

Original Article

Opposite effects of TNF- α on proliferation via ceramide in MDA-MB-231 and MCF-7 breast cancer cell lines

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Abstract: Aim: The goal of this study was to explore different effects of TNF- α on MDA-MB-231 and MCF-7 breast cancer cell lines. Materials and methods: Breast cancer cell lines were treated with TNF- α . Cell viability and signal mechanism were evaluated by MTT, flow cytometry, qRT-PCR, immunocytochemistry or Western blot, with the assistance of ASM and SPHK1 inhibitor. Results: TNF- α reduced proliferation and enhanced apoptosis in MCF-7, which was opposite in the MDA-MB231 cell line. Additionally, TNF- α increased ASM expression in MCF-7, as well as ceramide. In MDA-MB231, both ASM and SPHK1 protein level were increased, but ceramide decreased. Bcl-2 was decreased while Bax increased in MCF-7 cell. For MDA-MB231 cells, only PCNA was found to be increased after TNF- α treatment. ASM inhibitor or SPHK1 inhibitor addition could prevent the TNF- α -induced outcome. Conclusion: TNF- α exerts opposite function to the proliferation of MDA-MB-231 and MCF-7 breast cancer cell lines and ceramide maybe crucial during the process.

Keywords: TNF- α , opposite effects, MCF-7, MDA-MB-231, proliferation

Introduction

Systemic chemotherapy has proven efficient along with surgery and radiotherapy for the management of breast cancer patients [1]. However, controversy has been emerging in recent years regarding the sensitivity and specificity of chemotherapy. The cytological factors for enhancing the sensitivity to chemotherapy drugs and several factors have provided a desirable outcome [2].

TNF- α (Tumor necrosis factor alpha), named for its antitumor properties, is an intricate linker between inflammation and cancer through mediating the process of apoptosis and cell-mediated immunity. Interestingly, TNF- α has been shown to play two very opposing roles in different cell types. In one aspect, it is capable of inhibiting proliferation and inducing apoptosis. In 2006, Li YC et al. demonstrated an inducement effect of TNF- α on apoptosis of breast cancer cell lines [3]. In 2012, Su K et al. observed that TNF- α treatment attenuated the adhesion and proliferation of MCF-7 cells [4]. In 2014, Peng Z et al. suggested that TNF- α might

be involved in attenuation of growth, proliferation, and promotion of apoptosis in glioma cells [5]. In other aspects, TNF- α is also reported as a tumor promoter, with functions in promotion and progression of tumors, such as tumor migration, proliferation, matrix degradation, tumor metastasis, invasion, and angiogenesis [6, 7]. In 2014, Mori T et al. reported that TNF- α promotes osteosarcoma progression by maintaining tumor cells in an undifferentiated state [8]. So far, the features and mechanisms of TNF- α involvement remains elusive.

In this study, the effects of TNF- α on the proliferation of breast cancer cell lines with different genetic characteristics were explored and possible mechanisms were interpreted regarding differential regulation.

Materials and methods

Cell culture

Breast cancer cell lines MCF-7 and MDA-MB-231 were maintained in Dulbecco's modified eagle medium (DMEM, Invitrogen/Life Techno-

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logies) supplemented with 100 mL/L fetal bovine serum (FBS; Invitrogen/Life Technologies), streptomycin (100 mg/mL), and penicillin (100 IU/mL). All cells were incubated in a humidified atmosphere of 50 mL/L CO₂ at 37°C.

MTT assay

The effect of various concentrations of TNF- α (10, 20, 50 ng/mL) on cell proliferation was examined by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO) assays. In brief, the cells were plated in 96-well plates and incubated with MTT solution (SigmaAldrich, ST) for 24 or 48 hours. MTT solution (final concentration 0.5 mg/ml) was then added and samples were incubated for a further 4 hours at 37°C. The medium was then removed and 150 μ L DMSO (dimethyl sulfoxide) was added to dissolve the formazan dye crystals for 15 minutes. Finally, the optical density was determined at 490 nm using a Benchmark Plus microplate reader (Bio-Rad, Hercules, CA). Three independent experiments were performed in quintuplicate.

