Pro-adhesive phenotype of normal endothelial cells responding to metastatic breast cancer cell conditioned medium is linked to NFκB-mediated transcriptomic regulation

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Abstract. Tumor microenvironment is an important promoter of tumorigenesis in all forms of breast cancer and has been associated with the risk of metastasis in the different breast cancer subtypes including the more frequent luminal subtypes that encompass 60% of cancer patients. Adhesive properties of endothelial cells (ECs) are strikingly affected during cancer cell dissemination and are related to functional changes of adhesion receptors. The contribution of tumor secreted factors to tumor-EC adhesion represents a therapeutic opportunity for breast cancer metastasis. Conditioned medium (CM) of tumor cells can be used as a model to study the role of the secreted molecules to the tumor microenvironment. We explored transcriptomic changes associated to a pro-adhesive phenotype in primary human umbilical vein endothelial cells (HUVECs) treated with CM of the breast cancer cell line ZR75.30 or with TNF for 3 h. Selected genes were used to validate the microarray through RT-qPCR. The bioinformatic analysis identified NFκB as the main regulator of the pro-adhesive phenotype and this was confirmed by pharmacological inhibition of NFκB pathway with BAY 11-7085. The changes induced by ZR75.30-CM mimic those promoted by TNF and display changes in the expression of genes related to inflammatory response, wound healing, extracellular matrix, cytokines, metabolism and cell communication. Despite the abundance of G-CSF, IL-8, IL-6 and VEGF in the ZR75.30-CM and the confirmed activation of STAT3 and VEGFR2 pathways, our results suggest dominance of NFκB as a central controller of the transcriptomic response of ECs to breast cancer cells leading to expression of cell adhesion receptors.

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Introduction

Breast cancer is one of the most frequent causes of death among female population worldwide (1). Metastasis is responsible for >90% of breast cancer mortalities but it is one of the least understood stages of tumor development. Primary luminal A and B subtypes of breast cancer represent 60% of the tumors (2). From the two luminal subtypes, luminal B is the most aggressive tumor (2,3). Following initial clinical response, 40-50% of these patients present recurrence with metastases (4).

The crucial role of the tumor microenvironment in cancer development and metastasis has recently been highlighted (5). Virchow provided the first evidence of the interaction between normal tissue and tumor formation, postulating that cancer originates at sites of chronic inflammation (6,7).

The tumor microenvironment and chronic inflammation share several soluble molecules, such as cytokines, growth factors and metalloproteases, as well as a variety of distinct cell types, including endothelial cells (ECs) (8). Recruitment of ECs by tumors is essential in metastasis during tumor vascularization and because they regulate the intra- and extravasation of tumor cells (9). For a circulating tumor cell to exit the circulatory system (extravasation), it must first bind to a blood vessel wall by one of two mechanisms of arrest: physical occlusion or cell adhesion. The relative prevalence of these mechanisms depends on the biology of the tumor and the diameter of the local post-capillary venule (10). The extravasation can vary depending on the cancer cell type and the extravasation site

or target organ, suggesting that it is determined not only by the metastatic potential of the tumor cell, but also by the endothelial response to the unique local endothelial microenvironment (11). In this process soluble factors derived from both cell types serve in the communication between tumor and ECs.

During metastasis, ECs function not simply as static structural cells of perfusing vessels but also as active stromal regulatory cells with privileged access to the tumors (12). Inflammatory cytokines, including TNF, IL-1, IL-6 and chemokines as IL-8, may also promote adhesion and extravasation by increasing vascular permeability and promoting the survival of tumor cells in the blood circulation. Adherent tumor cells are dependent on chemokine and cytokine gradients to direct their migration through EC monolayers. Several inflammatory cytokines can act at a distance promoting a pro-adhesive phenotype characterized by an increase of adhesion molecules on the apical surface of ECs in target organs. Interestingly, a variety of cytokines can be found in the circulation of cancer patients, and the expression of chemokines and their receptors correlate with the aggressiveness of the tumor (13). The migratory arrest of cancer cells depends on the quality and quantity of adhesion molecules expressed on ECs, as well as the adhesion molecule repertoire on the cancer cells (9). In fact, in many cancer types, cell adhesion molecules (CAMs) are frequently associated with metastatic progression and adhesion to EC walls in distant organ sites (14).

Changes in the gene expression of tumor-associated endothelial cells (TAECs) have been postulated to affect cancer cell fate (15,16). Analyzing the contribution of tumor secreted factors to endothelial-recruitment *in vivo* has proven to be difficult and conditioned medium (CM) secreted by different tumor cell lines have been used as model systems, promoting angiogenesis and a pro-adhesive phenotype in human umbilical vein endothelial cells (HUVECs) as well as TNF (17-19). Luminal A and B forms of breast cancer are the most common presentations of the disease and despite the effective treatment; the recurrence in the B subtype is associated with metastasis in most of the patients. Therefore we analyzed the endothelial transcriptome in response to CM from the human luminal B metastatic breast cancer cell line ZR75.30. Bioinformatic analysis implicated NFκB as a key molecular regulator of the vascular pro-adhesive phenotype activated by CM dominating over other cytokine, chemokine and growth factor signaling pathways.

Materials and methods

Cell culture. The breast cancer cell lines MCF-7 (luminal A), ZR75.30 (luminal B) and the monocyte cell line U937 were obtained from ATCC (Manassas, VA, USA) and maintained in RPMI-1640 medium supplemented with 10% FBS (both from Gibco-BRL, Grand Island, NY, USA), penicillin 10,000 U/ml, streptomycin 10 mg/ml and amphotericin B 25 μg/ml (PAA Laboratories GmbH/GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) in a humidified atmosphere of 5% CO_2 at 37˚C.

HUVEC primary culture. HUVECs were isolated and cultured as previously reported (20) by mixing cells from at least three human umbilical cords. The resulting cell cultures were maintained in M199 medium (Gibco-BRL) supplemented with 10% FBS, 2 mM L-glutamine (Gibco-BRL), 20 μg/ml endothelial mitogen (Biomedical Technologies, Inc., Stoughton, MA, USA), 5 U/ml heparin (Laboratorios PISA S.A. de C.V), penicillin 10,000 U/ml, streptomycin 10 mg/ml and amphotericin B 25 μg/ml, under a humidified atmosphere of 5% CO_2 at 37˚C. All HUVEC cultures used for the experiments were at the third passage. The local Ethics and Research Committees of the Hospital General Dr Manuel Gea González, Ministry of Health (Mexico) approved this protocol (11-62-2014), and all participants signed an informed written consent form.

Conditioned medium. CM was isolated as previously described (17). Briefly, breast cancer cell lines were cultured in 100-mm plates until they reached 80% confluence. The cell layer was first washed 10 times with 10 ml of PBS/RPMI-1640 (1:1 v/v) without phenol red (Laboratorios Microlab S.A. de C.V., D.F. Mexico, Mexico) to remove serum components. Then, cells were maintained in 8 ml of serum-free RPMI without phenol red, after 48 h the culture medium was collected and lyophilized. The resulting powder was dissolved in water (1/10 of the original volume) and dialyzed using a PM-3 Ultrafiltration Membrane (EMD Millipore, Billerica, MA, USA). The solution was filtered through a 0.22-μm Millex-GS syringe filter unit, and a protease inhibitor cocktail was added (cOmplete™ Protease Inhibitor Cocktail; Roche Applied Science, Indianapolis, IN, USA). The protein concentration was determined using the Bradford reagent assay (Bio-Rad, Hercules, CA, USA). The resulting concentrated preparation was maintained at 4˚C until further use.

Bio-Plex assay. CM (50 μl), was analyzed with the Bio-Plex suspension array system (Bio-Rad) against 26 proteins, following the manufacturer's instructions.

