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Alterations in tumor necrosis factor signaling pathways are associated with cytotoxicity and resistance to taxanes: a study in isogenic resistant tumor cells

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Abstract

Introduction: The taxanes paclitaxel and docetaxel are widely used in the treatment of breast, ovarian, and other cancers. Although their cytotoxicity has been attributed to cell-cycle arrest through stabilization of microtubules, the mechanisms by which tumor cells die remains unclear. Paclitaxel has been shown to induce soluble tumor necrosis factor alpha (sTNF- α) production in macrophages, but the involvement of TNF production in taxane cytotoxicity or resistance in tumor cells has not been established. Our study aimed to correlate alterations in the TNF pathway with taxane cytotoxicity and the acquisition of taxane resistance.

Methods: MCF-7 cells or isogenic drug-resistant variants (developed by selection for surviving cells in increasing concentrations of paclitaxel or docetaxel) were assessed for sTNF- α production in the absence or presence of taxanes by enzyme-linked immunosorbent assay (ELISA) and for sensitivity to docetaxel or sTNF- α by using a clonogenic assay (in the absence or presence of TNFR1 or TNFR2 neutralizing antibodies). Nuclear factor (NF)- κ B activity was also measured with ELISA, whereas gene-expression changes associated with docetaxel resistance in MCF-7 and A2780 cells were determined with microarray analysis and quantitative reverse transcription polymerase chain reaction (RTqPCR).

Results: MCF-7 and A2780 cells increased production of sTNF- α in the presence of taxanes, whereas docetaxel-resistant variants of MCF-7 produced high levels of sTNF- α , although only within a particular drug-concentration threshold (between 3 and 45 nM). Increased production of sTNF- α was NF- κ B dependent and correlated with decreased sensitivity to sTNF- α , decreased levels of TNFR1, and increased survival through TNFR2 and NF- κ B activation. The NF- κ B inhibitor SN-50 reestablished sensitivity to docetaxel in docetaxel-resistant MCF-7 cells. Gene-expression analysis of wild-type and docetaxel-resistant MCF-7, MDA-MB-231, and A2780 cells identified changes in the expression of TNF- α -related genes consistent with reduced TNF-induced cytotoxicity and activation of NF- κ B survival pathways.

Conclusions: We report for the first time that taxanes can promote dose-dependent sTNF- α production in tumor cells at clinically relevant concentrations, which can contribute to their cytotoxicity. Defects in the TNF cytotoxicity pathway or activation of TNF-dependent NF- κ B survival genes may, in contrast, contribute to taxane resistance in tumor cells. These findings may be of strong clinical significance.

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Introduction

Taxanes are a family of chemotherapy drugs used to treat various human cancer types [1-6]. The most common family members include paclitaxel and docetaxel, which block microtubule depolymerization, inducing cell-cycle arrest at mitosis and multinucleation of tumor cells [7,8]. Taxanes also reduce tumor angiogenesis and cell migration, while stimulating the immune system against neoplasms [9-11].

Another mechanism for taxane cytotoxicity may involve tumor-necrosis factor (TNF)- α production, because paclitaxel has been shown to augment TNF- α levels in murine macrophages [12]. TNF- α is a membrane-integrated cytokine (mTNF- α) generally produced in activated macrophages and monocytes [13], which can be released from cells in a soluble form (sTNF- α) by the action of the metalloproteinase ADAM-17 [14]. The release of sTNF- α from cells can then induce cell death or a cell-survival response, depending on the receptor to which it binds: TNFR1 or TNFR2, respectively (reviewed in [15]). Although mTNF- α binds with equal affinity to both receptors, sTNF- α preferentially binds to TNFR1, which has a death-effector domain that induces caspase-8 cleavage and apoptosis [16,17]. Unlike TNFR1, TNFR2 does not contain a death domain. Limited reports suggest that TNFR2 activation promotes cell death, although the mechanism for this is poorly understood and may require the presence of TNFR1 [18,19]. Nevertheless, TNFR2 has been shown to induce NF- κ B activity and cell survival [20].

Many mechanisms associated with resistance to taxanes have been identified *in vitro*, such as overexpression of the drug-efflux pump Abcb1, β -tubulin gene mutations, or overexpression of β -tubulin (type III); however, their clinical relevance remains unclear [21,22]. Multiple mechanisms of taxane resistance likely occur simultaneously in cells [23]. To restore tumor sensitivity to taxanes appreciably in cancer patients, all clinically relevant mechanisms of docetaxel resistance must be identified.

To better understand the various pathways associated with taxane resistance, our laboratory selected MCF-7 breast tumor cells for survival in increasing concentrations (doses) of paclitaxel (MCF-7_{TAX-1} cells) [24] or docetaxel (MCF-7_{TXT} cells) [23]. Increased Abcb1 expression and decreased taxane uptake occurred on acquisition of taxane resistance in these cells, but a pan-ABC transporter inhibitor that restored taxane uptake had no or only a partial effect on drug sensitivity in these cells [23]. Therefore, additional mechanisms must contribute to taxane resistance, and these cell lines may serve as an attractive tool for assessing the possible role of TNF- α and other pathways in taxane cytotoxicity or resistance.

This study reveals for the first time that docetaxel concentrations of 3 nM or greater induce tumor necrosis factor (TNF) expression in MCF-7 cells, and that acquisition of docetaxel resistance can be temporally correlated with elevations in cellular TNF- α levels resistance to TNF- α cytotoxicity, degradation of TNFR1, and promotion of TNFR2-induced survival pathways through the activation of NF- κ B. In further support of the role of TNF in taxane cytotoxicity and resistance, we also report that both paclitaxel and docetaxel can induce TNF- α expression in A2780 ovarian carcinoma cells. In addition, we report the consistent alteration in networks of TNF-related genes on acquisition of docetaxel resistance in breast and ovarian tumor cells.

Materials and methods

Cell culture and maintenance

MCF-7 cells from the American Tissue Culture Collection (catalog number HTB-22) were cultured or selected for survival in increasing doses of docetaxel or paclitaxel, as previously described [23,24]. The initial concentrations of docetaxel and paclitaxel used to begin selection (dose 1) were 0.51 and 0.56 nM, respectively. Cells selected to docetaxel concentrations of 1.11 nM (dose 8, MCF-7_{TXT8}), 3.33 nM (dose 9, MCF-7_{TXT9}), 5.00 nM (dose 10, MCF-7_{TXT10}), 15 nM (dose 11, MCF-7_{TXT11}), and 45 nM (dose 12, MCF-7_{TXT12}) were used in this study. Numbers in subscripts of cell-line names refer to the maximal docetaxel dose level to which the cells were exposed. The paclitaxel-resistant cell line used in this study was selected in an identical manner to a final concentration of 6.64 nM paclitaxel (MCF-7_{TAX-1} cells; hyphenated number indicates the first cell-line selection, not drug dose). MCF-7 cells were also "selected" in the absence of taxanes to passage numbers similar to those of drug-selected cells to control for genotypic or phenotypic changes associated with long-term culture ("co-cultured control" MCF-7_{CC} cells). A2780 ovarian carcinoma cells from the European Collection of Cell Cultures were also selected for resistance to docetaxel in an identical manner (A2780_{DXL} cells), including the creation of "co-cultured control" A2780_{CC} cells (Armstrong *et al.*, unpublished data).

Measurement of sTNF- α and sTNFR1 in cell-culture media

Concentrated proteins from the medium of 2 million MCF-7_{CC}, MCF-7_{TXT}, or A2780 cells (grown in culture in the absence or presence of various concentrations of paclitaxel or docetaxel) were assessed for levels of sTNF- α or sTNFR1 by using ELISA kits from R&D Systems, following the manufacturer's instructions.

Clonogenic assays

Cellular sensitivity to TNF- α or docetaxel was assessed by using a clonogenic assay, as described previously

[24]. Docetaxel resistance factors for the cell lines were determined by dividing the median inhibitory concentration (IC_{50}) for docetaxel in the taxane-resistant cell lines by the IC_{50} for MCF-7_{CC} cells. In some experiments, cells were exposed to 1 μ g/ml cycloheximide, TNFR1 or TNFR2 neutralizing antibodies from R&D Systems (both at 5 μ g/ml), or a peptide from Calbiochem Laboratories (La Jolla, CA), which potently blocks NF- κ B function by inhibiting translocation of the NF- κ B complex into the nucleus [25] (SN-50, 7 μ g/ml). A control peptide at the same concentration (SN-50 M) was used in the latter experiments to assess the specificity of NF- κ B inhibition.

Immunoblotting analysis

MCF-7_{CC}, MCF-7_{TXT}, and MCF-7_{TAX-1} cells were incubated in the absence or presence of 20 ng/ml TNF- α for 24 hours. Cells were extracted in RIPA buffer, and 100 μ g of extract proteins assessed for the expression of specific proteins by using standard immunoblotting procedures, as previously described [24]. Antibodies used in these experiments included TNFR1-, TNFR2-, and I κ B-specific antibodies from Cell Signaling Technology (Danvers, MA) and a mouse-derived GAPDH antibody from Santa Cruz Laboratories. Densitometric quantitation of bands generated by the I κ B antibody was performed by using AlphaEaseFC software (Alpha Innotech, San Leandro, CA). Band intensity was normalized relative to GAPDH band intensity.

Quantification of TNFR1 and TNF- α transcript levels by RTqPCR

The levels of TNFR1 and TNF- α transcripts in MCF-7_{CC} and MCF-7_{TXT10} cells were assessed as described previously [26] by using the following primers: TNFR1: forward, 5'-ACTGCCTCAGCTGCTCCAAAT-3'; reverse, 5'-CCGGTCCACTGTGCAAGAA-3', TNF- α : forward, 5'-TCTTCTCGAACCCCGAGTGA-3', reverse, 5'-GGAGCTGCCCT-CAGCTT-3'; and S28: forward, 5'-TCCATCATCCGCAATGTAAAAG-3', reverse, 5'-GCTTCTGCGTCTGACTCCAAA-3'.

Measurement of NF- κ B activity

MCF-7_{CC} and MCF-7_{TXT} cells were cultured in the presence or absence of 50 nM docetaxel for 24 hours. The activity of the NF- κ B p65 and p50 subunits in 10 μ g of nuclear extracts was assessed as outlined in the TransAM NF- κ B Family ELISA kit (Active Motif, Carlsbad, CA). Readings at 450 nm were normalized to the sum of all readings on the plate to compare across triplicate experiments.

