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Whole Transcriptomic Analysis of Apigenin on TNFα Immuno-activated MDA-MB-231 Breast Cancer Cells

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Abstract. *Background: Triple-negative breast cancer is categorized by a lack of hormone receptors, inefficacy of anti-estrogen or aromatase inhibitor chemotherapies and greater mortality rates in African American populations. Advanced-stage breast tumors have a high concentration of tumor necrosis factor-α (TNFα) throughout the tumor/stroma milieu, prompting sustained release of diverse chemokines (i.e. C-C motif chemokine ligand 2 (CCL2)/CCL5). These potent chemokines can subsequently direct mass infiltration of leukocyte sub-populations to lodge within the tumor, triggering a loss of tumor immune surveillance and subsequent rapid tumor growth. Previously, we demonstrated that in the MDA-MB-231 TNBC cell line, TNFα evoked a rise in immune signaling proteins: CCL2, granulocyte macrophage colony-stimulating factor, interleukin (IL)1α, IL6 and inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKBKε) all of which were attenuated by apigenin, a dietary flavonoid found in chamomile and parsley. Materials and Methods: The present work elucidates changes evoked by TNFα in the presence or absence of apigenin by examining the entire transcriptome for mRNA and long intergenic non-coding RNA with Affymetrix Hugene-2.1_ST human microarrays. Differential gene-expression analysis was conducted on 48,226 genes. Results: TNFα caused up-regulation of 75 genes and down-regulation of 10. Of these, apigenin effectively down-regulated 35 of the 75 genes which were up-regulated by TNFα. These findings confirm our previous work, specifically for the TNFα-evoked spike in IL1A vs. untreated controls [+21-fold change (FC), p<0.0001] being attenuated by apigenin in the presence of TNFa (−15 FC vs. TNFα, p<0.0001). Similar trends were seen for apigenin-mediated down-regulation of TNFα-up-regulated transcripts: IKBKE (TNFα: 4.55 FC vs. control, p<0.001; and TNFα plus apigenin: −4.92 FC, p<0.001), CCL2 (2.19 FC, p<0.002; and −2.12 FC, p<0.003), IL6 (3.25 FC, p<0.020; and −2.85 FC, p<0.043) and CSF2 (TNFα +6.04 FC, p<0.001; and −2.36 FC, p<0.007). In addition, these data further establish more than a 65% reduction by apigenin for the following transcripts which were also up-regulated by TNFα: cathepsin S (CTSS), complement C3 (C3), laminin subunit gamma 2 (LAMC2), (TLR2), toll-like receptor 2 G protein-coupled receptor class C group 5 member B (GPRC5B), contactin-associated protein 1 (CNTNAP1), claudin 1 (CLDN1), nuclear factor of activated T-cells 2 (NFATC2), C-X-C motif chemokine ligand 10 (CXCL10), CXCL11, interleukin 1 receptor-associated kinase 3 (IRAK3), nuclear receptor subfamily 3 group C member 2 (NR3C2), interleukin 32 (IL32), IL24, slit guidance ligand 2 (SLIT2), transmembrane protein 132A (TMEM132A), TMEM171, signal transducing adaptor family member 2 (STAP2), mixed lineage kinase domain-like pseudokinase (MLKL), kinase insert domain receptor (KDR), BMP-binding endothelial regulator (BMPER), and kelch-like family member 36 (KLHL36). Conclusion: There is a possible therapeutic role for apigenin in down-regulating diverse genes associated with tumorigenic leukocyte sub-population infiltration by triple-negative breast cancer. The data have been deposited into the Gene Expression Omnibus for public analysis at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120550.*

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Key Words: Triple-negative breast cancer, apigenin, TNFα, differential gene expression analysis, immune signaling.

Breast cancer is often sub-categorized as being either hormone receptor-positive or -negative, to which the latter group if devoid of estrogen and progesterone receptors and human epidermal growth factor receptor 2 (HER2) is further classified as triple-negative breast cancer (TNBC). TNBC is non-responsive to estrogen receptor modulators (*e.g.* raloxifene, tamoxifen) and aromatase inhibitors, can be quite aggressive and is often associated with lower overall survival odds, particularly in African Americans (1). While the standard treatment for TNBC includes surgery, radiation and various types of chemotherapies (*e.g.* taxanes, doxorubicin,

or docetaxel/cyclophosphamide), new drug approaches or use of effective complementary and alternative medicines are needed. Possible drug candidates to treat aggressive TNBC are those that target phosphoinositide 3-kinase (PI3K), poly (ADP-ribose) polymerase (PARP), luminal androgen receptor (LAR) (2, 3), cytokine modulators, vaccines (4) and immune-therapies such as checkpoint antagonistic antibodies against programmed cell death-1 receptor/programmed death-ligand 1 (5, 6).

The immune system plays a critical role in both risk and mortality outcome associated with diverse human cancer types including TNBC. Initially, immune deficiency in immunocompromised individuals can play an essential role in tumor development. While a healthy immune system can target and destroy cancer cells through MHC class I chain-related molecules (MICs), cluster of differentiation (CD)4^+/ $CD8^+$ T- and natural killer cell activities, immunosuppression can hamper these biological responses and amplify the risk of developing spontaneous or carcinogen-induced tumors (7-10). Once a tumor is established, continual evasion of the immune system is part of malignant disease, carried out by the actions of chemotactic tumor-promoting proteins [*e.g.* tumor necrosis factor-α (TNFα), and C-C motif chemokine ligand 2 (CCL2)] that direct inward migration of leukocyte sub-populations (LSPs) bearing CCR2/CCR5 receptors to the tumor microenvironment. Types of LSPs can include tumor-associated macrophages, tumor-associated neutrophils, T-regulatory cells, myeloid-derived suppressor cells, metastasis-associated macrophages and cancer-associated fibroblasts, all of which collectively foster tumor growth, stem cell survival, angiogenesis, metastasis (11) and overexpression of programmed cell death 1 and programmed death ligand 1 (12), all of which inactivate the normal tumor immune response (13-17).