Sample treatment. For adhesion assay, microarrays and western blots, confluent HUVECs were treated with 9 μg/ml of the indicated CM or with 10 ng/ml of human recombinant TNF (R&D Systems, Inc., Minneapolis, MN, USA) for the indicated time frames depending on the experiment. At the end of the treatments, the corresponding assay was performed as described. Inhibition of IKKs was performed by pre-incubating HUVECs for 1 h with 10 μM BAY 11-7085 (Calbiochem/Merck KGaA, Darmstadt, Germany). After pre-incubation, HUVECs were stimulated with 9 μg/ml of ZR75.30-CM. Electrophoretic mobility shift assay (EMSA) and IκBα western blotting were performed after 20 min, while CAM western blotting and cell adhesion were evaluated after 3 h, as described. For time course assay, HUVECs were starved 4 h previous to stimulation with 9 μg/ml of ZR75.30-CM, and cell lysates were used for western blot analysis.

Adhesion assay. This assay was performed as previously described (17), adherent cells were visualized in a TMS-F phase-contrast inverted microscope Nikon Eclipse TS100 (Nikon Instruments, Inc., Melville, NY, USA) and counted in a β counter (1600TR liquid scintillation analyzer; Canberra-Packard, Meriden, CT, USA).

RNA isolation and microarrays. TRIzol reagent (Invitrogen Corp., Camarillo, CA, USA) was used to obtain total RNA

Table I. Primer sequences employed for qPCR.

Human gene	Forward primer 5'→3'	Reverse primer 5'→3'	Accession no.
ACTIN	TCCCTGGAGAAGAGCTACGA	AGCACTGTGTTGGCGTACAG	NM_001101
ICAM-1	GACCAGAGGTTGAACCCCAC	GCGCCGGAAAGCTGTAGAT	NM_000201
SELE	CCGAGCGAGGCTACATGAAT	GCATCGCATCTCACAGCTTC	NM_000450
VCAM-1	TGTTTGCAGCTTCTCAAGCTTTTA	GTCACCTTCCCATTCAGTGGA	NM_001078
NFKBIA	CTCCGAGACTTTCGAGGAAATAC	GCCATTGTAGTTGGTAGCCTTCA	NM_020529
CCL20	TGCTGTACCAAGAGTTTGCTC	CGCACACAGACAACTTTTTCTTT	NM_004591
TNFAIP2	GGCCAATGTGAGGGAGTTGAT	CCCGCTTTATCTGTGAGCCC	NM_006291
TRAF1	TCCTGTGGAAGATCACCAATGT	GCAGGCACAACTTGTAGCC	NM_005658
CXCL2	TGCCAGTGCTTGCAGAC	TCTTAACCATGGGCGATGC	NM_002089
PPP1R3C	GGTGGCACAGATAGTGATACCT	ACCATCATTGTTGTCCCAAAAGA	NM_005398
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG	NM_000600
MAP3K8	CTCCCCAAAATGGACGTTACC	GGATTTCCACATCAGATGGCTTA	NM_005204
CDKN1B	TAATTGGGGCTCCGGCTAACT	TGCAGGTCGCTTCCTTATTCC	NM_004064
NFKB2	GGGCCGAAAGACCTATCCC	CAGCTCCGAGCATTGCTTG	NM_002502
TGFB3	GGAAAACACCGAGTCGGAATAC	GCGGAAAACCTTGGAGGTAAT	NM_003239
SORBS1	CACAATCGAGAACAGCAAAAACG	ACCCGCCTACTGTCATCCTTT	NM_001034954

from three independent biological replicates of confluent HUVECs (60-mm plates) treated as indicated. The preparation of cRNA hybridization to Human Gene 1.0 ST and data analysis were performed according to Affymetrix™ recommendations. Differentially expressed genes were determined using the Partek® Genomics Suite software (Partek, Inc., St. Louis, MO, USA) with a $p<0.05$ and a differential fold change of 1.5 on either positive or negative directions. Gene Ontology (GO) classification was performed through the use of National Cancer Institute-Database for Annotation, Visualization and Integrated Discovery (NCI-DAVID) (http://david.abcc.ncifcrf.gov) (21) and the Search Tool for the Retrieval of Interacting Genes (STRING) software was used to build the functional gene association networks (string-db.org) (22). Enriched canonical pathways within the networks of differentially expressed genes were carried out using the Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems; www.ingenuity.com) and Protein ANalysis THrough Evolutionary Relationships (PANTHER) software (www.pantherdb.org) (23).

RT-qPCR. The generation of cDNA was performed using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 2 μg of total RNA as a template. PCR reactions were performed with Maxima SYBR-Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Inc.) on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a conventional amplification protocol. The housekeeping gene *ACTIN* was used as an internal control. Primer sequences used for gene expression analysis are shown in Table I. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (24).

Western blotting. Confluent cultures of HUVECs treated as indicated were lysed using RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA pH 8.0, 1 mM PMSF, 1X complete protease inhibitors cocktail, 2 mM Na_3VO_4, 10 mM Na_2MoO_4 and 5 mM NaF) and protein concentration was determined by Bio-Rad DC protein assay (Bio-Rad). Total cellular proteins (10-40 μg) were separated via SDS-PAGE, transferred onto PVDF (EMD Millipore), membranes blocked for 1 h at room temperature in TBS-Tween 0.1%, with 5% non-fat milk and probed overnight at 4˚C with specific antibodies against anti-VCAM-1, ICAM-1, E-selectin, IκBα, STAT3 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), phospho-STAT3 (Y705), phospho-VEGFR2 (Y1175), and VEGFR2 (all from Cell Signaling Technology, Inc., Danvers, MA, USA) and β-actin (Sigma-Aldrich, St. Louis, MO, USA). The membranes were incubated in the presence of HRP-conjugated secondary antibodies for 1 h at room temperature (Sigma-Aldrich), and the signals were detected using enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific, Inc.). The membranes were stripped as reported by Yeung and Stanley (25) and re-blotted. Optical densitometric scanning was performed using NIH ImageJ software.

Electrophoretic mobility shift assay. Nuclear protein extracts and cytoplasmic fractions from HUVECs and NFκB translocation to the nucleus were determined as previously described (26). Briefly, 10 μg of the nuclear protein extracts were incubated with γ-^{32}P-ATP-labeled oligonucleotide containing the consensus NFκB site (5'-AGTTGAGGGGA CTTTCCCAGGC-3') (Santa Cruz Biotechnology, Inc.) in the presence of 100-fold excess of unlabeled specific probe as specific competitor. Samples were fractionated on a 5% non-denaturing polyacrylamide gel in 1X Tris-borate-EDTA buffer and DNA-protein complexes visualized on a Storm PhosphorImager (Molecular Dynamics, San Francisco, CA, USA). For supershift analysis, 1 μg of anti-p65 antibody (Santa Cruz Biotechnology, Inc.) was incubated with the nuclear extract for 30 min at room temperature prior to adding the reaction mixtures.

