Molecular mechanism of gossypol mediating CCL2 and IL-8 attenuation in triple-negative breast cancer cells

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Abstract. Chronic inflammation associated with cancer is characterized by the production of different types of chemokines and cytokines. In cancer, numerous signaling pathways upregulate the expression levels of several cytokines and evolve cells to the neoplastic state. Therefore, targeting these signaling pathways through the inhibition of distinctive gene expression is a primary target for cancer therapy. The present study investigated the anticancer effects of the natural polyphenol gossypol (GOSS) in triple-negative breast cancer (TNBC) cells, the most aggressive breast cancer type with poor prognosis. GOSS effects were examined in two TNBC cell lines: MDA-MB-231 (MM-231) and MDA-MB-468 (MM-468), representing Caucasian Americans (CA) and African Americans (AA), respectively. The obtained IC_{50}s revealed no significant difference between the two cell lines' response to the compound. However, the use of microarray assays for cytokine determination indicated the ability of GOSS to attenuate the expression levels of cancer-related cytokines in the two cell lines. Although GOSS did not alter CCL2 expression in MM-468 cells, it was able to cause 30% inhibition in TNF-α-stimulated MM-231 cells. Additionally, IL-8 was not altered by GOSS treatment in MM-231 cells, while its expression was inhibited by 60% in TNF-α-activated MM-468 cells. ELISA assays supported the microarray data and indicated that CCL2 expression was inhibited by 40% in MM-231 cells, and IL-8 expression was inhibited by 50% in MM-468 cells. Furthermore, in MM-231 cells, GOSS inhibited CCL2 release via the repression of IKBKE, *CCL2* and *MAPK1* gene expression. Additionally, in MM-468 cells, the compound downregulated the release of IL-8 through repressing *IL-8*, *MAPK1*, *MAPK3*, *CCDC88A*, *STAT3* and *PIK3CD* gene expression. In conclusion, the data obtained in the present study indicate that the polyphenol compound GOSS may provide a valuable tool in TNBC therapy.

Introduction

Cancer evolves through different abnormal molecular signaling pathways, including chronic inflammation events. Dysregulation of the inflammatory process might lead to chronic inflammation that contributes to many diseases, including cancer, cardiovascular, diabetes, lung diseases, Alzheimer's, and autoimmune diseases (1,2). The relationship between cancer and inflammation is well-established (3). Chronic inflammation is a hallmark of tumorigenesis that significantly mediates various stages in cancer, including cellular changes, initiation, promotion, proliferation, survival, invasion, angiogenesis, and metastasis (1), leading to approximately 15 to 20% of all cancer-related deaths worldwide (4).

Meanwhile, breast cancer (BC) is a heterogeneous disease (5) that demonstrated its link to inflammation (4). In the United States, 15% of the diagnosed BC cases are classified as basal-like BC subtype according to the molecular profile (6,7). Triple-negative breast cancer (TNBC) is the most aggressive and metastatic subgroup that comprises up to 75% of the basal-like BC (8,9), and it is known to be more profound among African American (AA) patients than Caucasian American (CA) patients (10). In TNBC cells, the absence of three specific receptors: Estrogen (ER), progesterone (PR) and human epidermal growth factor (Her2/neu) is a difficult challenge in treating the disease (11,12). Even though, TNBC has an initial significant response to many chemotherapy agents (13), approximately 30% of the patients experience poor prognosis and treatment failure after repeated exposure to these agents, leading to a median survival of one year (14).

Chronic inflammation-associated cancer is characterized by the presence of leukocyte infiltration, prominently macrophages that produce chemokines and cytokines (15). These cytokines are the critical mediators of communication between neoplastic cells in the inflammatory tumor microenvironment (16). Particularly in BC, two chemokines, IL-8, and CCL2 regulate tumor angiogenesis (17,18), survival, and metastasis of cancer cells (19). In addition to their chemotactic

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role, these two chemokines enable the recruitment of other cells, including monocytes, neutrophils, T lymphocytes, and NK cells (20). Under normal conditions, the pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α), mediates the inflammatory pathway and controls inflamed cells (1). In an aggressive BC environment, TNF-α is excessively produced by the tumor-associated macrophages (TAMs) (21,22) and substantially upregulates many genes involved in cancer cell proliferation, invasion, and metastasis (23).

In normal tissue, homeostasis of cell number and rational cell functions are precisely controlled and maintained through the release of growth-promoting signals. Cell signal dysregulation and cell evolvement to the neoplastic state may impair growth suppressor genes that could lead to prolonging proliferative signaling, cell death resistance, angiogenesis, and even triggering invasion and metastasis (24). In the context of cancer, many signaling pathways, including JAK-STAT, MAPK, PI3K-AKT, NF-κB, Notch, and Wnt, have received substantial attention as targets in cancer therapeutic intervention. The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway is involved in the cellular response to cytokines and play a pivotal role in the growth factor signaling and apoptosis. Impaired JAK-STAT signaling can lead to tumorigenesis, either directly or indirectly (25). The Mitogen-Activated Protein Kinase (MAPK) pathway (also known as ERK pathway) is essential in stimulating survival, proliferation, migration, and cell adhesion through signals transduction from cytokines and growth factors (19). Parallel to the MAPK pathway, the activated phosphoinositide 3-kinase-protein kinase B (PI3K-AKT) pathway mediates cancer cell metabolism and regulates proliferation. Additionally, this pathway activates other signaling pathways, including Wnt and NF-κB (26), which in turn regulates different genes involved in inflammation, cell viability, proliferation, and apoptosis (27) in BC and many others cancer types.

Plant-derived compounds have been thoroughly screened for their potency in preventing and treating cancer (28). Numerous studies have demonstrated the medicinal importance of the polyphenol compound, gossypol (GOSS) (2,20-binaphthalene)-8,80-dicarboxaldehyde,1,10,6,60,7,70-hexahydroxy-5,50-diisopropyl-3,30-dimethyl, a minor constituent of cotton (*Gossypium hirsutum* L.) seeds (29-31). GOSS has various biological activities, including antifertility, antiviral, antimicrobial, and antioxidative activity (32). Moreover, the anti-proliferative, anti-metastatic, and apoptotic effects of GOSS have been documentd against several human cancers, including colon, prostate, glioma, adrenal, leukemia (24,33-37), in addition to breast cancer (28,38-40). The drug combination is critical to accomplish a synergistic therapeutic effect (41) and to overcome the resistance mechanisms of many diseases, including cancer (42). GOSS has been found to induce apoptosis in various types of human cancer cells in combination with low doses of dexamethasone (43), doxorubicin (44), taxanes (45), and valproic acid (46).

Many studies have demonstrated the anticancer effect of GOSS in BC, including the TNBC subtype, MDA-MB-231 (MM-231) cells. However, studying the racial perspective of the compound effects on MDA-MB-468 (MM-468), and its gene-related mechanism of action in comparison to MM-231 cells has never been addressed. Moreover, the potential effect of GOSS on the proinflammatory cytokines, IL-8 and CCL2 has not been reported prior to this work. Therefore, the current study is designed to compare the anticancer effect of GOSS on two TNF-α-stimulated human TNBC cell lines: MM-231 and MM-468, representing Caucasian (CA) and African American (AA) women, respectively (47). We hypothesized that GOSS could modulate the expression of genes involved in many cellular signaling pathways that mediate the regulation of diverse cancer-related cytokines/chemokines.