Identification of changes in gene expression associated with the acquisition of docetaxel resistance

Agilent 4 \times 44 k human genome oligonucleotide arrays were used to profile differences in gene expression

between MCF-7_{TXT} and MCF-7_{CC} cells at selection dose 10 and between docetaxel-resistant and wild-type A2780 ovarian carcinoma cells (A2780_{DXL} and A2780 cells, respectively) at the maximally tolerated dose by using MIAME standards [27]. RNA was isolated from each cell line by using RNeasy Mini kits (Qiagen, Mississauga, ON), and 500 ng of each RNA preparation was labeled and amplified by using Agilent Quick Amp labeling kits. The labeling and array hybridization procedures were performed as per the manufacturer's protocol for a two-color microarray experiment.

Identification of differences in gene expression associated with docetaxel resistance

The hybridized microarrays were scanned by using Agilent scanners and feature extraction software (version 10_7_3_1), and differentially expressed genes associated with the acquisition of docetaxel resistance were identified by using Partek Genomic Suite (Partek, Inc., St. Louis, MO). The background-corrected intensity values were used for analysis. A three-way ANOVA was performed to identify significant changes in gene expression by using the Method of Moments [28]. Genes with greater than twofold differences in gene expression were selected with a false discovery rate of either 0.05 or 0.01 [29]. The data from these array experiments were deposited in the National Centre for Biotechnology Information Gene Expression Omnibus database (accession number GSE26129) [30].

Network-based analysis of gene expression

To determine whether the previously described changes in gene expression associated with acquisition of docetaxel resistance in breast or ovarian tumor cells may reflect changes in the function of specific biochemical pathways in these cells, the genes identified as being associated with docetaxel resistance were subjected to functional-interaction (FI) network analysis [31]. In brief, the FI network covers ~50% of the human proteome representing more than 200,000 functional interactions. Pairwise shortest paths among genes of interest in the FI network were calculated and hierarchically clustered (based on the average-linkage method). Clusters were then selected containing more than 90% of altered genes. To calculate a *P* value for the average shortest path, we performed a 1,000-fold permutation test by randomly selecting the same number of genes from the biggest connected network component. A minimum spanning tree algorithm was used to find linkers that connected all genes of interest in one subnetwork [32]. We used the Markov Cluster Algorithm (MCL) [33] with inflation of 1.6 for network clustering. Only the biggest clusters with numbers of proteins not less than 2% of the total network were taken into

account. All network diagrams were drawn by using Cytoscape [34]. The functional enrichment analysis for pathways was based on a binominal test. A false discovery rate was calculated based on 1,000 permutations on all genes in the FI network. This network-based analysis was also applied to another dataset that documents differences in gene expression between docetaxel-resistant and parental MDA-MB-231 breast cancer cell lines (Gene Expression Omnibus (GEO) accession number GSE28784).

Confirmation of microarray-based changes in gene expression by reverse transcription quantitative polymerase chain reaction

A number of the TNF- α -related genes in these networks were further assessed for expression in wild-type and docetaxel-resistant MCF-7 and A2780 cells with reverse transcription quantitative polymerase chain reaction (RTqPCR) by using the primers depicted in Table 1 and the method described earlier.

Results

Docetaxel increases sTNF- α production in MCF-7_{CC} and A2780 cells

MCF-7_{CC} and A2780_{CC} cells secreted low levels of sTNF- α ($1.69 \times 10^{-18} \pm 0.40 \times 10^{-18}$ g/cell and $3.02 \times 10^{-18} \pm 0.28 \times 10^{-18}$ g/cell, respectively). These levels were not significantly changed when cells were treated with 0.1 to 1 nM docetaxel. In contrast, media extracted from MCF-7_{CC} cells treated with ≥ 3 nM docetaxel produced significantly elevated levels of sTNF- α (Figure 1a). A2780 cells produced even greater amounts of TNF- α in response to docetaxel (Figure 1a). Interestingly, the taxane paclitaxel (at concentrations ≥ 15 nM) induced even higher levels of sTNF- α production than docetaxel in A2780 cells (Figure 1b). Given the stronger induction of TNF- α by docetaxel in A2780 cells, we then assessed whether upstream mechanisms responsible for TNF- α induction in A2780

cells were similar to those of macrophages. Comparable to the induction of TNF- α expression by lipopolysaccharides in macrophages [35], we observed that TNF- α induction by docetaxel in A2780 cells was dependent on NF- κ B, because an inhibitor of this transcription factor (SN-50) significantly reduced the induction of TNF- α by docetaxel (Figure 1c). The basal amount of sTNF- α production and the magnitude of docetaxel-induced sTNF- α production varied between experiments (compare Figures 1a and 1c for 45 nM docetaxel). Nevertheless, the sTNF- α levels were consistently and substantially higher in cells treated with taxanes. The extent of TNF- α induction by the taxanes appeared to decrease at higher docetaxel concentrations, possibly because of other deleterious effects of these agents on cells at the higher doses.

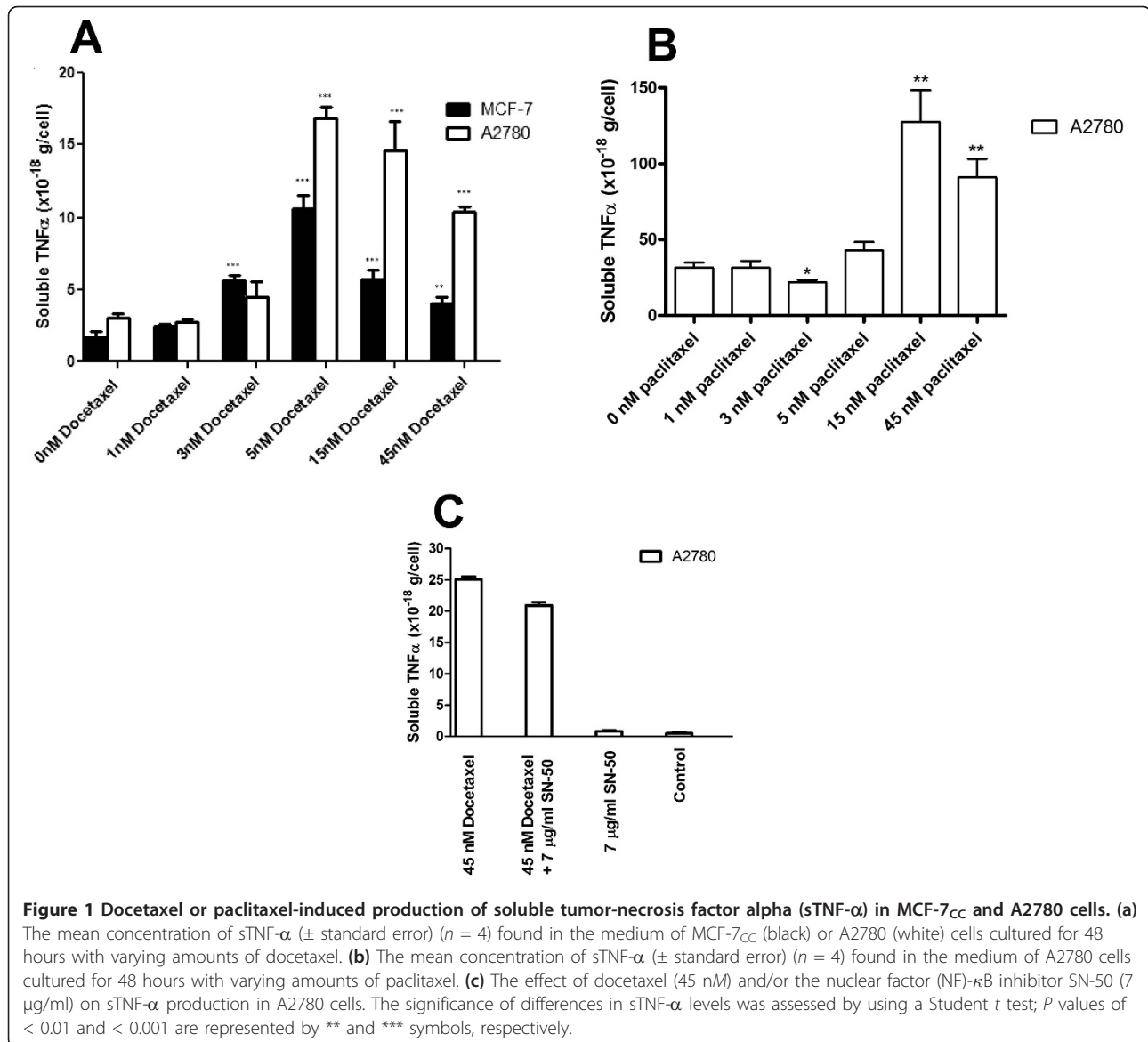
Selection of MCF-7 cells in increasing concentrations of docetaxel results in acquisition of progressive docetaxel resistance above a threshold dose

Increasing exposure of MCF-7 cells to docetaxel up to a concentration of 1.1 nM (dose 8, MCF-7_{TXT8} cells) did not affect docetaxel sensitivity (Figure 2). However, selection to 3.33 nM docetaxel (dose 9, MCF-7_{TXT9} cells) resulted in an 11.4-fold resistance to docetaxel. Above this threshold, resistance factors increased to 16.6, 32.8, and 184 for cells selected to final docetaxel concentrations of 5 nM (dose 10, MCF-7_{TXT10} cells), 15 nM (dose 11, MCF-7_{TXT11} cells), and 45 nM (dose 12, MCF-7_{TXT12} cells), respectively. Interestingly, MCF-7_{TXT} cells exhibited an even greater cross-resistance to paclitaxel, with resistance factors of 148 and 251 at selection doses 11 and 12, respectively [23]. The resistance factor for MCF-7 cells selected for resistance to paclitaxel at the maximally tolerated dose (MCF-7_{TAX-1} cells) was 42. These cells also exhibited strong cross-resistance to docetaxel (46-fold) [24]. In contrast, ovarian A2780 cells could be selected for resistance to considerably higher concentrations of docetaxel. A2780_{DXL} cells at their maximally tolerated dose

