RESEARCH ARTICLE

TNF differentially regulates ganglioside biosynthesis and expression in breast cancer cell lines

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Abstract

Gangliosides are glycosphingolipids concentrated in glycolipid-enriched membrane microdomains. Mainly restricted to the nervous system in healthy adult, complex gangliosides such as GD3 and GD2 have been shown to be involved in aggressiveness and metastasis of neuro-ectoderm derived tumors such as melanoma and neuroblastoma. GD3 synthase (GD3S), the key enzyme that controls the biosynthesis of complex gangliosides, was shown to be over-expressed in Estrogen Receptor (ER)-negative breast cancer tumors, and associated with a decreased overall survival of patients. We previously demonstrated that GD3S expression in ER-negative breast cancer cells induced a proliferative phenotype and an increased tumor growth. In addition, our results clearly indicate that Tumor Necrosis Factor (TNF) induced GD3S over-expression in breast cancer cells via NFkB pathway. In this study, we analyzed the effect of TNF on ganglioside biosynthesis and expression in breast cancer cell lines from different molecular subtypes. We showed that TNF up-regulated the expression of GD3S in MCF-7 and Hs578T cells, whereas no change was observed for MDA-MB-231. We also showed that TNF induced an increased expression of complex gangliosides at the cell surface of a small proportion of MCF-7 cells. These results demonstrate that TNF differentially regulates gangliosides expression in breast cancer cell lines and establish a possible link between inflammation at the tumor site environment, expression of complex gangliosides and tumor development.

Introduction

Gangliosides define as subclass of acidic glycosphingolipids (GSL) carrying one or more sialic acid residues in the carbohydrate moiety. Gangliosides are essential compounds of the outer leaflet of the plasma membrane, where they interact with phospholipids, cholesterol, and transmembrane proteins to form glycolipid-enriched microdomains [1] in which they interact with...
signaling molecules including receptors tyrosine kinases and integrins, and regulate signal transduction pathways involved in cell adhesion, proliferation, and recognition processes, [2–4]. The carbohydrate moiety of gangliosides is synthesized in the Golgi apparatus by specific glycosyltransferases (GT) and gangliosides are classified in four series according to the number of sialic acid residues linked to the lactosylceramide (Fig 1). Changes in ganglioside composition are observed between human tissues, complex gangliosides with two or more sialic acid residues being normally restricted to the nervous system [5,6]. Changes in the structure of gangliosides can also occur under pathological conditions [7–9] and a neo-expression of disialogangliosides such as GD2 and GD3 is observed in several cancers from neuroectoderm origin including melanoma and neuroblastoma, in which they play a key role in invasion and metastasis [10], and disialogangliosides are attractive targets for cancer immunotherapy [11,12].

In breast cancer, complex gangliosides GD3 and 9-O-acetyl-GD3 have been reported to be over-expressed in about 50% of invasive ductal breast carcinoma [13] and the GD3 synthase (GD3S) gene displayed higher expression among estrogen receptor negative breast cancer tumors [14], associated with poor pathohistological grading and a decreased free survival of patients [15]. We previously demonstrated that the expression of GD3S in breast cancer cells induced a proliferative phenotype and increased tumor growth due to the constitutive activation of c-Met receptor by GD2 ganglioside [16–18]. We also demonstrated that GD3S gene expression is up-regulated by TNF via the NFκB pathway and that estradiol repressed GD3S expression in estrogen receptor (ER) positive breast cancer cells by preventing NFκB nuclear translocation [19]. Moreover, GD2 ganglioside was recently identified as a new breast cancer stem cells specific marker [20].

Given the critical role of both GD2 ganglioside and inflammation in breast cancer aggressiveness [21], and in order to provide a general overview of the effect of inflammatory cytokines on ganglioside biosynthesis, we examined the effect of TNF on the expression of the main ganglioside-specific GT genes as well as cell surface gangliosides in breast cancer cells from different molecular subtypes.