Materials and methods

Materials. The compound GOSS (purity ≥90%) was purchased from Santa Cruz Biotechnology, Inc. Trypsin-EDTA solution 0.25% and Alamar Blue® (a sterile buffered solution of resazurin fluorescence dye) were purchased from Sigma-Aldrich; Merck KGaA. Dimethyl sulfoxide (DMSO), penicillin/streptomycin, and Dulbecco's Phosphate Buffer Saline (DPBS) were obtained from the American Type Culture Collection. Dulbecco's Modified Eagle Medium (DMEM), heat-inactivated fetal bovine serum (FBS), and cell culture plates were purchased from VWR International (Radnor). TNF-α, Human Cytokine Antibody Array kit (cat. no. AAH-CYT-1000), Human ELISA kits for C-C Motif Ligand 2 [CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), cat. no. ELH-MCP1] and Interleukin-8 (IL-8, also known as CXCL-8, cat. no. ELH-IL-8) were purchased from RayBiotech. TURBO DNA-free™ kit (cat. no. AM1907) was purchased from Life Technologies, Inc. TRIzol® reagent was purchased from Invitrogen; Thermo Fisher Scientific. An iScript™ cDNA Synthesis kit (cat. no. 170-8891), SsoAdvanced™ Universal SYBR® Green Supermix (cat. no. 1725271), Human PCR primers (*CCL2, IKBKE, IL-8, STAT3, MAPK1, MAPK3, CCDC88A, PIK3CD*, and *GAPDH*) were purchased from Bio-Rad Laboratories, Inc.

Cell culture. The two immortalized TNBC cell models: MM-231 (https://www.atcc.org/products/all/HTB-26.aspx) and MM-468 (https://www.atcc.org/Products/All/HTB-132.aspx), were purchased from ATCC. Both cell lines were grown in 75-cm TC-flasks at 37°C in humidified 5% CO_2 incubator and subculture as required, using trypsin/EDTA (0.25%). The routinely used DMEM growth medium contained 4 mM L-glutamine and was supplemented with 10% FBS (v/v), and 1% penicillin/streptomycin salt solution (100 U/ml and 0.1 mg/ml, respectively). The DMEM experimental medium was the same, except it was phenol-free and was supplemented with 2.5% FBS as previously reported by others (48).

Cell viability assay. In this experiment, cells were incubated overnight at 37°C at a density of $5x10^4$ cells/well in 96-well microplates. GOSS powder was reconstituted in DMSO. Both types of cells were treated for 24 h with 50 ng/ml of TNF-α, in addition to the compound (concentration ranges of 0-100 μM in MM-231 or 0-50 μM in MM-468 cells). Control wells were treated with DMSO at the highest used concentration (<0.1%). Blank wells were treated in the same manner but without cells. Alamar Blue® was used to determine cell viability, as described in our previous study (49). The fluorescent-fuchsia

dye of the reduced resazurin by viable cells was measured at an excitation/emission of 530/590 nm using a Synergy HTX Multi-Reader (BioTek).

Human cytokine/chemokine protein microarray. Cytokine expression microarray analysis was designed based on the data of the cytotoxicity assay. Four flasks for each cell line were incubated overnight with a density of $10x10^6$ cells/75-cm^2 TC flask using the experimental media with 2.5% FBS. Four flasks for each cell line were incubated overnight with a density of $10x10^6$ cells/75-cm^2 TC flask using the experimental media with 2.5% FBS. On the next day, the media were discarded, and the cells were treated with 50 ng/ml TNF-α and low concentrations of GOSS that slightly impacted the cell viability. MM-231 cells were treated as follows: TNF-α (50 ng/ml), GOSS (6.25 μM), and TNF-α + GOSS (50 ng/ml + 6.25 μM, respectively). Similarly, MM-468 cells were treated with TNF-α (50 ng/ml), GOSS (5 μM), and TNF-α + GOSS (50 ng/ml + 5 μM, respectively). In both experimental sets, control samples were exposed to only the solvent DMSO at a concentration of <0.1%. After a 24 h exposure period, the cell-free supernatant of each sample was collected, aliquoted, and stored at -80°C for later use. At the same time, the cells from each flask were pelleted and similarly stored at -80°C for RT-qPCR study. For each cell line, a semi-quantitative method using antibody-coated array membranes was established to measure chemokine/cytokine expression in the cell-free supernatants. The assay was established following the manufacturer protocols. Briefly, four membranes were carefully placed in four chamber-incubation trays and blocked with the provided buffer on a shaker for 30 min at RT. After that, the blocking buffer was decanted, and replaced with 1 ml cell-free supernatant from resting, GOSS-treated, TNF-α-stimulated or cotreated cells, and the four membranes were then kept overnight on a low-speed shaker at 4°C. On the following day, the supernatants were removed from each chamber, and the membranes were washed with the kit washing buffers. Next, 1 ml of freshly constituted biotinylated antibody cocktail was pipetted to each membrane and incubated at RT for 2 h, followed by washing with the same wash buffers. The membranes were incubated again for another 2 h with 2 ml of diluted horseradish peroxidase-conjugated streptavidin (HRP-Streptavidin) followed by the final washes. Cytokines intensities on the blots were detected as spots using a chemiluminescence cocktail. The blot images were captured using a Flour-S Max Multiimager (Bio-Rad Laboratories, Inc.), and the spot intensities were measured with the Quantity-One Software (Bio-Rad Laboratories, Inc.). The Excel-based data analysis was established, using the Human Cytokine Array software C1000 (CODE: S02-AAH-CYT-1000) from RayBiotech.

Human CCL2 and IL-8 chemokines ELISA study. Enzyme-Linked Immunosorbent Assay (ELISA) kits were used to measure the protein levels (pg/ml) for both CCL2 and IL-8 chemokines. Briefly, the standard curves, samples, and reagents were prepared at RT for both chemokines. Standards and samples of 100 μl each were incubated with the antibody pre-coated 96-well ELISA microplates for 2.5 h. The supernatant was replaced by 100 μl of the freshly

constituted biotinylated antibody for another hour, then removed. Streptavidin solution (100 μl) was added for 45 min, followed by the addition of 100 μl of the substrate reagent for 30-min incubation. Washes were always performed after each step according to the manufacturer's protocol. The reaction was terminated by the addition of 50 μl of a stop-solution, and the intensity for the chemokines and the standard were measured at 450 nm using a Synergy HTX Multi-Reader (BioTek).

RNA isolation and cDNA synthesis. In this assay, the previously-80°C-frozen cell pellets were used, as mentioned above in the Human cytokine/chemokine protein microarray study. The total RNA was extracted from each sample using 1 ml of Trizol® reagent and sonicated for 30 sec at RT using VirTishear mechanical homogenizer (LabWrench). Subsequently, 200 μl of chloroform was added to each sample, vortexed, incubated at RT for 2-3 min and centrifuged for 15 min at 10,000 x g and 2-8°C. The aqueous phase was separated and mixed with 500 μl of isopropyl alcohol to precipitate the RNA for different samples. The RNA pellets were then reconstituted in approximately 30-50 μl of nuclease-free water to measure the RNA concentration and purity in each sample using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.). Thereafter, the purified RNA was reverse transcribed into cDNA using an iScript™ cDNA Synthesis Kit and PCR run as follows: 46°C for 20 min and 95°C for 1 min.

Reverse transcription-quantitative PCR (RT-qPCR). In this study, we measured the expression of various genes involved in CCL2 and IL-8 regulation, using qPCR of the Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories, Inc.) (50). Briefly, the freshly synthesized cDNA (1 μl; 200 ng) was used for each sample in a final volume of 20 μl (10 μl of the 2x real-time Master Mix, 8 μl nuclease-free water, and 1 μl of primer mix). According to the manufacturer's protocol, the PCR run was performed as follows: Reactants were first incubated at 95°C for 2 min, and then 39 cycles of amplification (51) were carried out with each cycle consisting of denaturing at 95°C for 10 sec, annealing at 60°C for 30 sec, and melting curve at 65-95°C for 5 sec. All qPCR reactions were performed in triplicates for each primer. In this experiment, the used PCR primers were compatible with the different genes under investigation and are summarized in Table I. GAPDH was applied as a reference gene to normalize the mRNA levels for genes of interest.