Table 1 Primers selected for confirmation of changes in the expression of tumor necrosis factor- α -related genes by reverse transcription quantitative polymerase chain reaction

Gene	Forward primer	Reverse primer
S28	5'TCC ATC ATC CGC AAT GTA AAG-3'	5'-GCT TCT CGC TCT GAC TCC AAA-3'
TNFAIP3	5'-GAC CAT GGC ACA ACT CAT CTC A-3'	5'-GTT AGC TTC ATC CAA CTT TGC GGC ATT G-3'
TNFSF10	5'-CGT GTA CTT TAC CAA CGA GCT GA-3'	5'-ACG GAG TTG CCA CTT GAC TTG-3'
TNFSF13	5'-ACT CTC AGT TGC CCT CTG GTT G-3'	5'-GGA ACT CTG CTC CGG GAG ACT C-3'
TNFSF14	5'-TTT GCT CCA CAG TTG GCC TAA TC-3'	5'-CAA TGA CTG TGG CCT CAC CTT C-3'
TLR1	5'-GGT ACC AGG CCC TCT TCC TCG TTA G-3'	5'-TAG GAA CGT GGA TGA GAC CG TTT TT-3'
TLR6	5'-GCA AAA ACC CTT CAC CTT GTT TTT C-3'	5'-CCA AGT CGT TTC TAT GTG GTT GAG G-3'
BIRC3	5'-TGT TGG GAA TCT GGA GAT GA-3'	5'-CGG ATG AAC TCC TGT CCT TT-3'
TNFR1	5'-ACT GCC TCA GCT GCT CCA AAT-3'	5'-CCG GTC CAC TGT GCA AGA A-3'
TNF α	5'-TCT TCT CGA ACC CCG AGT GA-3'	5'-GGA GCT GCC CCT CAG CTT-3'

Reaction conditions were as described in Materials and methods, and expression was assessed relative to that of S28, the internal reference gene.



(405 nM) exhibited ~4,000-fold resistance to docetaxel (Armstrong *et al.*, unpublished data).

Effects of docetaxel on sTNF α in MCF-7_{CC} and MCF-7_{TXT} cell lines

MCF-7_{CC} and MCF-7_{TXT8} cells secreted low amounts of TNF- α ($11.5 \times 10^{-18} \pm 0.4 \times 10^{-18}$ g/cell and $5.5 \times 10^{-18} \pm 1.4 \times 10^{-18}$ g/cell, respectively). When these cell lines were exposed to 50 nM docetaxel, no significant difference in sTNF- α secretion was observed (Figure 3a). In contrast, untreated MCF-7_{TXT9} and MCF-7_{TXT10} cells secreted 31.8-fold and 18.2-fold higher levels of sTNF- α than did MCF-7_{CC} cells ($P < 0.0001$), and addition of 50 nM docetaxel increased sTNF- α production a further 1.62-fold and 1.27-fold, respectively ($P < 0.01$). sTNF- α

levels returned to basal levels in MCF-7_{TXT11} and MCF-7_{TXT12} cells, even after treatment with 50 nM docetaxel. No differences in sTNF- α levels were observed between MCF-7_{CC} and MCF-7_{TAX-1} cells, in the presence or absence of docetaxel (data not shown). TNF- α transcript levels in MCF-7_{TXT10} cells (relative to S28 expression) were 198.5 ± 30.5 higher than the levels of this transcript in MCF-7_{CC} cells (Figure 3b), suggesting that elevated secretion of sTNF- α is likely due to dramatically increased expression of TNF- α transcripts and protein.

MCF-7_{TXT} and MCF-7_{TAX-1} cells are resistant to TNF- α -induced cytotoxicity

TNF- α (10 ng/ml) reduced colony formation in a clonogenic assay by $79.8\% \pm 6.0\%$ and $66.6\% \pm 1.7\%$ for

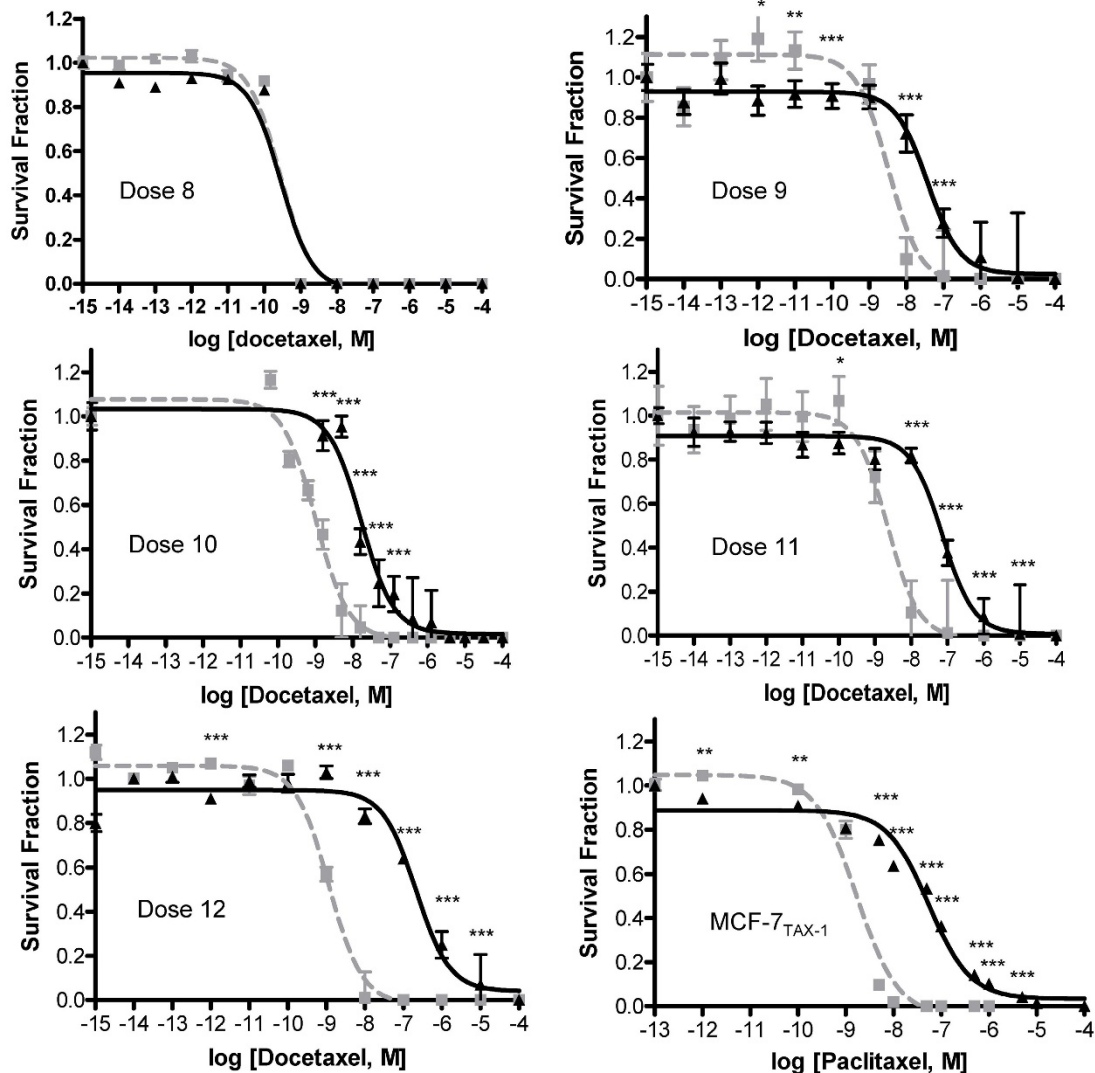


Figure 2 Acquisition of resistance to docetaxel or paclitaxel in MCF-7 cells. Sensitivity of MCF-7_{CC} cells (broken lines) and taxane-selected MCF-7 cells (solid lines) was measured after selection for survival to dose levels 8 (MCF-7_{TXT8}), 9 (MCF-7_{TXT9}), 10 (MCF-7_{TXT10}), 11 (MCF-7_{TXT11}), or 12 (MCF-7_{TXT12}), or at the maximally tolerated dose of paclitaxel (MCF-7_{TAX-1} cells). Mean survival fractions (\pm standard error) are plotted, and the significance of differences in docetaxel sensitivity between the taxane-selected and control cell lines was assessed by using a Student *t* test ($n = 5$); *P* values of < 0.05 , < 0.01 , and < 0.001 are represented by the *, **, and *** symbols, respectively.

MCF-7_{CC} and MCF-7_{TXT8} cells, respectively ($P < 0.0001$) (Figure 4a). In contrast, MCF-7_{TXT9}, MCF-7_{TXT10}, MCF-7_{TXT11}, and MCF-7_{TAX-1} cells all had similar levels of colony formation in the absence or presence of 10 ng/ml TNF- α , indicating substantial TNF- α resistance. TNF actually increased colony formation in MCF-7_{TXT12} cells, possibly because of a high level of activation of growth and survival pathways in these cells at the highest selection dose, some of which are TNF- α dependent (see Discussion). The cell lines were also cultured in the presence of varying concentrations of TNF- α . Colony formation was very strongly reduced in MCF-

7_{CC} cells in the presence of 50 or 100 ng/ml TNF- α ($P < 0.0001$) (Figure 4b). Reductions in colony formation were much smaller for MCF-7_{TXT10} cells treated with 50 ng/ml or 100 ng/ml TNF- α , again indicating resistance to TNF- α cytotoxicity in docetaxel-resistant cells. MCF-7_{TAX-1} cells treated with 10 ng/ml TNF- α formed similar numbers of colonies as did untreated cells, suggesting that these cells were also resistant to TNF- α . However, TNF- α concentrations of 50 or 100 ng/ml induced strong reductions in colony formation relative to MCF-7_{TXT10} cells, suggesting greater resistance to TNF- α in the former cell line than in the latter.

