamiflu and DANA on sialidase activity associated with TQ treatment of live BMC-2 macrophage cells, the 50% inhibitory concentration (IC_{50}) of each compound was determined by plotting the decrease in sialidase activity against the log of the agent concentration. As shown in Fig. 4b, Tamiflu had an IC_{50} of 0.0194 μM in comparison to an IC_{50} of 19.1 μM for DANA. We had previously reported that DANA has a limited inhibitory effect while Tamiflu has a potent inhibitory effect with an IC_{50} of 1.175 μM compared with oseltamivir carboxylate (IC_{50} of 1015 μM) for Neu1 sialidase activity associated with LPS treatment of live BMC-2 macrophage

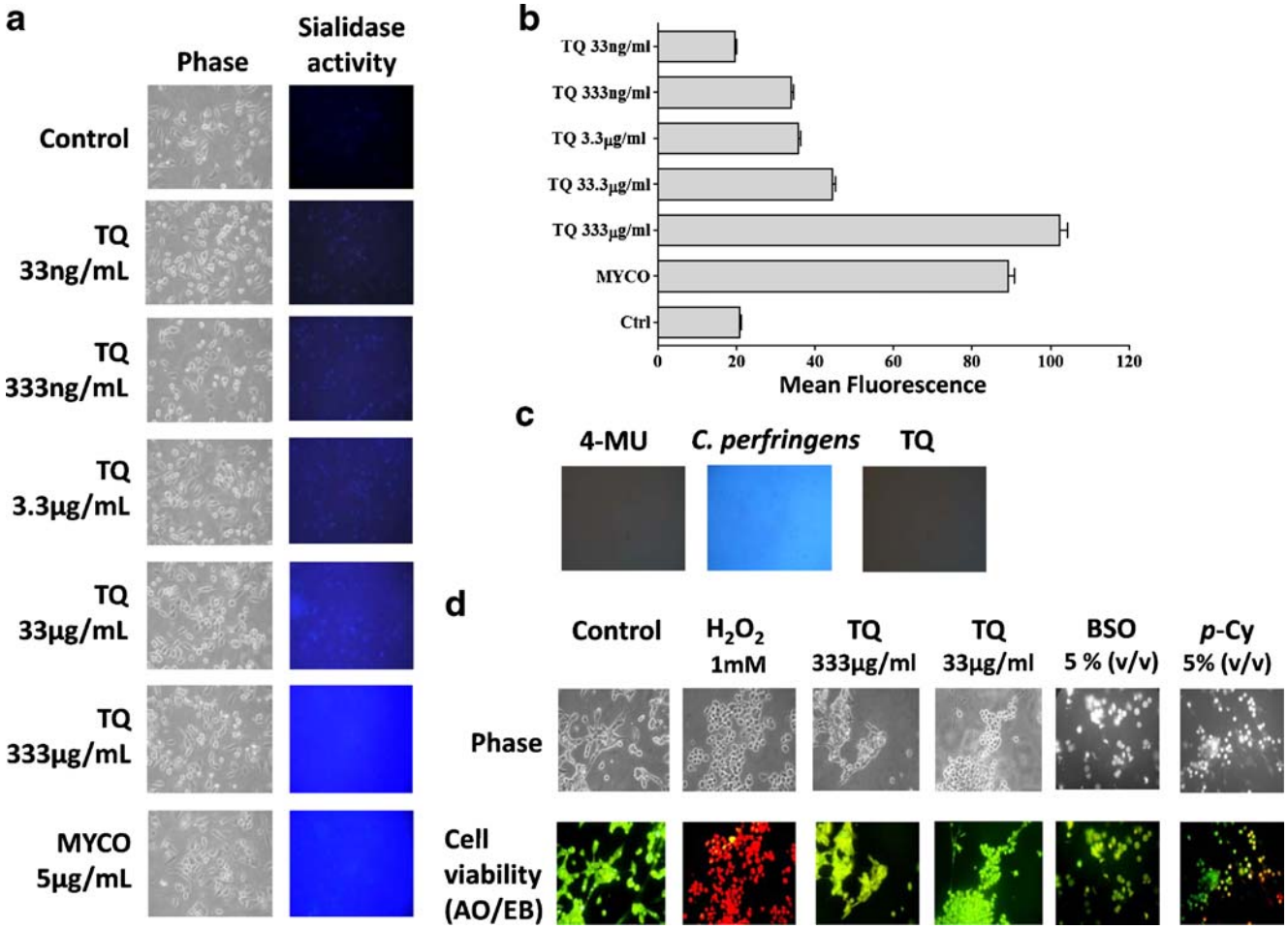


Fig. 2 TQ induces sialidase activity in BMC-2 macrophage cells in a dose dependent manner. **a** TQ-induced sialidase activity in BMC-2 cells at the indicated doses was measured as described in Fig. 1a. Sialidase activity associated with TLR-2 ligand MYCO (5 µg/ml killed *Mycobacterium butyricum* cells) treated live BMC-2 cells is used as a positive control. Fluorescent images were taken at 2 min after adding substrate using epi-fluorescent microscopy (40× objective). **b** The mean fluorescence surrounding the cells for each of the images was measured using Image J Software. The data are a representation of one out of three independent experiments showing similar results. **c** TQ alone does not hydrolyze substrate to give free 4-methylumbelliferone. A positive control neuraminidase (*Clostridium perfringens*) (2 µL; with a specific activity of 1 U per 1 mmole of *N*-acetylneuraminic acid per minute) was added to 2 µL of 4-MUNANA substrate. Fluorescent images were taken at 1 min after adding substrate using epi-fluorescent microscopy (40× objective). **d** Cell Viability in HEK-TLR4 cells treated with TQ, BSO and p-Cy. HEK-TLR4 cells were grown on 12 mm circular glass slides in medium containing 5% fetal calf serum and 0.8 µg/mL G418 for 24 h at 37°C in 5% CO_2. The medium was removed and cells were left alone (control), or treated with TQ, BSO, p-Cy or 1 mM hydrogen peroxide (H_2O_2) for 1 h. Cell viability was assessed by acridine orange/ethidium bromide (AO/EB) staining and fluorescence microscopy. The data represent one of two independent experiments showing similar results

cells [8]. We also show here that Tamiflu treatment of BMC-2 cells for 30 min followed with TQ stimulation had no marked effect on the morphology of the cells (Fig. 4d).

Neu4 sialidase activity is associated with TQ treatment of live macrophage cells

To identify the mammalian sialidase associated with TQ treatment of live macrophage cells, we used a recently developed assay to detect sialidase activity on the cell surface of viable cells [8, 9] using neutralizing antibodies against the four known human sialidases as described previously [8]. They are lysosomal Neu1 [21–27], cytosolic Neu2 and the plasma membrane bound Neu3 [28–30]. The fourth sialidase Neu4 is localized to either the mitochondrial [31] compartment or the lysosomal lumen [32]. As shown in Fig. 5a, rabbit anti-human Neu4 polyclonal antibody blocked TQ-induced sialidase activity in live human HEK-TLR4/MD2 cells compared with the TQ positive control. In contrast, antibodies against human Neu-1, -2 or -3 had no or limited inhibitory effect on TQ-induced sialidase activity in live HEK-TLR4/MD2 cells. In this sialidase assay, the activity is revealed by a fluorescence (λem 450 nm) surrounding the live cells treated

