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Diallyl disulfide inhibits TNFα induced CCL2 release through MAPK/ERK and NF-Kappa-B Signaling

D. Bauer, **N. Redmon**, **E. Mazzio**, **E. Taka**, **JS. Reuben**, **A. Day**, **S. Sadrud-Din**, **H. Flores-Rozas**, **KFA. Soliman**, and **S. Darling-Reed***

College of Pharmacy and Pharmaceutical Sciences, Florida A & M University, Tallahassee, Florida 32307, USA

Abstract

TNFα receptors are constitutively overexpressed in tumor cells, correlating to sustain elevated NFκB and monocyte chemotactic protein-1 (MCP-1/CCL2) expression. The elevation of CCL2 evokes aggressive forms of malignant tumors marked by tumor associated macrophage (TAM) recruitment, cell proliferation, invasion and angiogenesis. Previously, we have shown that the organo-sulfur compound diallyl disulfide (DADS) found in garlic (Allium sativum) attenuates TNFα induced CCL2 production in MDA-MB-231 cells. In the current study, we explored the signaling pathways responsible for DADS suppressive effect on TNFα mediated CCL2 release using PCR Arrays, RT-PCR and western blots. The data in this study show that TNFα initiates a rise in NFκB mRNA, which is not reversed by DADS. However, TNFα induced heightened expression of IKKε and phosphorylated ERK. The expression of these proteins corresponds to increased CCL2 release that can be attenuated by DADS. CCL2 induction by TNFα was also lessened by inhibitors of p38 (SB202190) and MEK (U0126) but not JNK (SP 600125), all of which were suppressed by DADS. In conclusion, the obtained results indicate that DADS down regulates TNFα invoked CCL2 production primarily through reduction of IKKε and phosphorylated-ERK, thereby impairing MAPK/ERK, and NFκB pathway signaling. Future research will be required to evaluate the effects of DADS on the function and expression of TNFα surface receptors.

Keywords

diallyl disulfide; tumor necrosis factor alpha; monocyte chemoattractant protein 1; nuclear factor kappa b; map kinase b

*Corresponding Author: Professor Selina Darling-Reed, PhD, Florida A&M University, College of Pharmacy and Pharmaceutical Sciences|Division of Basic Pharmaceutical Sciences, New Pharmacy Building Research Wing, Room 110, 1415 S. Martin Luther King Jr., Boulevard, Tallahassee, Florida 32307, t. 850.561.2786/850. 412.5078, f. 850.599.3347, selina.darling@famu.edu.

1. INTRODUCTION

Metastatic breast cancer brings together rapid tumor proliferation, detachment and development of secondary tumors with acquired characteristics of the primary tumor. Chemokines such as monocyte chemotactic protein-1 (MCP-1), known as CC chemokine-2 (CCL2) play a critical role in this process. These chemokines recruit monocytes that differentiate into tumor-associated macrophages (TAMs) which subsequently release substances needed for tissue remodeling, angiogenesis and metastasis [1–3]. TAMs can also directly release more TNFα, an inflammatory cytokine [4], furthering the processes of production/release of CCL2 in diverse tumor tissue.[5].

Previously we have shown that diallyl disulfide (DADS), found in garlic (Allium sativum), attenuates TNFα induced CCL2 production in human breast cancer cells. It is likely that TNFα induced CCL2 production occurs through up regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). These three components are concurrently expressed to a greater degree in aggressively advanced tumors marked by TAM recruitment [6–8] and elevated TNF-receptors (TNFRs) on diverse human cancers [9]. Drugs or compounds such as DADS that antagonize these effects are becoming significant therapeutic vehicles. These include infliximab (Remicade), adalimumab (Humira), anti-TNF antibodies [10–11], all of which prevent tumor infiltrating leucocytes [12]. Therefore, the purpose of this study was to determine if the garlic constituent diallyl disulfide, could impact the MDA-MB-231 cells since they are the most studied TNBC to date. In addition, many researchers have used this cell model because it mimics aggressive nature of clinical isolates and have displayed great ability to metastasize in xenograft models. Nakagawa et al. [13] demonstrated the ability of DADS to inhibit tumor growth through its modulation of the apoptotic genes in this cell line. Other studies have used this cell line to study the mechanisms involved in the immune response [14,15]. Moreover, in this study we further explore the mechanism behind the inhibitory effect by DADS on TNFα induced CCL2 release in human breast carcinoma cells, with focus on MAPK/ERK NFκB signaling.

2. MATERIALS AND METHODS

Cell lines, chemicals and reagents: Triple negative human breast tumor (MDA-MB-231) cells were obtained from American Type Culture Collection (Rockville, MD). Dulbecco's Modified Eagle Medium (DMEM) media, fetal bovine serum (FBS) and penicillin/streptomycin were all obtained from Invitrogen (Carlsbad, CA). Recombinant human TNFα was purchased from RayBiotech (RayBiotech Inc., Norcross, GA, USA). Diallyl disulfide (>80%) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Cell culture and Treatment

MDA-MB-231 cells were cultured in 75cm^2 or 175cm^2 flasks containing DMEM media supplemented with 10% FBS and 1% pen/strep (10,000u/ml penicillin G sodium, 10,000μg/ml streptomycin sulfate). Cells were grown in an environment of 37°C with humidified 95% air and 5% CO_2. Control cells received vehicle only (0.01% ethanol). DADS treated cells received 100 μM DADS in vehicle and 40ng of TNFα was given to TNFα-treated and co-treated cells. Cells were incubated for 24 hrs. after treatment. In this

study, 100μM was used as the dose for DADS concentration. Although some studies have shown that different concentrations of garlic components exert different responses, such as increasing proliferation and tumor growth, in our laboratory cell viability studies were conducted to determine a working concentration and we recently reported that the 100μM dose was the optimum dose for this cell line [16]. The present study is a continuation of the study performed by Bauer et al. [16]. Moreover, initial studies were done using both 100μM and 400μM with approximately the same effect on CCL2 release. Additionally in our previous studies, we demonstrated an optimum overall response using lower doses of DADS in MCF10A human epithelial cells [17]. Therefore, we used 100μM since it would be less likely to have toxic effects on the normal cells at this dose level.