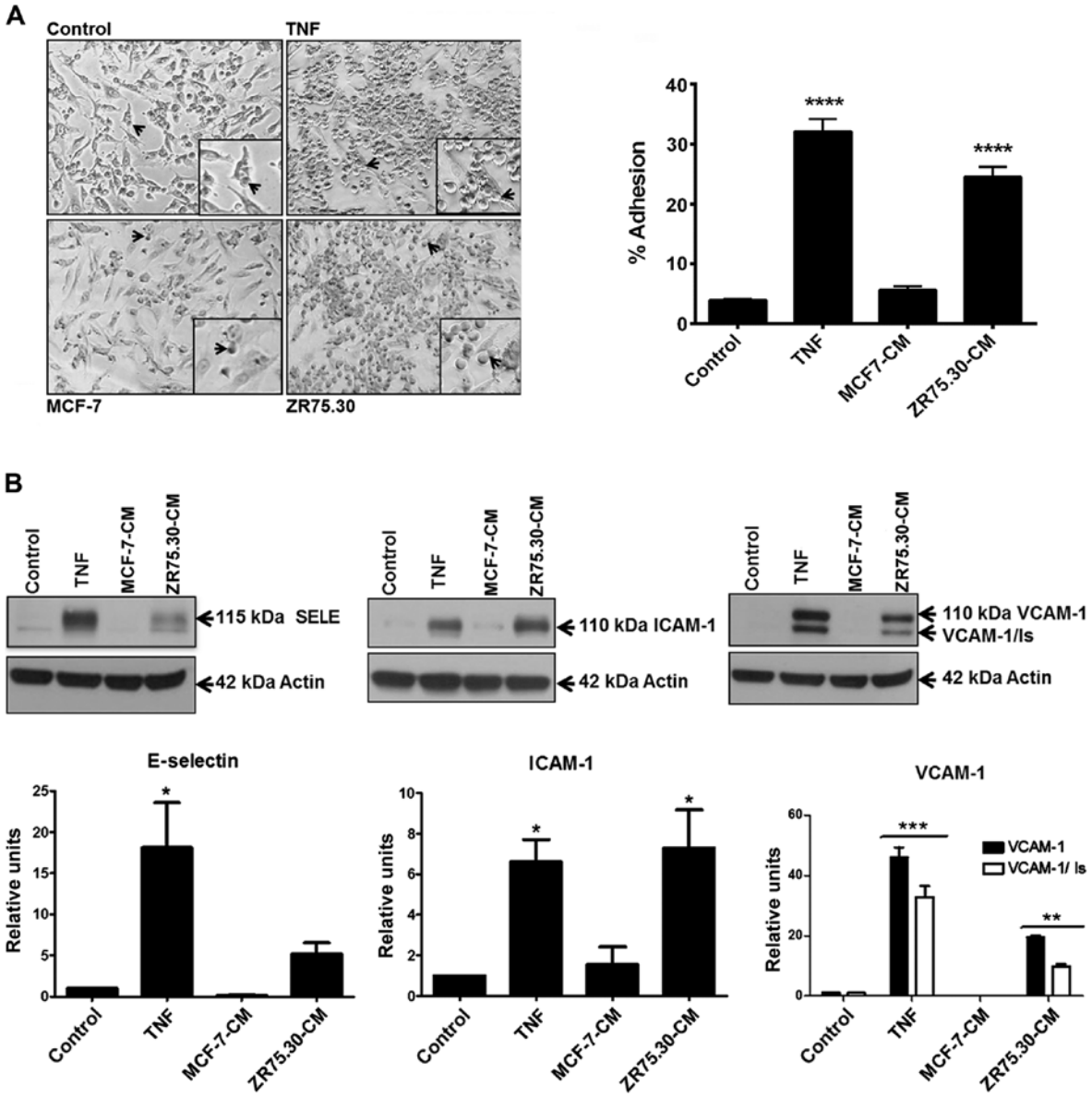

Figure 1. ZR75.30-CM promotes a pro-adhesive phenotype and expression of CAMs in HUVECs. (A) Micrographs of the adhesion assay. Control represents basal adhesion of U937 cells to confluent, untreated HUVECs. HUVECs were pre-treated for 3 h with TNF (10 ng/ml), ZR75.30-CM (9 μg/ml) or MCF-7-CM (9 μg/ml) prior to the addition of U937 cells to the HUVEC monolayer. The black arrows indicate U937 cells adhered to HUVECs. The micrographs were taken with x200 magnification. The percentage of U937 cells adhered to HUVECs was obtained as previously described (14). Data are presented as the means ± SE of the percentage of the total adherent cells in at least three independent experiments. ****p<0.0001. (B) A representative western blotting for ICAM-1, E-selectin and VCAM-1 adhesion molecules employing total HUVEC extracts from cells treated for 3 h with MCF-7-CM (9 μg/ml), ZR75.30-CM (9 μg/ml) or TNF (10 ng/ml). Untreated HUVEC extracts were employed as controls. Actin was used as a loading control. Histograms represent the means (ratio ICAM-1/actin, E-selectin/actin or VCAM-1/actin) ± SE of three independent experiments and are expressed as relative units. *p<0.05, **p<0.01, ***p<0.001. CM, conditioned medium; CAMs, cell adhesion molecules; HUVECs, human umbilical vein endothelial cells.

Statistical analysis. The results are expressed as the means ± SE, and statistical analyses were performed with GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). Multiple comparisons were analyzed using one-way ANOVA with Dunnett's post-hoc test and two-way ANOVA with Bonferroni correction. Significance was set at p<0.05.

Results

Differentially expressed genes associated with the pro-adhesive endothelial phenotype induced by ZR75.30-CM. Previous study by our group (18) showed that CM secreted from the breast cancer ZR75.30 cells promotes a pro-adhesive endothelial phenotype. Interestingly, CM secreted from the breast cancer MCF-7 cells did not promote this phenotype nor induced the expression of CAMs: ICAM-1, VCAM-1 and E-selectin, therefore we focus on characterizing the transcriptome of ECs treated with ZR75.30-CM (Fig. 1).

For the transcriptome analysis a new batch of CM was tested for induction of the pro-adhesive phenotype in ECs and for the content of soluble factors using a multiplex assay. We found enrichment in the inflammatory cytokines TNF, IFN-γ and IL-6, the hematopoietic cytokine G-CSF, the chemokine

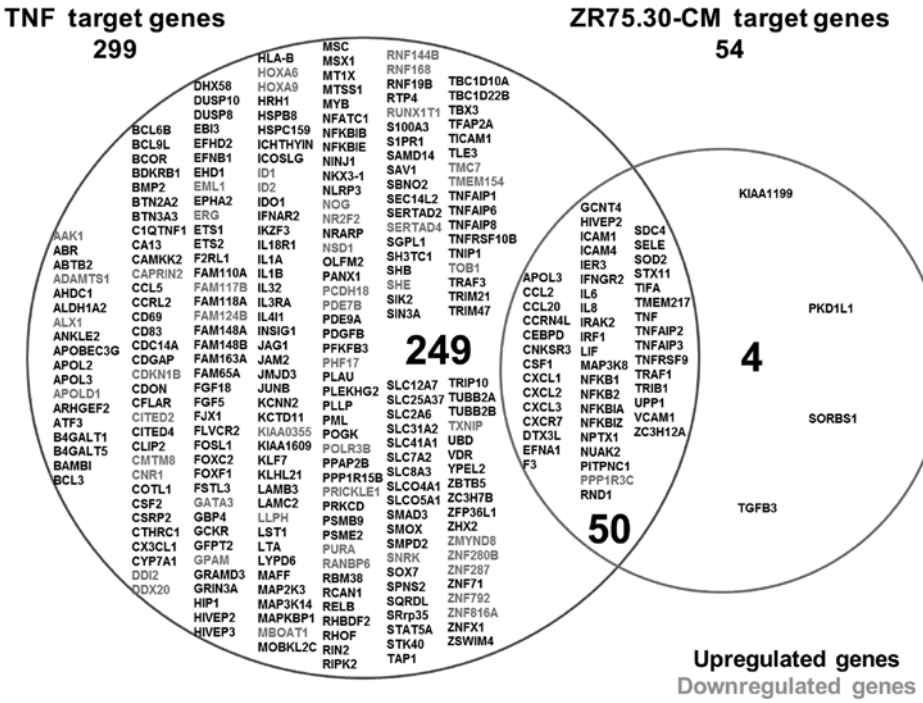

Figure 2. Genes with altered expression in HUVECs treated with ZR75.30-CM or TNF. Exposure to TNF (10 ng/ml) or ZR75.30-CM (9 μg/ml) for 3 h altered the expression of genes in HUVECs. Venn diagram was built with the results of the microarray analysis with Partek Genomics Suite software, used to determine differentially expressed genes using parameters of $p<0.05$ and a differential fold change of 1.5 in either direction. HUVECs, human umbilical vein endothelial cells; CM, conditioned medium.