**Materials and methods**

**Antibodies**

Anti-GM3 mAb GMR6 (mouse IgM), anti-GM2 mAb MK1-16 (mouse IgM) and anti-GD1b GGR12 (mouse IgG3) were purchased from AMS Biotechnology (Abingdon, UK). Anti-GD3 mAb R24 (mouse IgG3) and anti-GD2 mAb 14.G2a (mouse IgG2a) were purchased from Abcam (Cambridge, MA, USA). Fluorescein isothiocyanate (FITC)-conjugated cholera toxin B subunit from *Vibrio cholerae* used for GM1a expression analysis was from Sigma-Aldrich (Saint-Quentin Fallavier, France). Alexa Fluor® 488 donkey anti-mouse IgG (H+L) and Alexa Fluor® 488 anti-mouse IgM (μ-chain) were purchased from Molecular Probes Invitrogen (Cergy Pontoise, France).

**Cell culture**

The human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from LGC standards (Molsheim, France) and the American Type Cell Culture Collection (Rockville, MD, USA), respectively. The Hs578T human breast cancer cell line [23] was kindly provided by Dr Van Slambrouck (Department of Chemistry and Biochemistry, South Dakota State University, Brookings, SD 57007, USA). Cells were routinely grown in monolayer cultures in Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 g/L glucose, 2 mM L-glutamine, supplemented with 10% fetal calf serum (FCS) and 100 μg/mL penicillin-streptomycin (Lonza, Verviers, Switzerland).
Gangliosides are classified in 4 series according to the number of sialic acid residues linked to lactosylceramide (LacCer) [22]. The 0-series gangliosides are directly synthesized from LacCer and the precursors of other series are synthesized by specific sialyltransferases: ST3Gal V (GM3 synthase), ST8Sia I (GD3 synthase) and ST8Sia V (GT3 synthase), respectively. The elongation of precursors is performed by the sequential action of N-acetyl-galactosaminyltransferase (β4GalNAc T1), galactosyltransferase (β3Gal T4) and sialyltransferases (ST3Gal II and ST8Sia V). Cer, ceramide. Adapted from [4].

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Belgium) at 37˚C in an atmosphere of 5% CO₂. Cells were treated with 40 ng/mL TNF (Eurobio AbCys, Paris, France) during 12–24 h before qPCR analysis or during 48 h for other experiments.

RNA Extraction, cDNA Synthesis and Quantitative real-time Polymerase Chain Reaction (qPCR)

Total RNA was extracted from the different cell lines using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). The amount of extracted RNA was quantified using a DeNovix DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE, USA) and the purity of the RNA was checked by the ratio of absorbance at 260 nm vs. 280 nm. Total RNA was subjected to reverse transcription using the Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific, France) according to the protocol provided by the manufacturer. PCR reactions were performed using 2X SYBR® Green Universal QPCR Master Mix (ThermoFisher Scientific). Primer sequences (Eurogentec, Seraing, Belgium) used for the PCR reactions are given in Table 1. *HPRT* gene was used to normalize the expression of transcripts of interest. PCR reactions were performed using the Mx3005p Quantitative System (Stratagene) as previously described [16]. PCR conditions were as follows: 95˚C for 30 s, Tm ˚C for 60 s, 72˚C for 20 s (40 cycles). The analysis of amplification was performed using the Mx3005p software and relative quantification was performed using the method described by Pfaffl that takes in account the efficiency of each sequence amplification [24]. Student’s *t*-test was used for statistical analysis. A *p*-value < 0.05 was considered statistically significant.

### Table 1. Primer pairs used for qPCR experiments.

| Gene          | Glycosyltransferase     | Sense primer Antisense primer | Tm (˚C) |
|---------------|-------------------------|-------------------------------|---------|
| *HPRT*        |                         | 5’-GCCAGACTTTTGTGGATTTG-3’  | 58      |
| *ST3GAL5*     | GM3 synthase            | 5’-ATCCTGCTAGTGGGCTTTT-3’   | 51      |
| *ST8SIA1*     | GD3 synthase            | 5’-GGCGACTTCTCGTGGTTTAT-3’  | 60      |
| *B4GALNT1*    | GM2/GD2 synthase        | 5’-TGCGTACGTCATGCTGG-3’     | 51      |
| *B3GALT4*     | GM1/GD1b synthase       | 5’-TATGTGCTGTACGGTCTGCT-3’  | 60      |