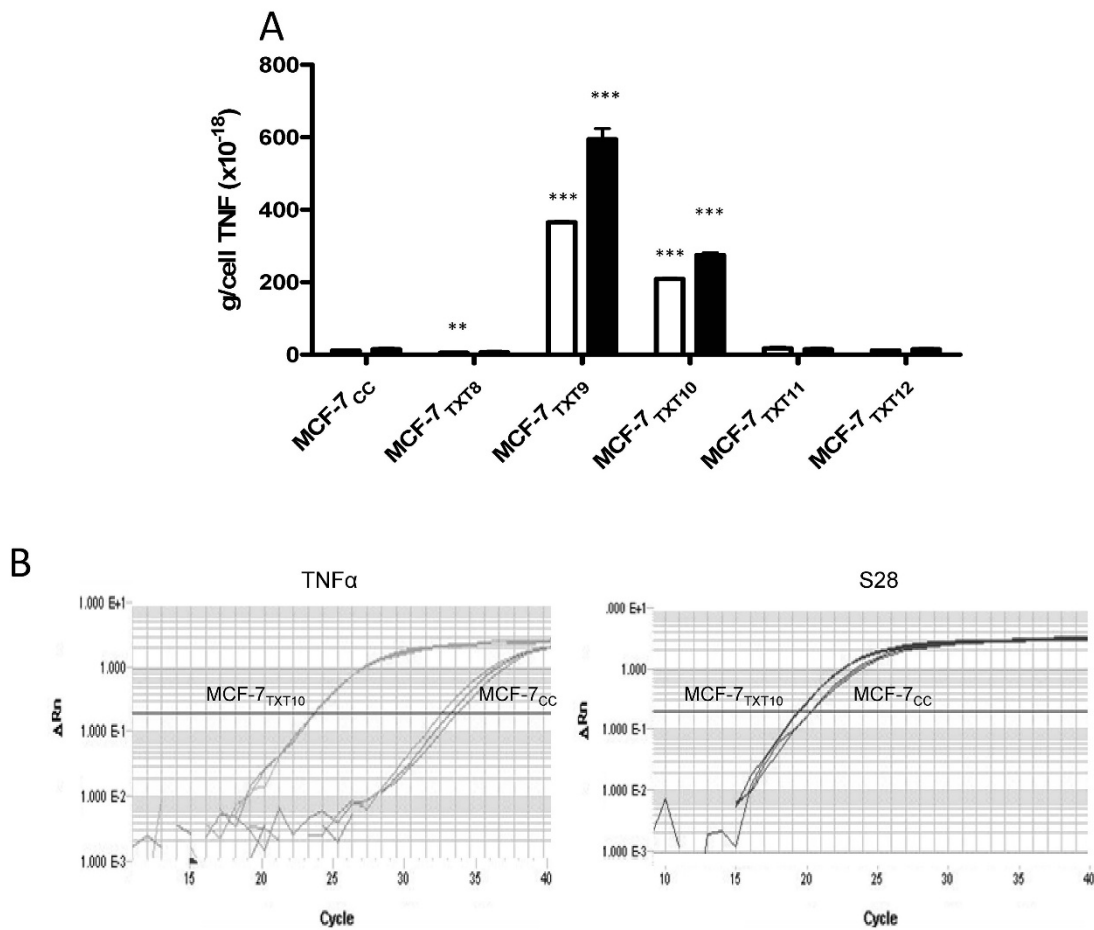


Figure 3 Levels of soluble tumor-necrosis factor alpha (sTNF- α) in the medium of MCF-7_{CC} and MCF-7_{TXT} cells on exposure to docetaxel. The ability of MCF-7 cells to produce sTNF- α was measured by using an enzyme-linked immunosorbent assay (ELISA) after selection for survival to docetaxel dose levels 8 (MCF-7_{TXT8}), 9 (MCF-7_{TXT9}), 10 (MCF-7_{TXT10}), 11 (MCF-7_{TXT11}), or 12 (MCF-7_{TXT12}), or to the maximally tolerated dose of paclitaxel (MCF-7_{TAX-1} cells). After selection, the cells at the various selection doses were assessed for their production of sTNF- α in the absence (white bars) or presence (black bars) of 50 nM docetaxel (a). The results presented are the mean levels (\pm standard error) for five independent experiments, and the significance of differences in sTNF- α levels between MCF-7_{CC} and MCF-7_{TXT} cells was assessed by using a Student *t* test; *P* values of < 0.01 and < 0.001 are represented by the ** and *** symbols, respectively. (b) Expression of TNF- α and S28 transcripts measured with RTqPCR by using cDNA preparations from MCF-7_{CC} and MCF-7_{TXT} cells (dose level 10).

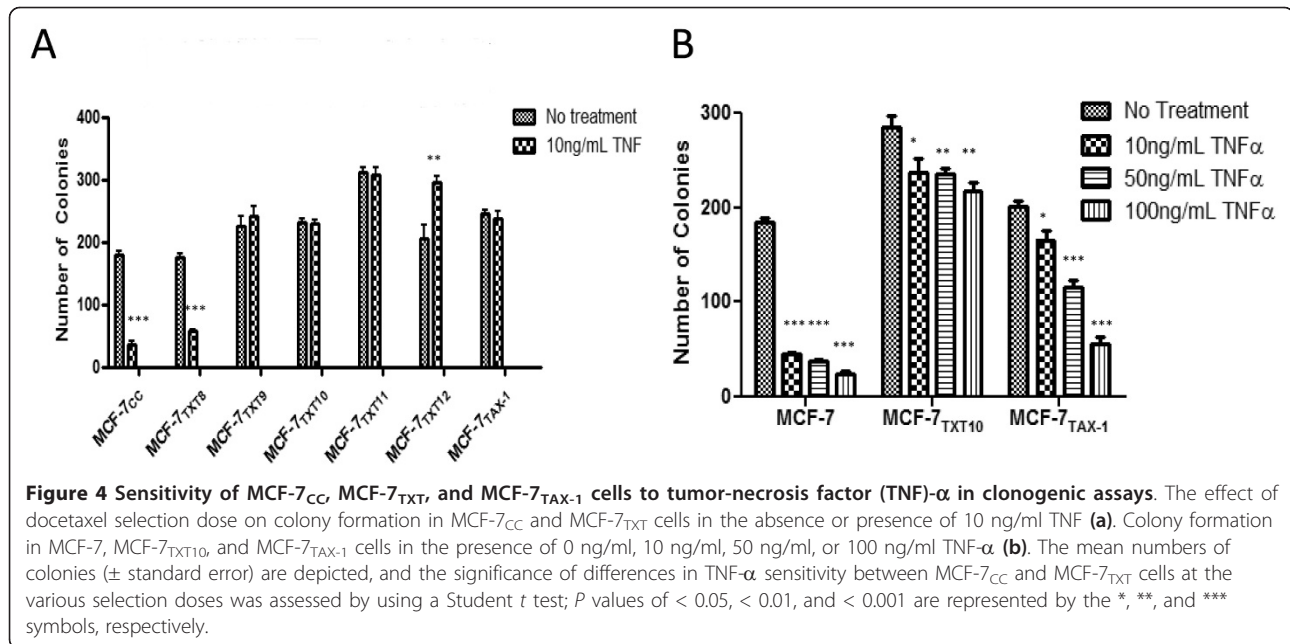
TNFR1 protein levels (but not transcript levels) are reduced on acquisition of docetaxel resistance in MCF-7 cells

Unlike TNFR2, the levels of TNFR1 protein (as measured in immunoblotting experiments) decreased on acquisition of docetaxel resistance at dose 9 (MCF-7_{TXT9} cells) and remained low in MCF-7_{TXT10} and MCF-7_{TXT12} cells (Figure 5a). Interestingly, RTqPCR analysis revealed no significant differences in TNFR1 transcript expression between these cell lines (Figure 5b). Similar soluble TNFR1 (sTNFR1) levels were observed in MCF-7_{CC} and MCF-7_{TXT8} cells (Figure 5c), although levels decreased in MCF-7_{TXT9} and MCF-

7_{TXT10} cells (*P* < 0.001). sTNFR1 levels then returned to those of MCF-7_{TXT8} cells as docetaxel-selective pressure was increased.

Induction of docetaxel resistance in MCF-7 cells through application of a TNFR1 neutralizing antibody

Significant differences in colony formation were observed between TNFR1 neutralizing antibody-treated MCF-7_{CC} cells and untreated cells when incubated with 1.23 nM (*P* < 0.0001), 0.41 nM (*P* = 0.0002), 0.14 nM (*P* = 0.0006), and 0.046 nM (*P* < 0.0001) docetaxel (Figure 5d). Nonlinear regression curve-fitting programs revealed that MCF-7_{CC} cells incubated with the TNFR1



neutralizing antibody were about 2.25-fold more resistant to docetaxel than were untreated cells, consistent with a role for the TNF- α pathway in docetaxel cytotoxicity.

Activation of NF- κ B on acquisition of docetaxel resistance

Unlike MCF-7_{TAX-1} cells, MCF-7_{TXT10} cells had 35% lower I κ B levels than did MCF-7_{CC} cells (*P* = 0.03) (Figure 6a). Measurement of NF- κ B binding in nuclear extracts from MCF-7 and MCF-7_{TXT8} cells revealed low binding of NF- κ B p65 and p50 subunits to the NF- κ B transcription factor binding site (Figures 6b and 6c). In contrast, nuclear extracts from MCF-7_{TXT9} and MCF-7_{TXT10} cells exhibited more than threefold higher levels of subunit binding to the NF- κ B sequence compared with equivalent extracts from MCF-7_{CC} cells (*P* < 0.05). This binding was reduced as cells were exposed to higher docetaxel selection doses. Interestingly, 50 nM docetaxel induced even higher levels of p65 and p50 subunit binding in MCF-7_{CC} and MCF-7_{TXT} cells, except when docetaxel selection doses were more than 15 nM (doses 11 and 12).