Flow cytometry for apoptosis

Quantitation of apoptosis in breast cancer cells induced by TNF- α was tested by flow cytometry. When cells treated for 24 or 48 hours, cells were collected at 1000 r for 5 minutes and washed twice with cold PBS. Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Oncogene Research Products, Boston, MA, USA) was used to measure apoptotic cells. According to the manufacturer's instructions, after a stain with 5 μ L Annexin V-FITC and 5 μ L propidium iodide for 15 minutes in the dark at room temperature, cells were immediately subjected to flow cytometry (Becton Dickinson) for apoptosis observation and relative quantitative analysis within 1 hour.

Immunocytochemistry and laser scanning confocal microscope detection

After 24 hours of treatment, cells (MDA-MB-231 and MCF-7) were harvested and fixed by 40 g/L formaldehyde for 30 minutes. Rinsed by PBS three times, anti-Cer antibody (1:500, Enzo Biochem, NY, USA) was added and incubated for 1 hour at room temperature. FITC labelled goat anti-mouse IgG secondary antibody (1:1000, sigma, St. Louis, MO) was then added and incubated for 15 minutes at room temperature.

After mount by 700 mL/L, results were identified under laser scanning confocal microscope then analyzed using IPP software 6.0 (Media Cybernetics, Bethesda, MD, USA).

RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

Total RNA were extracted using Qiagen RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Agarose gel electrophoresis can be used to detect the integrity and purification of RNA samples. The absorption under 260 nm was a good reflex of RNA concentration. About 5 μ g total RNA for each sample was reverse transcribed into first strand cDNA for qRT-PCR analysis. The qRT-PCR was performed at a final volume of 10 μ L which contained 5 μ L of SsoFast TM Eva Green Supermix (BIO-RAD), 1 μ L of cDNA (1:50 dilution), and 2 μ L each of the forward and reverse primers (1 mM). The steps of qRT-PCR were performed as follows: 94°C 2 min for initial denaturation; 94°C for 20 s, 58°C for 15 s, and 72°C for 15 s; 2 s for plate reading for 40 cycles; and melt curve from 65 to 95°C. GAPDH was used as quantity and quality control to normalize the gene expression.

Western blotting analysis

Cells were homogenized and lysed in radio-immunoprecipitation assay (RIPA) lysis buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM β -glycerophosphate, 2 mM sodium vanadate, and 1 mM protease inhibitor). The protein concentration was assayed using the micro BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Bedford, MA, USA), which was blocked with 5% non-fat milk in 0.1% Tween-20/Tris-buffered saline (TBS) and incubated with specific anti-ASM antibody (1:500, Abcam, USA), anti-SPHK1 antibody (1:300, Abcam, USA), anti-Bcl2 antibody (1:500, Abcam, USA), anti-Bax antibody (1:1000, Santa Cruz, CA), anti-PCNA antibody (1:1000, Santa Cruz, CA) and anti-GAPDH antibody (1:1000, Sigma St. Louis, MO) overnight at 4°C. The membrane was then incubated with a secondary antibody (1:10000) conjugated to horser-