Extraction and preparation of glycosphingolipids

Cultured cells were washed twice with ice-cold PBS and detached from T175 flasks with cell dissociation non- enzymatic solution. Cells were sonicated on ice in 200 μL of water. The resulting material was dried under vacuum and sequentially extracted by CHCl₃/CH₃OH (2:1, v/v), CHCl₃/CH₃OH (1:1, v/v) and CHCl₃/CH₃OH/H₂O (1:2:0.8, v/v/v). Supernatants were pooled, dried and subjected to a mild saponification in 0.1 M NaOH in CHCl₃/CH₃OH (1:1) at 37˚C for 2 h and then evaporated to dryness [25]. Samples were reconstituted in CH₃OH/H₂O (1:1, v/v) and applied to a reverse phase C₁₈ cartridge (Waters, Milford, MA, USA) equilibrated in the same solvent. After washing with CH₃OH/H₂O (1:1, v/v), GSL were eluted by CH₃OH, CHCl₃/CH₃OH (1:1, v/v) and CHCl₃/CH₃OH (2:1, v/v). The elution fraction was dried under nitrogen stream prior to structural analysis.
Mass spectrometry analysis of GSL

Prior to mass spectrometry analysis, GSL were permethylated according to Ciucanu and Kerek [26]. Briefly, compounds were incubated 2 h in a suspension of 200 mg/mL NaOH in dry DMSO (300 μL) and CH3I (200 μL). The methylated derivatives were extracted in CHCl3 and washed several times with water. The reagents were evaporated and the sample was dissolved in CHCl3 in the appropriate dilution. MALDI-MS and MS/MS analyses of permethylated GSL were performed on 4800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) mass spectrometer, operated in the positive reflectron mode. For MS acquisition, 5 μL of diluted permethylated samples in CHCl3 were mixed with 5 μL of 2,5-dihydroxybenzoic acid matrix solution (10 mg/mL dissolved in CHCl3/CH3OH (1:1, v/v)). The mixtures (1 μL) were then spotted on the target plate and air dried. MS survey data comprises a total of 50 sub-spectra of 1500 laser shots. Peaks observed in the MS spectra were selected for further MS/MS. CID MS/MS data comprises a total of 100 sub-spectra of 3000 laser shots. Two or more spectra can be combined post-acquisition with mass tolerance set at 0.1 Da to improve S/N ratio. The potential difference between the source acceleration voltage and the collision cell was set to 1 kV and argon was used as collision gas.

Flow cytometry analysis

Cells were plated in 6-well plates (2.5 × 10^5 cells/well). The next day, cells were treated with 40 ng/mL TNF for 48 h. Cells were detached by trypsin and incubated for 30 min at 4˚C with anti-gangliosides mAbs: anti-GM3 (1:100), anti-GM2 (1:100), anti-GD3 (1:100), anti-GD2 (1:100) or anti-GD1b (1:100), all diluted in PBS containing 0.5% bovine serum albumin (BSA; Sigma-Aldrich, Lyon, France). After washing with PBS/0.5% BSA, cells were incubated for 30 min on ice with Alexa Fluor® 488 anti-mouse IgM or IgG (dilution 1:500 in PBS/0.5% BSA). Controls were performed using secondary antibodies alone. To analyze GM1α expression, cells were incubated with a FITC-conjugated cholera toxin B subunit from Vibrio cholerae (1:1000) for 30 min at 4˚C. Controls were performed using non-stained cells. Cells were then subjected to flow cytometry analysis using a FACScalibur flow cytometer from Becton Dickinson (Le-Pont-de-Clai, France).