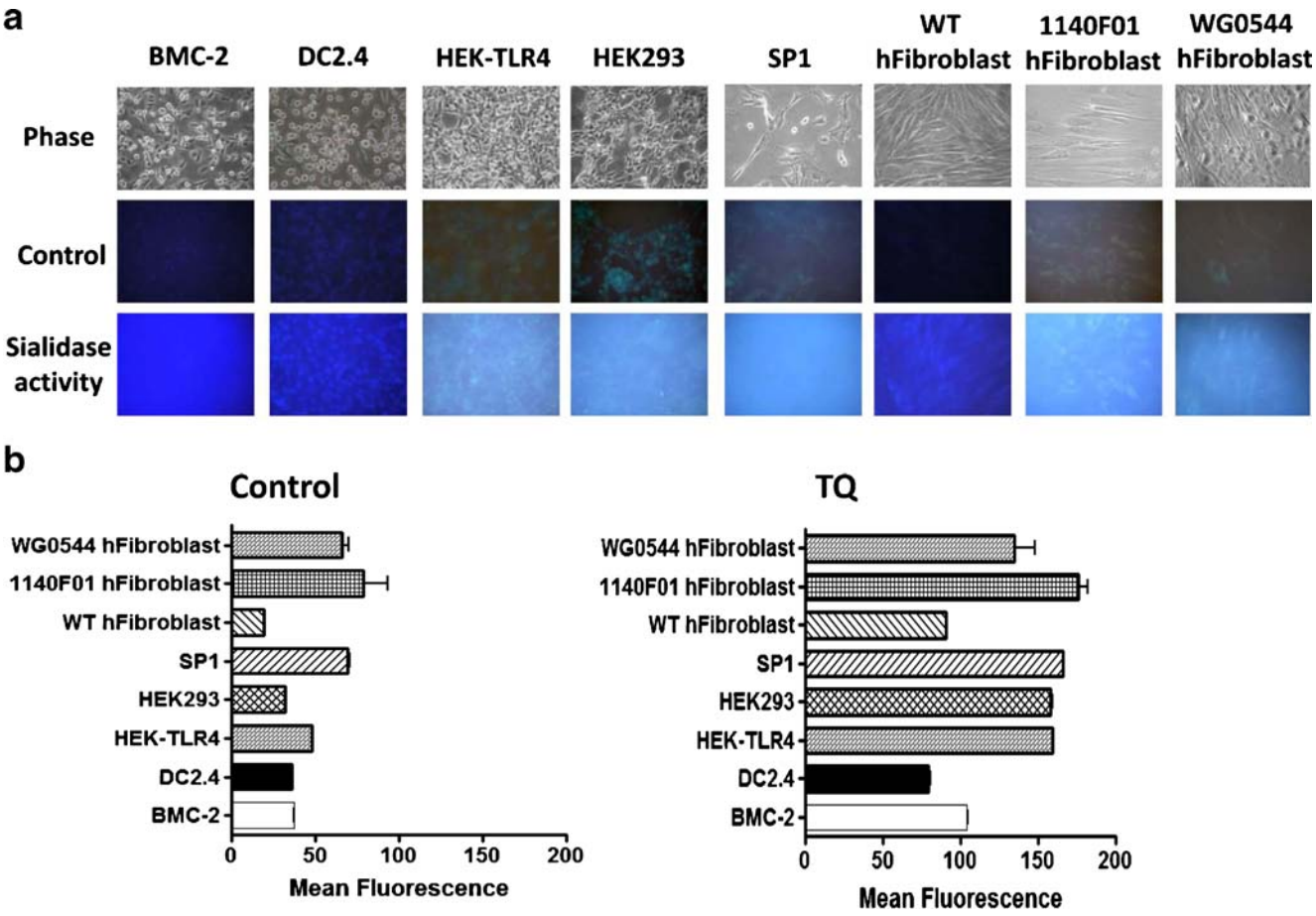


Fig. 3 **a** TQ induces sialidase activity in live BMC-2macrophage cells, DC2.4 dendritic cells, HEK-TLR4/MD2 cells, HEK293, SP1 cells and human WT and sialidosis fibroblast cells. TQ-induced sialidase activity in live indicated cells was measured as described in Fig. 1a. Fluorescent images were taken at 2 min after adding substrate using epi-fluorescent microscopy (40× objective). **b** The mean fluorescence surrounding the cells for each of the images was measured using Image J Software. The data are a representation of one out of three independent experiments showing similar results

with the fluorogenic sialidase substrate, 4MU-NeuAc (2′-(4-methylumbelliferyl)-α-*N*-acetylneuraminic acid) and caused by the emission of 4-methylumbelliferone. The data indicate that anti-Neu4 antibodies inhibited TQ-induced sialidase activity on the cell surface. Live cells treated with the substrate alone did not show any fluorescence surrounding the cells. Instead, they revealed an internal blue fluorescence indicating substrate internalization by the cell revealing internal sialidase activity. These results are consistent with our previous report [8].

To confirm these results, we used primary BM macrophages derived from the Neu4 knockout (Neu4 KO) mice [14] in addition to primary macrophage cells obtained from wild-type (WT) and hypomorphic cathepsin A mice with the secondary ~90% reduction of the Neu1 activity (NeuI KD) [15]. After 7 days in culture with conditioned medium containing monocyte colony stimulating factor (M-CSF) [16], the primary macrophage cells were treated with TQ to induce sialidase activity. The data indicated that TQ induced sialidase activity within 1 min in live primary WT and Neu1-deficient macrophage cells, but not in Neu4 KO cells (Fig. 5b). These results with the Neu1-deficient macrophage cells are consistent with the data in Fig. 3a using 1140F10 and WG0544 type 1 sialidosis human fibroblast cells. These observations suggest that TQ-induced sialidase activity in live primary Neu1-deficient macrophage cells and sialidosis fibroblast cells does not involve Neu1 sialidase. Taken together, the results with the anti-Neu4 antibodies and primary Neu4 KO macrophage cells clearly identify Neu4 sialidase associated with TQ treatment of live cells.

Neu4 sialidase is expressed on the cell surface in naive primary macrophage cells and type I sialidosis fibroblast cells

Since Neu4 sialidase is categorized as a lysosomal and mitochondrial storage enzyme and it is induced within a

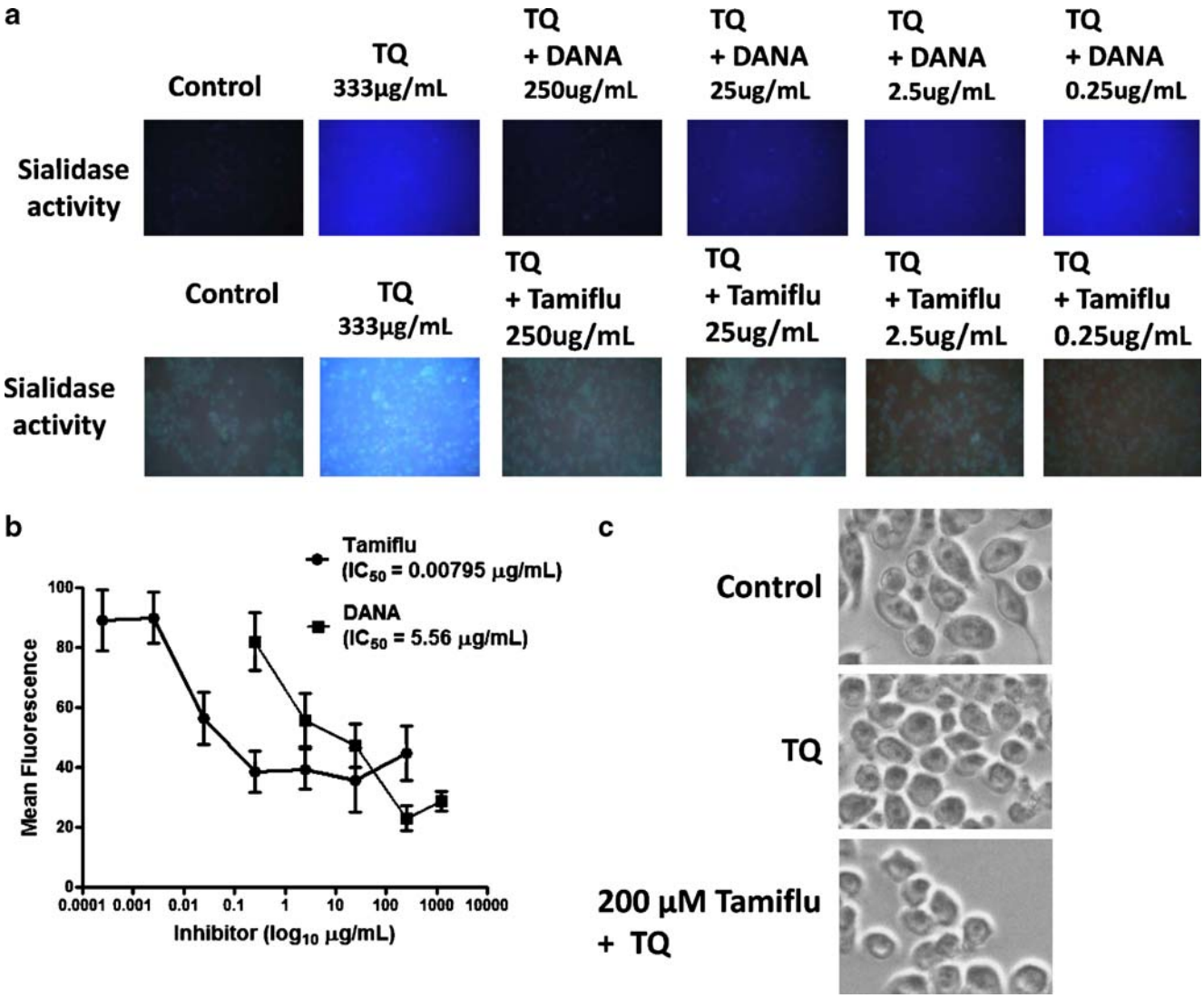


Fig. 4 **a** Tamiflu completely inhibits TQ-induced sialidase activity in BMC-2 macrophage cells in a dose dependent manner, while the neuraminidase inhibitor DANA has a limited inhibitory effect. TQ-induced sialidase activity in BMC-2 cells was measured as described in Fig. 1a. Fluorescent images were taken at 2 min after adding 0.318 mM 4-MU substrate together with TQ and indicated neuraminidase inhibitors (Tamiflu and DANA) using epi-fluorescent microscopy (40× objective). The data are a representation of one out of three independent experiments showing similar results. **b** The 50% inhibition concentration (IC_{50}) for Tamiflu and DANA on sialidase activity induced by TQ in live BMC-2 cells. The mean fluorescence surrounding the cells for each of the images was measured using Image J software. The 50% inhibitory concentration (IC_{50}) of each compound was determined by plotting the decrease in sialidase activity against the log of the agent concentration. **c** Cell morphology of BMC-2 cells treated with TQ and Tamiflu. BMC-2 cells were grown on a 24 well plate in DMEM medium containing 5% fetal calf serum for 6 h at 37°C in 5% CO_2. After removal of medium, 200 µM Tamiflu was added to the cells for 30 min followed with 100 µg/ml TQ. Pictures were taken immediately on inverted microscope under phase contrast using Micron photo software. This experiment was done in duplicate