Promotion of TNF- α cytotoxicity in MCF-7_{TXT10} cells by cycloheximide or a TNFR2 neutralizing antibody

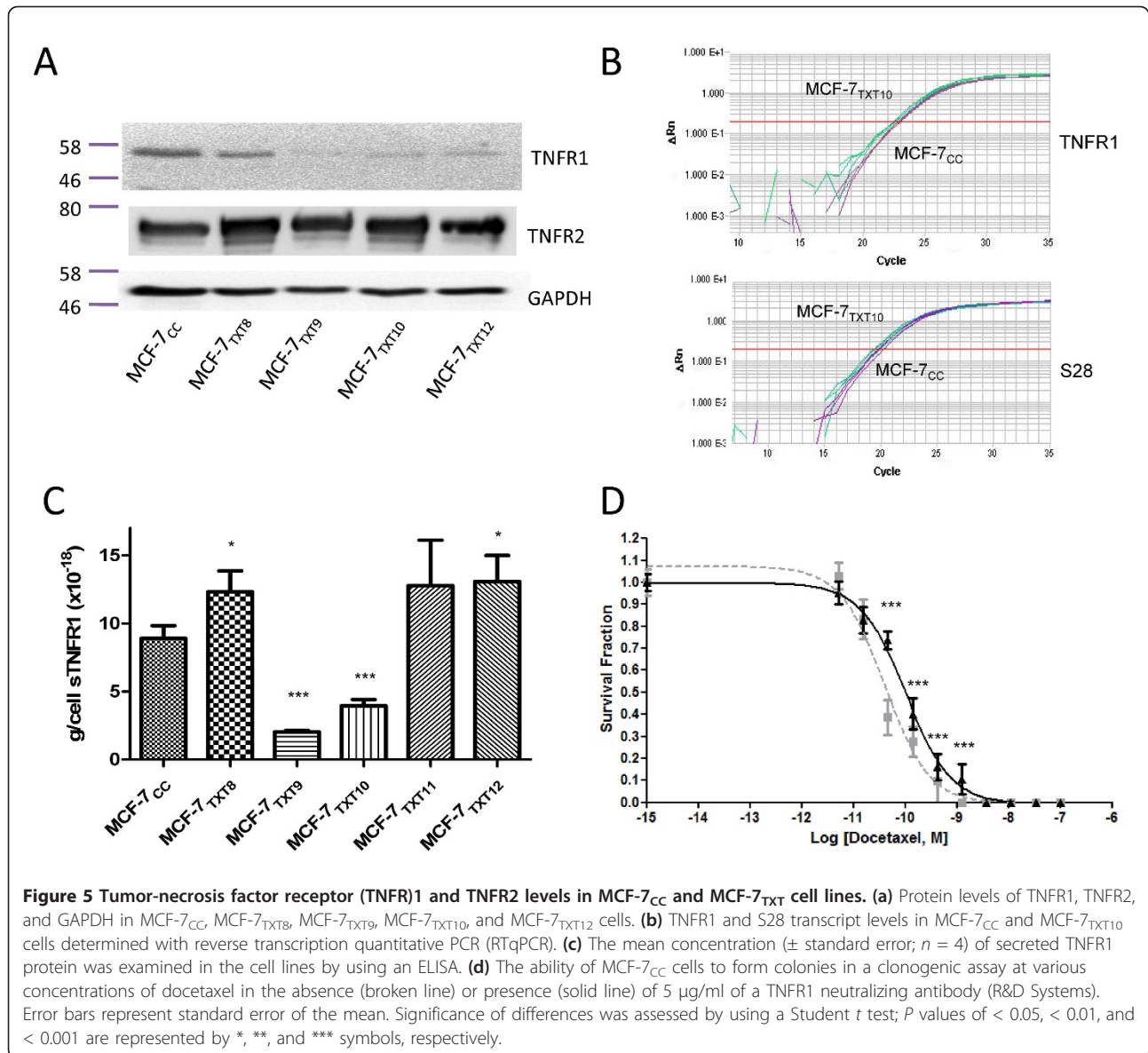
As previously observed, exposure of MCF-7_{CC} cells to 10 ng/ml TNF- α strongly decreased colony formation in a clonogenic assay, whereas MCF-7_{TXT10} cells exhibited significant resistance to TNF- α (Figure 7a). The addition of the protein-synthesis inhibitor cycloheximide, 5 μ g/ml, restored the ability of TNF- α to be cytotoxic to MCF-7_{TXT10} cells, while having only a small additional

effect on TNF- α cytotoxicity in MCF-7_{CC} cells. These observations suggested that a protein, possibly NF- κ B, is critical for maintaining resistance to TNF- α .

To test this hypothesis, and because NF- κ B is activated on TNF- α binding to TNFR2, resulting in enhanced expression of survival genes [20], we theorized that docetaxel cytotoxicity might be increased in MCF-7_{TXT10} cells on addition of a TNFR2-neutralizing antibody or an inhibitor of NF- κ B function. Supporting this conjecture, we observed a greater reduction in colony formation for TNFR2-neutralizing antibody-treated cells than untreated cells when treated with 41.2 nM (*P* = 0.0007), 13.7 nM (*P* = 0.005), 4.5 nM (*P* = 0.006), or 1.7 nM (*P* = 0.01) docetaxel (Figure 7b). Nonlinear regression curve-fitting for three independent experiments revealed that the TNFR2 neutralizing antibody rendered MCF-7_{TXT10} cells 2.13-fold more sensitive to docetaxel than were untreated cells. Moreover, as shown in Figure 7c the peptide SN-50, which contains the nuclear localization signal of NF- κ B and thus blocks the transcription factor translocation to the nucleus [25], increased docetaxel cytotoxicity to an even greater degree in MCF-7_{TXT10} cells (7.1-fold). In contrast, a control peptide (SN-50 M), in which critical basic amino acids within the nuclear localization signal are replaced with uncharged amino acids, had no effect on docetaxel sensitivity (Figure 7c).

Network-based analysis of genes associated with the acquisition of docetaxel resistance

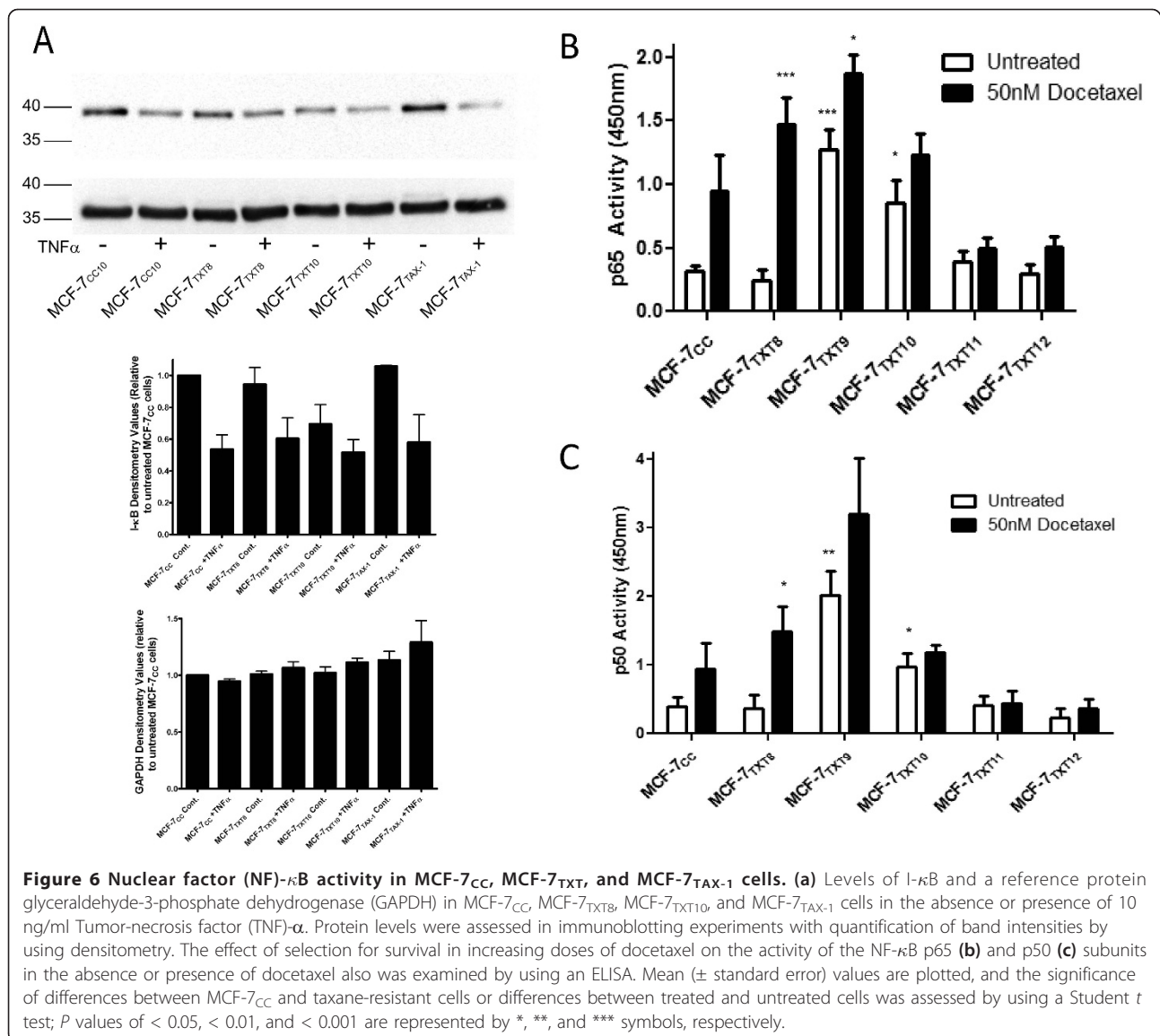
Assessment of microarray data by using an FI network approach (see Materials and methods) revealed 2,235



genes that were differentially expressed between parental and docetaxel-resistant MCF-7 breast cancer cell lines (fold-change > 2.0 and FDR ≤ 0.05). Of these, 834 (37.3%) were in the FI network, and hierarchic clustering reduced this to a set of 753 of the most interconnected candidates. This gene set was then used for further analyses. The average shortest-distance calculation showed that genes in this set were linked together much more tightly than would be expected by chance alone ($P < 0.001$), indicating that these differentially expressed genes occupy a small corner of the large FI network space. A subnetwork was built from these 753 genes by adding the minimum number of linker genes required to form fully connected networks involving these genes. The resulting networks consisted of 938

genes, 185 of which were linkers. A Markov clustering algorithm was then used to identify clusters of proteins (coded by the genes) that are highly interconnected with each other and less connected to the outside world. This algorithm identified 14 clusters consisting of more than 20 genes, including a cluster of 22 TNF-associated genes and eight linkers (Figure 8a).