Immunofluorescence staining

MCF-7 cells were plated on glass coverslips (ThermoFisher Scientific) in 6-well plates (2 × 10^5 cells/well). The next day, cells were treated with 40 ng/mL TNF for 48 h. Cells were then washed with DPBS and fixed 15 min in 4% paraformaldehyde at room temperature. After washing, cells were permeabilized in 0.5% Triton X-100 and blocked 1 h in a blocking buffer containing 2% BSA, 2% FCS and 0.2% gelatin in PBS. Cells were incubated for 1 h at room temperature with anti-GM3 (1:100), anti-GM2 (1:100), anti-GD3 (1:100), anti-GD2 (1:100) or anti-GD1b (1:50) antibodies diluted in blocking buffer. After three washes with PBS, cells were incubated 1 h with appropriate secondary antibodies (dilution 1:1000 in blocking buffer) and with the FITC-conjugated cholera toxin B subunit from Vibrio cholerae for GM1α analysis (1:4000). Cells were washed with PBS then with deionized water, and finally mounted in Fluorescent mounting medium (Dako, Carpenteria, CA, USA). The nuclei were stained with DAPI. Slides were examined under a Zeiss LSM 700 confocal microscope. The image acquisition characteristics were the same throughout the different conditions to ensure the comparability of the results and to allow the comparison of the fluorescence levels. For statistical analysis, images of ≥ 100 cells for each condition were collected based on the DAPI signal and were then analyzed for GD3 and GD2 expression using an automated algorithm on ImageJ.
software. Anova was used for statistical analysis. A $p$-value $< 0.05$ was considered statistically

**Results**

**MS analysis of GSL of breast cancer cells**

The composition of total GSL was determined by MALDI-TOF mass spectrometry after per-
methylation in three commonly used breast cancer cell lines, i.e. the luminal-A MCF-7 cells, the
basal-like MDA-MB-231 cells, whose ganglioside composition was already analyzed [16],
and the basal-like Hs578T cells, whose ganglioside profile appeared to be quite different from
the two other cell lines (Fig 2 and Table 2). Two ceramide isoforms are commonly expressed
in human tissues due to the substitution of the sphingosine moiety by palmitic acid C16:0 or
lignoceric acid C24:0. In MCF-7 cells, an additional ceramide isoform was observed with a
cerotic acid C26:0 linked to sphingosine. As expected, MCF-7 essentially expressed a-series
gangliosides, mainly $G_{M1a}$ whereas MDA-MB-231 cells mainly expressed $G_{M1b}$ as confirmed
by fragmentation of permethylated GSL using MALDI-TOF/TOF mass spectrometry (data
not shown). $G_{M3}$ and $G_{M2}$ were also expressed in both cell lines but in lower amounts. Both
cell lines also expressed low amounts of disialogangliosides. Signals at $m/z$ 2182.34 and
2294.46 corresponding to $G_{D1}$ isomers with 3 hexoses, one N-acetylated hexosamine and 2 N-acet-
ylneuraminic acid residues, were confirmed as and mixture of $G_{D1a}$ (90%) and $G_{D1b}$ (10%) in
MCF-7 cells and a mixture of $G_{D1a}$ (40%) and $G_{D1c}$ (60%) in MDA-MB-231 cells. Both cell
lines also expressed neutral GSL from globo-series such as $G_{b3}$ and $G_{b4}$. These results are in
agreement with previous published data [18,27]. The GSL composition of Hs578T cells was
never previously determined and appeared relatively simpler compared to the two other cell
lines. Hs578T cells mainly expressed the monosialoganglioside $G_{M2}$ but very low amounts of
$G_{M3}$ and $G_{M1b}$. Neutral GSL from globo-series such as $G_{b3}$ and $G_{b4}$ was also almost absent and
only a low amount of $G_{A2}$ was detected. Finally, Hs578T cells also expressed a low amount of
the disialoganglioside $G_{D2}$ as indicated by the signal at $m/z$ 2090.1.