2.2. Inhibition study

Cultured MDA-MB-231 cells were treated for 24 hours with DADS with and without TNFα treatment at above conditions. Additionally, cells were co-treated with inhibitors of JNK, MEK and p38 at concentrations of 10 μM, 2 μM and 2 μM, respectively. The inhibitors for JNK (SP600125), MEK (U0126) and p38 (SB202190) were purchased from Sigma Aldrich (St. Louis, MO). Cells were detached and the lysate collected.

2.3. ELISA: CCL2 detection

Supernatants from resting and stimulated (24 hrs) MDA-MB-231 cells were collected and centrifuged at 1000× g for 5 min at 4°C. Specific ELISA was performed using MCP-1/CCL2 ELISA kit (Raybiotech, Norcross, GA, USA) following 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 100μl of prepared biotinylated antibody mixture was added to each well. After 1 hour, 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 for 30 min followed by the addition of 50 μl stop solution. Plates were read at 450nm using a UV microplate reader.

2.4. Western Blot: MAPK/ERK pathway and IKKE

Total cell protein concentrations from MDA-MB-231 cells treated with DADS, with and without TNFα co-treatment for 24 hrs, was determined using a modified Bio-Rad "DC" protein assay (Bio-Rad Laboratories, Hercules, CA, USA). A series of concentration standards ranging from 0–20 μg/ml were prepared using IgG. Test samples were prepared by adding 5 μl of a 1/10 dilution of the cell lysate to 795 μl H_2O. The standards and samples were mixed with 200 μl Bio-Rad "DC" protein assay dye concentrate and thoroughly mixed by vortexing. Following incubation for 5 minutes at room temperature, the samples were vortexed again and 200 μl of each loaded into a 96-well plate. Protein concentrations were quantified at a wavelength of 595 nm with the Power Wave X 340 microplate reader equipped with KC4 v3.0 PowerReports software (Bio-Tek Instruments, Winooski, VT, USA).

Cell lysates were separated by electrophoresis on 10% SDS-polyacrylamide gels and then transferred to Immobilon-P PVDF membranes. Equal loading was verified by staining with Ponceau S (Sigma-Aldrich Chemical Co, St. Louis, MO). Blots were blocked at 4°C

overnight in 5% Carnation Instant Milk in Tris-buffered saline with 0.05% Tween 20 in PBS (PBST) and then incubated overnight at 4°C with mouse anti-human p38 MAPK, ERK and JNK affinity purified antibody (IMGENEX, San Diego, CA). Membranes were washed with PBST and incubated overnight with anti-goat IgG-horseradish peroxidase (Santa Cruz Biotechnology, CA) in PBST overnight at 4°C. Protein loading was monitored in each gel lane by probing the membranes with anti-GAPDH antibodies (R & D Systems, Minneapolis, MN). Immunoblot images were obtained using a Flour-S Max Multimager (Bio-Rad Laboratories, Hercules, CA). Lane density data was acquired with Quantity One Software (Bio-Rad Laboratories, Hercules, CA).

2.5. RT-PCR

MDA-MB-231 cells treated with or without DADS, were subcultured in 6-well plates until confluent. Cells were lysed with 1 ml Trizol reagent. Chloroform (0.2 ml) was added to the lysed samples, tubes were shaken, incubated at 15–30°C for 2–3 min and centrifuged at 10,000 × g for 15 min. at 2–8°C. The aqueous phase was transferred to a fresh tube and the RNA precipitated by mixing 0.5 ml isopropyl alcohol. After incubation the samples were centrifuged, the supernatant was removed and the RNA pellets were washed with 75% ethanol. The samples were mixed before being centrifuged at 7,500 × g for 5 min. at 2–8°C. The RNA pellet was dried and dissolved in RNase-free water and incubated for 10 min. at 55–60°C.

RT reaction—RNA (5 μg/10 μl) was heated for 10 min. then quenched on ice before use. The following components were added to the reaction: 10 μl heat denatured RNA, 3.0 μl 10 x PCR buffer, 2.5 μl 10 mM dNTPs, 6.0 μl 25 mM $MgCl_2$, 1.0 μl random primers, 0.5 μl SuperScript II reverse transcriptase and 17.0 μl water. Samples were allowed to sit for 10 min. at 25°C then incubated for 1 hr. at 42°C. The cDNA was denatured at 95°C and placed on ice. PCR reaction: The following components were mixed in a 0.5 ml PCR tube: 6.0 μl cDNA product, 1.5 μl 10 x PCR buffer, 0.2 μl Taq polymerase, 0.5 μl primer and 10.3 μl water. PCR will be performed with 30 cycles of denaturation: 30 sec. at 95°C; annealing: 45 sec. at 60°C; and extension 60 sec. at 72°C using BioDoc-it System (UVP, Upland CA, USA). cDNA synthesis and Real-Time PCR was performed using First Strand cDNA synthesis kit/SABiosciences RT2 qPCR Master Mix from Qiagen (Gaithersburg, Md., USA) according to manufacturers instructions.

2.6. Statistical Analysis

Statistical analysis on the data was determined by Graph Pad Prism 5.0. All data was expressed as mean ± standard error from at least 3 independent experiments. Differences between mean values were analyzed by a one-way analysis of variance (ANOVA) with Dunnett's Multiple Comparison test, *$p<0.05$; **$p<0.01$.

3. RESULTS

3.1 Effect of DADS on the TNF-mediated release of CCL2 in MDA-MB-231 cells

MDA-MB-231 cells were stimulated with TNFα at sub-lethal concentrations (40 ng/ml) and the time-dependent release of CCL2 was monitored. As indicated in Figure 1, the levels of

CCL2 remained unchanged for the first 1.25 hours, and began to accumulate after 3 hours with peak levels, approximately 3-fold higher than basal levels, achieved at 24 hours post induction. This time point provides a level of accumulation that is adequate for further analysis (Figure 1). As reported in the literature the induction of TNFα-induced CCL2 is mediated via MAPK signaling pathways (Figure 2A). To evaluate its effect on CCL2 release, cells were treated with DADS in the absence and presence of MAP kinase inhibitors. As shown in Figure 2, neither DADS nor the MAPK inhibitors in combination with DADS, affected CCL2 basal levels in the absence of TNFα-induction. Upon stimulation with TNFα, a significant increase in CCL2 was not prevented by inhibition of JNK (Figure 2). DADS alone reduced the accumulation of CCL2 in TNFα-induced cells, and further enhanced the reduction exerted by p38 and MEK1 inhibitors when co-treated. These data suggest a controlling role for TNFα-induced CCL2 via MAPK involving MEK and P38 signaling, but not JNK.