Statistical analysis. The quantitative data for this study were analyzed using GraphPad Prism 6.2 software. All data points were obtained from the average of at least two independent studies and are expressed as the mean ± SEM. For the viability assay, IC_{50}s values were determined by nonlinear regression model of log (inhibitor) vs. normalized response-variable slope on the software with the $R2$ best fit and the lowest 95% confidence interval. The significance of the difference between each control and its related treatment groups was determined using one-way ANOVA, followed by Bonferroni's multiple comparison tests. A statistical difference was considered significant at $P<0.05$ and less. For array blots and ELISA studies, independent (unpaired) Student's t-test was used to verify the significance of the difference between TNF-α vs. control groups, or between TNF-α vs. TNF-α + GOSS groups.

Table I. List of primers used in reverse transcription-quantitative PCR experiments.

Name	GenBank accession no.	Amplicon context sequence
MCP1/CCL2	NM_002982.4	ACTGAAGCTCGCACTCTCGCCTCCAGCATGAAAGTCTCTGCCGCCCTTCTGT GCCTGCTGCTCATAGCAGCCACCTTCATTCCCCAAGGGCTCGCTCAGCCAGA TGCAATCAATGCCCCAGTCACCTGCTGTTATAACTTCACCAATAGGAAGATCT CAGTGCAGAGGCTCGCGAGCTAT
IL-8	NM_000584.4	GAGCACTCCATAAGGCACAAACTTTCAGAGACAGCAGAGCACACAAGCTTC TAGGACAAGAGCCAGGAAGAAACCACCGGAAGGAACCATCTCACTGTGTG
IKBKE	NM_014002.4	GGCTTGGCTACAACGAGGAGCAGATTCACAAGCTGGATAAGGTGAATTTCAG TCATTTAGCCAAAAGACTCCTGCAGGTGTTCCAGGAGGAGTGCGTGCAGAA GTATCAAGCGTCCTTAGTCACACACGGCAAGAGGATGAGGGTGGTGCACGAG
MAPK1	NM_002745.4	TTCAGCTGGTCAAGATAATGCTTCCCTGGAAAGATGGGCCTGTTAGAAAGCAT TTCTGCCAGAATGCAGCCTACAGACCAAATATCAATGGACTTGGTGTAGCCCT TGGAATTCAACATAATTTCTGGAGC
MAPK3	NM_002746.3	TCTCCATCAGGTCCTGCACAATGTAGACATCTCTCATGGCTTCCAGGGTGGAC GCCCGCAGAATGTCTCGGATGCCGATGACATTCTCATGGCGGAAGCGCAGCA GGATCTGGATCTCCCGGAGCGTGCGCTGGCAGTAGGTCTGATGTTCGAAGGG GCTGATCTTCTTGATGGCCACGC
CCDC88A	NM_001365480.1	GGAGTTGGGCATTCTGGTTCATGAGTGAGGTACTTTGGGAATTAAGGGTGGA ATTTCAACCTGAAGCTTGGCATTCTGTGTTTGAAGAGTGGTATTCTGTTCTTG TAATGACACTGTCTGCCTCTGAAGTGCAAGAATCTGAGCCTGCAAATTATTGT TCTGTGTCT
STAT3	NM_139276.2	GGTGTCACACAGATAAACTTGGTCTTCAGGTATGGGGCAGCGCTACCTGGGT CAGCTTCAGGATGCTCCTGGCTCTCTGGCCGACAATACTTTCCGAATGCCTCCT CCTTGGGAATGTCAGGATAGAGATAGACCAGTGGAGACACCAGGATATTGGT
PIK3CD	NM_005026.4	GCGGCTGGAGTTCGACATCAACATCTGCGACCTGCCCCGCATGGCCCGTCTCT GCTTTGCGCTGTACGCCGTGATCGAGAAAGCCAAGAAGGCTCGCTCCACCAA GAAGAAGTCCAAGAAGGCGGACTGCCCCATTGCCTGGG
GAPDH	NM_002046.7	GTATGACAACGAATTTGGCTACAGCAACAGGGTGGTGGACCTCATGGCCCAC ATGGCCTCCAAGGAGTAAGACCCCTGGACCACCAGCCCCAGCAAGAGCACA AGAGGAAGAGAGAGACCCTCACTGCTGGGGAGTCCCTGCCACAC

Quantitative mRNA expression was analyzed using CFX 3.1 Manager software for Bio-Rad Laboratories, Inc. All genes were normalized against the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and verified with unpaired Student's t-test.

Results

GOSS decreases cell viability in MM-231 and MM-468 TNBC cells. The anticancer effects of GOSS were examined in the presence of 50 ng/ml of TNF-α in both MM-231 and MM-468 cell lines. As indicated in Fig. 1A and B, a highly significant cytotoxic effect (P<0.0001) was detected at all the tested concentrations ranges 12.5-100 μM in MM-231 cells and 1-50 μM in MM-468 cells. The obtained IC$_{50}$s (19.13±0.32 μM for MM-231 and 23.46±0.43 μM for MM-468) showed a slightly higher response to the compound in MM-231 cells. Based on the viability studies data, optimum doses of GOSS that causes less than 20% cell death and 50 ng/ml of TNF-α were determined for cytokine/chemokine expression study.

GOSS inhibits the release of CCL2 in MM-231 cells and IL-8 in MM-468 cells. To understand the mechanism of the

antitumor effects of GOSS on TNF-α-stimulated MM-231 and MM-468 TNBC cells, we measured the expression of different cytokines/chemokines released in the cell-free supernatants in the presence and the absence of GOSS and 50 ng/ml of TNF-α (52). Low concentrations of GOSS that slightly impacted the cell viability were used (6.25 μM in MM-231 cells and 5 μM in MM-468 cells).

The obtained results show that GOSS repressed the expression of two critical chemokines, CCL2 in MM-231 cells and IL-8 in MM-468 cells, as indicated by the red frames on both the maps and the blot arrays. In the cytokine blots, AAH-CYT-6, the expression of the cytokine was scarcely noticeable on most of blots presenting the supernatants of both control and GOSS-treated cells (Fig. 2A and B). High expression of CCL2 was observed in both TNF-α-treated cell lines (Fig. 2C1 and C2). The intensity of the spot was attenuated in MM-231 cells by the effect of 6.25 μM GOSS (Fig. 2D1), the observation that was not showed in MM-468 cells (Fig. 2D2).