**Effect of TNF on the expression of ganglioside GTs in breast cancer cell
lines**

The effect of TNF on the expression of glycosyltransferase genes involved in ganglioside bio-
synthesis was determined by qPCR for the three cell lines. The mRNA levels for each GT gene,
 i.e. $ST3GAL5$ encoding the GM3 synthase, $ST8SIA1$ encoding the GD3 synthase, $B4GALNT1$
 encoding the GM2/GD2 synthase and $B3GALT4$ encoding the GM1/GD1b synthase, were nor-
malized to the expression of $HPRT$ and reported to the expression of GT genes in non-treated
 cells (Fig 3). Our results confirmed the increased expression of $ST8SIA1$ after TNF treatment
in MCF-7 and Hs578T as previously reported [19] but no change in the expression of $ST8SIA1$
was observed in MDA-MB-231 cells. We also confirmed by qPCR the expression of $TNFR1$,
the gene encoding the TNF receptor 1, in the three cell lines (data not shown). TNF treatment
also increased the expression of $ST3GAL5$ in MCF-7 but no significant change of $ST3GAL5$
expression was observed in both Hs578T and MDA-MB-231 cells. In parallel, no significant
change of $B4GALNT1$ and $B3GALT4$ gene expression under TNF treatment was observed in
the three cell lines. These results indicate that TNF treatment could increase the biosynthesis
of b-series gangliosides, especially in MCF-7 cells, by increasing the expression of two essential
GT genes ($ST3GAL5$ and $ST8SIA1$) whereas TNF had no effect on the expression of these
genres in MDA-MB-231 cells.
Fig 2. Mass spectrometry (MALDI-TOF) analysis of permethylated GSL isolated from MCF-7, Hs578T and MDA-MB-231 cells. Blue circle, Glc; yellow circle, Gal; yellow square, GalNAc; purple diamond, Neu5Ac. Only masses corresponding to N-palmitoyl- (C16:0) or N-lignoceroyl- (C24:0) 2-amino-4-octadecene-1,3-diol (sphingosine) are indicated. Cer*: Ceramide d18:1/C16:0; Cer**: Ceramide d18:1/C24:0.

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Flow cytometry analysis of gangliosides at the cell surface of breast cancer cells

To establish the ganglioside profile of control and TNF-treated cells, we analyzed the composition of gangliosides at the cell surface using specific anti-ganglioside mAbs or cholera toxin B subunit for G<sub>M1a</sub> detection. The results presented in Fig 4A showed that MCF-7 cells expressed a limited number of gangliosides of the a-series, mainly G<sub>M1a</sub>. Low amounts of G<sub>M3</sub> and G<sub>M2</sub> were also detected confirming the results of MS analysis. Moreover, we were able to confirm the presence of low amount of G<sub>D1b</sub> but also of G<sub>D3</sub> and G<sub>D2</sub> in a very limited subpopulation of MCF-7 cells, whereas these b-series gangliosides were not detected by MS analysis. In agreement with the increased expression of GM3 synthase transcripts observed by qPCR, G<sub>M3</sub> and G<sub>M2</sub> expression was increased at the cell surface of MCF-7 cells after TNF treatment. Importantly and also in agreement with qPCR analysis, the subpopulation expressing G<sub>D3</sub> and G<sub>D2</sub> also appeared to be higher in MCF-7 treated cells than in control cells. In parallel, we analyzed the expression of gangliosides at the surface of Hs578T cells. Interestingly we could only detect

Table 2. Compositional assignments of singly charged sodiated molecular ions [M + Na]<sup>+</sup>, observed in MALDI-TOF mass spectrometry spectra of permethylated GSL from MCF-7, Hs578T and MDA-MB-231 cells.

