Diallyl Disulfide Inhibits TNFα-induced CCL2 Release by MDA-MB-231 Cells

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Abstract. *Monocyte chemotactic protein-1 (MCP-1/CCL2) is released by tumor tissues, serving as a potent chemokine enabling directional homing of mononuclear cells to tumor tissue, which subsequently differentiate into tumor-associated macrophages (TAMs) via TGFβ1 signaling. TAMs readily invade tumor tissue and continue to synthesize pro-oncogenic proteins including tumor growth factors, matrix proteases (metastasis), angiogenic factors (neovascularization) and CCL2. Substances, which can attenuate or block the initial release of CCL2 have been shown to prevent cancer-associated inflammative pro-oncogenic processes. In the current study, we investigated the effects of the organosulfur compound diallyl disulfide (DADS), a natural constituent of Allium sativum (garlic) on suppression of TNFα-induced release of CCL2 from triple-negative human breast tumor (MDA-MB-231) cells. Using an initial adipokine/chemokine protein panel microarray, the data show a predominant expression profile in resting/untreated MDA-MB-231 cells for sustained release of IL6, IL8, plasminogen Activator Inhibitor 1 and TIMP1/2. Treatment with TNFα (40 ng/ml) had no effect on many of these molecules, with a single major elevation in release of CCL2 (~1,300-fold up-regulation). TNFα-induced CCL2 release was reversed by a sub-lethal concentration of DADS (100 μM), evident in antibody based assays. These findings provide evidence to support another avenue of anticancer/chemopreventative properties attributable to garlic constituents through immunomodulation.*

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Cancer-associated inflammation involves complex collective events within the immune system which enable growth and metastasis of diverse cancers 1. In brief, human tumors of almost every type including gliomas, melanoma (1) lung (2) renal (3) prostate (4) and breast (5) produce and release high concentrations of CCL2, a most prolific tumor-promoting chemokine which attracts monocytes to the tumor area (3, 6) *via* monocyte G-coupled CCL2 receptors such as CCR2A/2B (7). Once monocytes arrive at the tumor site, transforming growth factor beta-1 (TGFβ1) and interleukin-8 assist with advanced differentiation whereby these cells acquire traits beneficial to tumor cells, with a phenotypic change leading them to be recognized as tumor-associated macrophages (TAMs) (8, 9). TAMs then embed within the tumor, and increase tumor growth by fostering production and release of tumor growth factors (tumor growth), matrix proteases (invasion), angiogenic factors (neovascularization) and mechanistic blocking of tumor reactive T-cells/reducing (immune evasion) (10-12).

Therapeutic targeting of either the monocyte CCR2 receptor or release of CCL2 constitutes a dynamic means of blocking recruitment and mobilization of infiltrating monocytes to the tumor site (13, 14). A number of studies have demonstrated efficacy of monoclonal antibody to CCL2 IgG1κ (carlumab) or broccoli-derived compounds (*i.e.* indole-3-carbinol and 3,3'-diindolylmethane) against deplete monocyte infiltration and thereby also reduce tumor growth and metastasis (14-16). In the current study, we investigated the effects of a primary organosulfur compound diallyl disulfide (DADS) constituent of Allium sativum (garlic) on suppression of TNFα-induced release of CCL2 from triple-negative human breast tumor (MDA-MB-231) cells.