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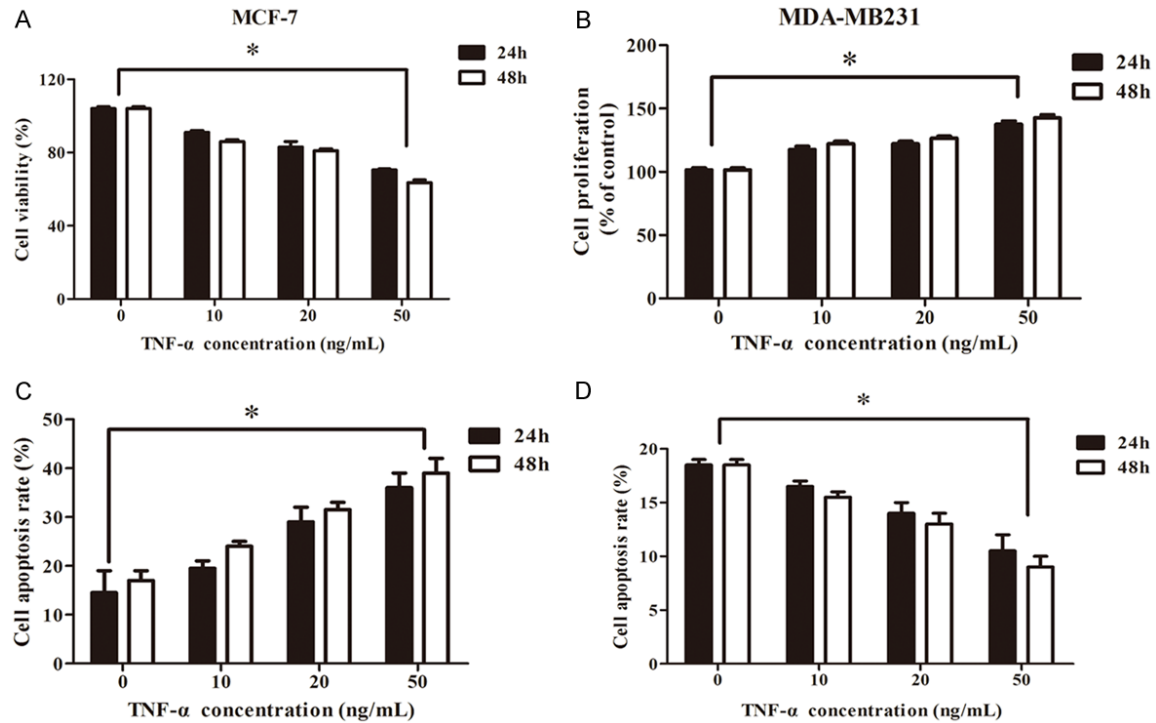


Figure 1. Effects of TNF- α on the proliferation and apoptosis of MCF-7 and MDA-MB231 cell lines. A. Effects of TNF- α on the proliferation of MCF-7 cell line. B. Effects of TNF- α on the proliferation of MDA-MB231 cell line. C. Effects of TNF- α on the apoptosis of MCF-7 cell line. D. Effects of TNF- α on the apoptosis of MDA-MB231 cell line.

adish peroxidase (HRP; 1:10000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and developed using a chemiluminescent substrate (PerkinElmer, Waltham, MA, USA) according to the manufacturer's recommendations. Finally, protein bands were visualized using Kodak X-Omat processor (Rochester, NY, USA).

Statistical analysis

All experiments to date were derived from at least 3 independent experiments and all values are expressed as the means \pm standard deviation (SD). Statistical analyses were performed using the SPSS 22.0 statistical software package (SPSS Inc., Chicago, IL). Any differences were assessed using the Student's t-test or one-way analysis of variance (ANOVA). $P < 0.05$ was accepted as significant.

Results

Opposite effects of TNF- α on the proliferation and apoptosis of MCF-7 and MDA-MB231 cell lines

As shown in **Figure 1**, MCF-7 and MDA-MB231 cell lines were sensitive to TNF- α treatment. At

the dose of 50 ng/mL TNF- α , cell proliferation and apoptosis showed significant difference to the control group ($P < 0.05$). For MCF-7 cells, TNF- α reduced proliferation and enhanced apoptosis (**Figure 1A, 1C**). In contrast, TNF- α improved proliferation and attenuated apoptosis of MDA-MB231 cell line (**Figure 1B, 1D**).

Different influence of TNF- α on ASM and SPHK1 expression

Using Western blots as described above, expression profiles of ASM and SPHK1 in MCF-7 and MDA-MB231 cell lines were examined. ASM and SPHK1 cell lines were found to display opposite changes both in mRNA and protein level. As shown in **Figure 2A**, after TNF- α treatment, the mRNA level of ASM, but not SPHK1 was increased in MCF-7 cells, but both of them were increased in MDA-MB-231 cells, in comparison with control group. Similarly, the protein expression of ASM was increased in MCF-7 cells while SPHK1 protein level showed little changes. But the ASM and SPHK1 protein level were all increased in MDA-MB-231 cells ($P < 0.05$, **Figure 2B, 2C**).