| Fatty acids | GSL | Calculated mono-isotopic molecular masses | MCF-7 | Hs578T | MDA-MB-231 |
|-------------|-----|---------------------------------|------|-------|-----------|
| 16:0        | LacCer | 1010.7485 | - | - | 1010.75 |
| 24:0        | LacCer | 1122.8743 | - | - | 1122.88 |
| 16:0        | G<sub>b3</sub> | 1214.8483 | - | - | 1214.85 |
| 24:0        | G<sub>b3</sub> | 1326.9741 | - | - | 1326.98 |
| 16:0        | G<sub>b2</sub> | 1255.8748 | - | 1255.85 | - |
| 24:0        | G<sub>b2</sub> | 1367.9736 | - | 1367.98 | - |
| 16:0        | G<sub>gm3</sub> | 1371.9222 | 1371.91 | 1371.90 | 1371.94 |
| 24:0        | G<sub>gm3</sub> | 1484.0480 | 1484.04 | 1484.03 | 1484.06 |
| 16:0        | G<sub>gb4</sub> | 1459.9746 | 1459.98 | 1459.96 | 1460.02 |
| 24:0        | G<sub>gb4</sub> | 1572.1004 | 1572.09 | 1572.08 | 1572.12 |
| 16:0        | G<sub>gm2</sub> | 1617.0485 | 1617.07 | 1617.04 | 1617.10 |
| 24:0        | G<sub>gm2</sub> | 1729.1743 | 1729.17 | 1729.17 | 1729.18 |
| 16:0        | G<sub>gd3</sub> | 1733.0958 | - | - | - |
| 24:0        | G<sub>gd3</sub> | 1845.2216 | - | - | - |
| 16:0        | G<sub>gm1a/b</sub> | 1821.1483 | 1821.17 (G<sub>gm1a</sub>) | 1821.26 (G<sub>gm1b</sub>) | 1821.18 (G<sub>gm1b</sub>) |
| 24:0        | G<sub>gm1a/b</sub> | 1933.2741 | 1933.28 (G<sub>gm1a</sub>) | 1933.26 (G<sub>gm1b</sub>) | 1933.31 (G<sub>gm1b</sub>) |
| 16:0        | G<sub>g22</sub> | 1978.2222 | - | 1978.24 | - |
| 24:0        | G<sub>g22</sub> | 2090.3480 | - | 2090.36 | - |
| 16:0        | G<sub>gt3</sub> | 2094.2695 | - | - | - |
| 24:0        | G<sub>gt3</sub> | 2206.3953 | - | - | - |
| 16:0        | G<sub>g7</sub> | 2025.2481 | - | - | - |
| 24:0        | G<sub>g7</sub> | 2137.3739 | - | - | 2137.40 |
| 16:0        | G<sub>g1a/b</sub> | 2182.3219 | 2182.34 (G<sub>g1a/b</sub>) | - | 2182.37 (G<sub>g1a/b</sub>) |
| 24:0        | G<sub>g1a/b</sub> | 2294.4477 | 2294.46 (G<sub>g1a/b</sub>) | - | 2294.49 (G<sub>g1a/b</sub>) |

Only masses corresponding to N-palmitoyl- (C16:0) or N-lignoceroyl- (C24:0) 2-amino-4-octadecene-1,3-diol (sphingosine) are indicated. Assignments were confirmed by mass spectrometry fragmentation of permethylated GSL by MALDI-TOF/TOF (data not shown). LacCer, lactosylceramide; Gb, globoside.

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an increased expression of $G_{D2}$ in TNF-treated cells (Fig 4B). Finally, MDA-MB-231 cells presented a ganglioside pattern similar to MCF-7 cells (data not shown), and no change of ganglioside expression was observed at their cell surface after TNF treatment, in agreement with qPCR analysis. These results indicate that the increased expression of GT genes induced by TNF in both MCF-7 and Hs578T cells led to an increased expression of disialoganglioside $G_{D2}$ at the cell surface.