Materials and Methods

Cell line, chemicals and reagents. Triple-negative human breast tumor (MDA-MB-231) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were all obtained from Invitrogen

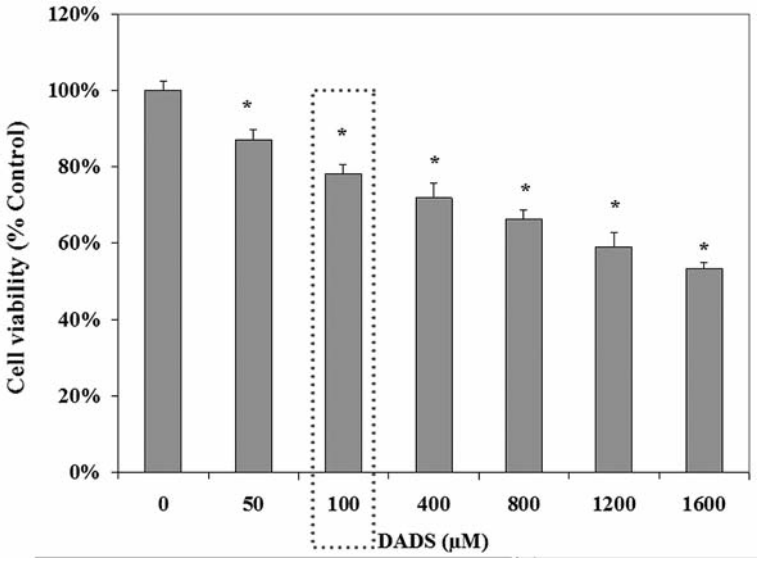

Figure 1. *The effect of DADS on cell viability of MDA-MB-231 cells at 5% CO_2/Atm for 24 hr. The data are presented as mean±S.E.M. (n=4). Significance of differences from the control were determined by a one-way ANOVA, with a Tukey post hoc test. *p<0.05 compared to control.*

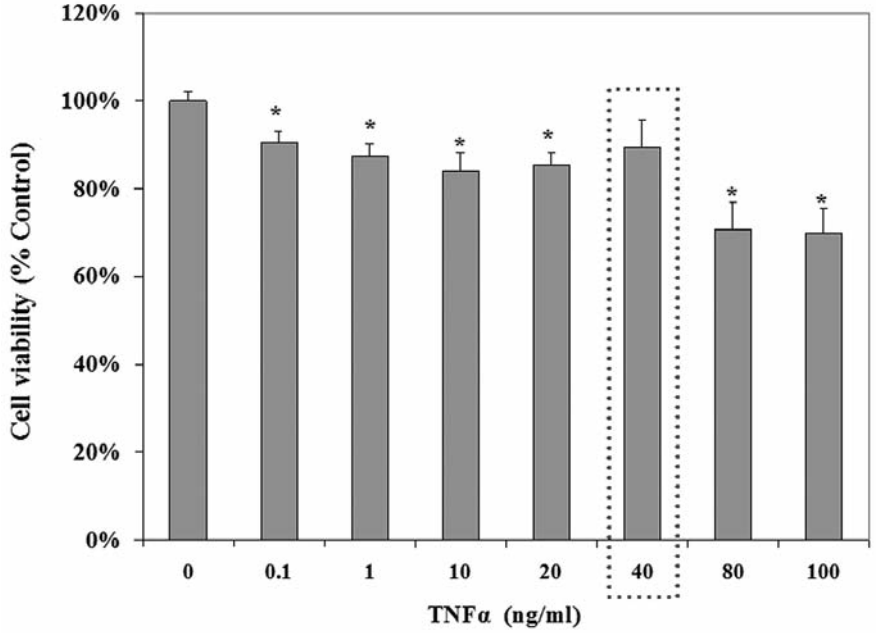

Figure 2. *The effect of TNFα on cell viability of MDA-MB-231 cells at 5% CO_2/Atm for 24 h. The data are presented as the mean±S.E.M. (n=4). Significance of differences from the control were determined by a one-way ANOVA, with a Tukey post-hoc test. *p<0.05.*

(Carlsbad, CA, USA). Recombinant human TNFα was purchased from RayBiotech (RayBiotech Inc., Norcross, GA, USA). DADS (>80% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. MDA-MB-231 cells were cultured in 75 cm^2 or 175 cm^2 flasks containing DMEM supplemented with 10% FBS and 1% 10,000 U/ml penicillin G sodium/10,000 μg/ml streptomycin sulfate. Cells were grown at 37˚C with humidified 95% air and 5% CO_2.