Apigenin is a natural compound which has the capacity to antagonize several mitigating events in human tumor development and tumor immune evasion. These include the capability to reduce inflammation, inhibit casein kinase 2 (CK2), matrix metalloproteinases 9 and 1, protein kinase C (18), cytochrome *P450 1A1* (19) topoisomerase I (20) tumor growth factors (21), CCL2-induced release by TNFα-activated breast cancer (22) and blockade of drug resistance-associated extrusion pumps (23, 24). In the current study, we expand on our previous work by evaluating the effects of apigenin on TNFα-treated MDA-MB-231 cells (22) by analyzing the whole transcriptome.

Materials and Methods

Cell line, chemicals, and reagents. TNBC 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 obtained from Invitrogen (Carlsbad, CA, USA). Recombinant human TNFα was purchased from RayBiotech (RayBiotech Inc., Norcross, GA, USA). Apigenin, CCL2 enzyme-linked immunosorbent assay (ELISA) kits, and recombinant IL1α were purchased from Abcam (Cambridge, MA, USA).

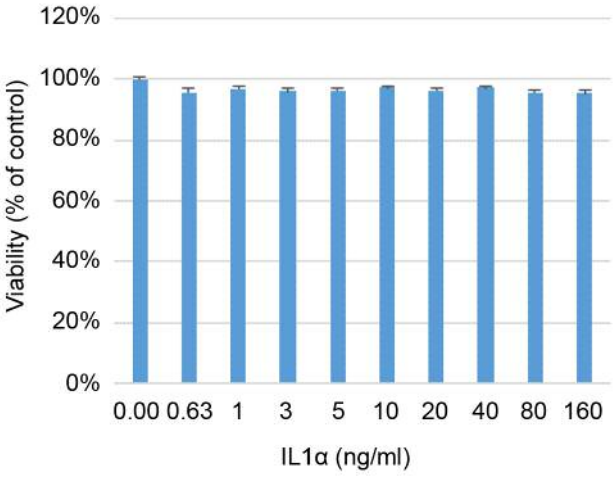

Figure 1. *The effect of interleukin 1α (L1α) on viability of MDA-MB-231 cells at 5% CO_2/Atm for 24 h. The data are the mean±S.E.M. (n=4) and presented as viability relative to that of the control. No significant differences from the control were found by one-way ANOVA.*

Cell culture. MDA-MB-231 cells were grown in high-glucose DMEM (with phenol red and glutamine) then supplemented with 10% FBS and 1% (10,000 U/ml) penicillin G sodium and (10,000 μg/ml) streptomycin sulfate. Cells were grown at 37˚C with humidified 95% air/5% CO_2 and sub-cultured every 3-5 days.

Cell viability assay. Cell viability was determined using the Alamar Blue assay. Briefly, 96-well plates were seeded with MDA-MB-231 cells at a density of 5×10^4 cells/100 μl/well. After 24 h, Alamar blue (0.1 mg/ml in Hank's balanced salt solution (HBSS)) was added at 15% v/v to each well and plates were incubated for a further 6-8 h. Quantitative analysis of dye conversion was measured using a Biotek Synergy multi-mode detection reader equipped with Gen5 software at 550/580 nm (excitation/emission) (BioTek Instruments, Inc., Winooski, VT, USA). All data are expressed as a percentage of live untreated controls.

CCL2 detection by ELISA. Supernatants from controls and TNFα-stimulated (24 h) MDA-MB-231 cells were collected and centrifuged at 100 × *g* for 5 min at 4˚C. ELISA was performed using MCP-1/CCL2 Human MCP1 ELISA Kit from Abcam (ab179886) (Cambridge, MA, USA) following the manufacturer's instructions. Briefly, 100 μl of supernatant from samples were added to 96-well plates pre-coated with the capture antibody, followed by a wash, second antibody, detection antibody and final reading at 450 nm using a Biotek Synergy multi-mode detection reader equipped with Gen5 software (BioTek Instruments, Inc.).