3.2 DADS does not affect the protein levels of MAPK factors, but attenuates the TNFα-dependent phosphorylation of ERK and the induction of IKKε in MDA-MB-231 cells

Based on the ability of DADS to enhance the effect of MAPK inhibitors we evaluated the protein expression levels of the signaling proteins involved in the pathway. Neither DADS, or TNFα, nor their combination considerably altered the protein levels of JNK1/2/3, p38 or ERK (Figure 3). Evaluation of the phosphorylation status of ERK reveals that DADS does not affect its phosphorylation status but significantly attenuates the TNFα-dependent phosphorylation. In addition, DADS also reduced TNFα-dependent induction of IKKε (Figure 3).

3.3 Evaluation of the effect of DADS on the mRNA profile of NFκB signaling

To investigate if DADS inhibition of CCL2 release in the presence of TNFα is mitigated by inhibition of NFκB signaling, we analyzed mRNA profiles of the pathway components using the NFκB Signaling Pathway RT^2 Profiler™ PCR Array PAMM-025Z (Qiagen, Gaithersburg, Md., USA) (Table 1). Briefly, the array includes genes involved with Rel, NFκB, and IκB families, NFκB-responsive genes, ligand, receptors, kinases and transcription factors that propagate the signal. The data showed no statistical differences for baseline values between controls vs DADS (Figure 4A) for any gene in the array. The effects of TNFα on mRNA expression showed significant elevation in Bcl10, IL1b, Csf1, Crebbp, Fas, Tnfrsf1a, IL-10, Ikbkγ and Tnfrsf1b (Figure 4B). Genes were entered into bio-informatic analysis (Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7) to identify major systems affected (Table 2), where classifications provided by DAVID enrichment scores are presented for those averaging less than $p<0.01$ [18, 19]. There were significant differences found between Control vs TNFα-treated groups in the upward direction showing statistical probabilities for: regulation of cytokine biosynthesis, positive regulator of NFκB signaling, cell death and Nod-Like receptor signaling. Effect of TNFα vs TNFα/DADS shows DADS downregulation of Ccl2, Casp8 and Tradd at equal to or greater than 2-fold with $p<0.05$ (Figure 4C). We further investigated the effect of DADS on the TNFα NFκB expression pattern using RT-PCR. As shown in Figure 5, there were no changes in the mRNA expression for NFκB1 corroborating the results of the mRNA profile analysis.

DISCUSSION

CCL2 is a cancer promoting chemokine with capacity to enhance malignant cell migration, proliferation and invasive properties [20]. This enhancement occurs by mobilization of monocytes, macrophages and other inflammatory components to infiltrate the tumor area [21] enabling differentiation into TAMs [22]. The potent effects of CCL2 on metastatic invasion are likely due to it association with the elevation of matrix metalloproteinases (MMPs) (e.g. MMP-1, MMP-9) [23], prooncogenic substances such as TNF-α, vascular endothelial growth factor (VEGF)-A, TGFβ1 and IL-8 which collectively assist differentiation of human monocytes into TAMS. In a self-perpetuating cycle, CCL2 release is indirectly controlled by TNFα via TNF-receptors (TNFRs), with both CCL2 and TNFα being highly co-expressed in many human cancers [9,24]. Overactive TNFα receptor signaling is associated with coordination of tumor angiogenesis and metastasis [5] necessitating the development of therapeutic anti-cancer drugs. These drugs were designed to sequester TNFα [25] or block TNFRs [26 27] such as infliximab (Remicade), adalimumab (Humira), all of which downregulate TAMS [10] or other tumor infiltrating leucocytes in the tumor microenvironment [12]. Likewise, drugs or agents that can suppress CCL2-CCR2 signaling, can block monocyte recruitment and inhibit metastasis *in vivo* [28].

DADS, one of the major organo-sulfur compounds in garlic, is becoming recognized as a potential cancer chemopreventive compound. DADS is effective against growth of diverse cancer cell types such as HT-29 [24] HL-60 [30] cultured human colon tumor cells (HCT-15) skin (SK MEL-2) and lung (A549) [31]. Preliminary studies in our lab have indicated that DADS can attenuate CCL2 release in TNFα stimulated human breast carcinoma cells. DADS has recently been shown to reduce migration and invasion of human colon cancer in part mediated by NF-κB, ERK1/2, JNK1/2 and p38 signaling. [32] In this study, we explore signaling involved with DADS ability to down-regulate CCL2 release in TNFα-stimulated MDA-MB-231 cells.

In tumor cells, elevated NFκB signaling is triggered by TNFα, corresponding to a rise in CCL2 and TAM recruitment, cell proliferation, invasiveness and angiogenesis.[6–8]. TNF-α activation of NFκB requires its translocation from the cytoplasm to the nucleus to function. The location of NFκB is controlled by IκBs, which binds NFκB and prevents nuclear uptake. Further downstream, IκBs are themselves regulated by phosphorylation which can trigger ubiquitin-dependent degradation. The phosphorylation of IκB by IκB kinase (IKK) occurs on IKKbeta, itself a component of IKK complexes housing regulatory subunits IKKα, IKKγ and NEMO. [33]. Phosphorylation enables the recognition by E3RS (IκB/β-TrCP) to E3 ubiquitin ligase, leading to degradation, and thereby breaking controlling elements for IκB, enabling rapid NFκB translocation to the nucleus to turn on proinflammatory molecules [34]. The data in this study suggest that TNFα initiated a rise in NFκB1/2 gene expression (confirmed by PCR Array PAMM – 025Z and RT-PCR), both sustained in the presence or absence of DADS. However, DADs reduced protein expression of IKKε, which could negatively control NFκB activation signaling, and account for loss of CCL2 protein expression.