Cell viability assay. Alamar Blue cell viability assay was used to determine cytotoxicity. Viable cells are capable of reducing resazurin to resorufin, resulting in fluorescence changes. Briefly, 96-well plates were seeded with MDA-MB-231 cells at a density of 5×10^4cells/100

Table I. *Array listing of 62 adipokines evaluated by protein microarray.*

Acronym	Description
41BB	Tumor necrosis factor (ligand) superfamily, member 9
ACE2	Angiotensin converting enzyme 2
ACRP30	ACRP30/adiponectin
ADIPSIN	Complement factor D
AGRP	Agouti related protein homolog
ANG2	Angiopoietin-2
ANG1	Angiopoietin-1
ANGPTL1	Angiopoietin-related protein 1
CRP	C-reactive protein
EBA78	C-X-C motif chemokine 5
FAS	Tumor necrosis factor receptor superfamily member 6
FGF6	Fibroblast growth factor 6
GROWTH HORMONE	Growth Hormone
HCC4	Human CC chemokine-4
IFNGAMMA	Interferon gamma
IGF1	Insulin-like growth factor 1
IGF1SR	Insulin-like growth factor 1 (soluble)
IGFBP1	Insulin-like growth factor-binding protein 1
IGFBP2	Insulin-like growth factor-binding protein 2
IGFBP3	Insulin-like growth factor-binding protein 3
IL10	Interleukin-10
IL11	Interleukin-11
IL12	Interleukin-12
IL1R4/ST2	Soluble IL-1 Receptor 4/ST2
IL1sRI	Soluble interleukin-1 receptor I
IL1α	Interleukin-1α
IL1β	Interleukin-1β
IL6	Interleukin-6
IL6sR	interleukin-6 soluble receptor
IL8	Interleukin-1α
INSULIN	Insulin
IP10	C-X-C motif chemokine 10
LEPTIN	Leptin
LEPTIN R	Leptin R
LIF	Leukemia inhibitory factor
LYMPHOTACTIN	Chemokine (C motif) ligand (XCL1)
MCP1	Chemokine (C-C motif) ligand 2
MCP3	Chemokine (C-C motif) ligand 7
MCSF	Macrophage colony-stimulating factor
MIF	Macrophage migration inhibitory factor
MIP1β	Macrophage inflammatory protein-1β
MSPα	Macrophage stimulating protein
OPG	Osteoprotegerin
OSM	Oncostatin M
PAII	Plasminogen Activator Inhibitor 1
PARC	p53-Associated parkin-like cytoplasmic protein
PDGFAA	Recombinant Human Platelet Derived Growth Factor-AA
PDGFAB	Platelet Derived Growth Factor-AB
PDGFBB	Platelet Derived Growth Factor-BB
RANTES	Chemokine (C-C motif) ligand 5
RESISTIN	Adipose tissue-specific secretory factor
SDF1	C-X-C motif chemokine 12
SAA	Serum Amyloid A
sTNF RI	Soluble TNF-Receptor Type I
sTNF RII	Soluble TNF-Receptor Type I I
TECK	C-C motif chemokine 25
TGFβ	Transforming growth factor, beta 1
TIMP1	Metalloproteinase inhibitor 1
TIMP2	Metalloproteinase inhibitor 2
TNFα	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor A

XEDAR: Tumor necrosis factor receptor superfamily member 27.