minute by TQ, we asked whether or not Neu4 is expressed on the cell surface. Using fluorescence microscopy, Neu4 was found on the cell surface in naive, paraformaldehyde fixed and non-permeabilized BMC-2 macrophage cells (Fig. 5c). The immunolocalization of Neu4 to the cell surface was also confirmed by flow cytometry using live human WT and WG0544 sialidosis fibroblast cells immunostained with anti-Neu4 antibodies and Alexa488 conjugated secondary F(ab')$_2$ antibodies (Fig. 5d).

Neu4 sialidase activation associated with TQ treated live cells involves the potentiation of GPCR-signaling via membrane targeting of Gαi subunit proteins and matrix metalloproteinase-9 (MMP-9) activation

The results shown in Fig. 4 indicate that DANA inhibits TQ-induced sialidase activity with an IC_{50} of 19.1 µM. DANA is also an inhibitor of plasma membrane Neu3 sialidase (PMGS) [33], which is specific for gangliosides as

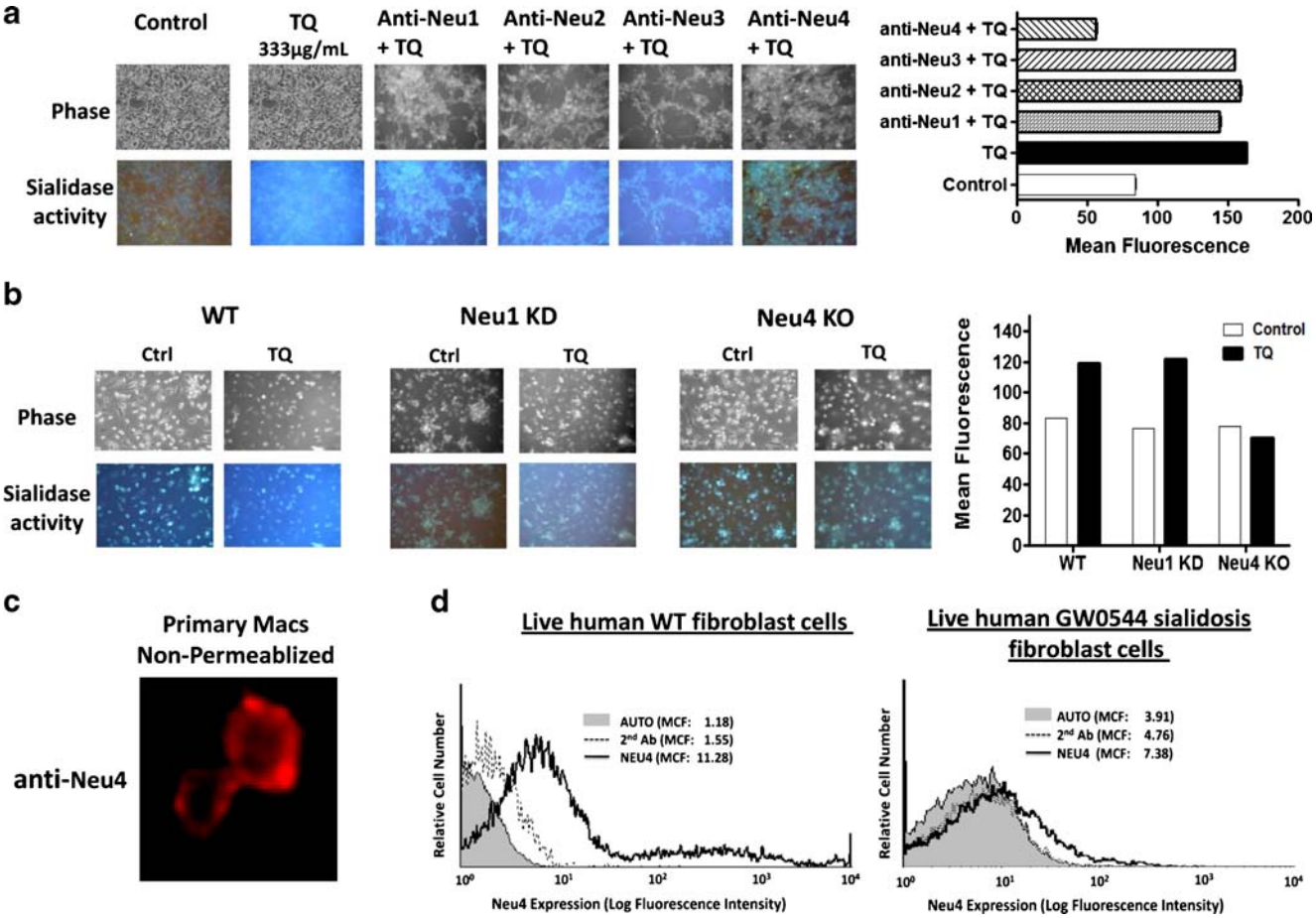


Fig. 5 **a** Anti-Neu4 neutralizing antibodies inhibit TQ-induced sialidase activity in live HEK-TLR4/MD2 cells. Cells were allowed to adhere on 12 mm circular glass slides in media containing 5% horse and 3% fetal calf sera and G418 for 24 h in a humified incubator at 37°C and 5% CO_2. After removing media, 0.318 mM 4-MUNANA substrate in Tris buffered saline pH 7.4 was added to live cells alone (Control), with 333 μg/ml TQ or with anti-Neu1 (25 μg/ml), anti-Neu2 (25 μg/ml), anti-Neu3 (25 μg/ml), or anti-Neu4 (25 μg/ml) neutralizing antibodies in combination with TQ using epi-fluorescent microscopy (40× objective). The substrate is hydrolyzed by cell surface sialidase to give free 4-methylumbelliferone, which has a fluorescence emission at 450 nm (*blue color*) following excitation at 365 nm. Fluorescent images were taken at 2 min after adding substrate using epi-fluorescent microscopy (40× objective). The mean fluorescence surrounding the cells for each of the images was measured using Image J software. The data are a representation of one out of four independent experiments showing similar results. **b** TQ induces sialidase activity in primary mouse BM macrophage cells. Primary BM macrophage cells obtained from normal, wild-type (WT), Neu1-CathA KD (Neu1 deficient and cathepsin A deficient), and Neu4 KO (Neu4 knockout) mice were cultured in conditioned medium supplemented with 20% (v/v) M-CSF, 10% FCS and Penn/Strep/Glut for 7 days to 8 days on circular glass slides in 24 well tissue culture plates. After removing media, 0.318 mM 4-MU substrate was added to cells as in Fig. 1. The mean fluorescence surrounding the cells for each of the images was measured using Image J software. The data are a representation of one out of two independent experiments showing similar results. **c** Neu4 on the cell membrane of primary BM macrophage cells. Untreated primary macs were fixed with 4% paraformaldehyde, nonpermeabilized and immunostained with anti-Neu4 antibodies followed with Alexa594 conjugated secondary antibody. Stained cells were visualized using epi-fluorescence microscopy at objective 40×. The cell image panel was enlarged with Adobe Photoshop. **d** Flow cytometry analysis of Neu4 expressed on the cell surface of live human WT and WG0544 sialidosis fibroblast cells. Histograms show staining with anti-Neu4 antibodies after incubation on ice for 15 min and followed with Alexa488 conjugated $F(ab')_2$ secondary antibodies for additional 15 min on ice. Control cells were stained with Alexa488 conjugated $F(ab')_2$ secondary antibodies for 15 min on ice or untreated cells (auto). Cells were analyzed by Beckman Coulter Epics XL-MCL flow cytometry and Expo32 ADC software (Beckman Coulter). Overlay histograms are displayed. Live untreated cells are represented by gray filled histogram. Control Alexa488 secondary antibodies treated live cells are represented by the unfilled gray dashed line. Live cells stained with anti-Neu4 antibodies are depicted by the unfilled histogram with the black line. The mean channel fluorescence (MCF) for each histogram is indicated for 30,000 acquired cells (80% gated)

well cytosolic sialidase Neu2 [34]. Since cholera toxin subunit B (CTX-B) targets GM1 ganglioside and blocks Neu3 interaction, we asked whether or not CTX-B would have any inhibitory effect on TQ-induced sialidase activity in live cells. The GM1 ganglioside specific CTX-B applied to BMC-2 cells had no inhibitory effect on TQ-induced sialidase activity (Fig. 6a). In addition, cholera toxin (CTX), an inhibitor of the active form of Gs (α subunit) of GPCR,