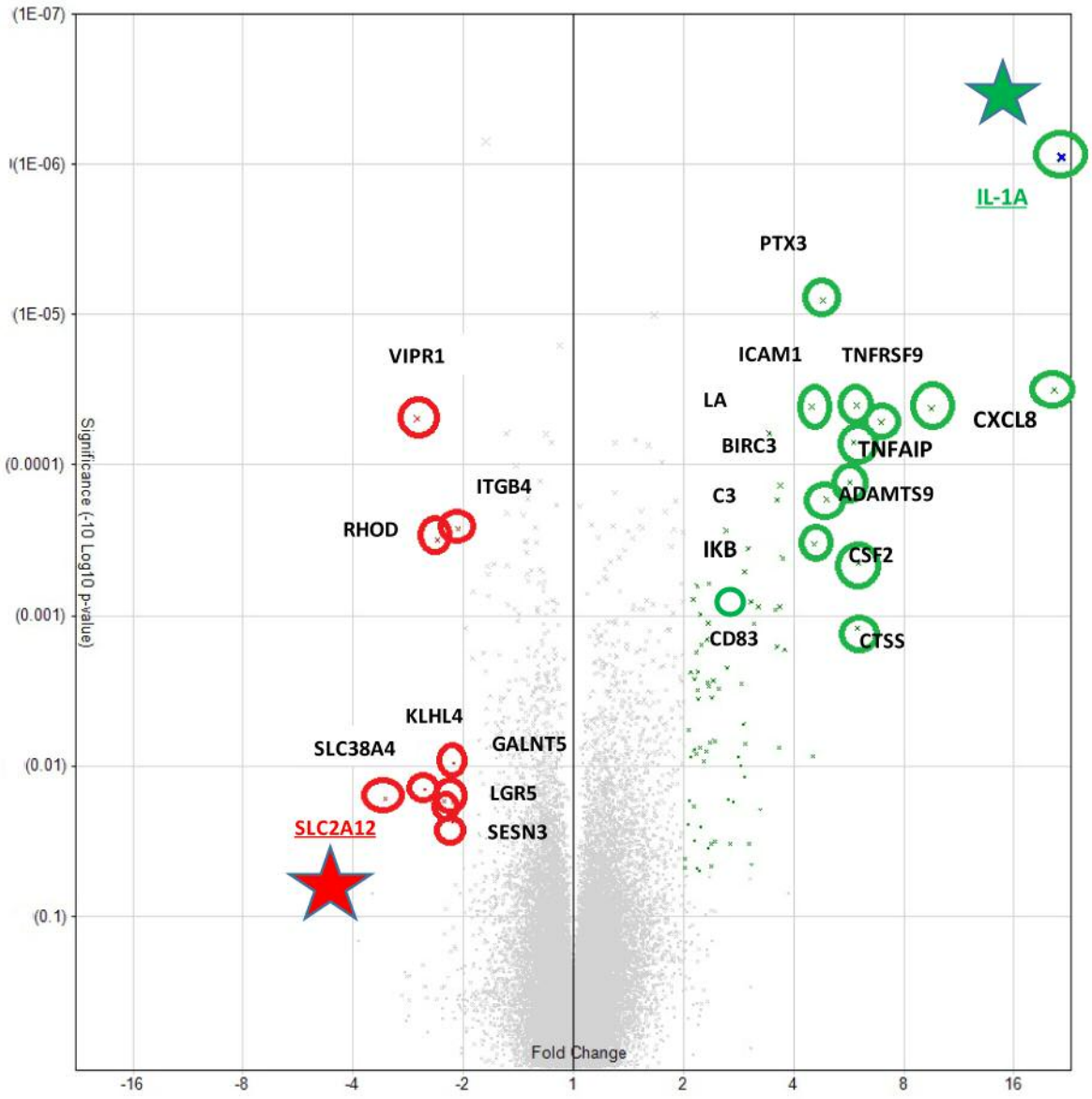

Figure 2. *Whole transcriptomic differential changes between MDA-MB-231 cells after 24 h of treatment with tumor necrosis factor-α (TNFα) (40 ng/ml). The data are presented as fold change (FC) and ANOVA p-value. Up-regulated transcripts are shown in the right-hand panel, and down-regulated transcripts are shown in the left-hand panel. Top differentially expressed genes are denoted by official gene symbol, with all data presented for differential changes in Table I.*

Reverse transcription polymerase chain reaction (RT-PCR). MDA-MB-231 cells were lysed with 1 ml Trizol reagent. Chloroform (0.2 ml) was added to the samples, which were then vortexed, 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 then transferred to a fresh tube, and the RNA precipitated by mixing 0.5 ml isopropyl alcohol. RNA was extracted and subject to iScript advanced RT to the reaction. The reverse transcription was performed for 30 min at 42˚C and RT inactivation for 5 min. at 85˚C. For PCR reaction, the following components were mixed in a 0.5 ml PCR tube: 5.0 µl cDNA product, 10 µl SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 1.0 µl inhibitor of nuclear factor kappa-B kinase subunit epsilon (*KBKE*) primer and 4 µl water. PCR was performed with 39 cycles of denaturation: 15 s at 95˚C; annealing: 30 s at 60˚C; and extension 60 s at 72˚C using a Bio-Rad CFX96 Real-Time System. cDNA synthesis and real-time PCR was performed using iScript™ advanced cDNA synthesis kit/advanced Universal SYBR® RT-PCR (Bio-Rad Laboratories, Inc.) was run and normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA for each sample.

Microarray WT 2.1 human datasets. After treatment, cells were washed three times in ice-cold HBSS, rapidly frozen and stored at −80˚C. Total RNA was isolated and purified using the TRIzol/

Table I. *Differential whole transcriptome pattern induced in MDA-MB-231 cells by tumor necrosis factor-α (TNFα) (40 ng/ml) relative to that of untreated controls. The data are presented as average (n=3) bi-weight × signal (log2).*