A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
a	POS	POS	NEG	NEG	41BB	ACE2	ACRP30	ADIPSIN	ARGP	ANG2	ANG1	ANGPTL4	CRP	EBA78
b	POS	POS	NEG	NEG	41BB	ACE2	ACRP30	ADIPSIN	ARGP	ANG2	ANG1	ANGPTL4	CRP	EBA78
c	FAS	FGF6	GROWTH H	HCC4	IFNg	IGFBP	IGFBP	IBFBp3	IGF1	IGF1 SR	IL1R4	IL1sRI	IL10	IL11
d	FAS	FGF6	GROWTH H	HCC4	IFNg	IGFBP	IGFBP	IBFBp3	IGF1	IGF1 SR	IL1R4	IL1sRI	IL10	IL11
e	IL12	IL1α	IL1β	IL6	IL6SR	IL8	INSULIN	IP10	LEPTINR	LEPTIN	LIF	LYMPHOTA	CCL2	MCP3
f	IL12	IL1α	IL1β	IL6	IL6SR	IL8	INSULIN	IP10	LEPTINR	LEPTIN	LIF	LYMPHOTA	CCL2	MCP3
g	MCSF	MIF	MIP1β	MSPα	OPG	OSM	PAII	PARC	PDGFAA	PDGFAB	PDGFBB	RANTES	RESISTIN	SAA
h	MCSF	MIF	MIP1β	MSPα	OPG	OSM	PAII	PARC	PDGFAA	PDGFAB	PDGFBB	RANTES	RESISTIN	SAA
I	SDF1	sTNFRII	sTNF RI	TECK	TGFβ	TIMP1	TIMP2	TNFα	VEGF	XEDAR	BLANK	BLANK	BLANK	POS
j	SDF1	sTNFRII	sTNF RI	TECK	TGFβ	TIMP1	TIMP2	TNFα	VEGF	XEDAR	BLANK	BLANK	BLANK	POS

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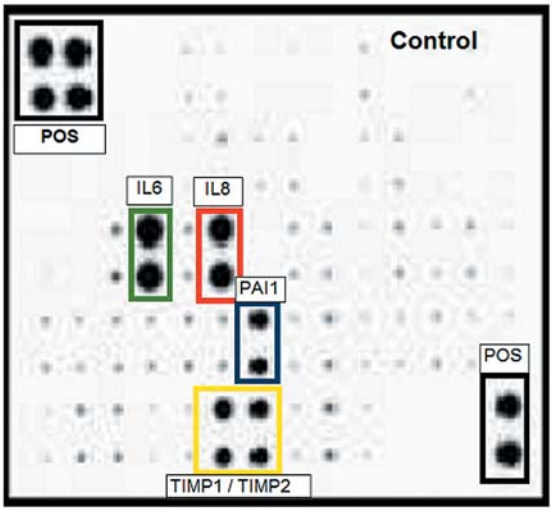

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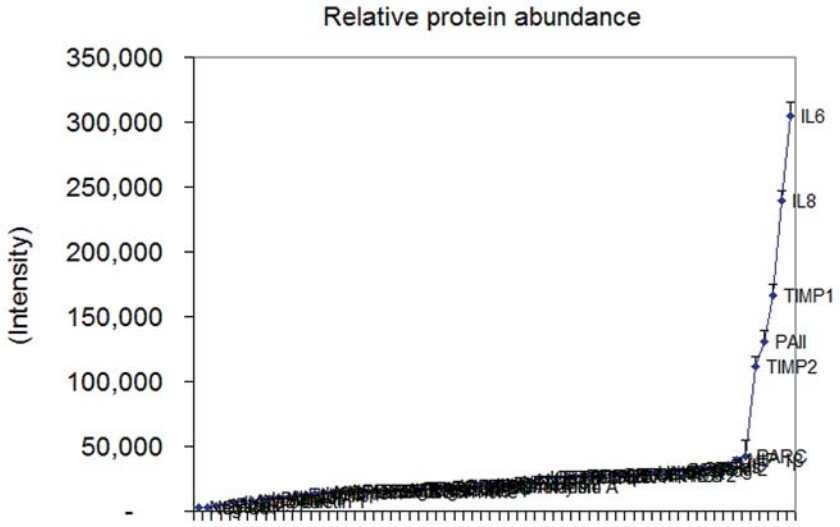