We used an identical approach to identify clusters of differentially expressed genes between wild-type and docetaxel-resistant A2780 ovarian carcinoma cells. Of 955 genes that were differentially expressed between the two cell lines, a network of 11 TNF-related genes and three linkers was identified (Figure 8b). When the same approach was used to identify networks of genes differentially expressed between docetaxel-sensitive and



docetaxel-resistant MDA-MB-231 cells (data obtained from GEO, accession number GSE28784), a cluster of 22 TNF-related genes and three linkers was identified (see Figure 8c).

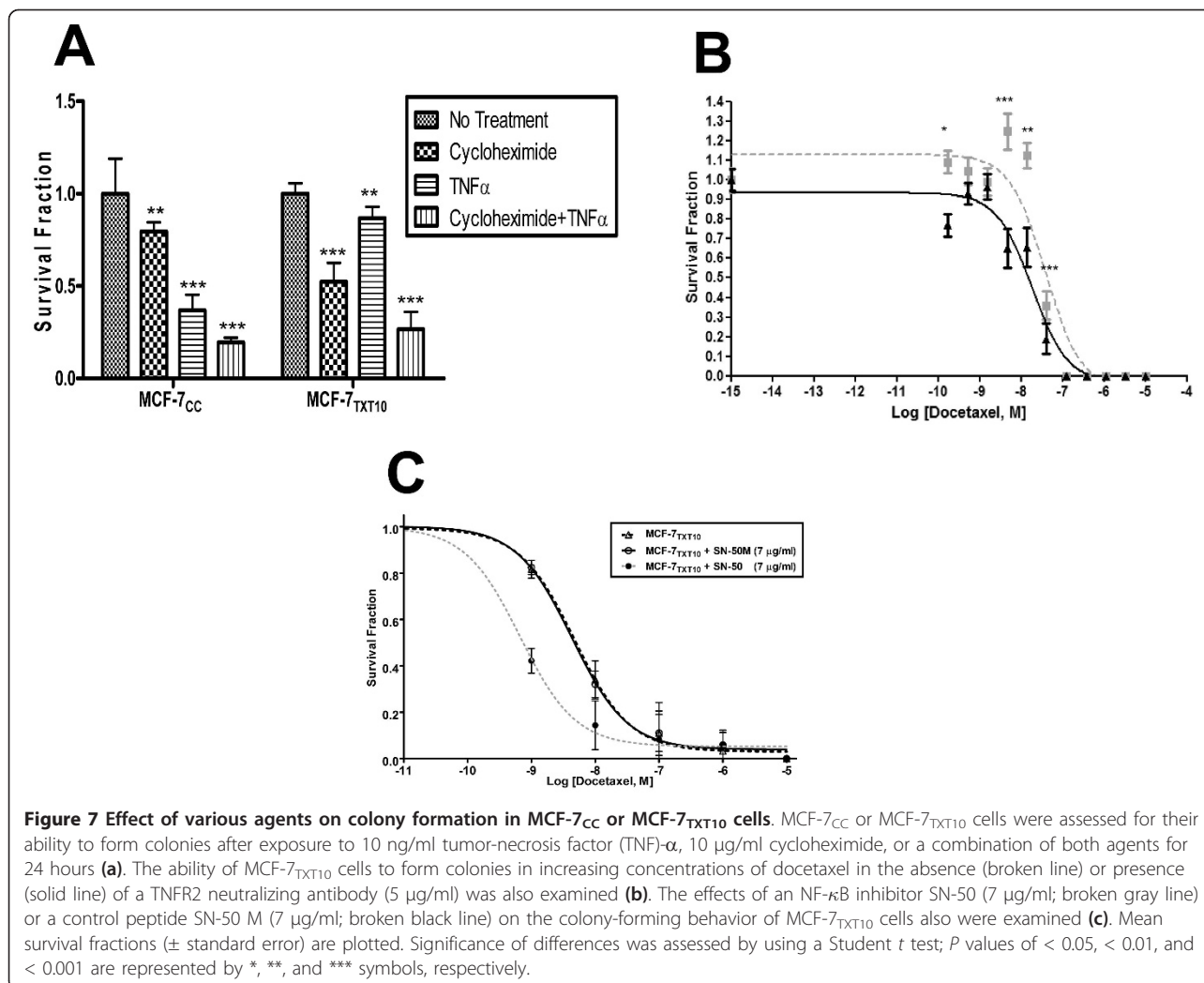
Confirmation of changes in the expression of TNF-α-dependent genes by RTqPCR

The expression of a selected number of genes within the previously identified TNF-α signaling networks was quantitatively assessed with RTqPCR. As shown in Figure 9, a generally strong concordance was noted between changes in gene expression identified by microarray analysis and those determined by RTqPCR (12 of 14 gene-expression changes assessed). Six TNF-α-dependent genes were confirmed to have altered expression on selection of MCF-7 cells for resistance to

docetaxel, including *TNFSF13*, *TNFSF10*, *TLR6*, *TNFAIP3*, *TNFSF14*, and *BIRC3* (the latter two genes being upregulated 30-fold and 21-fold, respectively). Three of these genes were also upregulated in A2780_{DXL} cells (*BIRC3*, *TLR6*, and *TNFSF10*, which increased expression almost 300-fold).

Discussion

Although taxanes are known to inhibit cell division by preventing microtubule depolymerization and inducing multinucleation [8,36], it is unclear whether these are their sole mechanisms of tumor cell growth arrest/death *in vitro* and *in vivo*. Paclitaxel has been shown to increase sTNF-α release from murine macrophages [12,37], although the levels used in those studies would be unachievable in patients, and docetaxel had no effect on TNF-α expression

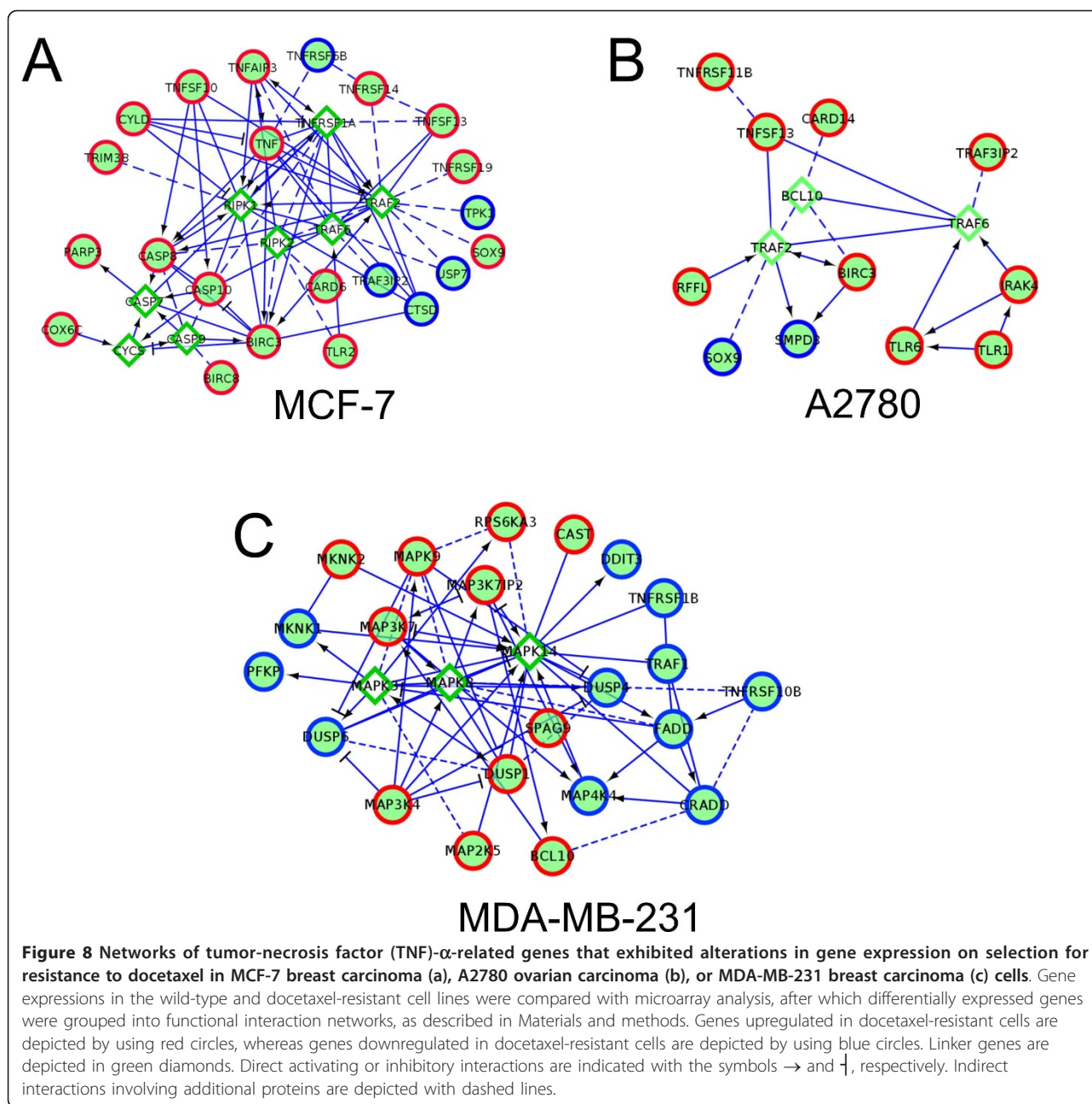


in the same study. In our study, we showed for the first time that docetaxel (at concentrations between 3 and 45 nM) can stimulate TNF- α production and sTNF- α release from both breast and ovarian tumor cells. Such concentrations are clearly in the range of plasma levels of docetaxel in breast cancer patients after docetaxel infusion (10 to 75 nM) [38] and are likely sufficiently high to induce TNF expression in even poorly vascularized tumors. This newly identified TNF-dependent mechanism of docetaxel action may also account for its reported immunomodulatory activity [11,39]. In addition, we show in this article that paclitaxel treatment (at 5 and 15 nM concentrations) can dramatically increase sTNF- α release from ovarian tumor cells.