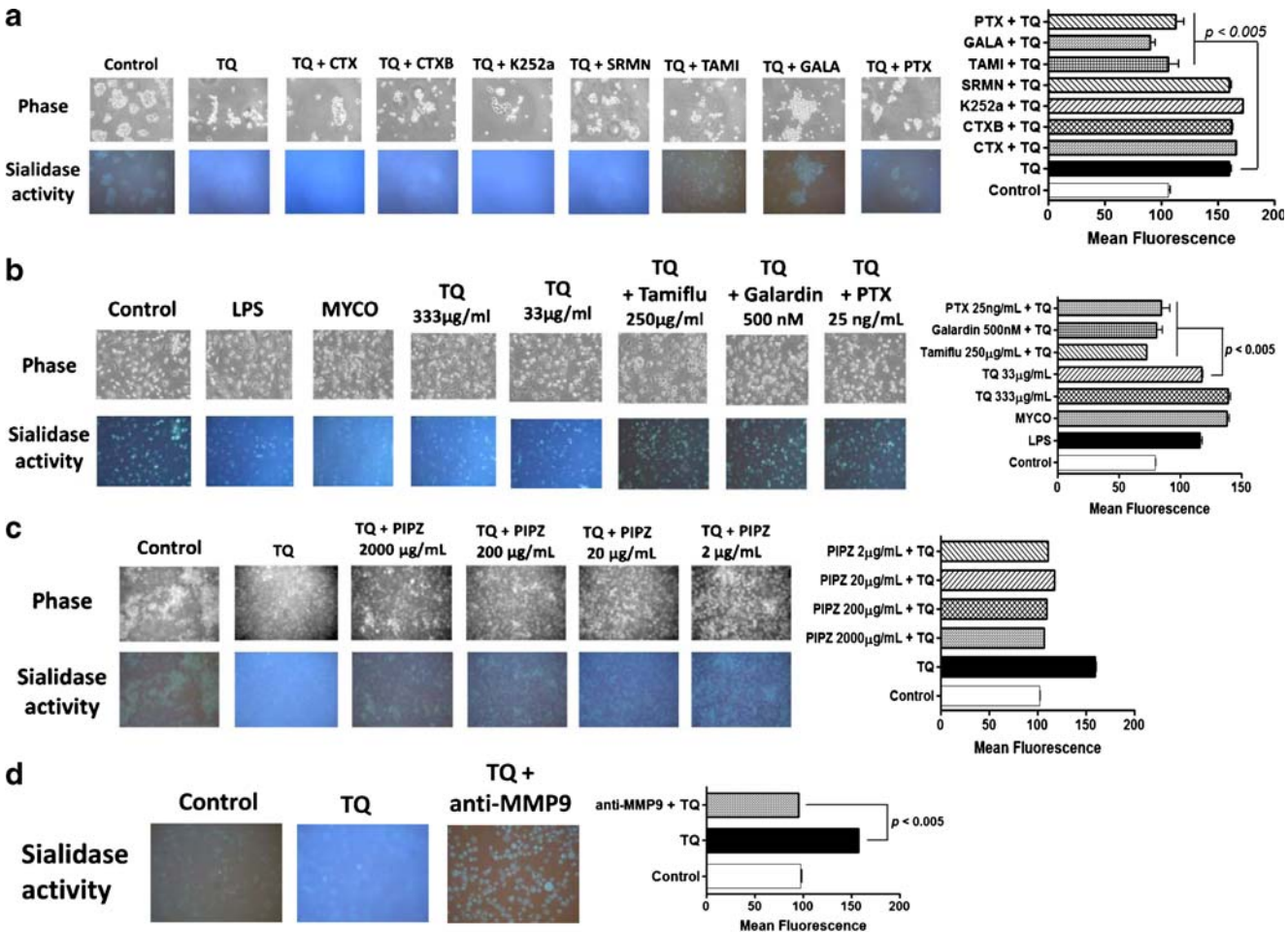


Fig. 6 **a** TQ-induced sialidase activity is blocked by specific inhibitors against GPCR Gαi subunit and MMP inhibitors in BMC-2 macrophage cells. Cells were incubated on 12 mm circular glass slides in medium containing 5% horse and 3% fetal calf serum for 5 h at 37°C. After removal of medium, 0.2 mM 4-MUNANA (4-MU) substrate in Tris buffered saline pH 7.4 was added with mounting medium to cells alone (Control), with 333 µg/ml TQ, or TQ in combination with 62.5 µg/ml cholera toxin (CTX), 0.625 mg/ml cholera toxin subunit B (CTXB), 0.2 µM K252a, 100 µM suramin (SRMN), 250 µg/ml Tamiflu (TAMI), 500 nM Galardin (GALA) or 25 ng/ml pertussis toxin (PTX). Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40× objective). The mean fluorescence surrounding the cells for each of the images was measured using Image J software. *P* values represent significant differences at 95% confidence using the Bonferroni's multiple comparison tests to compare all treatments. The data are a representation of one out of six independent experiments showing similar results. TQ-induced sialidase activity is blocked by specific inhibitors against GPCR Gαi subunit and MMP inhibitors in primary BM macrophage cells (**b**) and BMC-2 cells (**c**). Primary BM macrophage cells were obtained as described in Fig. 5b. TQ-induced sialidase activity in combination with the indicated inhibitors in primary BM macrophage cells and BMC-2 cells at the indicated doses was measured as described in Fig. 1a. Sialidase activity associated with TLR4 ligand LPS (5 µg/ml LPS) and TLR-2 ligand MYCO (5 µg/ml killed *Mycobacterium butyricum* cells) treated live BMC-2 cells were used as positive controls. C. MMP inhibitor Piperazine (PIPZ) was used to inhibit TQ-induced sialidase activity in BMC-2 cells in a dose dependent manner. Fluorescent images were taken at 2 min after adding substrate using epi-fluorescent microscopy (40× objective). The mean fluorescence surrounding the cells for each of the images was measured using Image J Software. The data are a representation of one out of three independent experiments showing similar results. **d** Anti-MMP-9 antibodies inhibited TQ-induced sialidase activity in BMC-2 cells. After removal of medium, cells were treated using 0.2 mM 4-MU and DAKO with cells alone (Control), 250 µg/ml TQ, or 250 µg/ml TQ and 25 µg/ml rabbit anti-human anti-MMP9 antibody. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40× objective). *P* values represent significant differences at 95% confidence using the Bonferroni's multiple comparison tests to compare all treatments. These figures represent one of three individual experiments

suramin, an inhibitor of protein tyrosine phosphatase and broad range GPCR and K252a, an inhibitor of protein tyrosine kinase had limited inhibitory effect on TQ-induced sialidase activity in live cells. In contrast, galardin (GM6001), a broad specific inhibitor of MMP-1, -2, -3, -8 and -9, and pertussis toxin (PTX), a specific inhibitor of G_{i2} and G_{i3} (α subunits) of G protein subtypes completely blocked TQ-induced sialidase activity in live BMC-2 (Fig. 6a) and primary BM macrophage cells (Fig. 6b). In addition, piperazine (a MMP inhibitor II), which specifically inhibits MMP-1, -3, -7 and -9 completely blocked TQ-induced sialidase activity when applied to BMC-2 cells (Fig. 6c).

Taken together, we deduce that a specific MMP(s) that may be playing a role in alliance with Gαi proteins activating sialidase was likely MMP-1, MMP-3 or MMP-9. When anti-MMP-9 antibodies were applied to BMC-2 cells, TQ-induced sialidase activity was completely blocked (Fig. 6d). These data collectively suggest an involvement of MMP-9 in TQ-induced activation of Neu4 sialidase.

To confirm these results, we used a human monocytic THP-1 cell line, which was pretreated with 10 ng/mL of 4-beta-phorbol 12-myristate 13-acetate (PMA) to induce differentiation of the monocytic cells to macrophages. These differentiated THP-1 cells were able to adhere to 12-mm circular glass slides in medium containing 10% fetal calf serum for 24 h. The cells were treated with 250 μg/mL TQ for 1 min in the presence or absence of antibodies or inhibitors, or left untreated as controls. The data shown in Fig. 7a indicate that antibodies specific for Neu4 and MMP-9, but not for Neu1 completely blocked TQ-induced sialidase activity in these live cells. In addition, Tamiflu, piperazine, galardin and the specifc inhibitor of MMP-9, but not the inhibitor of MMP-3 completely blocked sialidase activity when the cells were treated with TQ.