Figure 3. *A: Microarray layout. OSM, Oncostatin M; TPO, thrombopoietin; POS, positive control; NEG, negative control. Each protein is in duplicate. Positive controls are located in the upper left (n=4) and lower right (n=2) corners to insure equal distribution of supernatant (top). B: Microarray chemiluminescent spot intensity analysis of supernatant derived from resting MDA-MB-231 cells. POS controls are located in the upper left and lower right corners with dominant cytokines demarcated. C: Baseline cytokine release in untreated MDA-MB-231 cells corresponding to image. The data are presented as spot intensity and are the mean±S.E.M. (n=6). See Table I for cytokine abbreviations.*

μl/well. Cells were treated without or with either DADS (50 μM, 100 μM, 400 μM, 800 μM or 1.2 mM) or TNFα (0.1, 1, 10, 20, 40, 80, 100 ng/ml) for 24 h at 37˚C, 5% CO_2. Alamar blue (0.1mg/ml in HBSS) was added at 15% v/v to each well, and incubated for 6-8 hrs. Quantitative analysis of dye conversion was measured on a microplate fluorometer–Model 7620-version 5.02 (Cambridge Technologies Inc, Watertown, MA, USA) set at 550/580 (excitation/emission). The data were expressed as a percentage of live untreated controls.

Human adipokine obesity array. Sandwich-based obesity arrays purchased from RayBiotech (Norcross, GA, USA) consist of array membranes with 62 different proteins in duplicate. Each experiment was carried out in accordance with manufacturer's instructions. Briefly, antibody-coated array membranes were treated with 1 ml of medium from resting, DADS-treated (100 μM), TNFα-treated (40 ng) and co-treated cells and incubated overnight at 4˚C on a rocker/shaker. The medium was decanted, the membranes were washed with wash buffer and then incubated with 1 ml biotin-conjugated antibodies (overnight 4˚C). The mixture of biotin-conjugated antibodies were removed and membranes were incubated with horse radish peroxidase -conjugated streptavidin (2 h). After a final wash, membrane intensity was acquired using chemiluminescence and analyzed using Quantity One software (Biorad Laboratories, Hercules. CA, US) . Densities were measured as a percentage of the positive controls included on each membrane.

CCL2 detection by ELISA. Supernatants from resting and stimulated (24 h) MDA-MB-231 cells were collected and centrifuged at 100× *g* for 5 min at 4˚C. Specific ELISAs were performed using MCP-1/CCL2 ELISA kit (Raybiotech) following the manufacturer's instructions. Briefly, 100 μl of supernatants from samples and standards were added to 96-well plates pre-coated with capture antibody. After incubation, 100 μl of prepared biotinylated antibody mixture was added to each well. After 1 h, the mixture was decanted and 100 μl streptavidin solution was placed in each well and incubated. Substrate reagent (100 μl) was then added to each well followed by the addition of 50 μl stop solution 30 min later. The plate was read at 450 nm using UV microplate reader.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 3.0; GraphPad Software Inc. San Diego, CA, USA) with significance of difference between the groups assessed using a one-way ANOVA, followed by Tukey *post hoc* means comparison test , two way ANOVA or Student's *t*-test.

Results

Both DADS and TNFα initiated a mild loss of cell viability in MDA-MB-231 cells (Figures 1 and 2), respectively. Based on observations from cell viability assays, we elected to use 100 μM of DADS and 40 ng/ml of TNFα as our working concentrations for subsequent evaluation. In order to elucidate cytokines affected by DADS, TNFα or a co-treatment of TNFα with DADS *vs*. controls, a global assessment was carried out using sandwich-based obesity adipokine arrays from RayBiotech for detection of 62 proteins (Table I). A baseline profile was established for untreated resting MDA-MB-231 cells and presented as probe array layout in Figure 3a and the corresponding array blot in Figure 3b. These show a sustained elevated release of IL6, IL8, TIMP1/2 and PAII in untreated cells. The intensity analysis profile is presented in Figure 3c.