IKKi/IKKε plays an important role in carrying out TNFα signaling, via acting as a serine-threonine kinase [33]. It is capable of phosphorylating NFκB subunit RelA (also known as p65) correlating to NFκB activation [35], a rise in CCAAA/enhancer-binding protein (C/EBPδ) [36] and phosphorylation/rapid degradation of inhibitors of NFκB. Subsequent dissociation of the inhibitor/NFκB complex allows free NFκB translocation to the nucleus and initiates gene transcription. The ability of DADS to downregulate IKKε could in effect hamper TNFα induced IKKε-mediated NFκB activation [37]. This is an otherwise strong correlate to many human cancers, including, breast, ovarian, prostate, glial, [38, 39], esophageal, [40] and aggressive metastasis, tumor survival, [41] and poor clinical prognosis in diverse cancers [42]. Further, the correlation of IKKε with cell proliferation and transformation, has given rise to its being classified as oncogene [43]. Silencing or inhibition of IKKε results in inhibition of cell growth, proliferation, invasion, [44] clonogenicity, migration [45] and overcoming its contributory resistance to tamoxifen [46] in breast cancer, as well as cisplatin in ovarian tumors.[42] The identification of novel molecules that can inhibit IKKε is currently underway as a means to inflammatory processes associated with cancer progression.[47] Moreover, if DADS can reduce IKKε, this could also prevent events downstream to IKKε over expression such as activation of p52 NF-κB dimers [48], [49] estrogen receptor ERα activation, upregulation of cyclin D1 and chemotherapy resistance in breast cancer cells in particular to tamoxifen [50]. In the current study we were focused on IKKi/IKKε because it plays an important role in TNFα signaling. The presented data show a correlation between IKKε and cell proliferation and transformation as well as many different cancers. The data also show the involvement of IKKi/IKKε in tumor survival and aggressive metastasis. We are reporting that IKKε expression is reduced in this model, which is not isolated to this model but is important since this model has been known to be highly aggressive and has fewer treatment options. In the study we have not examined signaling molecules in other TNBC cell lines but we are planning to do so in future studies.

The data presented in this study suggest DADS can down-regulate IKK and CCL2 but the mechanism for this is unclear. It is possible that DADS could be down regulating the TNFα receptor complex, which would correlate to subcellular localization of NFκB, and its influence on induction of CCL2. The effect of DADS in this study, did not appear to involve mRNA of NFκB, but possibly reduced TNFRSF1A gene and adaptor protein tumor necrosis factor receptor (TNFR-associated death domain, TRADD), which are well known to “activate” via altering subcellular localization of NFκB. [51–53] Future research will be required to evaluate if the effects of DADS on CCL2 occur due to upstream events including TNFR down-regulation or potential involvement of AKT, which directly leads to up regulation of IKKε protein expression in MDA-MB-231 cells [54].

Down stream TNFα-triggered multiple signaling pathways that lead to expressed and secreted RANTES and CCL2 [55]. These pathways are believed to involve TNFR1 association with Jak2, c-Src, which could lead to CCL2 release through one or more of activating p38 MAPK, JNK, and Akt or activation of NFκB [56]. The data from this study show no change in the total proteins for ERK, P38, MEK and JNK, in the presence or absence of TNFα and DADS, however TNFα induction of CCL2 is mediated through signaling which involves MAPK phosphorylation signaling, which were further reduced in the presence of DADS. These findings suggest that level of control of DADS in CCL2

reduction is either or both occurring at the TNFα receptor or phosphorylation of ERK and P38, the former being confirmed by the data.

In summary, the findings of this study contribute to the body of work describing garlic as a chemopreventive agent, carrying diverse properties which range from carcinogen detoxification to cell-cycle arrest/apoptosis and a reduced expression of monocyte-chemoattractant protein [57]. DADS or any compound that can suppress TAM recruitment is considered an effective therapeutic approach in treatment of human cancers [58–59].

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Highlights

- DADS/TNFα decreased CCL2, Casp8, and Tradd gene expression in a TNBC cell line
- DADS/TNFα reduced IKKε protein expression in a TNBC cell line.
- DADS/TNFα reduced phosphorylated ERK protein expression in a TNBC cell line.

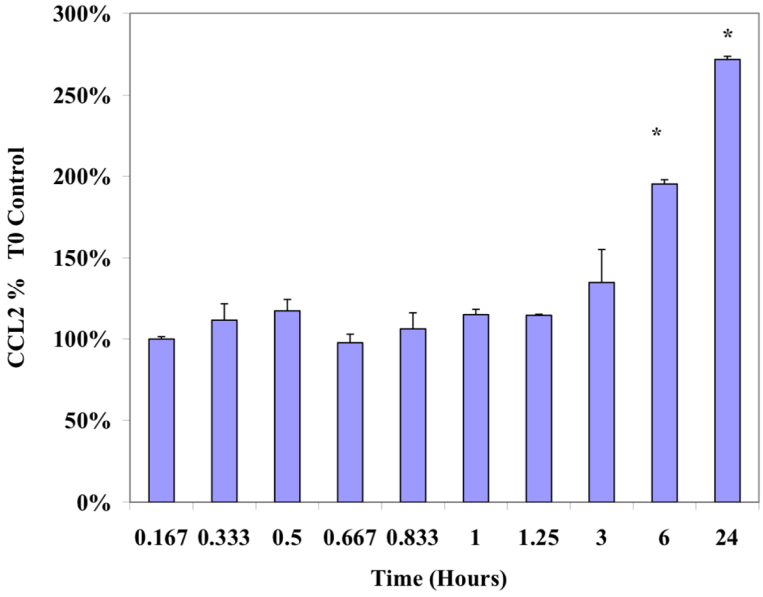


Figure 1. Time course of the TNFα-dependent accumulation of CCL2 in MDA-MB-231 cells

MDA-MB-231 cells were exposed to sublethal levels of TNFα (40ng/ml) and CCL2 release was monitored as described in Materials and Methods. The data is presented as % Ctrl at $Time_{zero}$, displayed as the Mean ± S.E.M., n=4. Differences from Control were determined using a determined by a one-way ANOVA, with a Tukey post hoc test. *p<0.05.