Our study also illustrates that the acquisition of docetaxel resistance in breast tumor cells temporally correlates with increased production and release of sTNF- α from cells, despite the ability of sTNF- α to be cytotoxic to cells [40]. However, the onset of docetaxel resistance

in MCF-7 cells (at docetaxel selection doses \geq 3.33 nM) also correlated with strongly reduced levels of TNFR1, which would block the ability of TNF- α to induce cell death. Although the mechanism responsible for TNFR1 reduction remains undefined, neither changes in TNFR1 transcript levels nor increased levels of sTNFR1 in the media were found, suggesting that the receptor was not shed from cells by the ADAM-17 protease [14]. In fact, MCF-7_{TXT9} and MCF-7_{TXT10} cells exhibited decreased levels of sTNFR1 in the medium in which it was grown. It is possible that increased levels of sTNF α produced by these cells bound to sTNFR1 in the medium, preventing its detection by the TNFR1 antibody. Taken together, our findings suggest that downregulation of TNFR1 occurs posttranscriptionally, because of either reduced translation of the *TNFR1* transcript or increased TNFR1 proteolysis.

A recent study [41] found that TNF- α or paclitaxel induced NF- κ B activity in C2C12 myotubes. However,



paclitaxel did not induce increased TNF- α production, and inhibition of TNFR1 blocked TNF- α -induced NF- κ B activation but did not abolish paclitaxel-induced NF- κ B activity [41]. It is important to note that, in these studies, TNF- α levels were assessed only 4 hours after treatment with paclitaxel (10 nM to 10 μ M).

Whereas docetaxel selection doses between 3 and 5 nM resulted in highly elevated sTNF- α production, higher selection doses (≥ 15 nM) did not. This was despite the ability of the drug to induce TNF- α production in wild-type cells over a large concentration range (Figure 1). This may be explained by the increased

expression of the Abcb1 drug transporter and reduced docetaxel uptake that we observed in MCF-7_{TXT11} and MCF-7_{TXT12} cells. Expression was maximal at the highest selection doses (≥ 15 nM) [42]. We propose that docetaxel accumulates at sufficient concentrations to induce production of sTNF- α in MCF-7_{TXT9} and MCF-7_{TXT10} cells. However, at or above 15 nM docetaxel, MCF-7_{TXT} cells exhibit reduced drug uptake, such that docetaxel accumulation is insufficient to stimulate TNF- α production.

The mechanism for resistance to taxanes and TNF- α in MCF-7_{TAX-1} cells appears to differ from that of

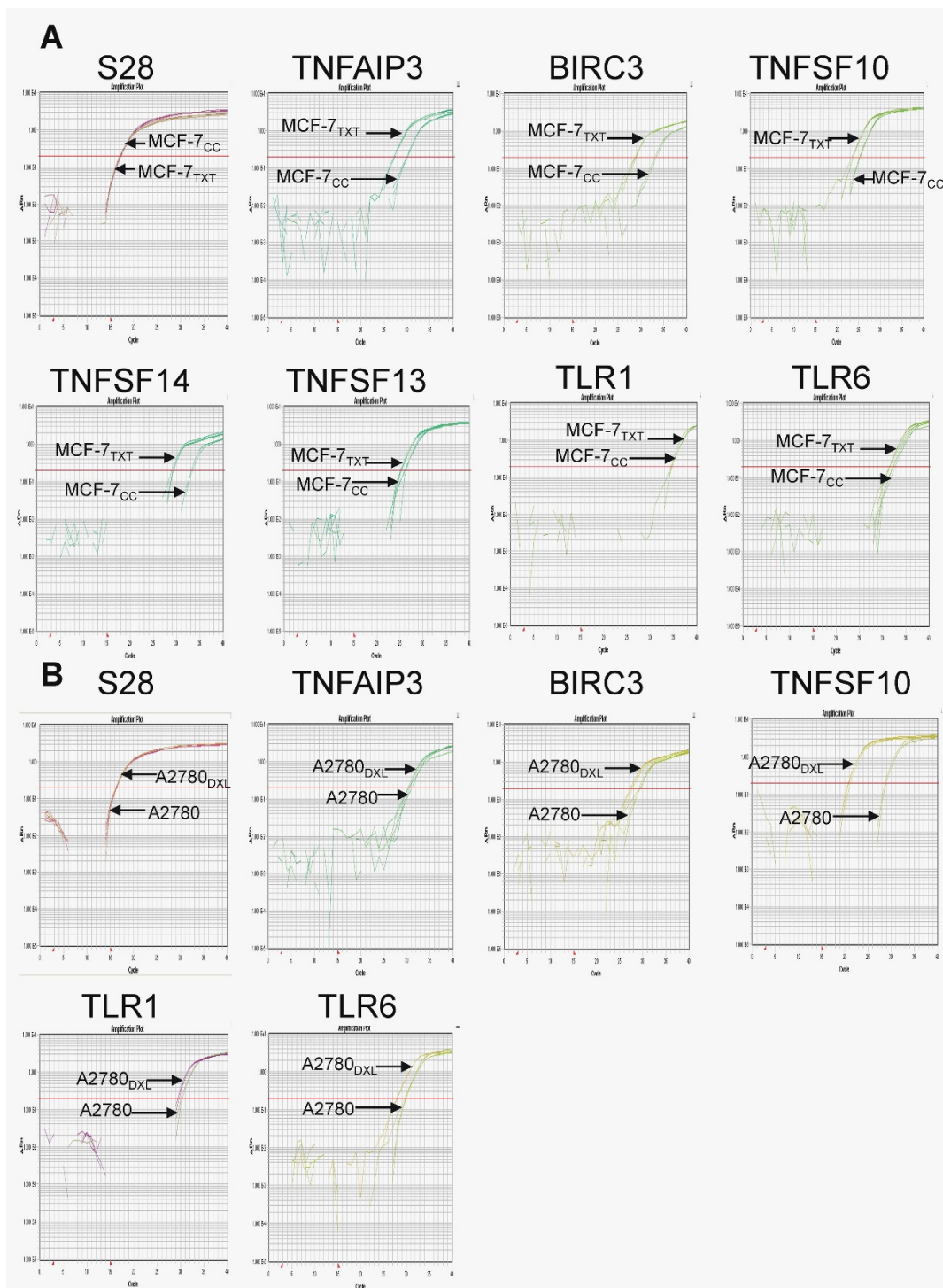


Figure 9 Use of reverse transcription quantitative PCR (RTqPCR) to assess differences in the expression of Tumor-necrosis factor (TNF- α)-related genes between MCF-7_{CC} and MCF-7_{TXT} cells (a) and between A2780 and A2780_{DXL} cells (b). For genes in which qPCR confirmed the changes in gene expression identified by cDNA microarray analysis, representative amplification plots are shown. S28 was used as the reference gene.

MCF-7_{TXT} cells. TNFR1 levels were equivalent in MCF-7_{TAX-1} and MCF-7_{CC} cells (data not shown), and I κ B levels were also unchanged during selection for paclitaxel resistance (Figure 6). Because only cells exposed to the maximally tolerated dose of paclitaxel were retained during selection of MCF-7_{TAX-1} cells, it is likely that cells selected at lower doses could have exhibited elevated production of TNF- α and TNF- α -mediated NF- κ B activation. However, survival by circumventing the TNF- α ability to stimulate TNFR1-induced cytotoxicity must lie downstream of the receptor. MCF-7_{TAX-1} cells are also high expressors of Abcb1 [24]. Interestingly, another paclitaxel-resistant MCF-7 cell line (MCF-7_{TAX-2} cells) [23] retained sensitivity to TNF- α (data not shown), suggesting that defects in the TNF- α pathway are not critical for taxane resistance *in vitro*. Nevertheless, considering that three of the four taxane-resistant cell lines exhibited alterations in TNF- α signaling and that docetaxel has been shown to increase sTNF- α levels in both breast and ovarian tumor cells, it appears that we have identified a common but unknown mechanism of taxane cytotoxicity and resistance that warrants further study for its potential clinical relevance.

To provide further support for a general involvement of the TNF- α pathway in docetaxel cytotoxicity and in the induction of docetaxel resistance, we also showed in this study that selection of breast and ovarian tumor cells for resistance to docetaxel results in changes in the expression of networks of genes related to TNF- α signaling (Figure 8 and Table 2). Quite strikingly, the vast majority of the upregulated genes depicted in Table 2 code for proteins that are TNF-ligand family members, TNF-receptor family members, TNF receptor-associated proteins, TNF-dependent activators of NF- κ B, or proteins that help promote degradation of the inhibitor of NF- κ B (I κ B). Other upregulated genes are TNF-dependent inhibitors of apoptosis. Downregulated genes code for proteins that inhibit the activation of NF- κ B or promote apoptosis. The net effect of the changes in gene expression would thus be to promote the ability of TNF to augment NF- κ B-dependent cell survival, while blocking its ability to induce tumor cell death via activation of TNFR1.

The findings of our study may have significant clinical relevance. A presentation at the 26th annual meeting of the European Association of Urology in 2011 [43] revealed that serum levels of proinflammatory cytokines, including TNF- α , increased 2 days after administration of docetaxel to patients with castration-resistant prostate cancer. Interestingly, these changes in cytokine expression correlated with the induction of apoptosis and with clinical response. In addition, a study presented recently at the American Association for Cancer Research, 101st Annual Meeting [44], revealed that, in patients with

serous epithelial ovarian carcinoma, pretreatment tumor expression of various genes within the TNF- α and NF- κ B signaling networks could be used to distinguish between responders and nonresponders to paclitaxel/carboplatin chemotherapy. It also was shown in a small study involving patients with locally advanced breast cancer that tumor levels of nuclear (activated) NF- κ B could be used to distinguish between responders and nonresponders to neoadjuvant anthracycline- and/or taxane-based chemotherapy regimens [45]. These and other studies strongly support the likely clinical significance of the findings. For example, because TNF- α has been shown to reduce tumor vascularization in mice through its effects on TNFR1-expressing endothelial cells [46], the reported ability of docetaxel to affect tumor angiogenesis [10] may be through an ability of the drug to promote sTNF α -mediated decreases in tumor vascularization. Moreover, one of the well-established dose-limiting toxicities associated with docetaxel chemotherapy in breast cancer patients is fatigue [47], and high TNF levels have been shown to correlate with fatigue onset in cancer patients [48]. Given our findings of docetaxel-induced TNF- α production, perhaps these two phenomena are linked.