**Immunocytochemistry analysis of gangliosides in MCF-7 cells**

In order to confirm the results obtained by flow cytometry, control and TNF-treated MCF-7 were analyzed by immunofluorescence and confocal microscopy. As shown in Fig 5A, $G_{M1a}$ was highly expressed at the plasma membrane of MCF-7 cells, while lower amounts of $G_{M3}$ and $G_{M2}$ could be observed. The expression of $G_{D3}$ and $G_{D2}$ was also very low in control MCF-7 cells. After 48 h of incubation with TNF, $G_{M3}$ and $G_{M2}$ levels at the plasma membrane were higher, whereas no significant change was observed for $G_{M1a}$, which remained highly expressed after TNF treatment. Finally, the analysis of $G_{D2}$ expression in Hs578T cells allowed us to confirm the increased expression of $G_{D2}$ at the cell surface of treated cells observed by flow cytometry (Fig 5B). To quantify variations in ganglioside expression in MCF-7 cells after
TNF treatment, we analyzed the population expressing GD3 and GD2 using an automated algorithm on ImageJ software. For each condition, images of ≥ 100 control and treated cells were collected based on the DAPI signal for total cell counting. In the control cell population, GD3 and GD2 expressing cells were estimated at 4.0 ± 4.0% and 3.4 ± 2.3% of total cells, respectively, whereas TNF treatment extended these populations to 12 ± 3.5% and 12.7 ± 3.0% (Fig 5C).

Discussion

Complex gangliosides from b-series are oncofetal markers of human tumors of neuroectoderm origin such as melanoma, glioblastoma and neuroblastoma where they play a functional role in tumor growth and metastasis by mediating cell proliferation, migration and angiogenesis [7]. Previous studies have shown that the expression of ganglioside-specific GTs was also altered in breast cancer tumors. In particular, the GD3S was shown to display higher expression among ER-negative breast cancer tumors [14], associated with a lower survival rate of patients [15]. In parallel, the gangliosides GD3, as well as its derivatives 9-O-acetyl-GD3 and
9-O-acetyl-G_{T3} are over-expressed in about 50% of invasive ductal carcinoma, whereas they show a very restricted expression in normal breast tissues, [13]. In that context, we previously showed that the ectopic expression of the GD3S in breast cancer cells induced the accumulation of b- and c-series gangliosides at the cell surface together with the acquisition of a

Fig 5. Immunocytochemistry and confocal microscopy analysis of gangliosides expression. MCF-7 cells (A) and Hs578T cells (B) were treated with 40 ng/mL TNF for 48h. Control and treated cells were incubated with anti-G_{M3}, G_{M2}, G_{D3}, G_{D2} or G_{D1b} specific antibodies and revealed with Alexa Fluor® 488 conjugated anti-mouse IgG or IgM. G_{M1a} ganglioside was revealed with the FITC-conjugated cholera toxin B subunit from Vibrio cholerae. The nuclei were counter stained with DAPI. Bars: 25 μm. For statistical analysis (C), images of ≥ 100 MCF-7 cells for each condition were collected based on the DAPI signal and were analyzed for G_{D3} and G_{D2} expression using an automated algorithm on ImageJ software. *: p < 0.05, **: p < 0.01 vs. untreated (control).

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proliferative phenotype and enhanced tumor growth [16,17], due to the specific and constitutive activation of c-Met receptor in the absence of ligand, and subsequent activation of the PI3K/Akt and Erk/MAPK pathways in GD2 expressing breast cancer cells [18]. Ganglioside GD2 was also identified as a putative marker of CD44hiCD24lo breast cancer stem cells (CSC) capable of initiating tumors, and several GT genes involved in GD2 biosynthesis (ST3GAL5, B4GALNT1, and ST8SIA1) are highly expressed in CSC [20]. Moreover, the induction of epithelial–mesenchymal transition (EMT) in transformed human mammary epithelial cells dramatically increased GD3S as well as GD2 expression [28], confirming the role of GD3S and GD2 in breast cancer progression.