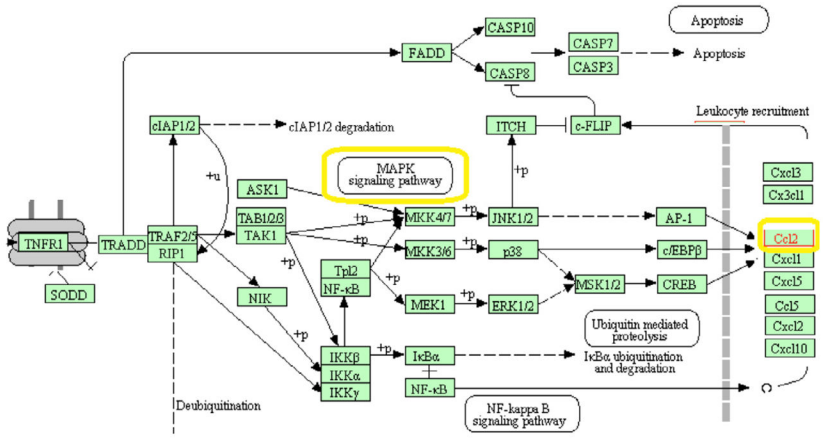


Figure 2A

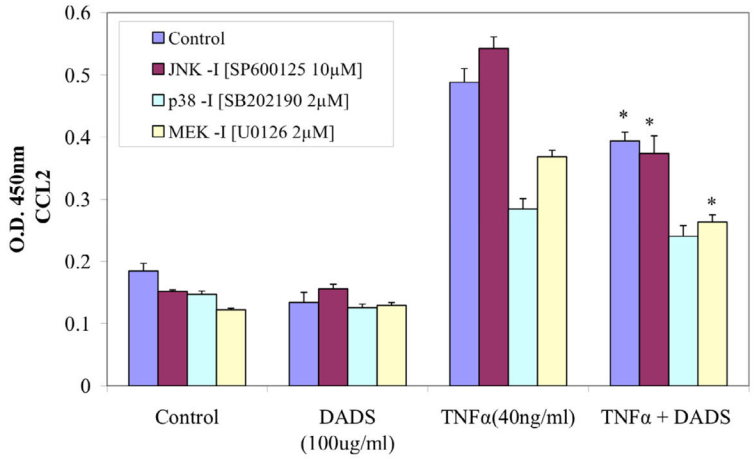


Figure 2B

Figure 2.

Figure 2A. Kegg Diagram interconnecting MAPK signaling with CCL2 release. CCL2 release is initiated by TNFα acting on TNFR1 and contributes to leukocyte recruitment. The factors analyzed in this study are highlighted.

Figure 2B. Effect of DADS and MAPK signaling inhibitors on CCL2 release in MDA-MB-231 cells. Additive or synergistic effects of MAPK inhibitors on DADS-treated (100μM), TNFα-treated (40ng/ml) and co-treated MDA-MB-231 cells after 24 hrs. The data are presented as CCL2 release (OD 450nm) and represent the Mean ± S.E.M. n=4. Significance of differences from the control in both groups was determined by T-test. *$p<0.05$.

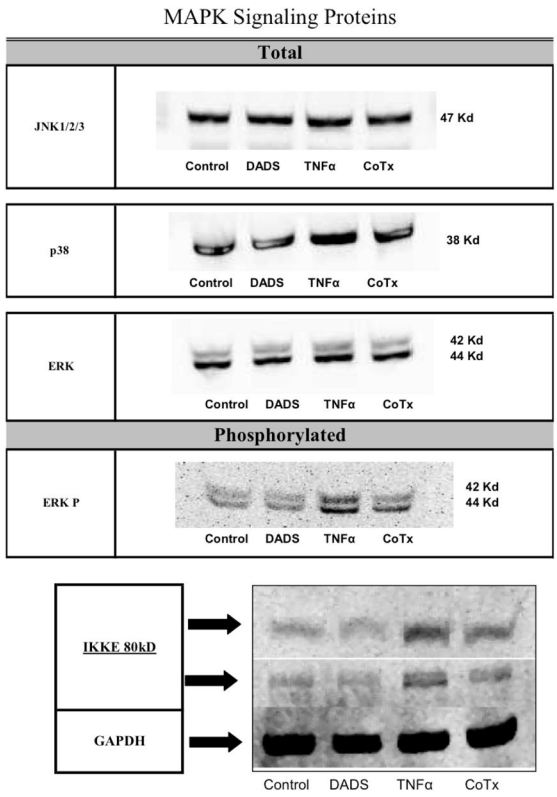


Figure 3. Evaluation total or phosphorylated proteins involved with NFκB Signaling Pathway

DADS-treated (100μM), TNFα-treated (40ng/ml) and co-treated MDA-MB-231 cell lysates were evaluated for the protein levels of JNK1/2/3, p38, ERK and IKKε. Phosphorylation status of ERK was also determined as described in the Materials and methods.