Since most studies describing the expression of MMP-9 on the cell surface use growth factors or phorbol esters, we asked whether or not MMP-9 is expressed on the cell surface in live, naive and untreated THP-1 cells. The immunolocalization of MMP-9 as well as Neu4 on the cell surface of these cells was confirmed by flow cytometry using live cells immunostained with anti-MMP-9 or anti-Neu4 antibodies followed with Alexa Fluor488 conjugated $F(ab')_2$ secondary antibodies. The data shown in Fig. 7b clearly indicate that 30,000 acquired live, untreated mono-

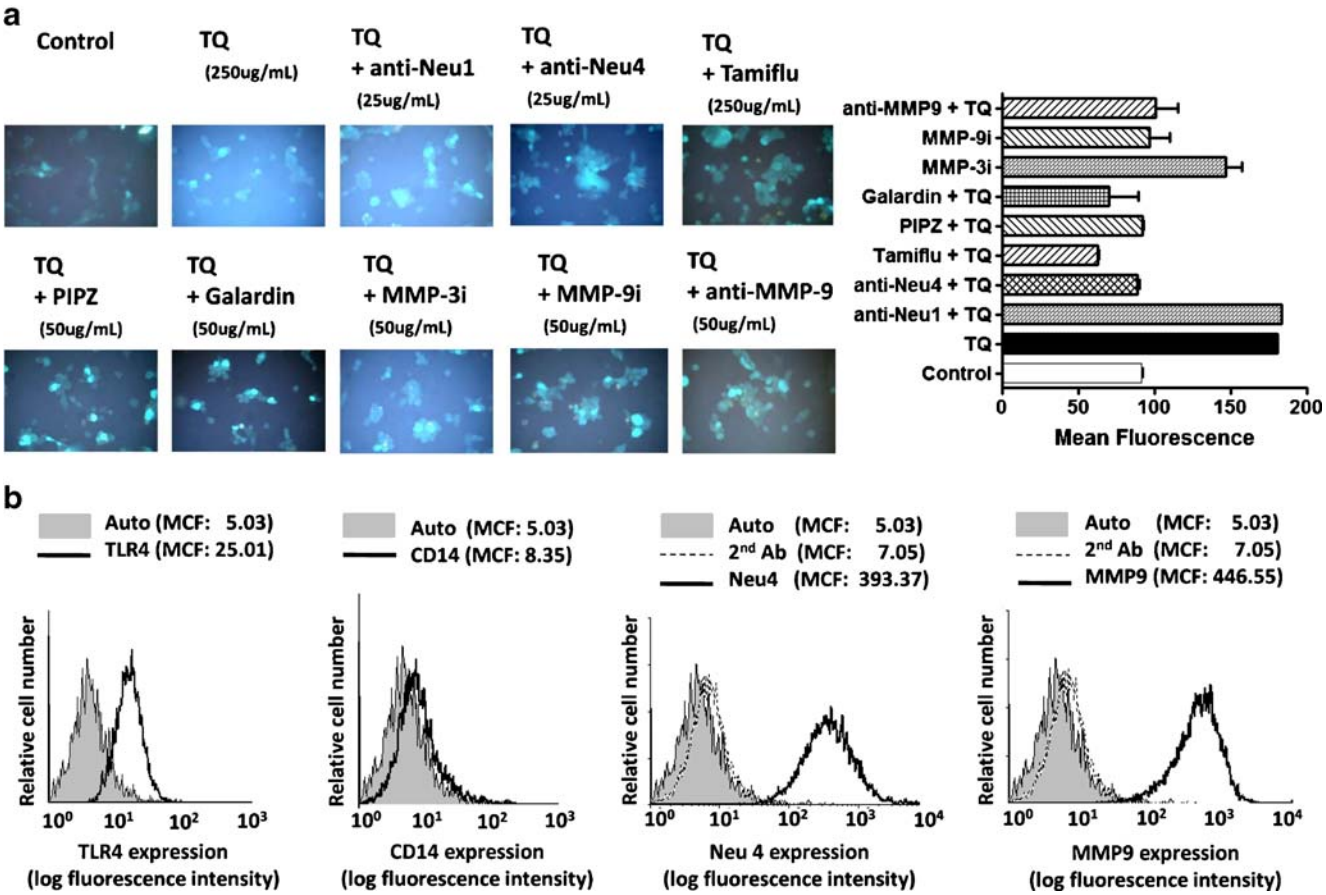


Fig. 7 **a** TQ-induced sialidase activity is blocked by specific inhibitors against GPCR Gαi subunit and MMP inhibitors in human THP-1 monocyte cells. TQ-induced sialidase activity in combination with the indicated inhibitors in THP-1 cells was measured as described in Fig. 1a. **b** Flow cytometry analysis of Neu4 and MMP-9 expressed on the cell surface of live human THP-1 cells. Histograms show staining with fluorescein (FITC) conjugated anti-TLR4, FITC conjugated anti-CD14, anti-Neu4, anti-MMP-9 antibodies after incubation on ice for 15 min and followed with Alexa488 conjugated $F(ab')_2$ secondary antibodies for additional 15 min on ice. Control cells were stained with Alexa488 conjugated $F(ab')_2$ secondary antibodies for 15 min on ice or untreated cells (auto). Cells were analyzed by Beckman Coulter Epics XL-MCL flow cytometry and Expo32 ADC software (Beckman Coulter). Overlay histograms are displayed. Live untreated cells are represented by gray filled histogram. Control Alexa488 secondary antibodies treated live cells are represented by the *unfilled gray dashed line*. Live cells stained with anti-Neu4 antibody are depicted by the unfilled histogram with the *black line*. The mean channel fluorescence (MCF) for each histogram is indicated for 30,000 acquired cells (80% gated)

cytic THP-1 cells showed significant immunostaining for both Neu4 and MMP-9 expression on the cell surface.

TQ-induced Neu4 desialylation of natural gangliosides and mucin substrates

Since Neu4 is expressed on the cell surface of THP-1 cells as well as on WT and sialidosis fibroblast cells, we asked whether TQ-induced Neu4 sialidase activity is able to hydrolyze sialic acids from natural gangliosides and mucin substrates in addition to our observations with the artificial 4-MUNANA substrate. Here, we utilized a standardized sialic acid kit that uses an enzyme coupled reaction in which the free sialic acid is oxidized resulting in the development of the Oxi-Red probe to give fluorescence (ex/em 535/587). The sialic acid concentration of the test samples was calculated from a standard curve after subtracting background and using the sample volume in the assay. When live BMA macrophage cells were treated with TQ in the presence of total gangliosides or mucin for 5 min, the substrate surrounding the live cells was tested for the released sialic acids. We were not able to detect free sialic acids from either natural substrate (data not shown). This may be due to the detection sensitivity of the kit (1 μM concentration of free sialic acid). Instead, we treated 10^6 THP-1 cells with TQ for 5 min or left them untreated as controls. The cells were washed once, pelleted and lysed in lysis buffer. To 25 μL of cell lysates was added 25 μL of 500 μg/mL mucin or total ganglioside extract for 30 min and the free sialic acid released was measured according to protocol of the sialic acid (NANA) assay kit (BioVision Research Products, Mountain View, CA). As shown in Fig. 8a, TQ-induced Neu4 sialidase activity hydrolyzes sialic acid from total gangliosides extracts from porcine brain and mucin substrates.

Comparison of Neu3 and Neu4 mRNA and protein levels in human monocytic THP-1 cells

We also questioned whether there might be an indirect influence due to the plasma membrane-associated Neu3 sialidase when the total gangliosides substrate was used. Immunocytochemistry analyses shown here demonstrate a higher proportion of Neu4 to Neu3 expression on the cell surface of BMC-2 macrophages and primary mouse BM macrophage cells (Fig. 8b). In addition, we performed flow cytometry to detect Neu3 and Neu4 protein expressions on the cell surface of live human monocytic THP-1 cells. As shown in Fig. 8c, Neu3 was very minimally expressed on the cell surface compared to a very high expression of Neu4. These latter observations with Neu3 are consistent with other observations reporting that Neu3 is not always detected on the cell surface [35], but may be localized partially on the cell surface as a peripheral membrane protein [36]. Using western blot analyses on cell lysates from naive (media control) and 5-min TQ-treated THP-1 cells, the additional intracellular localization of Neu3 and Neu4 protein expressions validated the predicted absolute amounts of these proteins in THP-1 cells (Fig. 8e). Surprisingly, Neu3 protein was very minimally expressed in the cell lysates compared to β-actin as an internal protein control. To further analyze the Neu3 protein in the cell lysates, we immunoprecipitated it with anti-Neu3 antibodies and the immunocomplexes were isolated using protein A magnetic beads, resolved by SDS-PAGE and the blot probed with anti-Neu3 antibodies. The data shown in Fig. 8e indicate the presence of Neu3 protein in the cell lysates after immunoprecipitation. In contrast, the Neu4 protein is abundantly present in the cell lysates from naive, unstimulated and TQ-treated cells. Stomatos *et al.* have also shown using western blotting analyses that Neu3 protein is minimally expressed in primary human monocytes derived from peripheral blood mononuclear cells, but it increases in macrophages differentiated from these monocytes [37]. They did not analyze Neu4 protein expression in these cells, perhaps because the amount of Neu4-specific RNA declined by 6.7-fold during monocytic cell differentiation into macrophages [37]. Using human THP-1 cells, Liang *et al.* could not detect Neu4 specific-RNA in these cells as well as in the THP-1-derived macrophages, while Neu3 mRNA decreased by 3–4-fold during this differentiation process [22].