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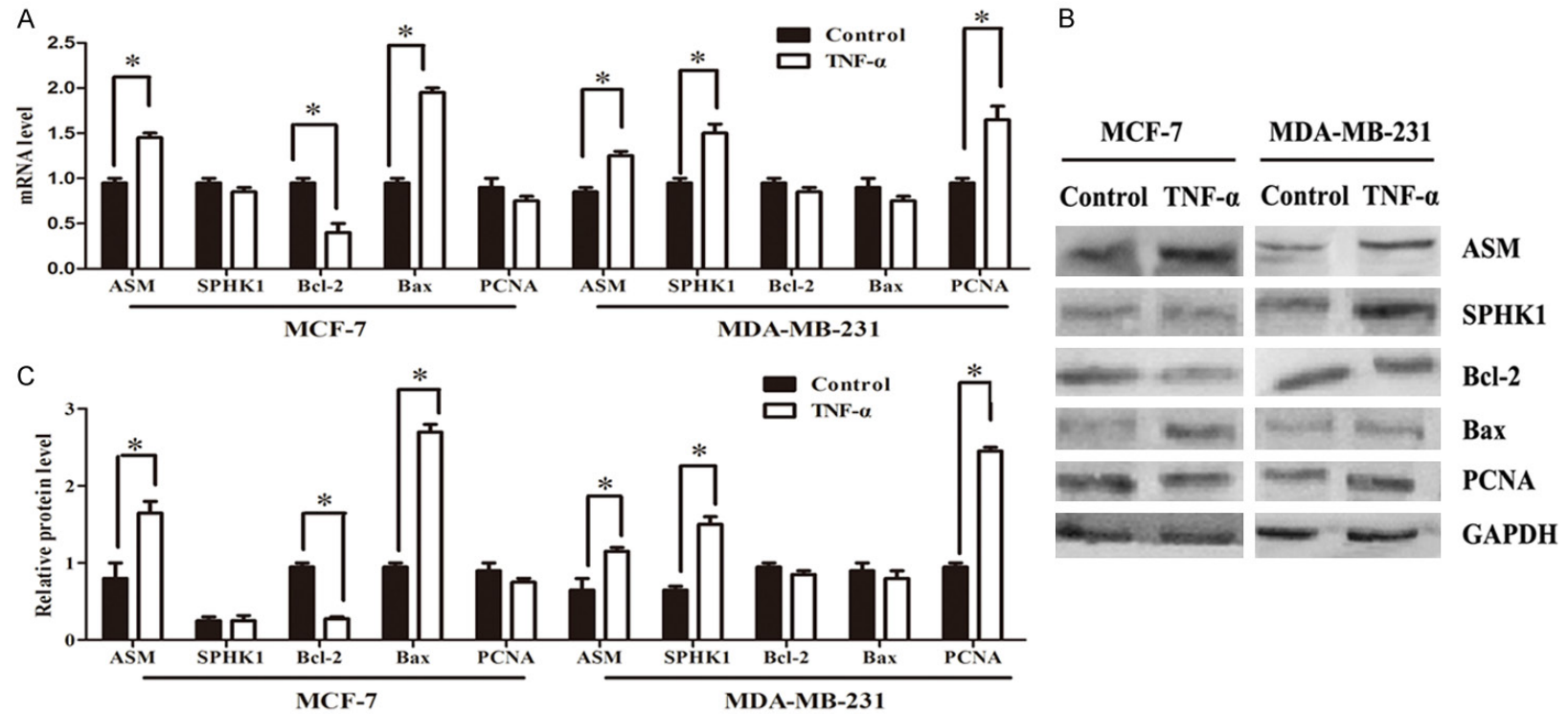


Figure 2. Influence of TNF- α on ASM and SPHK1 expression. A. Influence of TNF- α on the mRNA level of ASM and SPHK1. B. Western blot results of TNF- α treatment to the expression of ASM and SPHK1. C. Data analysis of TNF- α treatment to ASM and SPHK1 protein level.