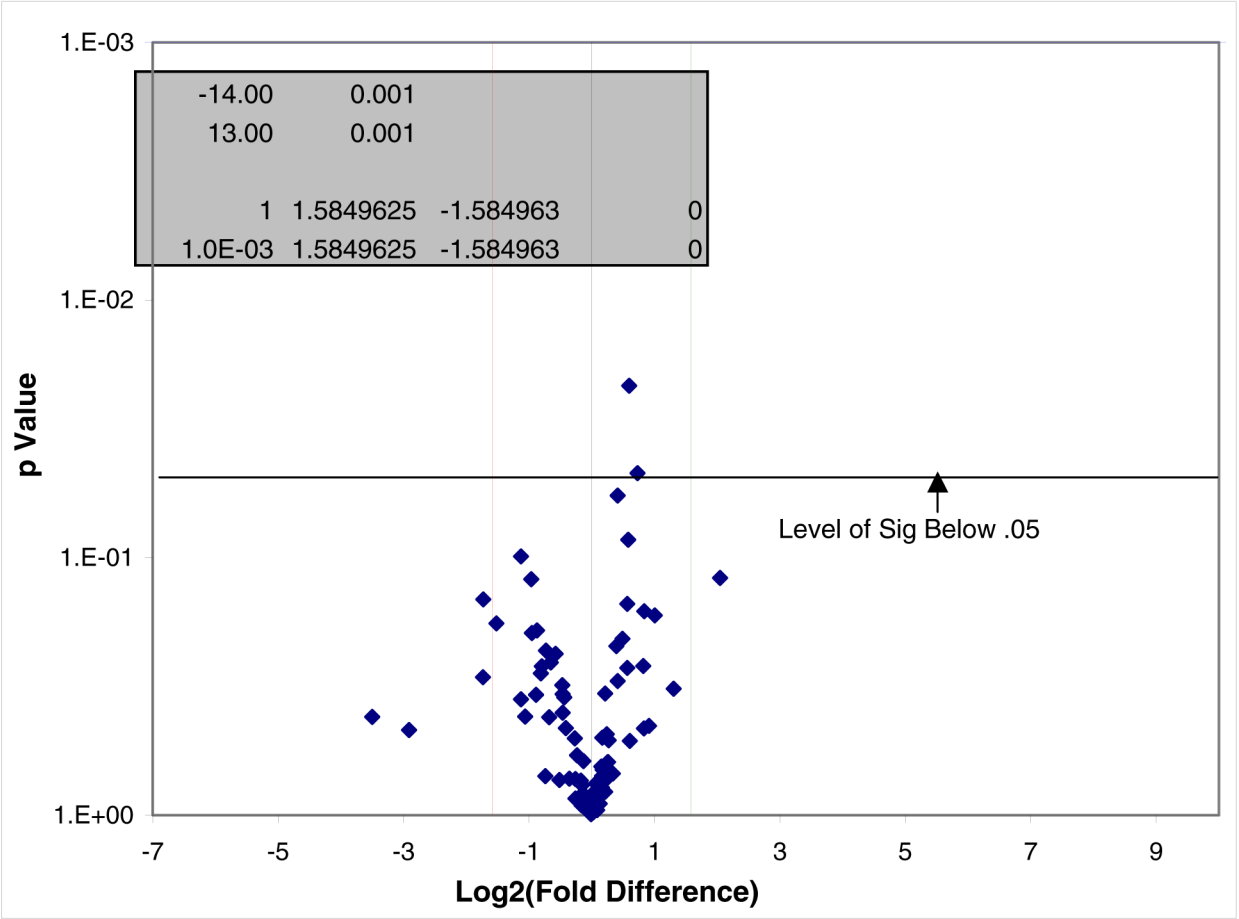
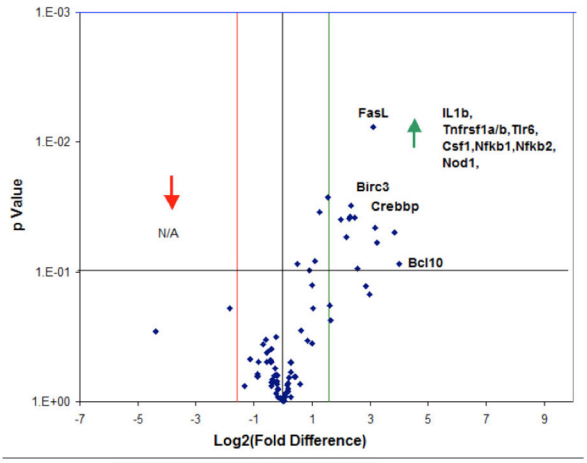
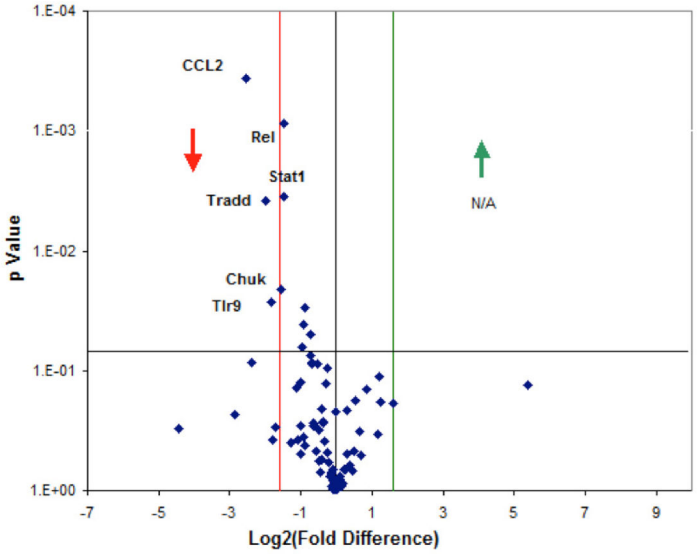


Figure 4A



		Volcano Plot	
Well	Symbol	Log_2(FC)	p Value
E02	Myd88	1.11	0.083
E03	Nfkb1	1.25	0.034
F09	Tlr6	1.55	0.027
C10	Ifng	1.98	0.040
E04	Nfkb2	2.19	0.054
E06	Nod1	2.29	0.039
E12	Ripk2	2.31	0.037
A09	Birc3	2.35	0.031
G03	Tnfrsf1b	2.46	0.038
D01	Ikbkg	2.57	0.094
D02	Il10	2.85	0.129
G02	Tnfrsf1a	2.99	0.150
C06	Fasl	3.12	0.008
B08	Crebbp	3.17	0.045
B09	Csf1	3.23	0.059
D04	Il1b	3.85	0.049
A05	Bcl10	4.02	0.086

Figure 4B



		Volcano Plot	
Well	Symbol	Log₂ (FC)	pValue
A03	Atf1	-4.45	0.302
G02	Tnfrsf1a	-2.86	0.232
B02	Ccl2	-2.55	0.000
B01	Casp8	-2.40	0.086
G07	Tradd	-2.00	0.004
F10	Tlr9	-1.82	0.027
B07	Chuk	-1.55	0.021
F03	Stat1	-1.48	0.001
E08	Rel	-1.48	0.004

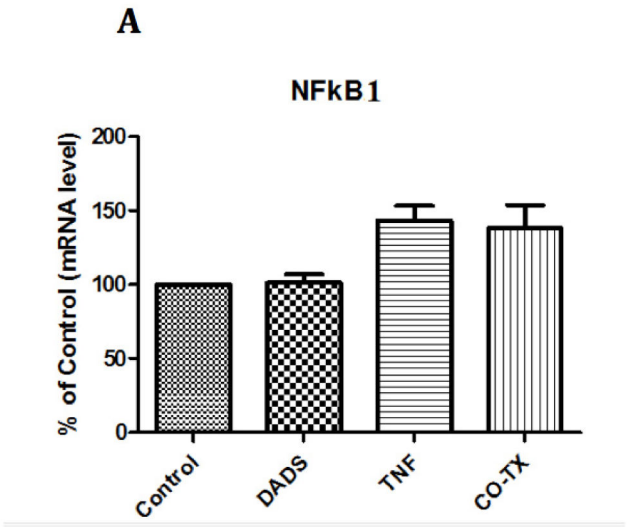
Figure 4C.