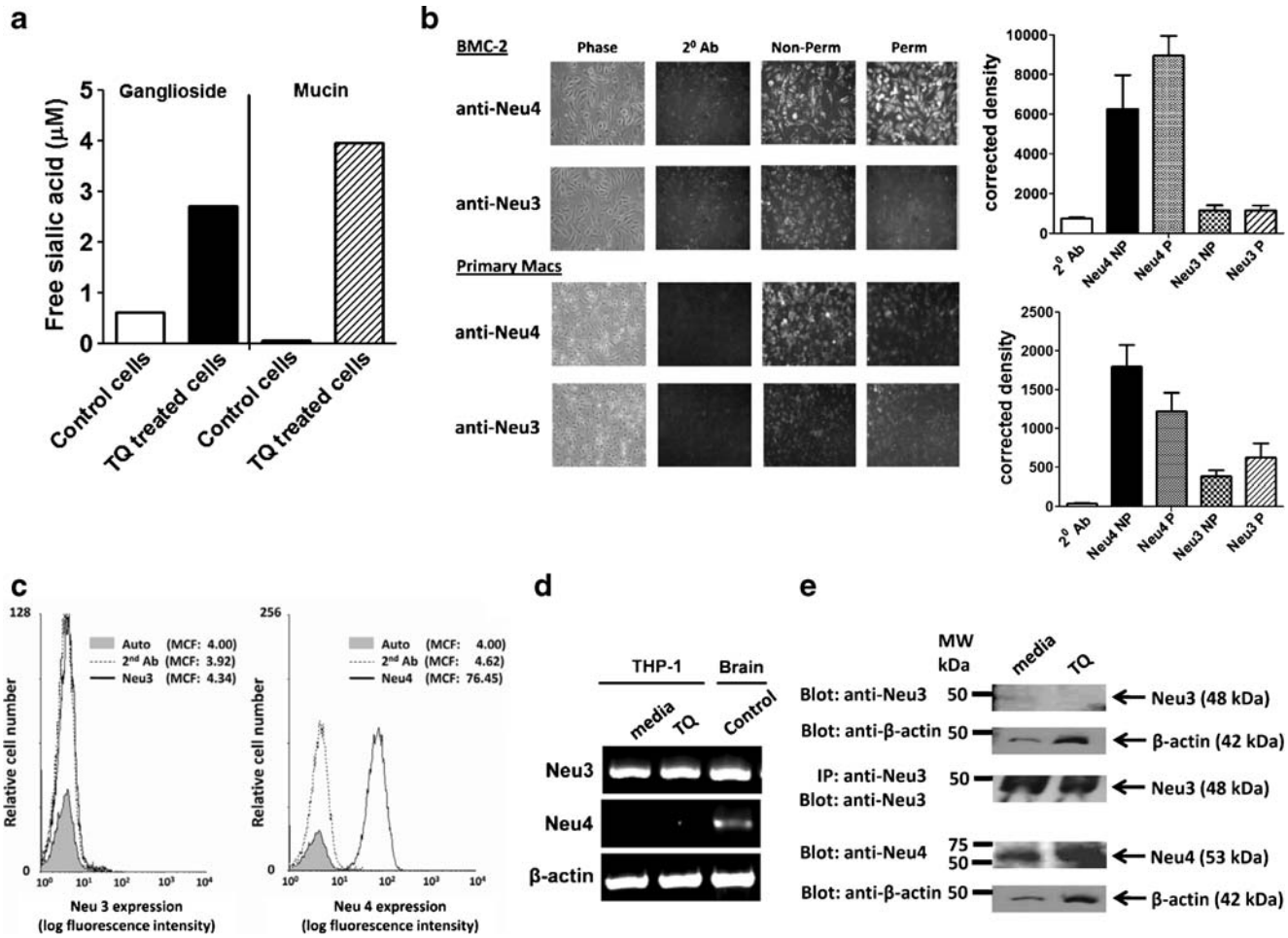
To determine whether the increased TQ-induced sialidase activity in THP-1 cells that was seen using 4-MUNANA and the natural substrates (Fig. 8a) was associated with increased expression of mRNA for Neu3 and Neu4, the relative amounts of these mRNAs in naive, untreated and 5-min TQ-treated THP-1 cells compared to RNA encoding β-actin was determined by RT-PCR. We used primers for Neu3 and Neu4 as described according to Bigi *et al.* [18]. We also used human brain total RNA as a positive control to detect mRNAs for Neu3 and Neu4 according to Shiozaki *et al.* [38] for Neu4 and Hasegawa *et al.* [39] for Neu3. As shown in Fig. 8d, the amount of RNA encoding Neu3 was clearly very high in relation to the amount of mRNA for β-actin, and there were no differences in the amount of Neu3 mRNA between the naive and TQ-treated cells. In contrast, the amount of RNA encoding Neu4 was absent in these cells whether they were treated with TQ or not. Taken together, these results are not only consistent with other reports [22], but also redefine the usefulness of mRNA expression values for Neu3 and Neu4 in a broad range of applications. The data provide evidence to support the premise that there is definitively no correlation between mRNA and protein values for Neu3 and Neu4 at least for human monocytic THP-1 cells.

Conclusions

Here, we report for the first time a novel activation of Neu4 sialidase on the cell surface of live macrophage, dendritic, and fibroblast cells by thymoquinone derived from the extract of *Nigella sativa* black seed (cumin) oil. The findings also provide evidence for the potentiation of GPCR-signaling via membrane targeting of Gαi subunit proteins and matrix metalloproteinase-9 activation in inducing Neu4 sialidase by TQ on the cell surface. Central to this process is that Neu4 in alliance with GPCR-signaling Gαi subunit proteins and MMP-9 is expressed on the cell surface of human monocytic THP-1 cells, human WT and WG0544 sialidosis type I fibroblast cells and BMC-2 macrophage cells. This tripartite alliance would actually make Neu4 readily available to be induced by TQ. Our data support this premise, because the Neu4 sialidase activity induced by TQ treated live cells occurs within a minute. How Neu4 sialidase is rapidly induced by MMP-9 together with GPCR Gαi subunit proteins remains unknown. Perhaps, it can be speculated that TQ binding to the cell surface GPCR Gαi subunit proteins may lead to GPCR-signaling sufficient to activate MMP. It is well known that agonist-bound GPCRs have been shown to activate numerous MMPs [40], including MMP-3 [41], MMPs 2 and 9 [42, 43], as well as members of the ADAM family of metalloproteases: ADAM10, ADAM15, and ADAM17 [44, 45]. However, the precise molecular mechanism(s) underlying GPCR-mediated MMP activation still remains unknown.

In this report, the data reveal an unpredicted expression of Neu4 on the cell surface of monocytic and fibroblast cells. Others have shown that Neu4 is a lysosomal lumen sialidase [19] as well as a mitochondrial sialidase [31] encoded by the *NEU4* gene on human chromosome-2. With regard to the human NEU4 ortholog, it appears to consist of two isoforms differing in their possession of 12N-terminal amino acid residues within the mitochondria. These Neu4 isoforms are also differentially expressed in a tissue-specific manner. In the brain, muscle and kidney, they contain both the long and short forms, while in the liver and the colon they are predominantly the short form as revealed by RT-PCR [46]. Both isoforms of Neu4 possess broad substrate specificity, including activity towards mucin. In addition, Neu4 may be involved in cell apoptosis or neural differentiation [46]. Their data suggest that the long isoform of mitochondrial Neu4 probably regulates the level of ganglioside GD3, which is known to be an apoptosis-related ganglioside. We also tested whether TQ-induced Neu4 sialidase activity in human monocytic THP-1 cells had natural substrate specificity for total ganglioside from procine brain extract and for mucin from bovine submaxillary glands (type I-S). TQ-induced sialidase activity in the cell lysates clearly showed substrate specificity for ganglioside and mucin (Fig. 8c). The