IL-8, the growth factor VEGF and, to a lesser extent, the anti-inflammatory cytokine IL-1Ra for ZR75.30-CM. MCF-CM contained lower amounts of these factors. The molecules with the highest differences between ZR75.30-CM and MCF-7-CM were VEGF (>39,592 vs. 14,231 pg/ml), G-CSF (>28,728 vs. 9,594 pg/ml), IL-8 (>24,800 vs. 8,429 pg/ml), IL-6 (2,891 vs. 1,081 pg/ml), IFN-γ (2,068 vs. 1,315 pg/ml) and TNF (1,274 vs. 771 pg/ml). For VEGF, G-CSF and IL-8 from ZR75.30-CM, the values were above of detection range. Results from the composition analysis were similar to those previously reported (18). We used TNF as a positive control for the induction of a pro-adhesive endothelial phenotype accompanied by CAM expression. ZR75.30-CM altered the expression of 54 genes in ECs (53 upregulated and 1 downregulated), whereas TNF treatment altered the expression of 299 genes (249 upregulated and 50 downregulated). Of the total number of genes affected, 50 were common to both treatments, 249 were exclusive to the TNF treatment and 4 were exclusive to the ZR75.30-CM treatment (Fig. 2). Among the 10 genes most upregulated by ZR75.30-CM, are genes related to chemotaxis and inflammation (*CCL20*, *CXCL2*, *CXCL1*, *F3*, *IL-6*, *IL-8* and *TNFAIP3*) and cell adhesion (*VCAM-1*, *SELE* and *RND1*). Likewise, the most upregulated genes after TNF treatment correspond to cell adhesion process (*VCAM-1*, *SELE*, *ICAM-4* and *EFNA1*), chemotaxis and inflammation (*CCL20*, *CCL5*, *CXCL2*, *TNFAIP3* and *TNFAIP2*) and IFN-γ (*IRF1*) (Fig. 3A). All genes affected by both treatments showed significantly higher expression levels with TNF compared to those with ZR75.30-CM with the exception of *CXCL2*, for which ZR75.30-CM induced higher expression (Fig. 3A). The genes most repressed by TNF were *PPP1R3C*, *PRICKLE1*, *ALX1*, *RUNX1T1*, *ZNF792*, *ID2*, *MBOAT1*, *ID1*, *RANBP6* and

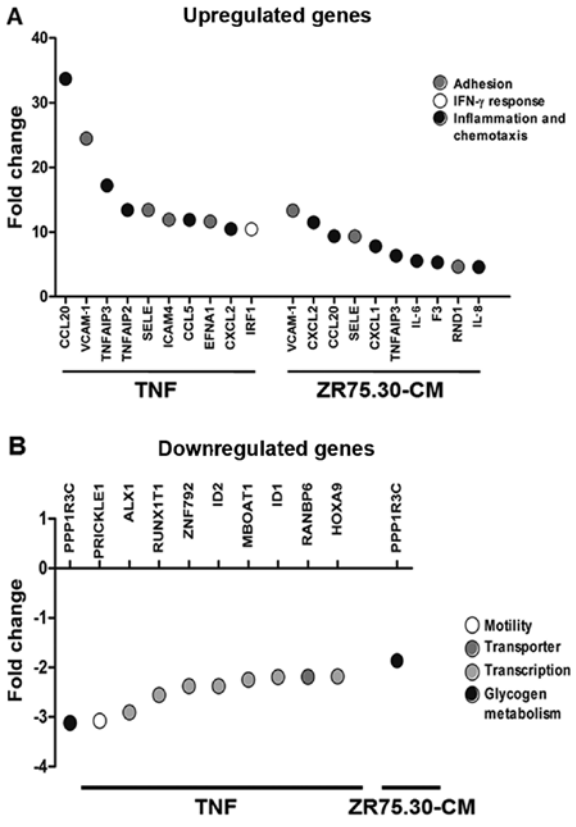

Figure 3. Up- and downregulated genes in HUVECs treated with ZR75.30-CM or TNF. (A) Top 10 genes upregulated in HUVECs treated with TNF or ZR75.30-CM presented from the highest to the lowest fold change. (B) Top 10 genes downregulated in HUVECs stimulated with TNF or ZR75.30-CM. Only one gene was specifically downregulated by ZR75.30-CM. Scale of darkness corresponds to different cellular processes. HUVECs, human umbilical vein endothelial cells; CM, conditioned medium.

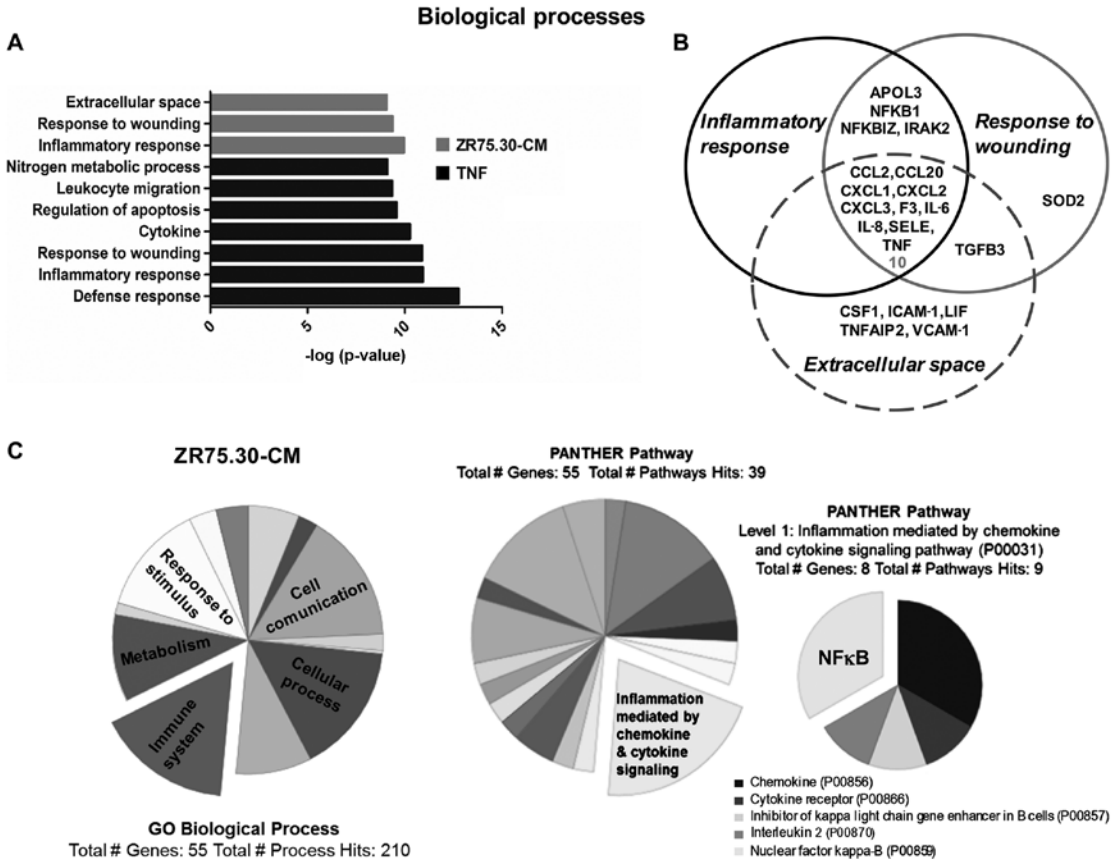

Figure 4. Biological processes and pathways altered in HUVECs treated with ZR75.30-CM. (A) Biological processes according to the NCI-DAVID software ordered by p≤e-10. (B) Venn diagram of genes grouped in ZR75.30-CM biological processes according to NCI-DAVID. (C) ZR75.30-CM PANTHER analysis of biological processes and pathways. The most representative pathway (inflammation mediated by chemokine and cytokine signaling) and its regulators are shown (small pay). HUVECs, human umbilical vein endothelial cells; CM, conditioned medium.

HOXA9. Interestingly, *PPP1R3C* showed the strongest downregulation with TNF and it was the only downregulated gene with ZR75.30-CM (Fig. 3B).

Bioinformatic analysis implicates the NFκB pathway as a central regulator of the gene expression pattern in ECs treated with ZR75.30-CM. The induction of a pro-adhesive phenotype directed the initial approach of this study, however, the bioinformatic analysis of the microarray by NCI-DAVID software associated the expression profile with other cellular processes such as: inflammatory response (p-values of 1.1e-10 for ZR75.30-CM treatment and 1.2e-11 for TNF), response to wounding (p-value of 4.1e-10 for ZR75.30-CM treatment and 1.3e-11 for TNF) and extracellular space (8.5e-10 for ZR75.30-CM) (Fig. 4A). While SELE was present in the three processes identified by NCI-DAVID, *ICAM-1* and *VCAM-1* were present only in the extracellular space process. The genes associated to these processes also shared chemokines, cytokines and components of the NFκB pathway known to regulate and promote a pro-adhesive phenotype (Fig. 4B).