Meanwhile, the intensity of IL-8 was low on the blots presenting the supernatants of both control and GOSS-treated cells (Fig. 3A and B). Indeed, TNF-α has increased IL-8 expression in both cell lines, as indicated on the AAH-CYT-7
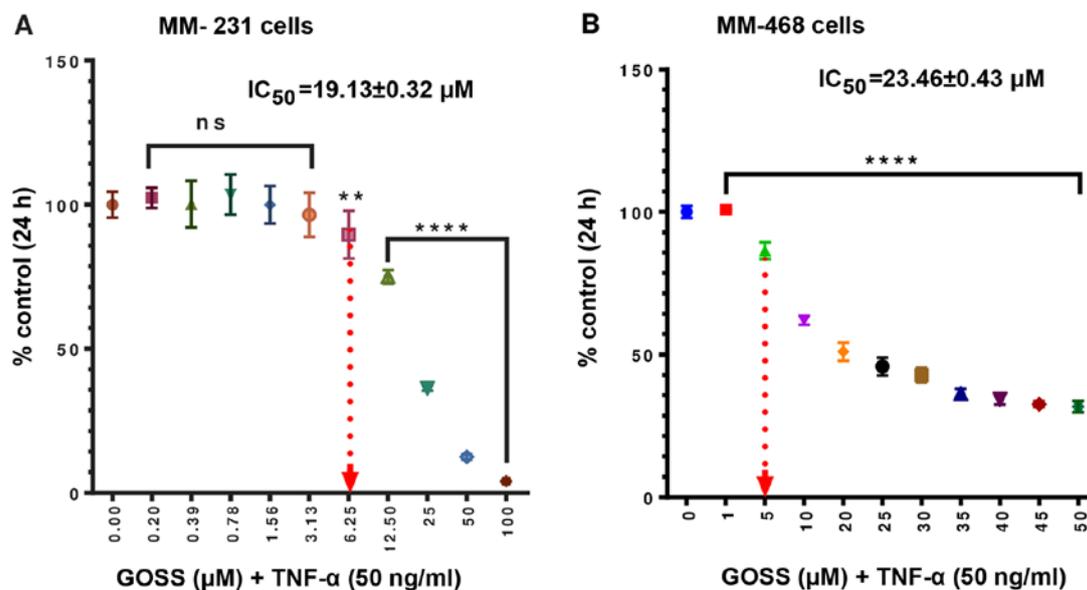
Figure 1. Effect of GOSS and TNF-α on the viability of TNF-α-stimulated MM-231 and MM-468 cells. In combination with 50 ng/ml TNF-α, cells were treated for 24 h with GOSS at concentration ranges of (A) 0-100 μM in MM-231 and (B) 0-50 μM in MM-468 cells. On the x-axis, the arrows represent the designated concentrations to be used in the following cytokine expression study. The percentages of cell survival compared with the control were calculated. The data points are presented as the mean ± SEM of three independent studies (n=6 each). **$P<0.01$, ****$P<0.0001$. The significance of the difference between the control and treated groups was determined using one-way ANOVA, followed by Bonferroni's multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference. GOSS, gossypol; ns, not significant; TNF-α, tumor necrosis factor α.

blots (Fig. 3C1 and C2). The compound GOSS did not reduce the cytokine expression in MM-231 cells (Fig. 3D1). However, an observed decrease was detected in MM-468 cells in the presence of 5 μM GOSS (Fig. 3D2).

Both chemokines, CCL2 and IL-8, were quantified as a percentage relative to their control. TNF-α has induced an 8-fold increase in CCL2 expression in MM-231 cells ($P<0.001$) (Fig. 4A). The data indicated that the presence of both GOSS and TNF-α significantly attenuated CCL2 by 30% ($P<0.01$). Similarly, in MM-468 cells (Fig. 4B), a more than 8-fold increase in the expression of IL-8 ($P<0.01$) was quantified in TNF-α-stimulated cells and then inhibited by 60% in the presence of GOSS ($P<0.05$). The results show that the GOSS compound did not attenuate IL-8 expression in MM-231 cells (Fig. 4C), nor CCL2 in MM-468 (Fig. 4D). Also, no changes were detected when we compare the control to the GOSS-treated cells in both chemokines of interest.

We further validated the microarray data for only the significantly attenuated chemokines. Protein expression quantification for CCL2 (pg/ml) and IL-8 (ng/ml) was measured using two independent ELISA assays. Overall, the data for cytokine arrays and ELISA were consistent in both cell lines. The assay indicated a significant increase in CCL2 ($P<0.001$) and IL-8 ($P<0.01$) in TNF-α-treated vs. resting cells (Fig. 4E and F). The simultaneous presence of GOSS and TNF-α attenuated the expression of CCL2 by ~40% in MM-231 cells ($P<0.001$), and IL-8 expression was also reduced by 50% in MM-468 cells ($P<0.05$).

GOSS alters the expression of genes regulating the release of CCL2 in MM-231 cells and IL-8 in MM-468 cells. RT-qPCR was performed to determine the relevance of our data and to elucidate the mechanism by which GOSS impact CCL2 and

IL-8 regulation in MM-231 and MM-468 cells, respectively (Figs. 5 and 6). Profiling the normalized expression of mRNAs in both cell lines provides precise insights into the influence of the compound on signaling pathways-related genes.

The RT-qPCR data presented in Figs. 5A and 6A indicated that mRNAs expressions of *CCL2* and *IL-8* were consistent with those of cytokine microarray and ELISA protein studies in both MM-231 and MM-468 cell lines, respectively. The mRNA's data showed that both cell lines responded to TNF-α and TNF-α + GOSS. In TNF-α-stimulated MM-231 cells, *CCL2* mRNA expression exhibited a highly significant 32-fold up-regulation ($P<0.0001$) (Fig. 5A and Table II). This gene was dramatically repressed by 80% in the presence of 6.25 μM GOSS ($P<0.0001$). Furthermore, GOSS repressed two more genes, *IKBKE* and *MAPK1*, which are involved in the signaling pathways of TNF-α-induced CCL2 release. *IKBKE* showed a significant increase in expression of ~11-fold by TNF-α that was repressed to ~53% by GOSS (Fig. 5B and Table II). The detected up-regulation in *MAPK1* was non-significant in the TNF-α-stimulated cells. However, GOSS was able to suppress significantly ($P<0.0001$) its expression by ~39% (Fig. 5C and Table II).

Similarly, in TNF-α-stimulated MM-468 cells, IL-8 mRNA was upregulated by ~65-fold ($P<0.0001$) followed by 60% downregulation ($P<0.001$) when combined with 5.0 μM GOSS (Fig. 6A and Table III). Moreover, a significant fold-increase ($P<0.01$-$P<0.0001$) was also found in five more genes (Fig. 6B-F and Table III), including *MAPK1*, *MAPK3*, *CCDC88A*, *STAT3*, and *PIK3CD* that showed the highest increase (3-fold). GOSS repressed the expression of these five genes by almost 40-50%, as shown in Fig. 6B-F and Table III.

Indeed, the obtained data in this study, including those of the cytokine microarray blots, ELISA, and PCR evaluation,
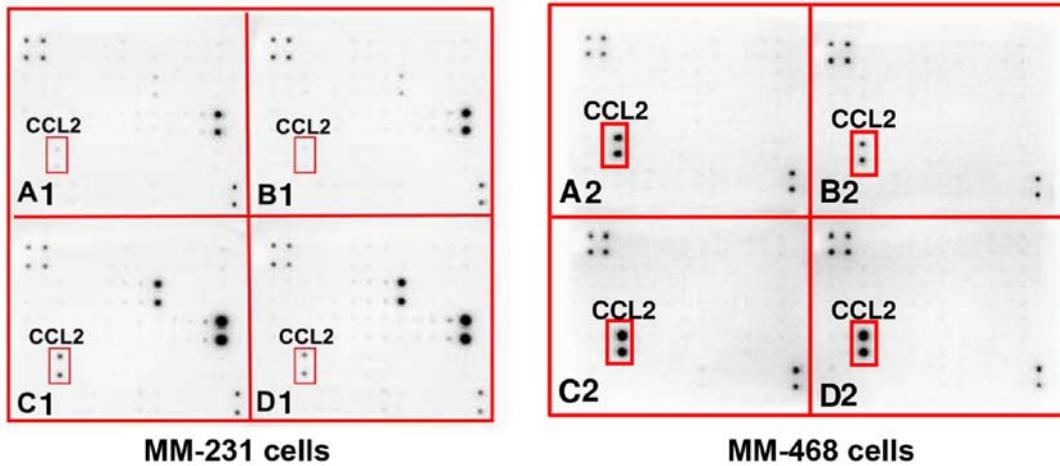
Figure 2. Effect of GOSS on cytokine expression in TNF-α-stimulated triple-negative breast cancer cells. The human protein microarray AAH-CYT-6 was used to measure chemokine/cytokine expression in the cell-free supernatant. (A1-D1) The four blots represent the supernatants of the following MM-231 cell treatments: (A1) Control, (B1) 6.25 µM GOSS, (C1) 50 ng/ml TNF-α and (D1) GOSS + TNF-α-treated. (A2-D2) The four blots represent the supernatants of the following MM-468 cell treatments: (A2) Control, (B2) 5 µM GOSS, (C2) 50 ng/ml TNF-α and (D2) GOSS + TNF-α-treated. Microarray chemiluminescence spotted the changes in only CCL2 cytokine expression that was red-framed on the AAH-CYT-6 array map as well as on all blots. GOSS, gossypol; TNF-α, tumor necrosis factor α.