Fig. 8 **a** TQ-induced sialidase activity hydrolyzes sialic acid from natural gangliosides and mucin. THP-1 cells (1×10^6 cells) were treated for 5 min with 250 μg/mL TQ in 57% DMSO and Tris buffer pH 7.4 or left untreated as controls. The cells were washed once, pelleted and lysed in lysis buffer. To 25 μL of cell lysates was added 25 μL of 500 μg/mL mucin or total ganglioside extract for 30 min and the free sialic acid released was measured according to protocol of the sialic acid (NANA) assay kit (BioVision Research Products). The kit utilizes an enzyme coupled reaction in which the free sialic acid is oxidized resulting in the development of the Oxi-Red probe to give fluorescence (ex/em 535/587) measured using a Varioskan spectrophotometric microplate reader (Thermo Electron Corp., Finland). The sialic acid concentration of the test samples in μM are calculated from a standard curve after subtracting background from control and the sample volume used in the assay. **b** Immunocytochemistry of Neu3 and Neu4 localization in permeabilized and non-permeabilized BMC-2 macrophage cells and primary BM macrophages. BMC-2 macrophage cells or primary BM macrophage cells were cultured on 12 mm circular glass slides for 24 h. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-X in Tris buffered saline or left non-permeabilized and immunostained with specific antibodies against Neu3 and Neu4 followed with respective Alexa Fluor conjugated secondary antibodies. Stained cells were visualized using an epi-fluorescent microscope with a 40× objective. Control images had secondary antibodies (2nd Ab) without the primary antibodies. Quantitative analysis was done by assessing the density of cell staining corrected for background in each panel using Corel Photo Paint 8.0 software. Each bar in the figures represents the mean corrected density of culture cell staining±SEM for equal ell density (5×10^5 cells) within the respective images.The data are a representation of one out of two independent experiments showing similar results. **c** Flow cytometry analysis of Neu3 and Neu4 expressed on the cell surface of live human THP-1 cells. Histograms show staining with fluorescein (FITC) conjugated anti-Neu3 and anti-Neu4 antibodies after incubation on ice for 15 min and followed with Alexa488 conjugated $F(ab')_2$ secondary antibody for additional 15 min on ice. Control cells were stained with Alexa488 conjugated $F(ab')_2$ secondary antibody for 15 min on ice or untreated cells (auto). Cells were analyzed by Beckman Coulter Epics XL-MCL flow cytometry and Expo32 ADC software (Beckman Coulter). Overlay histograms are displayed. Live untreated cells are represented by *gray filled histogram*. Control Alexa488 secondary antibody treated live cells are represented by the *unfilled gray dashed line*. Live cells stained with anti-Neu3 or anti-Neu4 antibodies are depicted by the unfilled histogram with the black line. The mean channel fluorescence (MCF) for each histogram is indicated for 30,000 acquired cells (80% gated). D. Expression of RNA-specific Neu3 and Neu4 in naive and TQ-treated THP-1 cells. THP-1 cells were cultured in medium or in the presence of 100 μg/mL of TQ for 5 min (TQ). Neu3 and Neu4 mRNA was evaluated by RT-PCR analysis using total RNAs from naive and TQ-treated cells. Amplification of β-actin mRNA served as an internal control for RNA loading. Clontech human brain total RNA was used as a positive control for Neu3 and Neu4 mRNA expression. The data are a presentation of one out of 12 independent experiments showing similar results. **e** Western blot and immunoprecipitation analyses of Neu3 and Neu4. THP-1 cells were treated with 100 μg/mL TQ for 5 min or left untreated as control (media). Cells were pelleted, lysed in lysis buffer and the protein lysates were resolved by SDS-PAGE. The blots were probed with antibodies against the indicated proteins. The blots were stripped and further probed with anti-β-actin antibodies. The protein lysates were also immunoprecipitated with anti-Neu3 antibodies for 18 h. Immunocomplexes were isolated using protein A magnetic beads, resolved by SDS-PAGE and the blot probed with primary anti-Neu3 anibodies followed with Clean-Blot IP Detection Reagent. The data are a representation of one out of five independent experiments showing similar results



flow cytometry analyses of Neu3 and Neu4 expression on the cell surface of live monocytic THP-1 cells also show a predominant Neu4 expression with negligible Neu3. Others have shown that unlike the bovine and mouse Neu3 sialidases, the human ortholog Neu3 is not always detected on the cell surface [35]. Although Miyagi *et al.* have recently eloquently reviewed this plasma membrane-associated Neu3 sialidase [47], others have suggested that Neu3 might be localized partially on the cell surface as a peripheral membrane protein and also in endosomal structures [36]. Their data were based on COS-7 cells transfected with *Mm*Neu3-HA and the cell-surface proteins were selected using biotinylated and isolated by avidin-biotin-affinity chromatography, solubilizing Neu3 from lipid bilayer by carbonate extraction or by Triton X-114 phase separation. Immunofluorescence localization experiments have been reported to show that Neu3 is localized at the plasma membrane and intracellularly in fixed, permeabilized COS-7 transfected with *Mm*Neu3-HA [36, 48] or with COS-7 cells transfected pCDNAI-NEU3 [49]. Using Western blot analyses of cell lysates from THP-1 monocytes during differentiation to macrophages, Liang *et al.* have shown that up-regulation of Neu1 and down-regulation of Neu3, whereas Neu3 mRNA decreased by 2–3-fold as revealed by RT-PCR data [22]. These latter results on Neu3 expression in THP-1 monocytic cells are consistent with our findings in concept, but they did not show direct evidence for Neu3 expression on the cell surface. Others have shown that T-cells have significant levels of Neu-1 and Neu-3 mRNAs, and western blot analyses and enzymatic comparison with recombinant sialidases have revealed that Neu-3 is induced as a major isoform in activated T-cells [50].

Furthermore, we have described in this report that there is no correlation between mRNA and protein values for Neu3 and Neu4 in human monocytic THP-1 cells. The RT-PCR data show that in naive THP-1 cells, the Neu3-specific RNA values were relatively high in comparison to the expression of β-actin as an internal control while Neu4-specfic RNA values were absence. However, western blot analyses of the cell lysates revealed that the Neu3 and Neu4 protein profiles did not correlate with the mRNA values. The results revealed a high Neu4 protein values and very low Neu3 protein expression. However, immunoprecipitation of Neu3 in the cell lysates revealed Neu3 protein (Fig. 8). Using primary human monocytes derived from peripheral blood mononuclear cells, Stamatos *et al.* have

also shown high Neu3 specific-RNA expression with relative amounts of Neu3 protein in the cell lysates [37]. However, others have shown that Neu3 mRNA expression is not universal in various cell lines [51]. Surprisingly, we have found a high Neu4 protein expression on the cell surface, which was not concomitant with a negligible Neu4 specific RNA. Taken together, these findings provide for the first time a varied post-transcriptional mechanism for these two mammalian sialidases. Although there have been attempts to correlate protein values with mRNA expression levels (see review [52]), one could speculate on the reason (s) for the poor protein and mRNA correlations for Neu3 and Neu4 in monocytic THP-1 cells. It would appear that Neu3 protein levels may have significant short half-lives compared to Neu4 as a result of varied protein synthesis and degradation. The cell may control the rates of degradation or even synthesis for Neu3 and Neu4 even when they have similar functions. Another possibility for high Neu4 protein values with no mRNA expression may lie in the levels of different protein subunits within protein complexes. This might be the case for Neu4 (long form) and Neu4 (short form) [18]. Although the long form of Neu4 localizes in and binds to the outer mitochondrial membrane and the short form is associated with the endoplasmic reticulum [18], it is unclear what is the precise nature of Neu4 isoform expression on the cell surface as observed in this report.

Seyrantepe *et al.* have demonstrated that Neu4 is ubiquitously expressed in human tissues and has broad substrate specificity for sialylated oligosaccharides, glycoproteins, and gangliosides [19]. They have shown that Neu4 is localized in the lysosomes by the mannose 6-phosphate receptor and does not require association with other proteins for enzymatic activity. Our data provide evidence for an unprecedented activation of Neu4 by TQ through GPCR-signaling via pertussis-sensitive Gαi proteins activation in inducing MMP-9. Using specific MMP-9 inhibitor, Neu4 sialidase activity associated with TQ treated live cells is completely blocked (Fig. 7a). Flow cytometry analyses also reveal that both Neu4 and MMP-9 are expressed on the cell surface of live macrophage and fibroblast cells. How MMP-9 influences the activation of Neu4 remains yet unknown. Our data also indicate that Neu4 is expressed on the cell surface of human sialidosis type I fibroblast cells, which are genetically deficient in Neu1. Indeed, TQ induces Neu4 sialidase activity in these live sialidosis type I fibroblast cells. These data are surprising, because primary structural analyses of Neu4 rules out the possibility of it being a transmembrane protein [18]. Thus, the subcellular distribution of Neu4 (short form) and Neu4 (long form) and their membrane anchoring mechanism still remains to be determined. Using protein extraction with Triton X-114 and sodium carbonate and cross-linking experiments, Bigi *et al.* demonstrated that both forms of NEU4 are actually extrinsic membrane proteins, anchored via protein-protein interactions [18]. Confocal microscopy and subcellular fractionation experiments have revealed that the long form localizes in the mitochondria bound to the outer mitochondrial membrane, while the short form is associated with the endoplasmic reticulum [18]. Interestingly, we provide data to suggest that Neu4 is also an extrinsic cell surface membrane protein, and the data suggest that Neu4 may be anchored there via its interaction with matrix metalloproteinase-9.