Gene symbol	Description	Expression		
		TNFα	Control	FC
*IL1A**	Interleukin 1 alpha	6.26	1.83	21.57
*CXCL8**	Chemokine (C-X-C motif) ligand 8	7.57	3.2	20.61
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	6.76	3.51	9.51
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	5.89	3.1	6.94
*CSF2**	Colony stimulating factor 2 (granulocyte-macrophage)	5.07	2.47	6.04
*CTSS**	Cathepsin S	8.38	5.8	5.97
ICAM1	Intercellular adhesion molecule 1	9.33	6.76	5.95
BIRC3	Baculoviral iap repeat containing 3	8.84	6.3	5.84
*ADAMTS9**	Adam metallopeptidase with thrombospondin type 1 motif 9	7.05	4.54	5.69
*C3**	Complement component 3	4.67	2.37	4.92
PTX3	Pentraxin 3, long	9.03	6.76	4.81
*IKBKE**	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	6.58	4.39	4.55
*LAMC2**	Laminin, gamma 2	8.6	6.43	4.48
CD83	CD83 molecule	5.95	4.03	3.78
*TLR2**	Toll-like receptor 2	5.76	3.86	3.73
TRAF1	Tnf receptor-associated factor 1	4.98	3.1	3.68
*GPRC5B**	G protein-coupled receptor, class c, group 5, member b	5.72	3.84	3.66
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in b-cells 2 (p49/p100)	6.06	4.19	3.65
*CNTNAP1**	Contactin associated protein 1	5.01	3.17	3.6
*CLDN1**	Claudin 1	5.03	3.18	3.6
IRAK2	Interleukin 1 receptor-associated kinase 2	5.85	4.02	3.56
TNIP1	Tumor necrosis factor, alpha-induced protein 1	7.59	5.81	3.43
*IL6**	Interleukin 6	4.7	3	3.25
NUAK2	NUAK family kinase 2	5.06	3.37	3.21
NFKBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	6.6	4.96	3.12
SNORD18C; RPL4	Small nucleolar RNA, C/D box 18c; ribosomal protein l4	5.24	3.62	3.07
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, alpha	8.06	6.45	3.06
NFATC2*	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	5.46	3.87	3.01
*SERPINB2; SERPINB10**	Serpin peptidase inhibitor, clade B (ovalbumin), member 2, and member 10	5.35	3.76	3.01
LOC100287834	Uncharacterized LOC100287834	10.9	9.34	2.94
*CXCL10**	Chemokine (C-X-C motif) ligand 10	3.21	1.66	2.94
*CXCL11**	Chemokine (C-X-C motif) ligand 11	4.36	2.81	2.93
ERN1	Endoplasmic reticulum to nucleus signaling 1	4.81	3.26	2.92
C15orf48; MIR147B	Chromosome 15 open reading frame 48; microRNA 147b	5.63	4.1	2.88
KRTAP2-3	Keratin-associated protein 2-3	4.86	3.34	2.87
LOC541472	Uncharacterized LOC541472	2.98	1.48	2.83
*IRAK3**	Interleukin 1 receptor-associated kinase 3	4.3	2.85	2.73
*NR3C2**	Nuclear receptor subfamily 3, group C, member 2	4.57	3.15	2.68
*IL32**	Interleukin 32	4.47	3.07	2.63
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	10.11	8.73	2.61
*IL24**	Interleukin 24	5.14	3.83	2.49
*DRAM1**	DNA-damage regulated autophagy modulator 1	4.44	3.15	2.44
TNFAIP2	Tumor necrosis factor, alpha-induced protein 2	5.43	4.14	2.44
*TNFSF15**	Tumor necrosis factor (ligand) superfamily, member 15	4.02	2.75	2.41
*SLIT2**	Slit guidance ligand 2	6.22	4.97	2.39
CD44	CD44 molecule (Indian blood group)	4.53	3.28	2.38
*AFAP1-AS1**	AFAP1 antisense RNA 1	6.32	5.09	2.34
TRAF3	Tnf receptor-associated factor 3	5.45	4.23	2.34
NAV3	Neuron navigator 3	6.9	5.68	2.34
G0S2	G0/G1 switch 2	8.95	7.73	2.34
*TMEM132A**	Transmembrane protein 132A	4.69	3.47	2.33
*TMEM171**	Transmembrane protein 171	6.95	5.74	2.32
ICAM2	Intercellular adhesion molecule 2	6.69	5.49	2.31
ZC3H12C	Zinc finger CCCH-type containing 12C	6.77	5.58	2.27
MMP9	Matrix metallopeptidase 9	3.54	2.38	2.24

Table I. *Continued*

Table I. *Continued*

Gene symbol	Description	Expression		
		TNFα	Control	FC
CXCL2	Chemokine (C-X-C motif) ligand 2	4.04	2.89	2.23
ZC3H7B	Zinc finger CCCH-type containing 7B	5.92	4.77	2.22
*STAP2**	Signal transducing adaptor family member 2	5.96	4.81	2.22
SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	2.98	1.85	2.19
*RHBDF2**	Rhomboid 5 homolog 2 (Drosophila)	5.44	4.31	2.19
*CCL2**	Chemokine (C-C motif) ligand 2	2.9	1.77	2.19
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	6.69	5.57	2.18
*MLKL**	Mixed lineage kinase domain-like	6	4.89	2.16
LACC1	Laccase (multicopper oxidoreductase) domain containing 1	6.35	5.24	2.16
*KDR**	Kinase insert domain receptor	4.98	3.88	2.14
*BMPER**	Bmp binding endothelial regulator	4.41	3.32	2.14
LOC105369848	Uncharacterized LOC105369848	3.21	2.11	2.14
IL23A	Interleukin 23, alpha subunit p19	3.22	2.13	2.13
PPIF	Peptidylprolyl isomerase f	7.96	6.87	2.12
LAMB3	Laminin, beta 3	8.61	7.55	2.09
RLN3; IL27RA	Relaxin 3; interleukin 27 receptor, alpha	5.35	4.29	2.09
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	4.39	3.33	2.08
POU2F2	Pou class 2 homeobox 2	3.82	2.78	2.06
KLHL36	Kelch-like family member 36	4.37	3.36	2.02
MIR4454	MicroRNA 4454	5.04	4.02	2.02
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	2.57	3.59	–2.03
ITGB4	Integrin beta 4	6.3	7.35	–2.07
KLHL4	Kelch-like family member 4	5.61	6.7	–2.12
GALNT5	Polypeptide n-acetylgalactosaminyltransferase 5	4.72	5.8	–2.13
SESN3	Sestrin 3	2.59	3.69	–2.14
LGR5	Leucine-rich repeat containing G protein-coupled receptor 5	2.11	3.27	–2.25
RHOD	Ras homolog family member d	4.73	5.97	–2.36
SLC38A4	Solute carrier family 38, member 4	3.46	4.81	–2.54
VIPR1	Vasoactive intestinal peptide receptor 1	4.79	6.21	–2.68
SLC2A12	Solute carrier family 2 (facilitated glucose transporter), member 12	3.06	4.77	–3.26