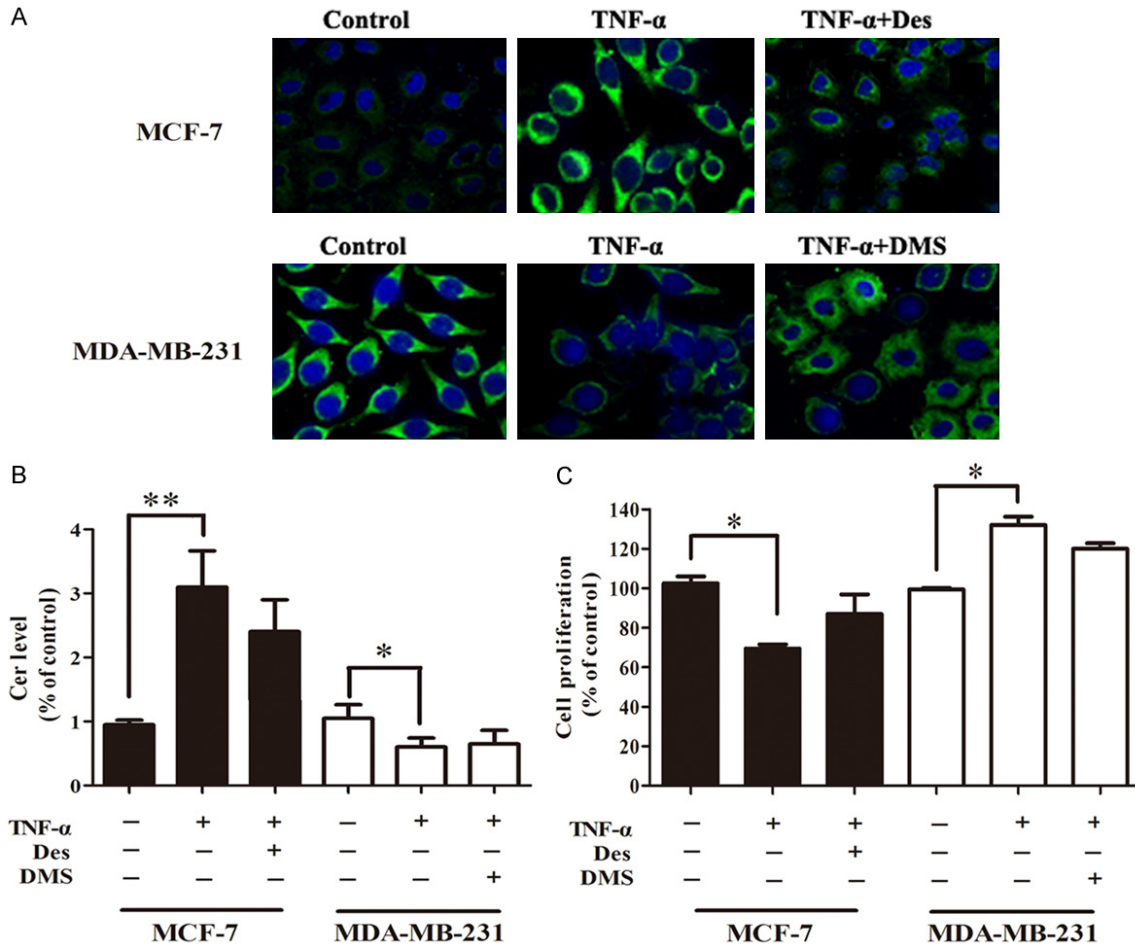


Figure 3. Involvement of ASM, SPHK1 and Cer in MCF-7 and MDA-MB-231 proliferation regulation. A. Picture referring the effects of TNF- α , Des or DMS on Cer expression under laser scanning confocal microscope. B. Semi-quantitative analysis of TNF- α , Des or DMS treatment to Cer expression. C. Effects of TNF- α , Des and DMS on the proliferation of MCF-7 and MDA-MB-231.

Different influence of TNF- α on Bcl-2 family and PCNA protein

Expression of Bcl-2, Bax, and PCNA proteins was analyzed to determine whether the differences in cell sensitivity to apoptosis could be correlated to the differential expression of these proteins. The results show that protein expression of Bcl-2 was decreased and Bax was increased in MCF-7 cell, while proliferating cell nuclear antigen (PCNA) shows no changes. For MDA-MB231 cells, only PCNA was found to be more than 2-fold increased after TNF- α treatment (Figure 2B, 2C).

Involvement of ASM, SPHK1 and Cer in MCF-7 and MDA-MB-231 proliferation regulation

After 24 hours of treatment of TNF- α (50 ng/mL), the Cer content was increased in MCF-7 cells. But when cells were pretreated with ASM

inhibitor desipramine (Des, 10 μ mol/L) for 1 hour, the TNF- α -induced enhancement of Cer level was attenuated. Furthermore, Des could also reverse the TNF- α induced decrease of proliferation in MCF-7 (Figure 3).