Chronic inflammation in the tumor micro-environment is known to play an important role in cancer progression and NFκB was proposed as an important actor of inflammation-driven malignancy [21]. Several GTs were previously described to be regulated by TNF [29]. As an example, we showed that TNF up-regulates ST3GAL4 expression through an intronic ATF2-responsive element, resulting in sialyl-Lewis^x antigen over-expression in lung epithelial cells [30]. Concerning GT genes involved in ganglioside biosynthesis, a functional NFκB binding site at -777/-762 pb upstream the ATG was shown to be essential for ST8SIA1 transcription in melanoma cells [31] and inflammatory cytokines including TNF and IL-6 enhanced GD3S gene expression in melanocytes [32]. We also reported that TNF enhanced GD3S expression in ER-negative breast cancer cells via NFκB pathway [19]. However, the effect of TNF on the expression of other ganglioside-specific GT genes in breast cancer cells was still unknown. In the present study, we examined the effect of TNF on the expression of the main ganglioside-specific GT genes as well as ganglioside expression in three breast cancer cells from different molecular subtypes. The most potent effect of TNF was observed for MCF-7 cells, in which TNF significantly enhanced the expression of genes encoding the GD3S (ST8SIA1) and also the GM3 synthase (ST3GAL5) that synthesizes G\textsubscript{M3}, the precursor of both G\textsubscript{M2} and G\textsubscript{D3}. In contrast, the expression of the other ganglioside-specific GTs genes B4GALNT1 and B3GALT4 was not significantly modified after TNF treatment, showing that TNF controls the expression of enzymes involved in the first steps of a- and b-series biosynthesis in MCF-7 cells. It was previously reported that exposure of mouse mammary carcinoma cells to TGF-β and TNF induced EMT and generated cells with a CSC phenotype [33]. It was also reported that the mRNA levels for ST3GAL5, ST8SIA1, but also for B4GALNT1 and ST3GAL2 were increased in human breast CSC model induced through EMT [34]. The ER-positive MCF-7 cells belongs to luminal breast cancer subtype with an epithelial phenotype [35] and the changes in the expression of GT genes that we observe in this study could be therefore associated with TNF-induced EMT of MCF-7 cells.

TNF treatment also significantly enhanced the expression of GD3S gene in Hs578T but the expression of the other GTs was not modified. The triple negative Hs578T cells were previously shown to over-express GD3S when treated by TNF [19]. In contrast with observations made in MCF-7 cells, TNF had no effect on the expression of the GM3 synthase. Surprisingly, no change in the expression of all GTs genes tested was observed for the triple negative MDA-MB-231 cells, which are of the same molecular subtype than Hs578T cells, and known to express the TNF receptor 1 (TNFR1) [36]. We also confirmed the expression of TNFR1 by qPCR in the three cell lines (data not shown). These results indicate that inflammation differently modulates the expression of ganglioside-specific GT genes in breast cancer cells.

The effect of TNF treatment on cell surface ganglioside expression was in parallel analyzed by flow cytometry and confocal microscopy. As expected, an increased expression of G\textsubscript{M3} and G\textsubscript{M2} was observed in TNF-treated MCF-7 cells that fitted well with the increased of GM3 synthase gene expression. G\textsubscript{M3} is the precursor for the biosynthesis of G\textsubscript{M1a} and the high and maybe saturated expression of G\textsubscript{M1a} in control conditions could also explain the accumulation...
of GM2 under TNF treatment. In renal carcinoma cells, TNF increased the expression of GM2 by enhancing the mRNA level of B4GALNT1 encoding the GM2/GD2 synthase, and resulted in T cell death and immune dysfunction [37]. As a result of the increased expression of GD3S, a significant increase of GD2 was observed by flow cytometry in both TNF-treated MCF-7 and Hs578T cells and confocal microscopy analysis allowed us to confirm the expression of GD2 in about 13% of MCF-7 cells. Confocal microscopy also revealed an intracellular labeling of GD3, presumably in the Golgi apparatus, showing the presence of the GD2 precursor in TNF-treated cells. These results demonstrate for the first time that TNF can modify the expression of gangliosides and increase GD2 expression at the cell surface of MCF-7 and Hs578T breast cancer cells. It was previously shown that TNF/TGFβ treatment of mouse mammary carcinoma cells induced EMT and generated breast cancer stem cells with a claudin-low molecular subtype characterized by the expression of mesenchymal and stem cell-associated markers, and associated with a poor prognosis [33]. According to the role of complex gangliosides, especially GD2, in breast cancer cells EMT and aggressiveness, the increased expression of GD2 in breast cancer cells under TNF treatment could establish a link between the presence of pro-inflammatory cytokines at the tumor site environment, expression of complex gangliosides and EMT, resulting in more aggressive cells with increased tumorigenicity and increased resistance to treatment.

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