*Genes also significantly down-regulated when treated with 40 μM apigenin. FC: Fold change relative to untreated controls; all FC had an ANOVA significance value of less than 0.05.

chloroform method, the quality was assessed, and concentration was equalized to 82 ng/μl in nuclease-free water. Whole-transcriptome analysis was conducted according to the GeneChip™ WT PLUS Reagent Manual for Whole Transcript (WT) Expression Arrays (Affymetrix/Thermo Fisher Scientific Waltham, MA, USA). Briefly, RNA was synthesized to first-strand cDNA, second-strand cDNA and followed by transcription to cRNA. cRNA was purified and assessed for yield, before second cycle single-stranded cDNA synthesis, hydrolysis of RNA and purification of second cycle single-stranded cDNA. cDNA was then quantified for yield and equalized to 176 ng/ml. Subsequently, cDNA was fragmented, labeled and hybridized onto the arrays before being subject to fluidics and imaging using the Gene Atlas (Affymetrix/Thermo Fisher Scientific). The array data quality control and initial processing from CEL to CHP files were conducted using expression console, before data evaluation using the Affymetrix transcriptome analysis console. Samples were run in triplicates (n=3). The data have been deposited into the Gene Expression Omnibus for public analysis at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120550

Statistical analysis. Data analysis on microarrays was conducted using Affymetrix® Transcriptome Analysis Console (TAC) Software, with parameter set at: Array Type: HuGene-2_1-st, Genome Version: hg19 (*Homo sapiens*) Condition (Comparisons): API; Control; Cotx; TNF, gene-level fold change <–2 or >2, gene-level *p*-value <0.05, ANOVA Method: empirical Bayes statistics for differential expression. Other statistical analysis was performed using GraphPad Prism (version 3.0; GraphPad Software Inc. San Diego, CA, USA) with the significance of the difference between the groups assessed using a one-way ANOVA, followed by Tukey *post-hoc* comparison of means test.

Results

A non-lethal working concentration in MDA-MB-231 cells was established for TNFα, apigenin (as previously reported) (22) and IL1α (Figure 1) where the sub-lethal working concentrations were set at the following: 40 μM apigenin and 40 ng/ml TNFα. To investigate TNFα-mediated induction of

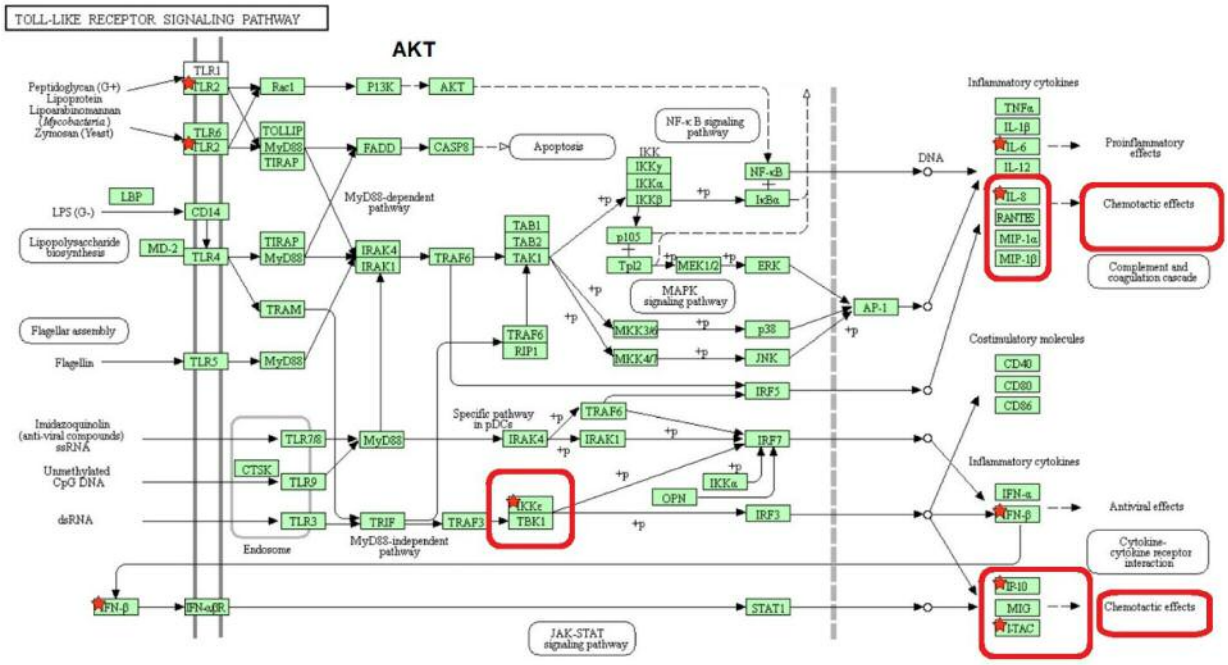

Figure 3. *Differential whole transcriptome assay where stars denote genes up-regulated by tumor necrosis factor-α (TNFα) (40 ng/ml) and down-regulated by apigenin (40 μM). Official gene symbols were uploaded from the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 where annotation files representing pathways show significant changes to the KEGG pathway for Toll-like receptor pathways.*