Finally, a previous clinical study used a TNF-decoy receptor (entanercept) to permit patients to tolerate higher doses of docetaxel without significant toxicity [49]; however, given our findings, it is not surprising that these blockers would create a greater tolerance to docetaxel, unfortunately at the likely expense of lesser anti-tumor efficacy. Our findings further question the utility of administering docetaxel to cancer patients on TNF- α blockers for treatment of co-morbid inflammatory diseases.

Conclusions

Our study provides evidence for the first time that taxanes can induce sTNF- α expression in two tumor cell lines of different tissue origin. Although this would promote the cytotoxicity of docetaxel, continued exposure to the drug appears to result in a downregulation of TNF- α -mediated cytotoxicity, while promoting TNF- α -dependent activation of NF- κ B-dependent cell-survival pathways and the inhibition of apoptosis. In addition, although drug-resistance studies often involve selection of cells to maximally tolerated drug doses [50,51], our study illustrates the critical role that the drug-selection dose may play on the mechanisms by which tumor cells acquire chemotherapy resistance. At lower doses of taxanes (3 to 5 nM), TNF- α -mediated activation of NF- κ B-dependent cell-survival pathways appears to be an important mechanism of taxane resistance, whereas at selection doses \geq 15 nM docetaxel, the drug induces overexpression of Abcb1, resulting in reduced

Table 2 Tumor-necrosis factor α (TNF α)- and nuclear factor (NF)- κ B-related genes associated with the acquisition of docetaxel resistance in MCF-7 breast tumor cells, MDA-MB-231 breast tumor cells, and A2780 ovarian carcinoma cells

Gene	Direction and magnitude of change in expression ^a	Role of gene product	Refs.
Changes in the expression of TNFα- or NF-κB-related genes in MCF-7_{TX10} cells relative to MCF-7_{CC10} cells			
<i>TNF</i>	Increased 12.6, 1.43	Binds to TNFR1 to promote cell death and to TNFR2 to stimulate expression of NF- κ B-dependent survival genes	[54,55]
<i>TNFSF10</i>	Increased 6.88	TNF-like ligand that binds to Dcr2 to activate NF- κ B-dependent survival genes; also called TRAIL	[56]
<i>TNFRSF19</i>	Increased 16.5, 1.64	Member of the TNF-receptor family that binds to lymphotoxin alpha, activates NF- κ B-mediated transcription	[57]
<i>TNFRSF14</i>	Increased, 4.24	TNF-receptor family member, interacts with members of the TNFR-associated factor (TRAF) family and activates NF- κ B and AP-1	[58]
<i>TNFSF13</i>	Increased, 3.07	Binds to TNFRSF13B to promote NF- κ B activation; also called APRIL	[59]
<i>BIRC3</i>	Increased, 52.2	Member of a family of proteins that inhibits apoptosis by binding to TNFR-associated factors TRAF1 and TRAF2	[60]
<i>TLR2</i>	Increased, 5.19	Stimulates NF- κ B activation	[61]
<i>TRIM38</i>	Increased, 4.22, 7.28	Removes Lys63-linked ubiquitin chains from TRAF2 and TRAF6, negatively regulating NF- κ B activity	[62]
<i>TNFRSF6B</i>	Decreased, -2.02	Member of the TNF-receptor superfamily, which, on binding of TNF, inhibits cell proliferation and induces apoptosis	[63]
<i>TRAF3IP2</i>	Decreased, -2.14	Associates with and activates I- κ B kinase, leading to the liberation of NF- κ B from its complex with I- κ B	[64]
Changes in the expression of TNFα- or NF-κB-related genes in A2780_{DXL} cells relative to A2780 cells (MTD)			
<i>TNFSF13</i>	Increased, 2.10	Binds to TNFRSF13B to promote NF- κ B activation; also called APRIL	[59]
<i>TNFRSF11B</i>	Increased, 2.01	Decoy receptor for the TNF-related apoptosis-inducing ligand TRAIL; confers resistance to TNF- and TRAIL-induced apoptosis	[65]
<i>CARD14</i>	Increased, 2.19, 1.31	Associates with guanylate kinase members that interact with BCL10 and activate NF- κ B	[66]
<i>IRAK4</i>	Increased, 3.70, 3.86	Required for the optimal transduction of IL-1-induced signals, including the activation of IRAK-1, NF- κ B, and JNK	[67]
<i>TLR1</i>	Increased, 2.79	Acts via MYD88 and TRAF6 to stimulate NF- κ B activation, cytokine secretion, and the inflammatory response	[68]
<i>TLR6</i>	Increased, 2.26, 1.57	Also acts via MYD88 and TRAF6 to stimulate NF- κ B activation, cytokine secretion, and the inflammatory response	[68]
<i>BIRC3</i>	Increased, 8.83	Member of a family of proteins that inhibits apoptosis by binding to TNFR-associated factors TRAF1 and TRAF2	[60]
<i>RFFL</i>	Increased, 2.15, 2.13	Endosome-associated ubiquitin ligase for RIP and regulates TNF-induced NF- κ B activation	[69]
<i>TRAF3IP2</i>	Increased, 2.16	Associates with and activates I- κ B kinase, leading to the liberation of NF- κ B from its complex with I- κ B	[64]
<i>SMPD3</i>	Decreased, -1.59, -2.03	Translocates to the plasma membrane in response to TNF- α in a time- and dose-dependent manner	[70]
<i>SOX9</i>	Decreased, -30.8	Protein whose expression and activity is negatively regulated by TNF-dependent NF- κ B activation	[71]
Changes in the expression of TNFα- or NF-κB-related genes in docetaxel-resistant MB-231 cells relative to A2780 cells			
<i>CAST</i>	Increased, 2.44	Inhibits degradation of NF- κ B to prolong NF- κ B activation	[72]
<i>BCL10</i>	Increased, 3.48	Potent activator of NF- κ B activity	[73]
<i>RPS6KA3</i>	Increased, 5.95	Phosphorylates I- κ B and activates NF- κ B in response to TNF	[74]
<i>MAP2K5</i>	Increased, 3.54	A survival protein highly expressed in MCF-7 breast tumor cells resistant to etoposide and TNF- α	[75]
<i>MKNK2</i>	Increased, 1.76	Promotes TNF- α biosynthesis at the posttranscriptional levels	[76] [75]
<i>TNFRSF10B</i>	Decreased, -1.67	Also known as TRAIL receptor 2; stimulates apoptosis via FADD	[77]
<i>FADD</i>	Decreased, -2.00	A component of the caspase 8-activating complex induced by TNF- α binding to TNFR1	[78]
<i>CRADD</i>	Decreased, -2.43	An adaptor protein that promotes TNF α -induced apoptosis through interaction with the TNFR1-interacting protein RIP	[79]
<i>TRAF1</i>	Decreased, -2.39	Negatively regulates the ability of TNFR2 to promote cell proliferation and NF- κ B activation in T cells	[80]
<i>MKNK1</i>	Decreased, -2.00	Promotes TNF- α -mediated mRNA degradation	[81]

Full-genome oligo-based microarray experiments were performed comparing differences in gene expression between wild-type and docetaxel-resistant MCF-7 cell lines. Differentially expressed genes were then classified into various functional interaction networks, as described in Materials and methods. Information on the identities and roles of genes associated with TNF- α signaling are presented in tabular form, with particular emphasis on the products of genes that are known to play a role in the induction of NF- κ B-dependent survival genes or in the inhibition of apoptosis. ^aNumbers represent the fold increase (positive numbers) or decrease (negative numbers) in expression of the gene as revealed by one or more oligo probes on the Agilent 44K human microarrays.

accumulation of docetaxel into cells and a consequent reduction in docetaxel-stimulated TNF- α production. Since the concentration of docetaxel within patient tumors is typically lower than that present in the vasculature, perhaps the pathways associated with resistance to lower concentrations of docetaxel are of greater clinical relevance. This may explain why Abcb1 inhibitors have had little ability to reverse resistance to taxanes in cancer patients [52,53]. In addition, given that some cancer patients with inflammatory diseases may be taking TNF blockers, this newly identified role for TNF in docetaxel cytotoxicity may be of particular importance, suggesting that these blockers may compromise the efficacy of docetaxel chemotherapy.

Abbreviations

A2780_{DXL}: docetaxel-resistant A2780 cells (405 nM); ANOVA: analysis of variance; DISC: death-inducing signaling complex; ELISA: enzyme-linked immunosorbent assay; MCF-7_{CC} cells: co-cultured control MCF-7 cells propagated in the absence of drug; MCF-7_{TAX-1}: first MCF-7 cell-line selection with paclitaxel; MCF-7_{TAX-2}: second MCF-7 cell-line selection with paclitaxel; MCF-7_{TX18}: docetaxel-resistant MCF-7 cells selected to dose 8 (1.11 nM); MCF-7_{TX9}: docetaxel-resistant MCF-7 cells selected to dose 9 (3.33 nM); MCF-7_{TX10}: docetaxel-resistant MCF-7 cells selected to dose 10 (5.00 nM); MCF-7_{TX11}: docetaxel-resistant MCF-7 cells selected to dose 11 (15 nM); MCF-7_{TX12}: docetaxel-resistant MCF-7 cells selected to dose 12 (45 nM); mTNF- α : soluble tumor necrosis factor alpha; RTqPCR: reverse transcriptase quantitative polymerase chain reaction; NF- κ B: nuclear factor-kappaB; sTNF α : soluble tumor necrosis factor alpha; TNF: tumor necrosis factor; TNF- α : tumor necrosis factor alpha; TNFR1: tumor necrosis factor receptor 1; TNFR2: tumor necrosis factor receptor 2.

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Authors' contributions

JAS performed the majority of the experiments associated with this investigation and wrote the manuscript. KR performed ELISA and RTqPCR experiments and assisted in preparing the manuscript. SA and CL generated the A2780_{DXL} cell line and assisted with microarray experiments. BG conducted the microarray experiments and data analysis and deposited microarray data in the Gene Expression Omnibus database. JAS, SLH, and AT performed the clonogenic assays. IK and LS performed the FI network analyses. AMP participated in the conception and design of the study, assisted in the interpretation of the data, reviewed the manuscript, and made final manuscript revisions. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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