Figure 3. Effect of GOSS on cytokine expression in TNF-α-stimulated triple-negative breast cancer cells. The human protein microarray AAH-CYT-7 was established to measure chemokine/cytokine expression in the cell-free supernatant. (A1-D1) The four blots represent the supernatants of the following MM-231 cell treatments: (A1) Control, (B1) 6.25 µM GOSS, (C1) 50 ng/ml TNF-α and (D1) GOSS + TNF-α-treated. (A2-D2) The four blots represent the supernatants of the following cell treatments: (A2) Control, (B2) 5 µM GOSS, (C2) 50 ng/ml TNF-α, and (D2) GOSS + TNF-α-treated. Microarray chemiluminescence determined the changes in only IL-8 chemokine expression that was red-framed on the AAH-CYT-7 array map as well as on all blots. GOSS, gossypol; TNF-α, tumor necrosis factor α.

Figure 4. Cytokine array quantification of chemokines inhibited by GOSS in TNF-α-stimulated triple-negative breast cancer cell lines.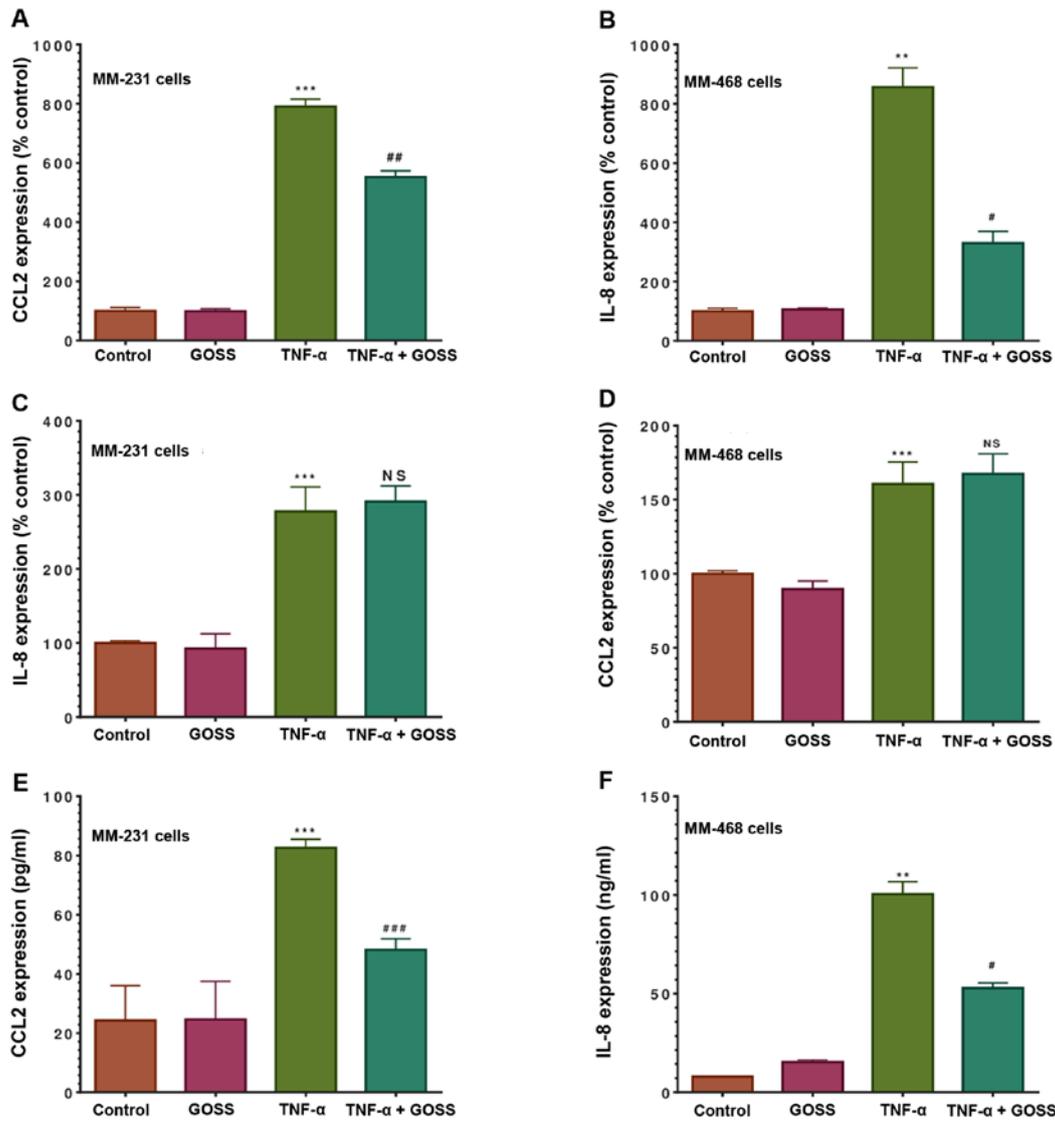 The effect of GOSS on the extracellular cytokine expression was assessed as follows: CCL2 release from (A) MM-231 cells and (D) MM-468 cells, IL-8 release from (B) MM-468 and (C) MM-231 cells. ELISA quantifications (E) for CCL2 released from MM-231 cells and (F) for IL-8 released from MM-468 cells. The normalized data show the cytokine expression in four sets of experimental cell supernatants, namely control, GOSS-treated (6.25 μM in MM-231 cells and 5 μM in MM-468 cells), 50 ng/ml TNF-α-stimulated, and co-treated cells (TNF-α + GOSS). The data are presented as the mean ± SEM of three independent experiments. The dot intensities are expressed as percent relative to control. The significant difference between TNF-α-stimulated cells vs. resting cells (*) or between TNF-α-stimulated vs. TNF-α + GOSS-treated cells (#) groups was determined by an unpaired t-test. P<0.05 was considered to indicate a statistically significant difference. **P<0.01, ***P<0.001, ##P<0.01 and ###P<0.001. NS, not significant; GOSS, gossypol; TNF-α, tumor necrosis factor α.
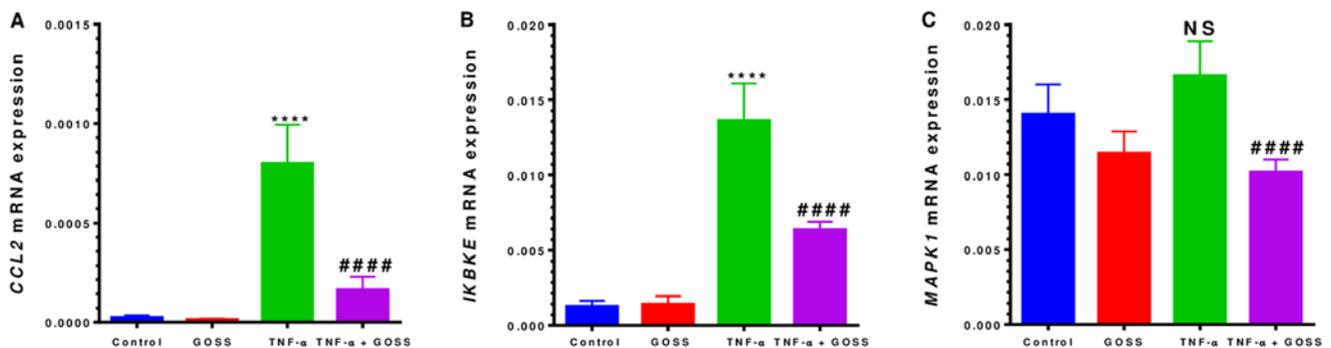