In a similar analysis using PANTHER software the most representative biological process was the immune system (Fig. 4C). Among the main pathways identified by IPA software we found granulocyte adhesion and diapedesis (p-value of 1.72e-12) as part of ZR75.30-CM treatment (Table II). NFκB emerged as the principal regulatory molecule with the highest score (p-value of 8.93e-38) in ZR75.30-CM treatment analyzed

Table II. Top five canonical pathways altered in HUVEC microarrays with each treatment according to IPA analysis.

IPA analysis		
Pathways	P-value	No. of genes
ZR75.30-CM		
Hepatic fibrosis/hepatic stellate cell activation	3.46e-15	12
Atherosclerosis signaling	3.34e-14	11
Granulocyte adhesion and diapedesis	1.72e-12	11
Role of macrophages, fibroblasts and ECs in rheumatoid arthritis	2.01e-12	13
Role of IL-17A in arthritis	2.18e-12	8
TNF		
Role of macrophages, fibroblasts and ECs in rheumatoid arthritis	1.5e-15	29
Hepatic fibrosis	2.03e-12	18
TNFR2 signaling	3.93e-12	10
Role of IL-17A in arthritis	1.49e-11	12
TREM1 signaling	5.92e-11	13

HUVEC, human umbilical vein endothelial cell; IPA, Ingenuity Pathway Analysis; CM, conditioned medium; ECs, endothelial cells.

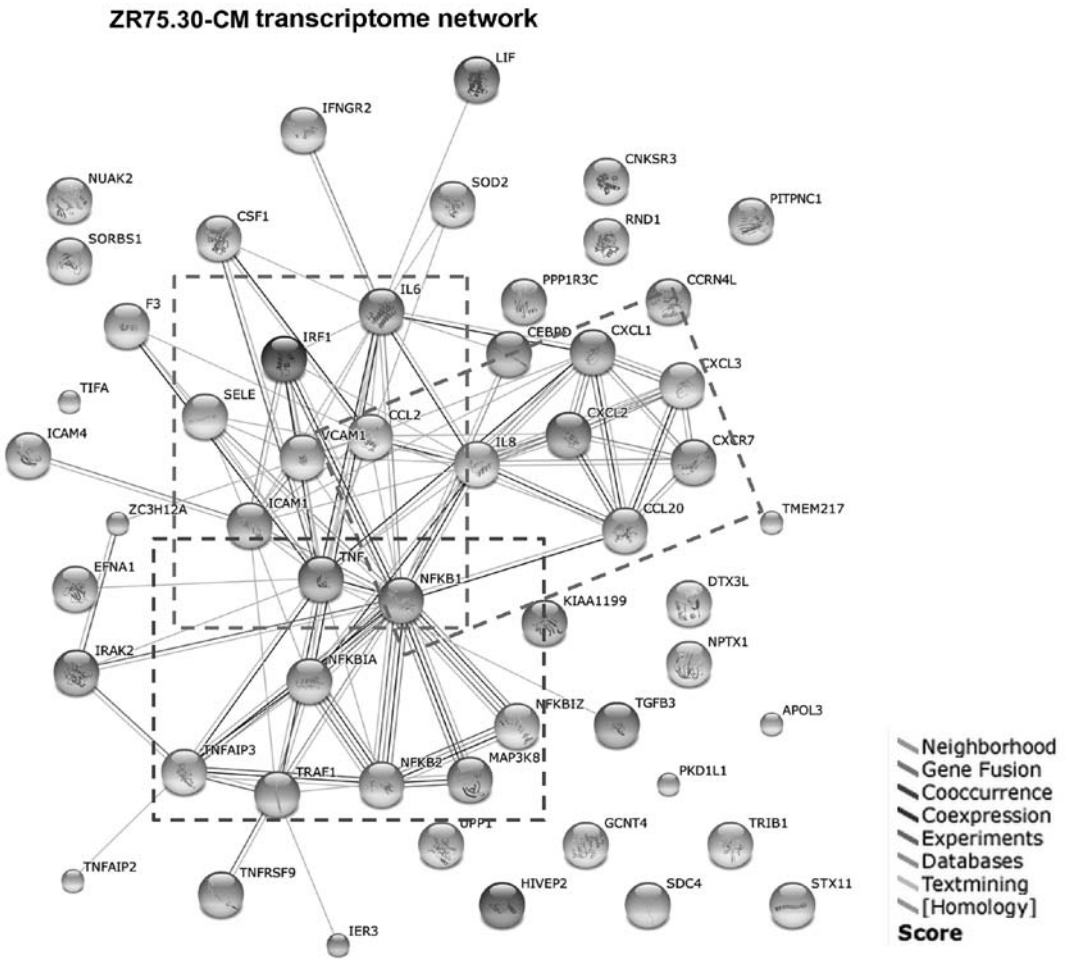

Figure 5. Functional gene association network of ZR75.30-CM transcriptome. The network was built on STRING software with a confidence of $p<0.05$. The complete transcriptome data were used (54 genes). Major interaction clusters of genes are shown in dashed rectangles. Each interaction line represents evidence obtained from different sources (score legends). CM, conditioned medium.

by IPA and PANTHER (Table III and Fig. 4C). Bioinformatic analysis of TNF-treated ECs also showed NFκB as the main regulator and shared some biological processes and pathways with ZR75.30-CM treatment (data not shown). Finally, we generated a functional gene association network with the STRING software. The resulting network (Fig. 5) confirmed a cluster of interactions between *TNF*, *NFKB*, and *CAMs* and revealed a second cluster of interactions among chemokines and cytokines. These two clusters are interconnected through *TNF*, *NFKB*, *IL-6*, *IL-8*, *CCL2* and *CCL20*.

Validation of the transcriptomic response of ECs to ZR75.30 breast cancer CM. To validate the data obtained in the microarray and to compare the expression levels of selected genes between treatments, we used real-time PCR to quantify the mRNA of 15 genes involved in processes like cell adhesion (*VCAM-1*, *SELE*, *ICAM-1*), NFκB pathway (*NFKBIA*, *NFKB2*, *MAP3K8*, *CDKN1B*), chemotaxis and inflammation (*CXCL2*, *CCL20*, *TGFB3*, *IL-6*, *TNFAIP2*, *TRAF1*) and metabolism (*PPP1R3C*, *SORBS1*). Two of these genes were induced exclusively with ZR75.30-CM (*TGFB3* and *SORBS1*), one with TNF (*CDKN1B*) which was repressed. Real-time PCRs validated the overexpression of the genes reported in the microarray. However, downregulation of *PPP1R3C* and *CDKN1B* genes

Table III. Top five molecular regulators altered in HUVEC microarrays with each treatment when analyzed by IPA or PANTHER software.

IPA analysis			PANTHER analysis	
Regulators	P-value	No. of genes	Regulators	No. of genes
ZR75.30-CM				
NFκB complex	8.93e-38	32	NFκB	3
TNF	1.29e-35	40	Chemokines	2
IL-1B	1.14e-34	33	Cytokine receptor	1
TRADD	1.98e-33	15	NFKBIA	1
NFKBIA	1.79e-32	27	IL-2	1
TNF				
TNF	1.11e-61	119	NFκB	7
NFκB complex	2.42e-56	77	Chemokines	4
IL-1B	3.50e-42	74	IL-2	4
LPS	5.49e-41	99	Cytokine receptor	2
CD40LG	1.20e-39	54	NFKBIA	2

HUVEC, human umbilical vein endothelial cell; IPA, Ingenuity Pathway Analysis; CM, conditioned medium.