Table II. *Biological process analysis: Comparing controls vs. tumor necrosis factor-α (TNFα)-treated MDA-MB-231 cells. Up-regulated genes were imported into Gene Ontology Consortium at geneontology.org using the Panther Classification system enrichment analysis by Go-Slim Biological Process. Classifications identified show TNFα largely affected cytokine-mediated signaling pathways (A), being further defined as primarily involved with immune cell migration and regulation of angiogenesis (B).*

Analysis type: PANTHER overrepresentation test
Annotation version and release date: Go Ontology database released 2018-08-09
Analyzed list: Up-regulated by TNFa *vs*. control
Reference list: *Homo sapiens* (all genes in database)
Annotation data set: Go Biological process complete
Test type: Fisher's exact with false discovery rate (FDR) multiple correction
Displaying only results with an FDR<0.05

	Reference list	Uploaded data set
Mapped IDs:	21,042 out of 21,042	66 out of 68
Unmapped IDs:	0	9
Multiple Mapping information	0	2

A. PANTHER GO-Slim Biological Process	Homo sapiens (REF)
Cytokine - mediated signaling pathway	60

B. Go Biological Process Complete	Homo sapiens (REF)	#	Expected	Fold enrichment	Raw *p*-Value	FDR
T cell extravasation (leukocyte migration, motility)	351	10	1.17	8.57	2.93E-07	3.46E-05
(T cell/lymphocyte migration)	21	4	0.07	58.94	1.18E-06	3.58E-03
Regulation of vascular would healing (response to stress)	1407	20	4.55	4.4	1.24E-08	2.06E-06
Regulation of angiogenesis	282	10	0.91	10.97	3.15E-08	4.77E-06

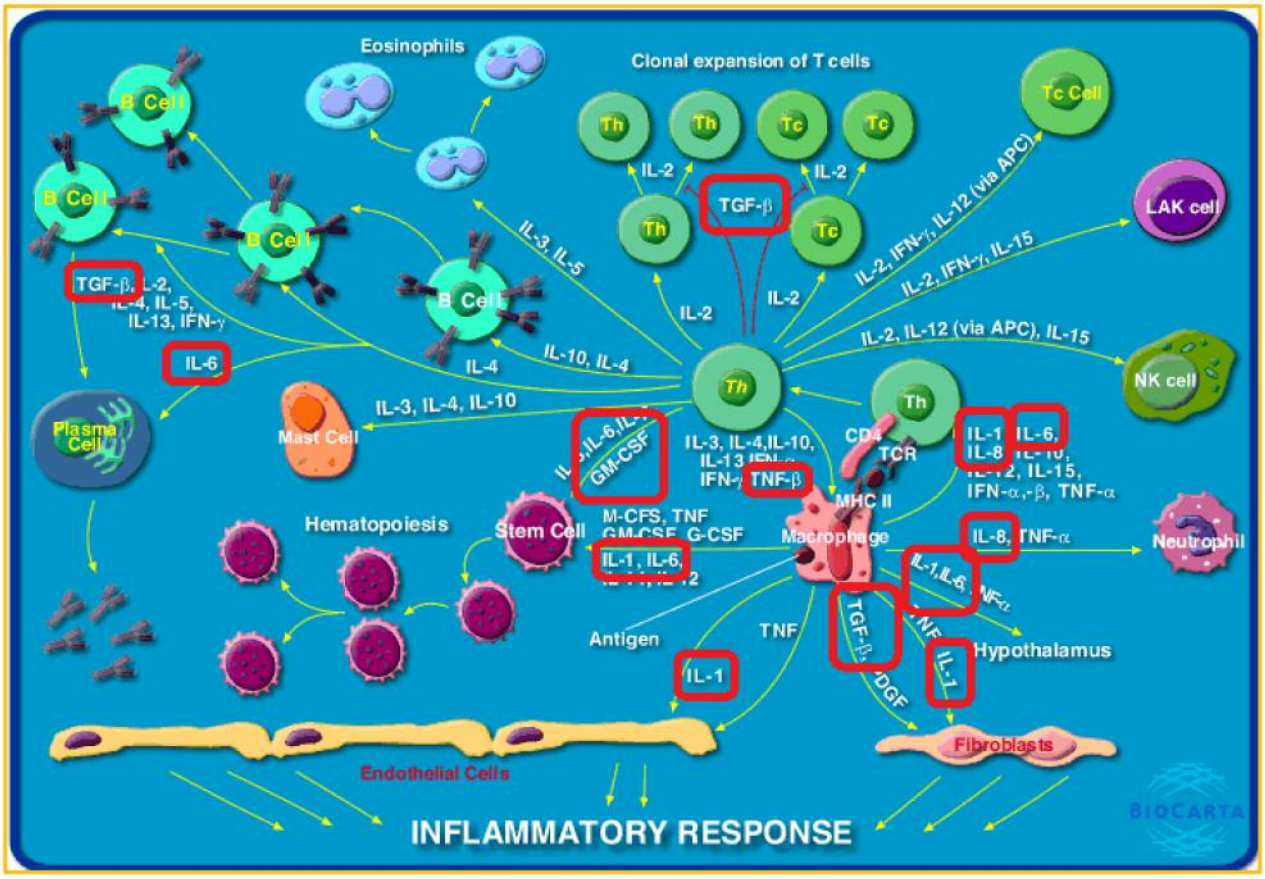

Figure 4. *Differential whole transcriptome assay highlighting genes in MDA-MB-231 cells up-regulated by tumor necrosis factor-α (TNFα; 40 ng/ml) and down-regulated by apigenin (40 μM). Official gene symbols were uploaded from the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 where annotation files representing pathways show significant changes to the Biocarta Inflammatory response pathway. Th: T-Helper cells; LAK: lymphokine-activated killer cell; Tc: T-cytotoxic cells; IL: interleukin; TGF: transforming growth factor; IFN: interferon; PDGF: platelet-derived growth factor; APC: antigen-presenting cell; NK: natural killer.*