Figure 5. Effect of GOSS on proinflammatory cytokine-related gene expression determined using reverse transcription-quantitative PCR in TNF-α-stimulated MM-231 cells. GAPDH-normalized data indicated a significant increase in (A) CCL2 and (B) IKBKE mRNA expression in TNF-α-stimulated MM-231 cells compared with control cells. The same genes, as well as (C) MAPK1, were significantly inhibited in the presence of 6.25 μM GOSS. The data points are presented as the mean ± SEM of three independent experiments. The significance of the difference was analyzed by an unpaired t-test. P<0.05 was considered to indicate a statistically significant difference. ****P<0.0001 vs. control; ####P<0.0001 vs. TNF-α. NS, not significant; GOSS, gossypol; TNF-α, tumor necrosis factor α.
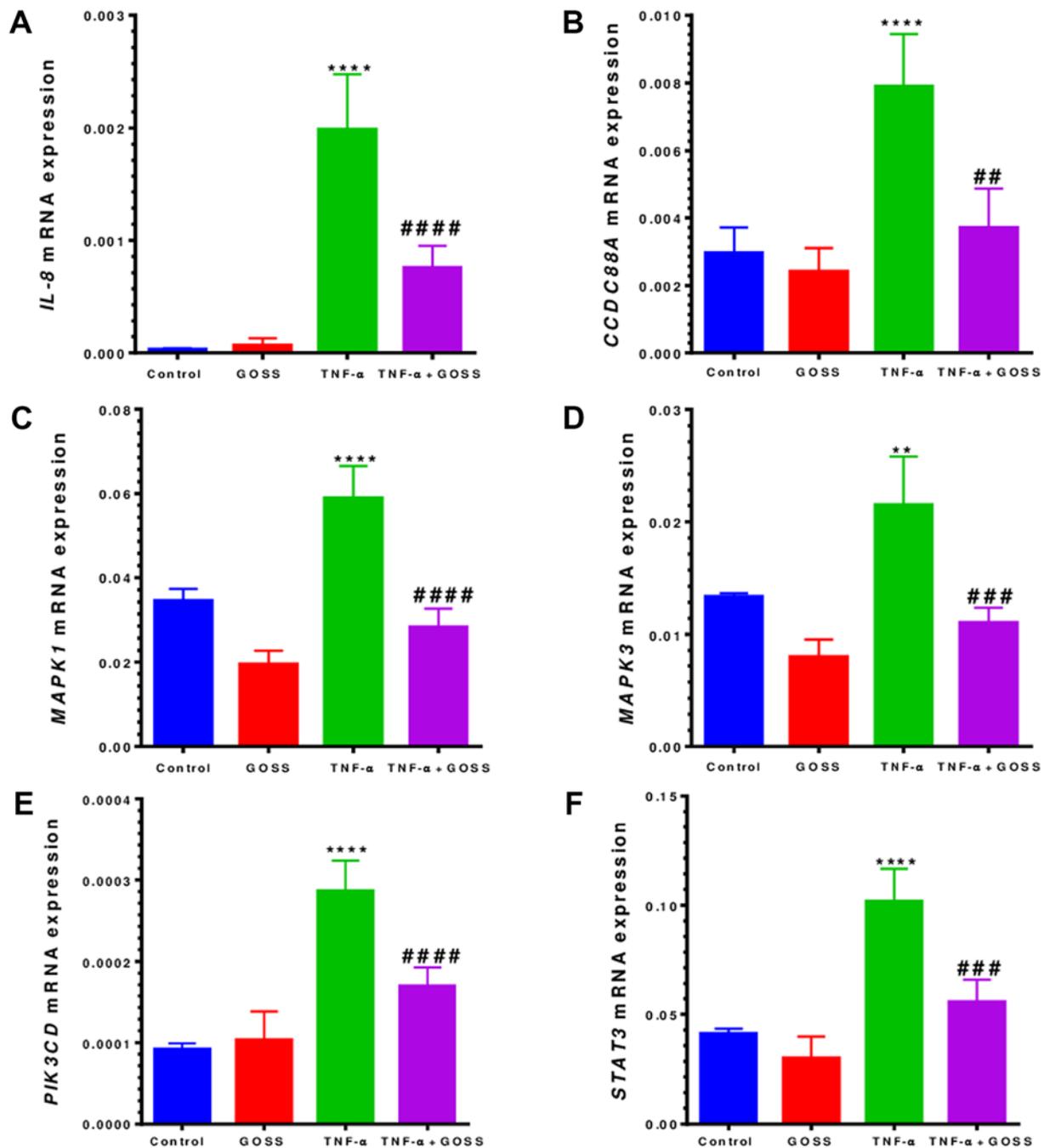
Figure 6. Effect of GOSS on proinflammatory cytokine-related gene expression in TNF-α-stimulated MM-468 cells according to reverse transcription-quantitative PCR. GAPDH-normalized data indicated a significant increase in six genes in TNF-α-stimulated vs. control cells as follows: (A) IL-8, (B) CCDC88A, (C) MAPK1, (D) MAPK3, (E) PIK3CD and (F) STAT3 genes. The same genes were significantly inhibited in the presence of 5 μM GOSS. The data points are presented as the mean ± SEM of three independent experiments. The significance of the difference between TNF-α-stimulated vs. control cells as well as TNF-α-activated vs. TNF-α + GOSS was analyzed by an unpaired t-test. P<0.05 was considered to indicate a statistically significant difference. **P<0.01 and ****P<0.0001 vs. control; ##P<0.01, ###P<0.001 and ####P<0.0001 vs. TNF-α. GOSS, gossypol; TNF-α, tumor necrosis factor α.

have validated the up-regulation of CCL2 and IL-8 pro-inflammatory proteins and their mRNAs and assured GOSS ability to inhibit both of them.

Discussion

One of the significant goals in anticancer drug development is targeting signaling pathways regulating cancer cells' survival and proliferation through the inhibition of distinctive gene expressions. The present study examined the anticancer

mechanism of the natural polyphenol GOSS in two racially different TNBC cell models. The cytotoxic effects of GOSS with a slight but significantly higher response against MM-231 cells than MM-468 TNBC cells indicate possible different mechanistic effects between the two cell lines. However, the effect of gossypol on normal breast cells was not established; a previous study on another cell line indicated the safety of the gossypol at a similar dose (53). This compound repressed the TNF-α-mediated upregulation of the two BC-dominant proinflammatory chemokines (CCL2) in MM-231 and (IL-8)

Table II. Gene expression changes in MM-231 triple-negative breast cancer.