In cells treated with TNFα, there were no differential effects on abundantly-released proteins, however, a major elevation in CCL2 was observed (Figure 4) and this was significantly attenuated by DADS, as shown by dot blot intensity analysis (Figure 5a and b). In order to corroborate these findings, CCL2 was determined using an ELISA protocol (Figure 5c).

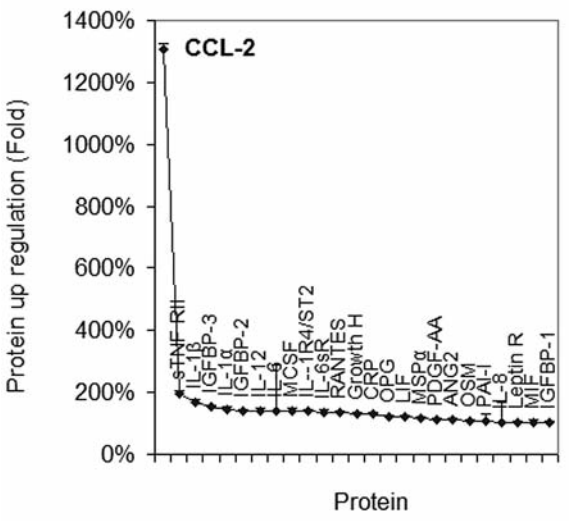

Figure 4. *TNFα induced cytokine expression by a dominant fold change in MDA-MB-231 cells. The data show a large differential up-regulation of CCL2 protein release amongst the 62 proteins evaluated. The data are presented as fold change and are the mean±S.E.M. (n=6).*

The findings from this study demonstrate that TNFα, induces up-regulation of CCL2 in human breast cancer cells which is blocked by DADS. Preliminary data from the antibody arrays also suggest a consistent elevation of sTNF receptor I by DADS, in both the control group (1.7 fold $p<0.05$) and TNFα-treated group (1.52-fold $p<0.001$), which could lead to attenuated TNF signaling at the receptor site (Figure 6). Future studies are required to further investigate the influence of DADS on TNF receptor signaling pathways.

Discussion

The data from this study show that CCL2 induced by TNFα is down-regulated by DADS in human breast carcinoma cells. It is well-known that tumor tissue can release promoting chemokines such as CCL2, amongst chemo-attractants and growth factors, which collectively enhance malignant cell migration, proliferation and invasive properties (6). CCL2 is responsible for triggering the recruitment and mobilization of monocytes, macrophages and other inflammatory components in order to infiltrate the tumor area (13). As in the case with breast cancer, CCL2 mobilizes $CD14^+$ $CD16^+$ monocytes (17) where it can bind to monocyte CCL2 receptors CCR2A/2B (6, 7) enabling differentiation into TAMs, which promote metastasis largely by matrix remodeling (18). TAMS surrounding the perimeter of tumor tissue will also exacerbate the rise in CCL2 by locally-positioned reactive macrophages, astrocytes, microglia immunocompetant/host cells (8, 19). The presence of TAM infiltrates are associated with many types of human cancer, as is an elevated expression of CCL2 as a correlate to poor treatment outcome (20, 21). CCL2 is involved in a number of additional

processes including up-regulation of the β-catenin/T-cell factor lymphoid-enhancing factor 1 transcriptional activation complex in breast tumor cells (22) and plays a role in almost every aspect of tumor progression from cell migration, cancer progression to epithelial-to-mesenchymal transition (23). Agents which can suppress CCL2–CCR2 signaling can block monocyte recruitment, inhibit metastasis *in vivo* (24) and are considered to be an effective therapeutic approach in treatment of human cancer (25, 26).