chemokines and the influence of apigenin, whole transcriptomic microarray analysis was conducted using the Affymetrix gene atlas system. The differences between TNFα and the untreated controls are shown by volcano plot (Figure 2) and listed in Table I. The data confirm a TNFα-evoked a rise in *IL1A, IKBKE, IL6, CCL2* and *CSF2*, as previously reported (22); in addition 75 genes were up-regulated and 10 down-regulated. Using GeneOntology, Panther Classification Systems, Annotation dataset, Panther Go-Slim biological process, the results show cytokine-mediated signaling to be the primary processes affected by the presence of TNFα, with a false-discovery rate value of less than 0.05 (Table II) and the top two events affected by TNFα being T-cell extravasation (leukocyte migration: T-cell migration, lymphocyte migration), and vascularization (regulation of angiogenesis, and vascular development) (Table II).

While the differences between the effects of TNFα and TNFα combined with apigenin were vast, many effects were under the control of apigenin alone. The interfering variables were eliminated, and analysis was performed for transcriptional changes due to TNFα alone, and which were directly influenced by the presence of apigenin (Table III). The results corroborate our previous work in showing IKBKE to be a central controlling transcription factor as demonstrated in a Kegg overlay map (Figure 3) and Biocarta inflammatory scheme (Figure 4). Given that altered expression of *IL1A* was a primary outcome of TNFα treatment, we then evaluated whether IL1α alone was able to elicit the same effect in up-regulating release of CCL2. The data confirm this to be the case (Figure 5A); both effects were reduced by apigenin with correlating changes for the *IKBKE* transcript (Figure 5B). These findings show that TNFα can up-regulate *IL1A*, which is likely to perpetuate a

Table III. *Differential whole transcriptome changes: Genes up-regulated by 40 ng/ml tumor necrosis factor-α (TNFα) and which were down-regulated by 40 μM apigenin in MDA-MB-231 cells. The data are fold change (FC) in expression. *See Table I for full gene names.*

Gene*	TNFα		TNFα+apigenin		Effect of apigenin reduction in expression
	FC	*p*-Value	FC	*p*-Value	
IL1A	21.57	<0.001	−15.16	<0.001	70%
CXCL8	20.61	<0.001	−2.22	<0.001	11%
CSF2	6.04	<0.001	−2.36	0.007	39%
CTSS	5.97	0.001	−3.96	<0.001	66%
ADAMTS9	5.69	<0.001	−2.11	<0.001	37%
C3	4.92	<0.001	−4.5	<0.001	91%
IKBKE	4.55	<0.001	−4.92	<0.001	108%
LAMC2	4.48	<0.001	−3.2	<0.001	71%
TLR2	3.73	<0.001	−2.93	<0.001	79%
GPRC5B	3.66	0.001	−3.44	0.001	94%
CNTNAP1	3.6	0.002	−2.45	<0.001	68%
CLDN1	3.6	<0.001	−2.53	<0.001	70%
IL6	3.25	0.020	−2.85	0.043	88%
SERP2; SERPB10	3.01	<0.001	−3.51	<0.001	117%
NFATC2	3.01	0.033	−4.02	0.009	134%
CXCL10	2.94	0.007	−2.45	0.010	83%
CXCL11	2.93	0.001	−3.46	<0.001	118%
IRAK3	2.73	0.017	−2.53	0.005	93%
NR3C2	2.68	0.033	−3.68	0.010	137%
IL32	2.63	0.002	−2.34	0.002	89%
SERPINE1	2.61	<0.001	−2.02	0.002	77%
IL24	2.49	0.003	−4.84	<0.001	194%
DRAM1	2.44	0.032	−2.19	0.035	90%
TNFSF15	2.41	0.003	−2.49	0.006	103%
SLIT2	2.39	0.004	−3	0.002	126%
AFAP1-AS1	2.34	0.035	−2.33	0.030	100%
TMEM132A	2.33	0.003	−2.1	<0.001	90%
TMEM171	2.32	0.001	−2.04	0.004	88%
STAP2	2.22	0.001	−2.22	<0.001	100%
CCL2	2.19	0.002	−2.12	0.003	97%
RHBDF2	2.19	0.003	−2.07	<0.001	95%
MLKL	2.16	0.001	−2.3	0.001	106%
KDR	2.14	0.032	−3.09	0.003	144%
BMPER	2.14	0.008	−3.02	0.001	141%
KLHL36	2.02	0.048	−2.66	0.005	132%