Control vs. TNF-α			TNF-α vs. TNF-α + GOSS		
Target gene	Fold (+)	P-value	Target gene	Inhibition (%)	P-value
CCL2	32.72	<0.0001	CCL2	79.46	<0.0001
IKBKE	10.60	<0.0001	IKBKE	53.06	<0.0001
MAPK1	1.18	0.0648	MAPK1	38.81	<0.0001

The left side of the table presents the genes that were upregulated (+ fold-changes) by 50 ng/ml TNF-α. By contrast, the right side presents the expression of genes that were downregulated (% inhibition) by 6.25 μM GOSS. P<0.05 was considered to indicate a statistically significant difference. GOSS, gossypol; TNF-α, tumor necrosis factor α.

Table III. mRNA gene expression changes in MM-468 triple-negative breast cancer.

Control vs. TNF-α			TNF-α vs. TNF-α + GOSS		
Target gene	Fold (+)	P-value	Target gene	Inhibition (%)	P-value
IL-8	64.6	<0.0001	IL-8	60.78	<0.0001
CCDC88A	2.66	<0.0001	CCDC88A	53.04	0.0013
MAPK1	1.70	<0.0001	MAPK1	51.90	<0.0001
MAPK3	1.61	0.0062	MAPK3	48.61	0.0002
PIK3CD	3.12	<0.0001	PIK3CD	40.70	<0.0001
STAT3	2.47	<0.0001	STAT3	45.28	<0.0001

The left side of the table presents the genes that are upregulated (+ fold-changes) by 50 ng/ml TNF-α. By contrast, the right side presents the expression of genes that are downregulated (% inhibition) by 5 μM GOSS. P<0.05 was considered to indicate a statistically significant difference. GOSS, gossypol; TNF-α, tumor necrosis factor α.

in MM-468. Mechanisms of inhibitions involved the attenuation of mRNA expression of divers, active genes crucial in regulating pivotal signaling pathways. GOSS impacted MAPK, JAK-STAT, and NF-κB pathways in both cell lines, in addition to the PI3K-AKT pathway only in MM-468 (Fig. 7).

The high expression of TNF-α has been reported in BC patients (21). Whether it is endogenous or is administered in a high dose, TNF-α can function as tumor necrosis or tumor-promoting factor, respectively (54). In cancer, the highly expressed TNF-α stimulates the release of many chemokines, including CCL2 and IL-8 (5,55-57), that are highly expressed in cancer.

The most attenuated chemokines, CCL2, and IL-8 have been known to share some common characters. Both chemokines are belonging to a superfamily of small, soluble, and secreted proteins (58). They both are released into the cancer microenvironment by the endothelial cells and fibroblasts (59,60) to activate lymphocytes and macrophages (61,62).

Treating MM-231 cells with TNF-α upregulated the expression of CCL2, a finding that is consistent with previous studies (17,63). In the presence of GOSS (low dose of 6.25 μM), CCL2 expression was dramatically downregulated in the TNF-α-activated cells. Cytokine CCL2 is the most cancer-related member of the CC chemokine family (64). In BC cells, upregulated CCL2 (65) promotes tumorigenesis and

metastasis, and its suppression significantly reduced tumor aggressiveness (66). In MM-231 and other malignant tumors, the upregulated CCL2 (51,59) was linked to the decreased survival of TNBC patients (6). Hence, the reduction of CCL2 expression following antibody administration or genetic mutation (67) led to reduced metastasis and enhanced survival (51), which hold promise in treating TNBC (6).

Previous investigations have highlighted the impact of CCL2 on different signaling pathways regulation. CCL2 and its main receptor CCR2 control BC cell survival through MAPK- and Smad3-dependent mechanisms (19). Moreover, in various tissues, CCL2 activates Notch signaling pathway and regulates cancer stem cells (68), which are vital for BC progression (69,70). CCL2 gene silencing in MM-231 led to various consequences, including inhibition of CCL2 expression, decreased cell proliferation, increased necrosis and autophagy, inhibition of self-renewal, and inhibition of the primary and secondary invasion of the TNBC xenograft tumor, and decreases M2 macrophage recruitment (6).

Our current RT-qPCR gene expression analyses indicate that TNF-α significantly increased the mRNA expression of two genes: CCL2 and its gene regulator IKBKE. At the same time, the data obtained show the ability of GOSS to attenuate the expression of these two genes as well as MAPK1. These three genes are involved in many signaling pathways,
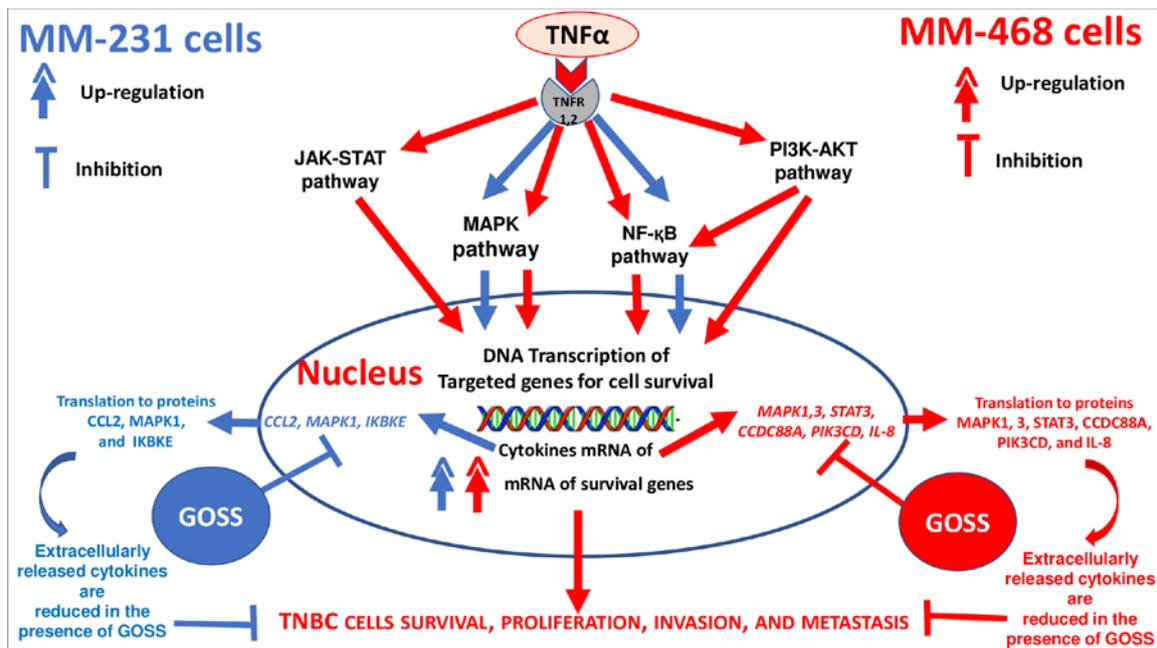
Figure 7. Schematic diagram illustrating pro-inflammatory genes involved in different signaling pathways that mediate the release of CCL2 and IL-8 in TNF-α-stimulated MM-231 (blue color) and MM-468 (red color) triple-negative breast cancer cells, respectively. In MM-231 cells, the most down-regulated genes by GOSS are *CCL2*, *IKBKE*, and *MAPK1*. In MM-468 cells, the most down-regulated genes are *IL-8*, *CCDC88A*, *MAPK1* and 3, *PIK3CD*, and *STAT3*. GOSS, gossypol; TNF-α, tumor necrosis factor α.

including the NF-κB, JAK-STAT, and MAPK, which regulate the expression of CCL2. Nevertheless, NF-κB and MAPK signaling pathways are used by both normal and cancer cells dominating these signaling pathways to enhance their survival and invasion (71).