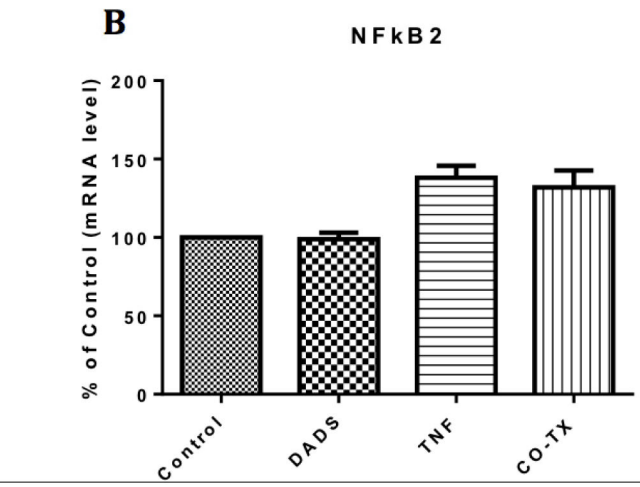
Figure 4.

Figure 4A. NFκB Signaling Pathway RT^2 Profiler™ PCR Array of DADS vs Control. Effect of DADS vs control in MDA-MB-231 cells, displayed on a volcano plot showing significance, fold change and direction. There were no significant differences found between these groups at $p<0.05$.

Figure 4B. NFκB Signaling Pathway RT^2 Profiler™ PCR Array of TNFα vs control. Analysis of TNFα vs control displayed on a volcano plot showing significance, fold change and direction. Transcriptome upward directional shifts (A), with significance and Log2 (Fold Change) listed along official gene symbols (B).

Figure 4C. NFκB Signaling Pathway RT^2 Profiler™ PCR Array of TNFα vs TNFα/ DADS. Effect of TNFα vs TNFα/DADS on gene expression: displayed on a volcano plot showing significance, fold change and direction. There were significant differences found between these groups using the PCR Array PAMM – 025Z in the downward direction (A), with significance and Log2 (Fold Change) listed along official gene symbols (B).





C

Symbol	Well	AVGΔC$_t$ (Ct(GOI)-AveCt (HKG))		2^-Δ Ct		Fold Difference	T-TEST	Fold Up- or Down-Regulation
		COTX	TNFA	COTX	TNFA	COTX/TNFA	p value	COTX/TNFA
Nfkb1	E03	5.49	5.51	2.2E-02	2.2E-02	1.01	0.9713	1.01
Nfkb2	E04	3.73	3.21	7.5E-02	1.1E-01	0.70	0.4640	-1.44

Figure 5. Effect of DADs on TNFα NF-Kappa B expression pattern using RT-PCR

The data represent the Mean ± S.E.M. n=3. There were no statistical differences found between the Control and TNFα controls ± DADS for NF-Kappa B1 (A) or NF-KappaB2 (B), also corroborating PCR expression arrays for both subtypes (C).

Table 1

Gene Table.

NFκB Signaling Pathway RT^2 Profiler™ PCR Array PAMM – 025Z plate layout. Well position, gene identifiers, official gene symbol and gene description.

Position	Unigene	GeneBank	Symbol	Description
A01	Mm.301626	NM_007428	Agt	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
A02	Mm.6645	NM_009652	Akt1	Thymoma viral proto-oncogene 1
A03	Mm.676	NM_007497	Atf1	Activating transcription factor 1
A04	Mm.209903	NM_009715	Atf2	Activating transcription factor 2
A05	Mm.239141	NM_009740	Bcl10	B-cell leukemia/lymphoma 10
A06	Mm.479217	NM_009742	Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a
A07	Mm.238213	NM_009743	Bcl2l1	Bcl2-like 1
A08	Mm.439658	NM_033601	Bcl3	B-cell leukemia/lymphoma 3
A09	Mm.2026	NM_007464	Birc3	Baculoviral IAP repeat-containing 3
A10	Mm.17629	NM_130859	Card10	Caspase recruitment domain family, member 10
A11	Mm.46187	NM_175362	Card11	Caspase recruitment domain family, member 11
A12	Mm.1051	NM_009807	Casp1	Caspase 1
B01	Mm.336851	NM_009812	Casp8	Caspase 8
B02	Mm.290320	NM_011333	Ccl2	Chemokine (C-C motif) ligand 2
B03	Mm.284248	NM_013653	Ccl5	Chemokine (C-C motif) ligand 5
B04	Mm.367714	NM_001033126	Cd27	CD27 antigen
B05	Mm.271833	NM_011611	Cd40	CD40 antigen
B06	Mm.486313	NM_009805	Cflar	CASP8 and FADD-like apoptosis regulator
B07	Mm.3996	NM_007700	Chuk	Conserved helix-loop-helix ubiquitous kinase
B08	Mm.392384	NM_001025432	Crebbp	CREB binding protein
B09	Mm.795	NM_007778	Csf1	Colony stimulating factor 1 (macrophage)
B10	Mm.4922	NM_009969	Csf2	Colony stimulating factor 2 (granulocyte-macrophage)
B11	Mm.1238	NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)
B12	Mm.420648	NM_007912	Egfr	Epidermal growth factor receptor
C01	Mm.181959	NM_007913	Egr1	Early growth response 1
C02	Mm.378990	NM_011163	Eif2ak2	Eukaryotic translation initiation factor 2-alpha kinase 2
C03	Mm.490895	NM_007922	Elk1	ELK1, member of ETS oncogene family
C04	Mm.24816	NM_010169	F2r	Coagulation factor II (thrombin) receptor
C05	Mm.5126	NM_010175	Fadd	Fas (TNFRSF6)-associated via death domain
C06	Mm.3355	NM_010177	Fasl	Fas ligand (TNF superfamily, member 6)
C07	Mm.246513	NM_010234	Fos	FBJ osteosarcoma oncogene
C08	Mm.276389	NM_010442	Hmox1	Heme oxygenase (decycling) 1
C09	Mm.435508	NM_010493	Icam1	Intercellular adhesion molecule 1
C10	Mm.240327	NM_008337	Ifng	Interferon gamma
C11	Mm.277886	NM_010546	Ikbkb	Inhibitor of kappaB kinase beta