For MDA-MB-231 cells, the Cer concentration was reduced by TNF- α , which could be prevented when SPHK1 inhibitor dimethylsphingosine (DMS, 10 μ mol/L) added. DMS could reverse the TNF- α induced increase of proliferation in MDA-MB-231 cells. Moreover, pretreatment of DMS could significantly weakened TNF- α -induced improvement of MDA-MB-231 cell proliferation (Figure 3).

Function of Bcl-2, Bax, and PCNA protein in apoptosis regulation of MCF-7 or MDA-MB-231

When MCF-7 cells were pretreated with ASM inhibitor desipramine (Des, 10 μ mol/L), the de-

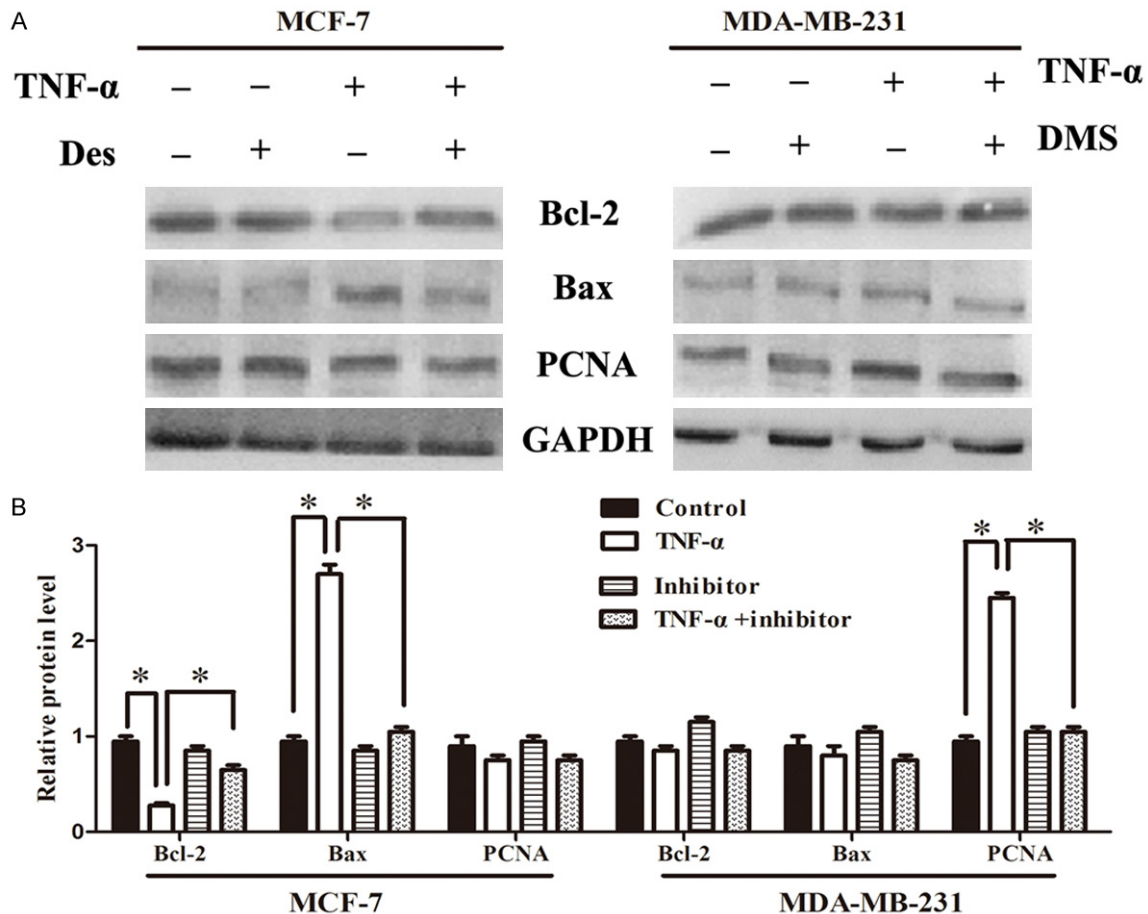


Figure 4. Function of ASM or SPHK1 in regulating the apoptosis of MCF-7 or MDA-MB-231. A. Effects of TNF- α , Des and DMS on Bcl-2, Bax and PCNA expression of MCF-7 and MCD-MB-231. B. Date analysis of TNF- α , Des and DMS treatment to Bcl-2, Bax and PCNA protein level.

creased expression of Bcl-2 triggered by TNF- α was improved. In contrast, TNF- α -induced Bax enhancement was attenuated. For MDA-MB-231 cell line, the increased expression of proliferating cell nuclear antigen (PCNA) induced by TNF- α could be prevented when SPHK1 inhibitor dimethylsphingosine (DMS, 10 μ mol/L) added (**Figure 4**). The possible signal pathway is summarized in **Figure 5**.