The gene *IKBKE* (also known as IKKε) is a member of the IKK family of kinases (72). Extensive studies have reported the correlation between *IKBKE* down-regulation and the decrease of CCL2 release (49,73). Our current data supported these previous findings and interpreted the mechanism of CCL2 inhibition by GOSS. In TNBC cells, the *IKBKE* gene is expressed in 60% of BC tissue (74) and acts as an oncogene to support cell viability. Furthermore, the up-regulation of *IKBKE* enhances resistance to anticancer drugs and protects the BC cells from Tamoxifen-induced apoptosis (75). Indeed, *IKBKE* is an upstream regulator of the transcription factor NF-kB and JAK-STAT pathways, which have been actively involved in the pathogenesis of TNBC (76-79). Expectedly, the silencing of *IKBKE* in BC cells has been reported to reduce the NF-κB activity and inhibit proliferation, clonogenicity, migration, and invasion (80-84).

GOSS inhibited the expression of *MAPK1* (also known as *ERK*) that was highly expressed in unstimulated cells. The obtained data are consistent with those from previous studies (85,86) that related the lower survival rate in TNBC to highly expressed *MAPKs*. These serine/threonine protein kinases are involved in various intracellular metabolism, including differentiation, proliferation, apoptosis, and cellular stress responses (87,88). Deregulated ERK signaling pathway leads to uncontrolled cell proliferation and a reduction in apoptosis (89). In MM-231 BC cells, upregulated p38 pathway is linked to the increased proliferation and migration (71) and

contribute to cancer cell invasion (90). Therefore, highlighting GOSS effects on *MAPKs* implies its particularity for TNBC.

Our data indicated that TNF-α-stimulated the release of IL-8 In MM-468 cells, the finding that agrees with a previous study (56). Indeed, a significant positive relationship has been reported between IL-8 release and TNF-α that can synergistically affect cancer initiation and progression (55). The double-functional IL-8 can boost the immunoregulatory ability or alter the cell microenvironment to enhance tumorigenesis (91). The chemokine IL-8 is a member of the CXC chemokine family of multi-functional proinflammatory cytokines (92). IL-8 is highly expressed in many types of tumors and has the ability to promote cancer cell proliferation (93). In BC patients, elevated levels of serum IL-8 is a predictive marker (55,94). Moreover, IL-8 is positively correlated with angiogenesis and metastasis (95) and contributes to multidrug resistance in human BC cells (96). Particularly in TNBC, IL-8 is highly expressed compared with other BC subtypes (97), and it is associated with a poor prognosis (55,92,98). Meaningfully, the poorer prognosis and treatment resistance are linked to IL-8 and its highly expressed receptors, CXCR1 (IL-8R$_A$) and CXCR2 (IL-8R$_B$) in BC patients (95,99,100). Therefore, targeting CXCR signaling is considered a promising approach in *in-vivo* models of BC (101) as it attenuates the distress effect of IL-8 (102). Here, the TNF-α-stimulated IL-8 expression was followed by a substantial attenuation of the chemokine in the presence of only 5 μM of GOSS.

In MM-468 cells, GOSS repressed many highly expressed genes, including *IL-8*, *MAPK1*, *MAPK3*, *CCDC88A*, *STAT3*, and *PIK3CD*. The number and type of the affected genes in MM-468 were divergent from its counterpart mRNA data for MM-231. The repressed genes modulate major signaling pathways, including MAPK, PI3K-AKT, JAK-STAT, and

NF-κB. Those oncogenic signaling pathways regulate IL-8 expression and potentiate tumor cell migration or invasion (103,104). The data indicated high expressions of *MAPK1* and *MAPK3* in unstimulated MM-468 cells; an observation which is consistent with its counterpart MM-231 and agrees with those previously reported findings (85,86). The reported link between *IL-8* mRNA stabilization, its protein production, and the MAPKs (105-107) is compatible with GOSS effects in our MM-468 study.

Furthermore, GOSS inhibited the expression of the *CCDC88A* gene [also known as AKT phosphorylation enhancer (APE), or AKT-binding protein Girdin], that is highly expressed in BC and other types of cancer (108). The gene augmenting the PI3K-AKT signaling pathway and regulate cell migration (109), proliferation, and apoptosis (110). Also, upregulated AKT expression enhances the NF-κB signaling pathway, promoting tumor progression and metastasis (111). GOSS repressed the overexpression of *PIK3CD,* the gene encoding phosphoinositide 3-kinase (*PI3K*). The activated *PI3K* signaling is involved in metabolism and is essential in promoting tumorigenesis and cell proliferation (112-114). In normal cells, PI3K-AKt/mTOR signaling pathways are critical for cell survival and proliferation. Meanwhile, PI3K, AKT, and mTOR proteins are highly upregulated in BC and other cancers. Inhibitors of these kinases are found to induce apoptosis and to synergize the effect of anticancer drugs to inhibit the tumor progression (112-114). Thus, the obtained data indicate GOSS targeting ability of both *PIK3CD* and *CCDC88A* by indirectly attenuating NFκB and, consequently, the overexpression of IL-8 in MM-468 BC cells. The mRNA expression inhibition of the signal transducer and activator of transcription3 (*STAT3*) signifies the GOSS role against BC. *STAT3* is a member of the STAT family proteins, and it is highly triggered in more than 50% of BC patients (115). Furthermore, Targeting *STAT3* impacts other cancer-associated pro-inflammatory cytokines (116) and many genes linked to apoptosis, proliferation, and angiogenesis (117). Moreover, phosphorylation of STAT3, AKT, and ERK1/2 stimulate BC cells to undergo epithelial-mesenchymal transition, up-regulate the expression of IL-8 and stimulate BC metastasis (104), inhibiting the mRNA expressions of these proteins in MM-468 cells may further elucidate previously reported anticancer mechanisms of GOSS through inhibiting their phosphorylation (37). Thus, inhibiting STAT3 activation by GOSS is a substantial target in treating different types of cancer.

In summary, the comparative effect of gossypol on different cytokines release from TNBC cells has never been reported before. Also, gossypol effects on the aggressive TNBC of MM-468 cells, derived from AA women (118), are not reported. The current study elucidated a novel mechanism targeting TNBC and highlight the possible gene-related signaling pathways. However, lacking protein production assessments, such as using Western blotting, is a limitation in this research and needs further broader range investigation. The polyphenol compound, GOSS, impacts MM-231 and MM-468 cells differently. In MM-231 cells, the compound attenuated the expression of the cytokine CCL2 through influencing its encoding gene, *CCL2,* as well as two more regulatory genes (*IKBKE* and *MAPK1*), which are involved in NF-κB, JAK-STAT, and MAPK signaling pathways. Similarly,

GOSS repressed the cytokine IL-8 in MM-468 cells by targeting IL-8 mRNA expression. However, more genes were inhibited by GOSS, including *MAPK1*, *MAPK3*, *PIK3CD*, *STAT3*, and *CCDC88A*. These genes are regulating different interactive signaling pathways mediating IL-8 releases such as PI3K-AKT, JAK-STAT, and MAPK. In conclusion, the data obtained in this study indicate that the polyphenol compound GOSS may provide a valuable tool in TNBC therapies.

Acknowledgements

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

The conceptualization of this study was accomplished by SSM and KFAS. The research methodology was designed by SSM, NOZ, PM, CC and KFAS. Data analysis was conducted by SSM, NOZ, PM, CC and KFAS. Funding acquisition was fulfilled by KFAS. Project administration was performed by KFAS, and study resources were collected by SSM, NOZ, PM, CC and KFAS. Software analysis of data and figures was conducted by SSM, NOZ, PM and CC, and supervision of the research was conducted by KFAS. Writing of the original draft was undertaken by SSM, and writing, review, and editing of the manuscript were carried out by SSM, NOZ, PM and KFAS. All authors read and approved the final manuscript and accept to be responsible for all aspects of the research in ensuring that the accuracy or integrity of any part of the work is properly considered.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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