Position	Unigene	GeneBank	Symbol	Description
C12	Mm.386783	NM_019777	Ikbke	Inhibitor of kappaB kinase epsilon
D01	Mm.12967	NM_010547	Ikbkg	Inhibitor of kappaB kinase gamma
D02	Mm.874	NM_010548	Il10	Interleukin 10
D03	Mm.15534	NM_010554	Il1a	Interleukin 1 alpha
D04	Mm.222830	NM_008361	Il1b	Interleukin 1 beta
D05	Mm.896	NM_008362	Il1r1	Interleukin 1 receptor, type I
D06	Mm.38241	NM_008363	Irak1	Interleukin-1 receptor-associated kinase 1
D07	Mm.152142	NM_172161	Irak2	Interleukin-1 receptor-associated kinase 2
D08	Mm.105218	NM_008390	Irf1	Interferon regulatory factor 1
D09	Mm.275071	NM_010591	Jun	Jun oncogene
D10	Mm.87787	NM_010735	Lta	Lymphotoxin A
D11	Mm.3122	NM_010736	Ltbr	Lymphotoxin B receptor
D12	Mm.15918	NM_011945	Map3k1	Mitogen-activated protein kinase kinase kinase 1
E01	Mm.8385	NM_011952	Mapk3	Mitogen-activated protein kinase 3
E02	Mm.213003	NM_010851	Myd88	Myeloid differentiation primary response gene 88
E03	Mm.256765	NM_008689	Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105
E04	Mm.102365	NM_019408	Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100
E05	Mm.170515	NM_010907	Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
E06	Mm.28498	NM_172729	Nod1	Nucleotide-binding oligomerization domain containing 1
E07	Mm.184163	NM_029780	Raf1	V-raf-leukemia viral oncogene 1
E08	Mm.4869	NM_009044	Rel	Reticuloendotheliosis oncogene
E09	Mm.249966	NM_009045	Rela	V-rel reticuloendotheliosis viral oncogene homolog A (avian)
E10	Mm.1741	NM_009046	Relb	Avian reticuloendotheliosis viral (v-rel) oncogene related B
E11	Mm.374799	NM_009068	Ripk1	Receptor (TNFRSF)-interacting serine-threonine kinase 1
E12	Mm.112765	NM_138952	Ripk2	Receptor (TNFRSF)-interacting serine-threonine kinase 2
F01	Mm.272675	NM_015747	Slc20a1	Solute carrier family 20, member 1
F02	Mm.7320	NM_016769	Smad3	MAD homolog 3 (Drosophila)
F03	Mm.487336	NM_009283	Stat1	Signal transducer and activator of transcription 1
F04	Mm.34580	NM_019786	Tbk1	TANK-binding kinase 1
F05	Mm.273024	NM_030682	Tlr1	Toll-like receptor 1
F06	Mm.87596	NM_011905	Tlr2	Toll-like receptor 2
F07	Mm.33874	NM_126166	Tlr3	Toll-like receptor 3
F08	Mm.38049	NM_021297	Tlr4	Toll-like receptor 4
F09	Mm.42146	NM_011604	Tlr6	Toll-like receptor 6
F10	Mm.44889	NM_031178	Tlr9	Toll-like receptor 9
F11	Mm.1293	NM_013693	Tnf	Tumor necrosis factor
F12	Mm.116683	NM_009397	Tnfaip3	Tumor necrosis factor, alpha-induced protein 3
G01	Mm.193430	NM_020275	Tnfrsf10b	Tumor necrosis factor receptor superfamily, member 10b
G02	Mm.474976	NM_011609	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a

Position	Unigene	GeneBank	Symbol	Description
G03	Mm.235328	NM_011610	Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b
G04	Mm.1062	NM_009425	Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10
G05	Mm.483369	NM_019418	Tnfsf14	Tumor necrosis factor (ligand) superfamily, member 14
G06	Mm.103551	NM_023764	Tollip	Toll interacting protein
G07	Mm.264255	NM_001033161	Tradd	TNFRSF1A-associated via death domain
G08	Mm.3399	NM_009422	Traf2	Tnf receptor-associated factor 2
G09	Mm.27431	NM_011632	Traf3	Tnf receptor-associated factor 3
G10	Mm.389227	NM_011633	Traf5	Tnf receptor-associated factor 5
G11	Mm.292729	NM_009424	Traf6	Tnf receptor-associated factor 6
G12	Mm.8038	NM_009539	Zap70	Zeta-chain (TCR) associated protein kinase
H01	Mm.391967	NM_007393	Actb	Actin, beta
H02	Mm.163	NM_009735	B2m	Beta-2 microglobulin
H03	Mm.304088	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
H04	Mm.3317	NM_010368	Gusb	Glucuronidase, beta
H05	Mm.2180	NM_008302	Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1
H06	N/A	SA_00106	MGDC	Mouse Genomic DNA Contamination
H07	N/A	SA_00104	RTC	Reverse Transcription Control
H08	N/A	SA_00104	RTC	Reverse Transcription Control
H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10	N/A	SA_00103	PPC	Positive PCR Control
H11	N/A	SA_00103	PPC	Positive PCR Control
H12	N/A	SA_00103	PPC	Positive PCR Control

Table 2

Function Categories Affected by TNF-alpha vs Control

Effect of TNFα vs Controls on targeted pathways using DAVID functional annotation statistical program. Gene-annotation enrichment analysis, functional annotation clustering on statistically differentially expressed genes. Statistical DAVID genes were analyzed for annotation cluster enrichment scores. The data represent the score, number of genes, shift direction and p-values (Fisher Exact/EASE Score).

Annotation Cluster 1	**Enrichment Score: 7.62**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	regulation of cytokine biosynthetic process	RT	6	2.50E-10	1.40E-08
Annotation Cluster 2	Enrichment Score: 7.02		Count	P_Value	Benjamini
GOTERM_BP_FAT	positive regulation of protein kinase cascade	RT	7	5.10E-12	8.5E-10
GOTERM_BP_FAT	regulation of interleukin-6 production	RT	6	6.5E-12	8.10E-10
GOTERM_BP_FAT	positive regulation of I-kappaB kinase/NF-kappaB cascade	RT	6	7.7E-12	7.70E-10
GOTERM_BP_FAT	regulation of stress-activated protein kinase signaling pathway	RT	5	1.40E-08	4.20E-07
Annotation Cluster 3	Enrichment Score: 4.08		Count	P_Value	Benjamini
GOTERM_BP_FAT	regulation of chemokine product	RT	4	3.30E-08	8.60E-07