While future research is required to specifically determine the pathways involved in these effects, it is believed that CCL2 release by human breast cancer cells can be initiated by pro-inflammatory cytokines which occur through nuclear factor κ-B (NF-κB), extracellular signal-regulated kinase signaling (ERK)

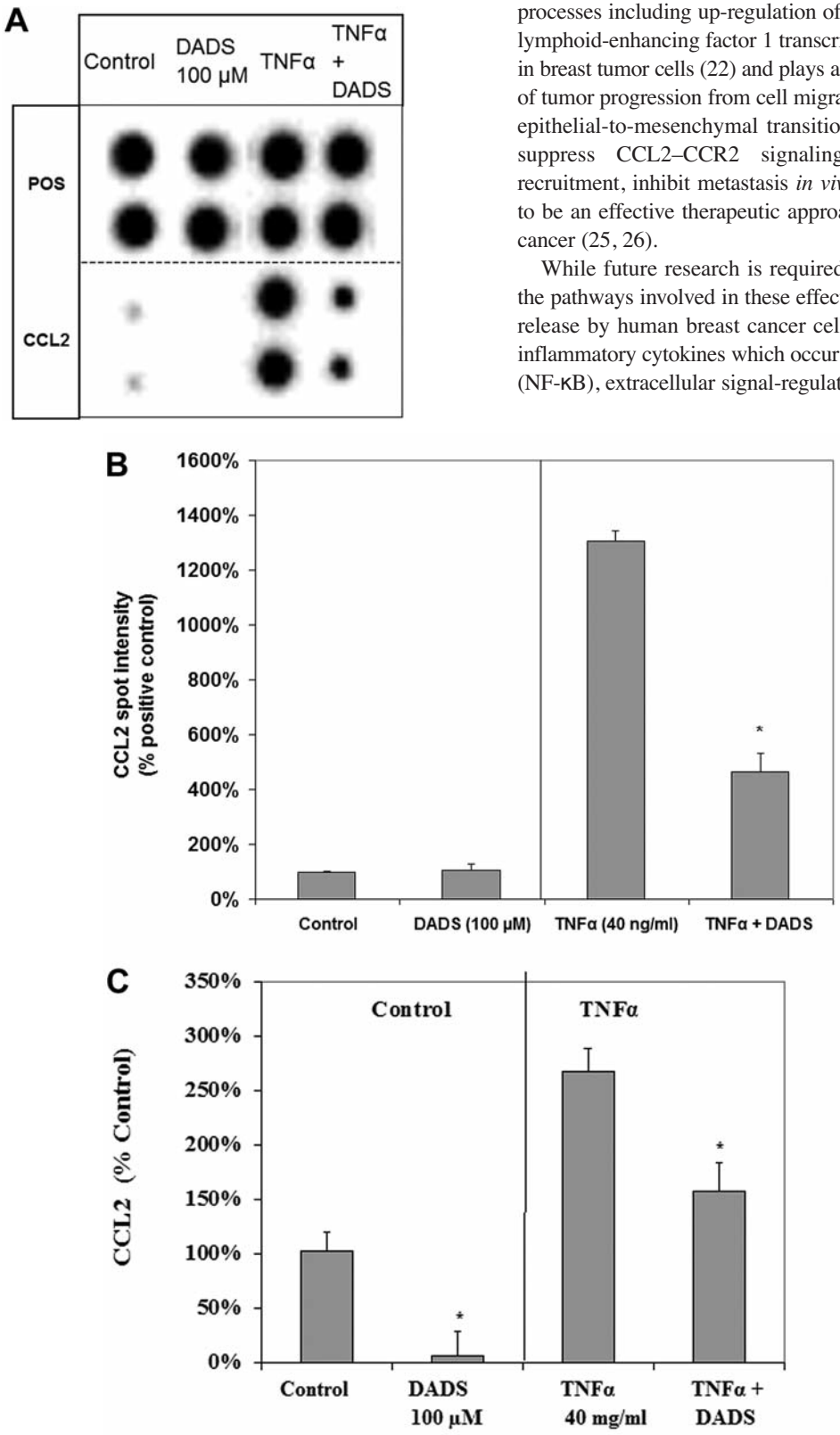

Figure 5. *Spot intensity analysis (A) of antibody- coated array membranes with quantitative analysis of chemiluminescent signal (B) and ELISA (C) for controls , DADS-treated (100 μM), TNFα-treated (40 ng/ml) and co-treated cells. The data are presented as the mean±S.E.M. (n=6) and significance of differences were determined by t-test. *p<0.05.*

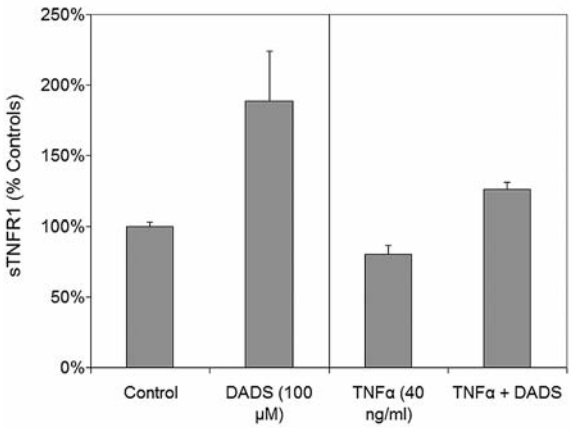

Figure 6. *Soluble TNFRI release in MDA-MB-231 cells for groups: control, DADS-treated (100 μM), TNFα-treated (40 ng/ml) and co-treated cells. The data are presented the mean±S.E.M. as percentage of control (n=6). Significance of differences from the controls in both groups were determined by t-test. *p<0.05.*

(27) or poly(ADP-ribose) polymerase-1 (PARP-1)/NF-κB signaling (28, 29). Moreover, elevated NF-κB signaling coincides with elevation of CCL2 and matrix remodeling processes (*e.g.* elevated expression of matrix metalloproteinases (MMPs) *e.g.* MMP1 and MMP9 (30). A reciprocal relationship may also exist where CCL2 stimulates MMP9 and MMP2, both induced by elevated circulating levels of TNFα (31). TNFα itself is released by cancer-associated fibroblasts and in some cases, tumor cells (32, 33), in particular breast cancer (34). Elevated levels of CCL2/TNFα/MMP9 also