Discussion

Tumor necrosis factor alpha (TNF- α) has been shown to possess both tumor promotion and tumor regression functions. However, the potential molecular basis for the differences in susceptibility to TNF- α among breast cancer cell variants remains unclear. In 2000, Zhou BP et al. reported that TNF- α exerted different function between MCF-7 and MCD-MB-453 cell lines. Unlike MCF-7 cell line, MCD-MB-453 cell line had no response to TNF- α treatment [9].

Similarly, in this study, we demonstrated that TNF- α could attenuate the proliferation of MCF-7 cells, but could heighten proliferation of MCD-MB-231 cells, indicating bidirectional regulation.

Ceramide (Cer), as a central mediator in sphingolipid metabolism and signaling pathways, plays important roles in regulating many fundamental cellular responses [10]. As usual, ceramide is referred to as a "tumor suppressor lipid", since it powerfully potentiates signaling events which drive apoptosis, cell cycle arrest, and autophagic responses. In the typical cancer cell, ceramide levels are usually suppressed [11]. As reported, Cer is mainly generated by hydrolysis of sphingomyelin through the action of ASM (acid sphingomyelinase) [12, 13]. In this study, TNF- α was shown to inhibit MCF-7 cell proliferation via ASM-Cer signal pathway, indicating the potential anti-cancer role of TNF- α to estrogen receptor positive breast cancer.

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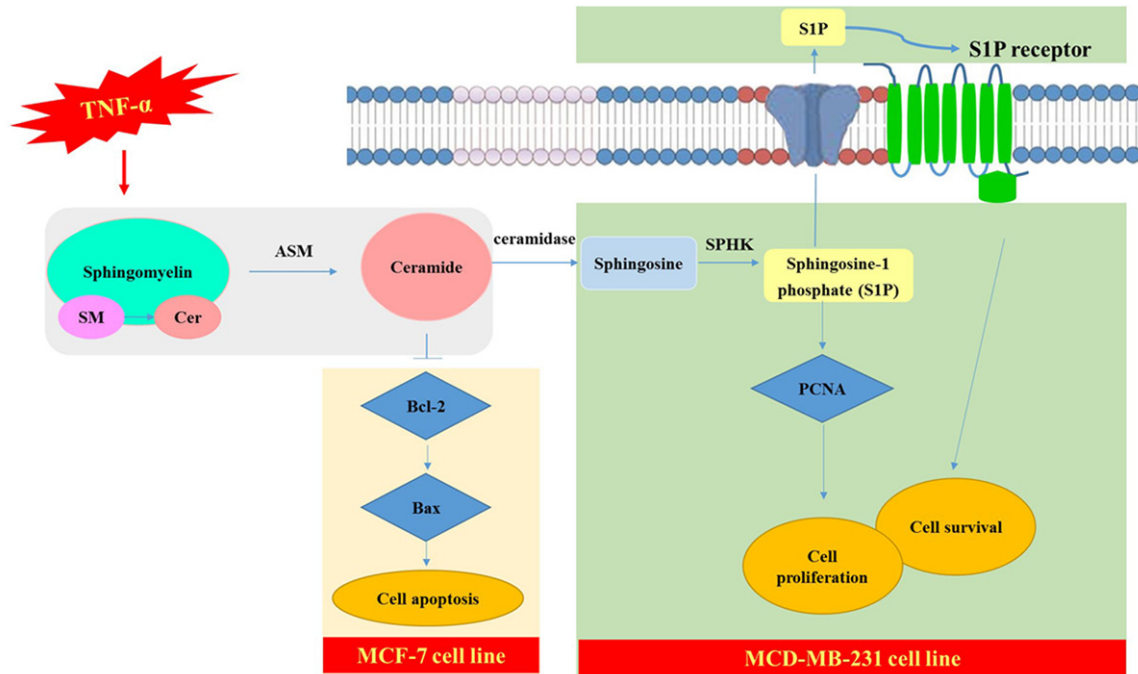


Figure 5. The regulation mechanism of TNF- α in MCF-7 and MCD-MB-231.