Seyrantepe *et al.* have also shown that Neu4 can completely eliminate undigested substrates of Neu1 and restored normal morphological phenotype of the lysosomal compartment in these Neu1-deficient sialidosis fibroblasts [19]. They observed that complete elimination of storage materials happened in 55% of sialidosis cells and in 25% of galactosialidosis cells (in addition 36% of galactosialidosis cells showed partially corrected phenotype), whereas only 3-5% of cells were transfected with Neu4 plasmid as assayed by immunohistochemistry. Most interestingly, TQ derived from the nutraceutical black cumin oil can activate Neu4 in these human Neu1-deficient sialidosis fibroblast cells through GPCR-signaling and MMP-9, and thus it would offer novel nutraceutical potential for therapy.

Since Neu1 sialidase is also a lysosomal enzyme, which has a unique orientation with the molecular multi-enzymatic complex that contains β-galactosidase and cathepsin A [15, 53–55] and elastin-binding protein (EBP) [56], we have recently shown that Neu1 and not Neu2, -3 and -4 forms a complex with TOLL-like receptors -2, -3 and -4 on the cell surface of naive macrophage cells [8]. The data provide evidence for a membrane controlling mechanism that is initiated by ligand binding to TLR-2, -3 and -4 to induce Neu1 sialidase activity within minutes in live primary bone marrow (BM) macrophage cells and macrophage and dendritic cell lines. The findings reveal an unprecedented mechanism of pathogen-molecule induced TLR activation and cell function [8]. Neu1 sialidase was proposed to be a key regulator of TLR activation to generate a functional receptor [8, 9]. Others have shown that Neu1 on the cell surface is tightly associated with a subunit of cathepsin A and the resulting complex influences cell surface sialic acid in activated cells and the production of IFNγ [23]. It has also been shown using Neu1-deficient mice that they produce markedly less IgE and IgG1 antibodies following immunization with protein antigens, which may be the result of their failure to produce IL-4 cytokine [57]. In another study, Neu1 was found to negatively regulate lysosomal exocytosis in hematopoietic cells where it processes the sialic acids on the lysosomal membrane protein LAMP-1 [24]. More recently, evidence is provided to suggest that cell surface Neu1 also activates phagocytosis in macrophages and dendritic cells presumably

through the desialylation of multiple receptors, including Fc receptors for immunoglobulin G (FcγR) [58].

Surprisingly, Tamiflu (oseltamivir phosphate) which is the ethyl ester pro-drug of oseltamivir carboxylate was found to be highly potent (IC_{50} of 0.00795 μg/mL; 0.019 μM) in inhibiting Neu4 activity induced by TQ treatment of live macrophage cells compared to an IC_{50} of 5.56 μg/mL (19.09 μM) for the neuraminidase inhibitor, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA). We have also shown that Tamiflu inhibited endotoxin LPS-induced Neu1 sialidase activity in live BMC-2 cells with an IC_{50} of 1.2 μM compared to an IC_{50} of 1,015 μM for its hydrolytic metabolite oseltamivir carboxylate [8]. Tamiflu blockage of LPS-induced Neu1 sialidase activity was not affected in BMC-2 cells pretreated with anticarboxylesterase agent clopidogrel. The antiplatelet agent clopidogrel has been previously determined to completely inhibit the hydrolysis of oseltamivir phosphate by carboxylesterase as much as 90% [59]. Other neuraminidase inhibitors like BCX1827, DANA, zanamivir and oseltamivir carboxylate had limited significant inhibition of the LPS-induced sialidase activity in live BMC-2 macrophage cells [8]. Studies using recombinant soluble human sialidases have shown that oseltamivir carboxylate scarcely inhibited the activities of the four human sialidases even at 1 mM [20], while zanamivir significantly inhibited the human Neu2 and Neu3 sialidases in the micromolar range. Furthermore, Nan *et al.* using lysates from mature dendritic cells have found that zanamivir completely inhibited Neu1 and Neu3 sialidase activity at 2 mM [23]. The reason(s) for this inhibitory potency of Tamiflu on Neu1 and Neu4 sialidase activity in live BMC-2 macrophage cells is unknown. For Neu1, however, it may be due to its unique orientation with the molecular multi-enzymatic complex that contains β-galactosidase and cathepsin A [15, 53–55, 60] and elastin-binding protein (EBP) [56], the complex of which would be associated within the ectodomain of TLR receptors [8]. Since Neu4 is not associated with other proteins for enzymatic activity [19], we think that Tamiflu might have unique inhibitory effects specifically for Neu1 and Neu4 sialidase activity.

Various studies have shown that the black seed (cumin) oil (BSO) has inhibitory properties such as anti-microbial, hypotensive, anti-nociceptive, anti-histaminic, immunomodulatory, anti-inflammatory, anti-tumour and anti-diabetic as well as many other characteristics [1]. We also determined whether BSO treatment of live macrophage cells in a dose dependent manner would inhibit Neu1 sialidase activity associated in response to TLR-4 ligand LPS. The live cell sialidase analyses (Fig. 1b) indicated this to be the case, suggesting that BSO has a very high potency of inhibition even at 5×10^{-7}% (v/v) of the LPS-induced Neu1 sialidase activity in live macrophage cells. In addition, para-cymene inhibited LPS-induced Neu1 sialidase activity in live macrophage cells. Surprisingly, both para-cymene and BSO had no inhibitory effects even at high doses on TQ-induced Neu4 sialidase activity in live macrophage cells (Fig. 1d). It is noteworthy that the BSO composition contains significant amounts of sterols consisting of β-sitosterol (69%), campesterol (12%) and stigmasterol (19%) as well as polyphenols and tocophenols. The seeds of *Nigella sativa*, known as black seed, black cumin and kalunji are commonly known to contain various compositions of protein (20%), fat (38%), moisture (5%), ash (4%), fibre (8%) and carbohydrate (32%). The seeds also contain 36–38% essential fixed oils, which are rich in fatty acids namely linoleic (C18:2), oleic (C18:1) and palmitic acids (C16:0). Of the many components of the essential fixed oils, the major ones are thymoquinone (28–57%), para-cymene (7–16%), carvacrol (6–12%), t-anethole (0.25–2.3%), 4-terpineol (2–7%) and longifoline (1–8%) [61] [62]. Thymoquinone is the main active constituent of the volatile oil extracted from *N. sativa* seeds [2, 63].

In conclusion, the data presented in this report suggest that thymoquinone (TQ), a derivative of the nutraceutical black seed (cumin) oil induces a vigorous Neu4 sialidase activity in live BMC-2 macrophage cells as well in live DC-2.4 dendritic cells, HEK-TLR4/MD2, HEK293, SP1 mammary adenocarcinoma cells, human WT and 1140F01 and WG0544 sialidosis fibroblast cells. This TQ-induced Neu4 sialidase activity hydrolyzes sialic acids not only from the artificial 4-MUNANA substrate but also natural gangliosides and mucins. The specific inhibitor of MMP-9 as well as anti-MMP-9 antibodies and anti-Neu4 antibodies, but not the specific inhibitor of MMP-3 completely block TQ-induced Neu4 sialidase activity in live THP-1 cells. Live fibroblast cells derived from sialidosis type 1 patients as well as human monocytic THP-1 cells express Neu4 and MMP-9 on the cell surface. Taken together, the results establish an unprecedented activation of Neu4 sialidase on the cell surface by TQ. The potentiation of GPCR-signaling by TQ via membrane targeting of Gαi subunit proteins and matrix metalloproteinase-9 activation may be involved in the activation process of Neu4 sialidase on the cell surface of live cells. The report also signifies a varied post-transcriptional mechanism for Neu3 and Neu4 mammalian sialidases even when they have similar functions. The cell may tightly control the rates of degradation or synthesis for Neu3 and Neu4 and this is not dependent on thymoquinone activation.

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Authors Contributions

M.R.S. and T.M.F. wrote the paper, designed and performed experiments; P.J. performed the free sialic acid assay and together with K.G. performed the flow cytometry; C.G. did the RT-PCR; A.G. did the Neu3 and Neu4 WB; R.B. generated HEK-TLR cells; S.R.A. helped with experiments and writing the paper; M.R.S. supervised the research design and the writing of the paper. All authors read and commented on the manuscript, and declare no competing financial interests.