coincide with expression patterns of vascular endothelial growth factor A, TGFβ1 and IL8 which collectively assist with differentiation of human monocytes into TAMS (9), and down-regulation of caspase-3 in cancer cells (35). This dynamic synergy can be potentiated by the direct role of CCL2 in epithelial-to-mesenchymal transition *via* its ability to up-regulate the transcription factor twist basic helix-loop-helix transcription factor 1, needed for extracellular matrix degradation and metastasis (36). These elements drive many pathological events associated with aggressive tumor pathology.

Garlic contains DADS, which was recently shown to reduce migration and invasion of human colon cancer, in part, mediated by attenuation of signaling pathways involving NF-κB, phosphatidylinositide 3-kinases, mitogen-activated protein kinases and p38 (37). These effects are consistent throughout the literature, where DADS has shown ability to inhibit growth of diverse cancer cell types such as HT-29 (38), HL-60 (39), HCT-15 (human colon tumor cells), SK MEL-2 (skin) and A549 (lung) (40). DADS can also act to suppress CCL2–CCR2 signaling, impede monocyte recruitment, and inhibit metastasis *in vivo* (24), a very effective therapeutic approach for treatment of human cancer (25, 26). The findings from this study contribute to this body of literature, demonstrating yet another potential avenue for DADS in mitigating tumor progression, which could possibly abrogate infiltration of TAMs that promote metastasis through down-regulating CCL2 expression.

Acknowledgements

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