Except for the anti-cancer function, Cer could also exerts opposing physiological role via C1P molecular (ceramide-1-phosphate). Cer metabolism by ceramidase can produce sphingosine, which can further be phosphorylated by sphingosine kinase 1 (SPHK1) to yield sphingosine-1-phosphate (S1P) [14]. S1P is implicated in many critical cellular processes. However, unlike ceramide, it promotes cell survival, proliferation, and migration, as well as angiogenesis and allergic responses [15]. In this study, TNF- α could promote MCD-MB-231 cell proliferation via ASM-Cer-SPHK1 signal pathway, indicating its role in progression of triple-negative breast cancer. Hence, the function of TNF- α to various breast cancer cell depends on the level of Cer. In MCF-7, Cer was significantly increased via ASM up-regulation, contributing to the inhibition of proliferation. In MDA-MB-231 cell line, the cell progression was related to the decline of Cer concentration. The increased Cer induced by ASM was subsequently transformed into S1P under the help of enhanced SPHK1 molecular.

Examination of several members of the Bcl-2 family, was carried out to evaluate the apoptosis regulation of MCF-7 or MDA-MB-231. The results indicate that Bcl-2 and Bax protein were

only associated with the modulation of apoptosis in MCF-7 cell, rather than MDA-MB-231 cell. Many studies confirm that Bcl-2 and Bax correlates with the resistance to apoptosis induced by a number of agents. In 2014, Nagahara Y et al. proved that the loss of Bcl-2 expression correlates with increasing sensitivity to apoptosis [16]. Berger A et al. exclaimed that kinase inhibitor BMS-345541-induced apoptosis was correlates with the activation of Bax [17]. Identically, in this study, TNF- α -induced apoptosis was related to the Bcl-2 decrease, as well as Bax increase. Moreover, the TNF- α -induced changes of Bcl-2 and Bax could be reversed by ASM inhibitor, indicating the possible regulation of TNF- α -ASM-Cer-Bcl-2/Bax signaling in MCF-7.

Proliferating cell nuclear antigen (PCNA) is a critical eukaryotic replication accessory factor that supports DNA binding in DNA processing, such as DNA replication, repair, and recombination [18]. Tahan SR et al. analyzed that a higher expression level of PCNA correlated with poor survival rate in breast cancer patients [19]. However, there are contradictory reports questioning the fidelity of PCNA as a clinical marker in tumor progression. In this study, expression of PCNA in MCF-7 or MDA-MB-231 was found to confer a significant improvement. Additionally,

increased expression of PCNA induced by TNF- α could be prevented when SPHK1 inhibitor dimethylsphingosine (DMS) added. Hence, the possible mechanism of TNF α -ASM-Cer-SPHK1-PCNA signal pathway in improving triple-negative breast cancer cell proliferation still needs further verification.

Cytological features of MCF-7 and MDA-MB-231 cell types might associated with the distinct reaction to TNF α . First of all, MCF-7, an estrogen receptor positive breast cancer cell line, has lower migration capability. In contrast, MDA-MB-231, a triple-negative breast cancer cell line, is negative for estrogen receptor (ER) and progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2). The prognosis and invasion ability of MDA-MB-231 cell line was much stronger than MCF-7 cell line. Moreover, different molecular expression, such as p53, caspase-3 and Rac3 gene is possible to bring out the functional distinction. In 2013, Gest C et al. found that Rac3 induces a different molecular pathway triggering breast cancer cell aggressiveness in MDA-MB-231 and MCF-7 breast cancer cell lines [20]. Nevertheless, the detailed mechanism leading to the variants remains to be determined.

Taken together, this study suggests the differences in susceptibility of breast cancer cell variants to TNF- α and ceramide level plays significant role in the process. In MCF-7, TNF- α induced ASM up-regulation maybe contributed to the enhancement of ceramide level, resulting in the proliferation depression. In MDA-MB-231, the ceramide level decline via SPHK1 may directly lead to proliferation.

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Disclosure of conflict of interest

None.

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