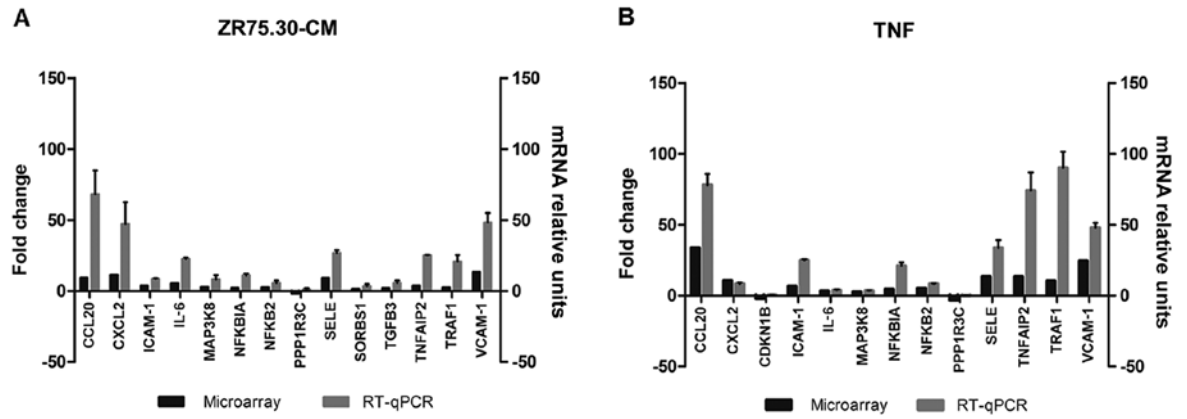

Figure 6. Validation of expression changes from ZR75.30-CM and TNF treatments in HUVECs. Comparison between microarray fold changes (black bars) against corresponding RT-qPCR $2^{-\Delta\Delta Cq}$ values (grey bars). (A) RT-qPCR of 14 altered genes in HUVECs treated for 3 h with ZR75.30-CM (9 μg/ml). (B) RT-qPCR of 13 altered genes in HUVECs treated for 3 h with TNF (10 ng/ml). β-actin was used to normalize the mRNA expression levels. The results were analyzed using the Livak method. Each experiment was performed in triplicate, and the results are expressed as relative units with error bars representing the means ± SE. CM, conditioned medium; HUVECs, human umbilical vein endothelial cells.

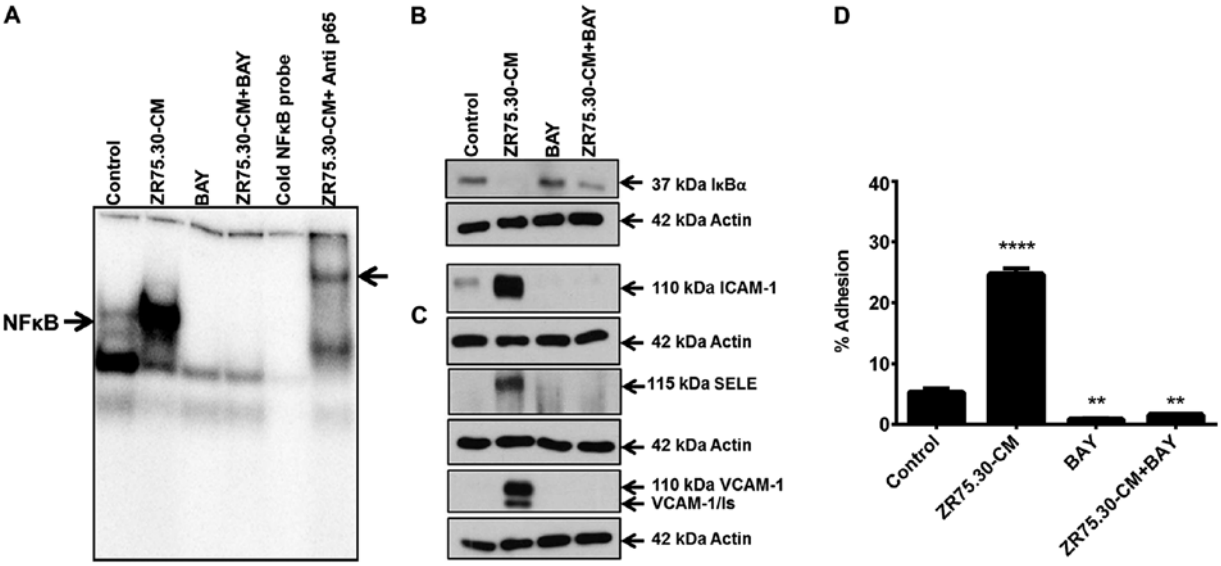

Figure 7. NFκB pathway inhibition prevents the molecular and pro-adhesive phenotype induced by ZR75.30-CM. (A) NFκB analyzed by supershift assay in nuclear extracts from HUVECs 20 min after treatment, using anti-p65 antibody. (B) Western blotting for IκBα in cytoplasmic fractions 20 min after treatment. (C) Western blotting for adhesion molecules in total protein extracts 3 h after treatment. (D) Percentage of U937 cells adhered to HUVECs, as described in Fig. 1. Data are presented as the means ± SE of the percentage of the total adherent cells. $^{**}p<0.01$, $^{****}p<0.0001$. BAY corresponds to HUVECs pre-treated with 10 mM of the IKK inhibitor BAY 11-7085 for 1 h prior to treatment with ZR75.30-CM (9 μg/ml). CM, conditioned medium; HUVECs, human umbilical vein endothelial cells.

observed in the microarray analysis was not replicated in the qPCR assays, although their expression level was lower than control for either TNF or ZR75.30-CM treatment (Fig. 6).

Mechanistic relevance of canonical NFκB pathway on the pro-adhesive endothelial phenotype in response to ZR75.30 breast cancer CM. As NFκB emerged as the principal regulator of gene expression changes after ZR75.30-CM treatment associated to the pro-adhesive phenotype, and to test the relevance of this pathway we used a pharmacological inhibition of IκBα phosphorylation by pre-treating ECs with BAY 11-7085 prior to stimulation with ZR75.30-CM. EMSA revealed a faint signal from a basal NFκB/DNA complex present in control cells. The presence of this complex increased significantly when ECs were treated with ZR75.30-CM (first and second lanes, respectively). The basal complex disappeared when ECs were treated with the inhibitor (third lane) and was barely visible in cells treated with ZR75.30-CM plus inhibitor (fourth lane). Excess unlabeled NFκB probe completely eliminated the signal, indicating the specific detection of this complex (fifth lane) (Fig. 7A). Supershift assays confirmed that ZR75.30-CM triggered canonical NFκB activation, evidenced by markedly enhanced supershifted band in the presence of anti-p65 (sixth lane) (Fig. 7A). Western blotting against IκBα showed that the basal level of expression in control cells disappeared when treated with ZR75.30-CM (first and second lane, respectively). A slight reduction in IκBα expression was observed in the presence of the inhibitor and

CM plus inhibitor (third and fourth lanes) (Fig. 7B). Western blotting revealed that the expression of ICAM-1, VCAM-1 and E-selectin increased with ZR75.30-CM treatment and that the expression was prevented when the cells were treated with the inhibitor (third and fourth lanes) (Fig. 7C). Finally, we confirmed the importance of the NFκB pathway in the adhesion process by showing that the pro-adhesive phenotype induced by ZR75.30-CM was prevented when the cells were treated with the inhibitor (Fig. 7D).

ZR75.30-CM activates early signaling events related to VEGF and STAT3 pathway. Despite the enrichment in VEGF (>39,592 pg/ml), G-CSF (>28,728 pg/ml), IL-8 (>24,800 pg/ml) and IL-6 (2,891 pg/ml) in ZR75.30-CM that could affect endothelial gene expression through STAT3, microarray analysis did not contain classic target genes related to these pathways. We verified that early signaling events of these systems were not impaired by analyzing the state of phosphorylation at residues related to functional activation of VEGFR2 (Y1175) and STAT3 (Y705) (Fig. 8A). In response to ZR75.30-CM VEGFR2 phosphorylation presents a biphasic response between 5-180 min. The signal presented a first peak after 5 min that reached a maximum at 30 min and returned to the value of the first peak after 1 h. STAT3 phosphorylation also presented a biphasic response between 5-180 min with a maximum signal at 30 min that became faint close to 120 min. Taken together these results indicate that early signaling of these two systems was not affected. However, when we analyzed the adhesion of HUVECs in response to recombinant human cytokines IL-6 and VEGF the adhesion was not significant (Fig. 8B).