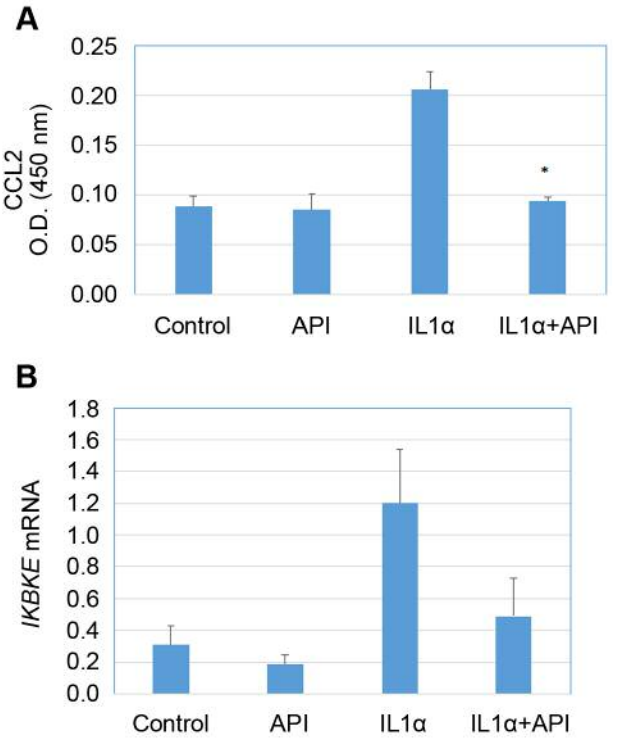

Figure 5. *A: Enzyme-linked immunosorbent assays were used to determine the effect of apigenin (API) on interleukin 1-alpha (IL1α)-induced and tumor necrosis factor alpha (TNFα) changes in C-C motif chemokine ligand 2 (CCL2) release by MDA-MB-231 cells. The data represent CCL2 concentration (pg/ml) and are presented as the mean±S.E.M. (n=3). B: Polymerase chain reaction analysis of inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKBKE) transcription in MDA-MB-231 cells with/without IL1α/API. The data represent normalized expression and are expressed as the mean±S.E.M. (n=3.) The significance of differences between the control and treated groups was determined by one-way ANOVA using the Tukey post-hoc test. *Significant differences between (IL1α vs. IL1α + API) groups at p<0.05.*

sustained release of CCL2, both of which can be down-regulated by apigenin.

Discussion

The data in this study show that *IL1A* was the transcript most highly up-regulated by TNFα (by 21.57-fold, $p<0.001$), which was suppressed by apigenin (by 15.16-fold, $p<0.001$) in MDA-MB-231 cells. TNFα along with IL1α, and corresponding cognate receptors are ubiquitous in the tumor milieu of highly invasive carcinomas (25) and correspond to larger tumor size, lymph node metastasis and overall poor prognosis (26, 27) and overexpression of certain chemokines (*e.g.* CCL2, IL8 and IL6). All of these cytokines were suppressed by apigenin in the presence of TNFα as demonstrated in this study. The capacity of apigenin to attenuate all of these pro-oncogenic processes simultaneously would, in theory, hamper infiltration of CCR2/CCR5 receptor-bearing LSPs or myeloid cells into the tumor milieu (28), which on their own amplify expression of tumor-promoting cytokines, growth/motility factor, and tumor evasion tactics (29-35).

While the data in this work show that apigenin can reduce a diverse range of TNFα-induced, tumor-promoting transcripts, often associated with tumor invasion, there was a near complete reduction of complement component C3, the

relevance of which needs further investigation. C3 is involved in the innate immune response, but high concentrations are found in the serum of patients with breast and lung cancer also considered a plausible diagnostic biomarker (36-39). The C3 convertase complex and initial cleavage of the C3 molecule activates the complement system, where downstream products (C3a and C3b) are deposited around solid tumors concomitant to overexpression of surface-expressed CD proteins that protect cells from complement-dependent cytotoxicity. Overexpressed C3s correspond to immune evasion (40) loss of dendritic cell-mediated cross-presentation (41) and antibody-dependent cell-mediated cytotoxicity (42). In contrast, chemotherapy drugs that reactivate the complement system-evoked cell death (43-45) and antibody-dependent cell-mediated cytotoxicity (46) or directly target C3 are considered a means of reactivating tumor immunosurveillance (47). There is a need to further investigate the role of apigenin in this process, as reducing C3 confers a protective effect against carcinogenesis.

In addition to *C3, CCL2, IL8, IL6* and *IL1A*, the data in this study support the notion that apigenin can reduce expression of numerous other oncogenes which drive metastatic tumor growth such as cathepsin S (CS) and laminin subunit gamma 2 (*LAMC2*) (48-52). These findings, as a whole, add to the existing body of literature demonstrating diverse anticancer effects for apigenin (27) reported to reduce tumor growth (53), migration, invasion, metastasis, and concomitant signaling targets [*e.g.* extracellular signal-regulated kinase, c-Jun *N*-terminal kinase activator protein 1, nuclear factor kappa-light-chain-enhancer of activated B cells (54, 55), mitogen-activated protein kinase, PI3K/protein kinase B (56), glycogen synthase kinase-3 beta (57), Janus kinases/signal transducer and activator of transcription proteins and WNT/beta-catenin pathways (58)]. Moreover, apigenin can also master expression of anticancer microRNAS such as *miR-520b* and *miR-101* (59) while sensitizing to the effects of diverse chemotherapy drugs (60) and overcoming resistance (58).

In summary, in this study we present a global view of the anticancer/anti-inflammatory properties of apigenin specific to the influence of TNFα in a TNBC cell model (Public Data set Gene Expression Omnibus file https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120550).

Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

Authors' Contributions

DB conducted the primary research, including all bench work studies pertaining to apigenin and TNFα in addition to article preparation. EM carried out the transcriptomic microarray study, including all quality control steps in addition to article preparation. KS provided oversight management including consultation, research direction in addition to the preparation of the article. All listed Authors declare consent to the article's publication.

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