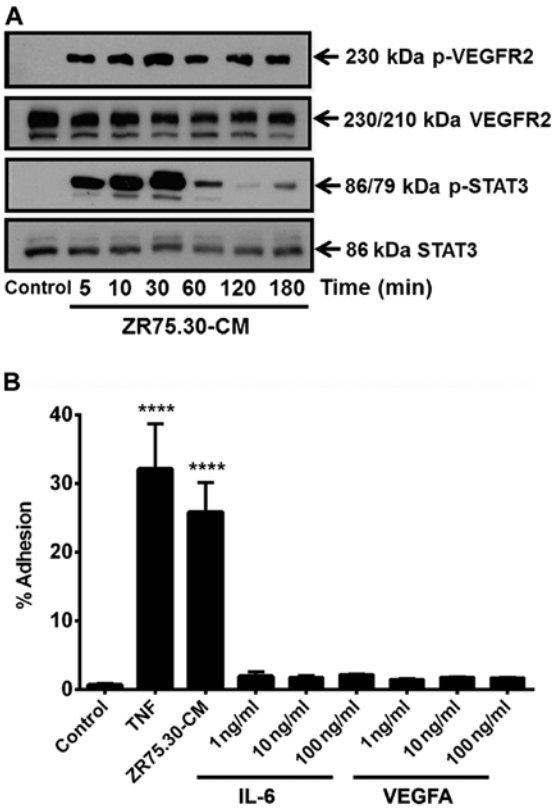

Figure 8. ZR75.30-CM induces VEGFR2 and STAT3 early activation but recombinant IL-6 and VEGF do not promote a pro-adhesive phenotype. (A) Total HUVEC extracts from cells treated with ZR75.30-CM (9 μg/ml) for different time periods (as indicated). VEGFR2 (Tyr-1175) and STAT3 (Tyr-705) phosphorylation was analyzed by western blotting. The membranes were stripped and analyzed with anti-VEGFR2 and anti-STAT3. Untreated HUVEC extracts were employed as controls. (B) HUVECs were pre-treated for 3 h with TNF (10 ng/ml), ZR75.30-CM (9 μg/ml), IL-6 (1, 10 and 100 ng/ml) or VEGFA (1, 10 and 100 ng/ml) prior to the addition of U937 cells to the endothelial monolayer. Percentage of U937 cells adhered to endothelial cells was obtained as in Fig. 1. Data are presented as the means ± SE of the percentage of the total adherent cells. ****p<0.0001. CM, conditioned medium; HUVEC, human umbilical vein endothelial cell.

Discussion

The tumor cell secretome consists of a complex mixture of cytokines and growth factors that contribute to the microenvironment associated with malignancy. These include paracrine and juxtacrine signals that may be involved at virtually any stage of tumorigenesis (27). These tumor soluble factors contribute to recruiting normal stromal cells into actions that favor tumorigenesis, such as normal ECs for the intra- and extravasation processes relevant in metastatic dissemination. Hematogenic dissemination of metastatic cells ends once the tumor cells attach to ECs in the target organs. Following adhesion to the apical membrane of the ECs, successful metastasis requires extravasation followed by metastatic cell proliferation in the stroma. In cell adhesion and extravasation many ligand-receptor interactions contribute to these processes, the endothelial repertoire of CAMs involved includes: selectins, integrins, cadherins, CD44 and members of the superfamily of CAMs (28). How normal cells integrate and priorize the information of a mixture of molecules present in the tumor microenvironment *in vivo* has been difficult to approach. However, treatment of normal or cancer cells with CM has been a useful strategy to perform this kind of studies (29). Considering that pro-inflammatory cytokines can induce a pro-adhesive endothelial phenotype, we postulated that the endothelial transcriptome associated to this phenotype induced by tumor secreted factors from the ZR75.30 breast cancer cell line could be similar to that induced by pro-inflammatory cytokines.

Microarray analysis was applied to further characterize this pro-adhesive endothelial state. Bioinformatic analysis with NCI-DAVID identified overlapping gene ontology profiles associated to inflammatory response, wound healing and extracellular space that was consistent with immune system and cell communication processes identified by PANTHER analysis. NFκB was identified as the principal molecular regulator by both IPA and PANTHER analysis. STRING gene interaction network analysis confirmed NFκB as a central hub related to CAMs and also revealed its connection with NFκB through *IL-6*, *IL-8*, *CCL2* and *CCL20*. Interestingly *CCL20* was the third highest gene expressed in response to ZR75.30-CM and the highest in response to TNF (Fig. 3).

These findings suggest that the EC expression profile in response to CM includes a group of genes associated to a transcriptome induced by inflammatory cytokines and moreover, this response appears to be regulated by NFκB.

TNF is recognized as a classic inducer of a pro-adhesive endothelial phenotype through NFκB activation (30-32); however, IL-6, IL-8, IFN-γ and VEGF can also promote

Table IV. Function of genes validated by RT-qPCR.

Gene	Fold change ZR75.30/TNF	Function	Refs.
CCL20	9.35/33.68	Chemokine involved in homing during metastasis and is a T-cell chemoattractant	(50)
		Actively released by ECs and epithelial cells, its high expression suggests a contribution to tumorigenesis	(51,52)
CXCL2	11.47/10.45	Chemokine related to atherosclerosis, angiogenesis and metastasis	(53)
		Part of a positive feedback loop with NFκB in cancer cells, leading to chemoresistance	(54)
		Its receptor is expressed by ECs, neutrophils, eosinophils and macrophages	
CDKN1B	(-)/-1.94	p27, CDK inhibitor. Binds to cyclin E-CDK2 or cyclin D-CDK4 complexes, controlling the cell cycle progression	(55)
ICAM-1	3.81/6.69	CAM related to inflammation	(47)
		Activates signaling pathways related to motility. Related to invasion and metastasis of BC	(48)
IL-6	5.52/3.46	Pro-inflammatory cytokine associated to growth signals, resistance against apoptosis, vascular inflammatory diseases as atherosclerosis and cancer progression	(33,56)
MAP3K8	2.86/2.79	Serine/threonine kinase activate in cancer	(57)
NFKB2	2.58/5.11	p52 subunit of NFκB complex	(58)
NFKBIA	2.13/4.46	Ubiquitin ligase that inhibits NFκB complex	(58)
PPP1R3C	-1.86/-3.11	Phosphatase involved in glycogen metabolism. Novel tumor suppressor candidate, its repression is associated to promoter methylation in MC	(59)
		In ECs its expression has been associated to angiogenesis	(60)
SELE	9.31/13.39	CAM expressed exclusively by ECs is related to inflammation process; it has been related with metastatic dissemination and angiogenesis	(48,61)
SORBS1	1.52/(-)	Is related to lipid anabolism. Is also associated with FAK and is a substrate of the c-Abl tyrosine kinase	(41)
TGFB3	2.0/(-)	Plays an important role in cellular differentiation and development. In ECs, this isoform participates in homeostasis and maintenance to shear stress	(42)
TNFAIP2	3.76/13.41	TNF-inducible protein 2 recently associated with cancer. Its overexpression is associated to microvessel density, migration and metastasis	(62)
TRAF1	2.46/10.36	Forms a heterodimeric complex required for TNF-mediated activation of MAPK8/JNK and NFκB. Interacts with IAPs to mediating the anti-apoptotic signal from TNF receptors	(63)
		TRAF1 has been associated with rheumatoid arthritis	(64)
VCAM-1	13.31/24.44	CAM related to inflammation, involving in the adhesion and tethering process of leukocytes	(65)
		Expressed by ECs and tumor cells, associating this expression to metastasis promotion of lung and bone targets and activation of PI3K/Akt and NFκB pathway	(49,66)

ECs, endothelial cells; CDK, cyclin-dependent kinase; CAM, cell adhesion molecule; BC, breast cancer; MC, melanoma cancer.

indirect NFκB activation (33-35). Early STAT3 phosphorylation was confirmed in response to ZR75.30-CM indicating intact signaling capacity (Fig. 8A). Similarly, the signaling activity of VEGF present in ZR75.30-CM was confirmed when we followed the phosphorylation state of VEGFR2 (Fig. 8A). However, these cytokines did not induce a significant pro-adhesive response compared to ZR75.30-CM or TNF (Fig. 8B). The NFκB-dependent transcription appears to be dominant over other transcription-initiating pathways after 3 h and shows a partial overlap with VEGF-dependent transcriptome in HUVECs at 0.5-6 h of exposure to VEGF. The genes shared between VEGF and ZR75.30-CM are: *F3*, *SELE*, *CEBPD*, *CXCL2*, *IL-8*, *NFKBIZ*, *CXCL1*, *CXCL3*, *MAP3K8*, *CCL2*, *VCAM-1*, *HIVEP2* and *CNKSR3* (36). In the case of VEGF transcriptome related to endothelial proliferation is probably a later event in time (24 h) and are therefore absent at the time point analyzed (3 h). In contrast, IL-6 and IL-8 pathways appear in our bioinformatic analysis (positions 28 and 45, respectively) with p<e-12. In fact, crosstalk between NFκB/STAT3 and NFκB/VEGF pathways has been reported (35,37) suggesting that a signaling crosstalk converged through NFκB gene expression in our model, perhaps which might priorize gene expression of this master regulator. A similar transcriptional dominance was reported in lymphatic ECs treated with MDA-MB231-CM were, the STAT3 activation prevailed (29).

The transcriptome induced in ECs by ZR75.30-CM shares 93% of its transcripts with TNF. Only four genes (7%) were exclusive to the ZR75.30-CM treatment: *KIAA1199*, *PKD1L1*, *SORBS1* and *TGFB3*. In breast cancer, increase of *KIAA1199* expression correlated with hypomethylation and NFκB binding in the *KIAA1199* regulatory region (38). In contrast, *KIAA1199* repression in colon cancer cells attenuates the Wnt pathway and reduces proliferation (39). Chimeric products

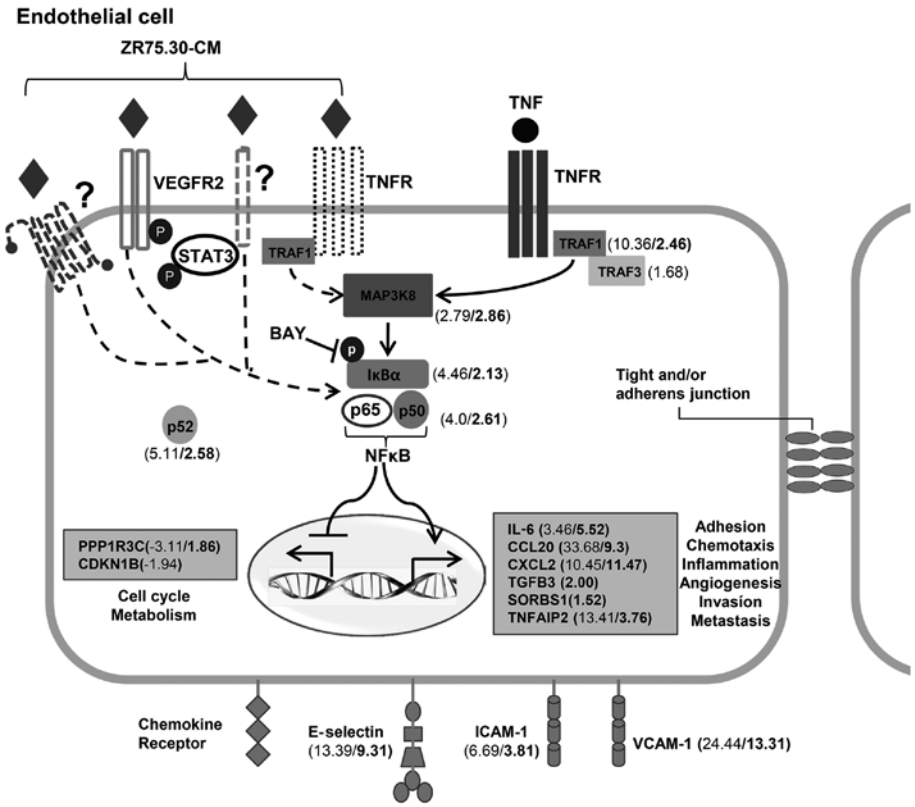

Figure 9. ZR75.30-CM treatment induces an adhesive response linked to canonical NFκB transcriptomic regulation in HUVECs. The scheme represents NFκB signaling mediated by both TNF and ZR75.30-CM. Gray rectangles represent genes with altered expression, the fold change of each molecule appears in parentheses, and the fold changes for ZR75.30-CM are in bold. The receptors for some of the ZR75.30-CM components and their specific activation are unknown (dashed lines). CM, conditioned medium; HUVECs, human umbilical vein endothelial cells.

of *PKD1L1* with *RIF1* have been identified in breast cancer cells (40). The function of *SORBS1* is related to lipid anabolism and this protein is also associated with FAK and is a substrate of the c-Abl tyrosine kinase (41). *TGFB3* is important in cellular differentiation and development, and in ECs, this isoform participates in homeostasis and maintenance when cells are subjected to shear stress (42). Hence, *SORBS1* and *TGFB3* appear to represent specific genes related to changes in cellular behavior that promote intercellular interactions.

Although the bioinformatic analysis indicates TNF/NFκB-activated pathway, only 50 of the 299 genes affected by recombinant TNF responded to ZR75.30-CM treatment (Fig. 3), suggesting that concomitant stimulation with the mixture restricted the expression pattern. The majority of the overlapping genes had the highest fold change with the TNF treatment. Among the genes repressed by TNF, the phosphatase *PPP1R3C* had the highest score, and was the only gene repressed in response to ZR75.30-CM treatment. IPA analysis indicates that the repression of this phosphatase is linked to TNF. Overall, the expression changes induced by ZR75.30-CM have a smaller magnitude than those induced by TNF.

There are several microarrays of ECs treated with different cytokines and growth factors. These treatments include TNF (31,43), IL-1 (44) and VEGF (36,45); however, expression profiles of ECs treated with IL-6, IL-8 and IFN-γ are scarce, and transcriptome data for ECs exposed to components of the tumor microenvironment are even more limited. A recent study in TAECs identified 49 genes to be associated with chronic inflammation diseases and cancer; 6 of them constituted an inflammation-related endothelial-derived gene signature (IREG) (46). Of these 49 genes *TNFAIP3* was the only one shared with ZR75.30-CM treatment. We found the gene variant *IRF1*, which is related to the *IRF7* variant from the IREG. We also identify inflammation-related diseases (Table II) as well as TNF and members of the NFκB family to be central regulators (Table III).

We validated expression changes in 14 of the 54 genes altered by ZR75.30-CM. Several of the validated genes are important endothelial physiological mediators and have also been associated with tumorigenesis and malignancy in a variety of cancer models (Table IV).

Among the validated genes are the CAMs ICAM-1, E-selectin and VCAM-1 whose expression has been used as marker of pro-adhesive endothelial phenotype. Expression of these three cell adhesion molecules has been associated to tumorigenesis and malignancy (47-49).

The bioinformatic analysis of microarrays in the present study indicates that NFκB is a central regulator of the pro-adhesive phenotype of HUVECs induced by breast cancer secreted factors (Fig. 9). To further verify the physiological and molecular relevance of NFκB, we used BAY 11-7085, a specific inhibitor of IKKs, to interfere with this pathway. We had previously shown that interference with NFκB activation in ECs treated with CM from human leukemia prevents NFκB activation and cell adhesion (17). The fact that we obtained

similar results with CM from a different neoplastic such as breast cancer suggests that NFκB-dependent activation may be common to epithelial and hematopoietic neoplastic diseases.

In conclusion, the endothelial transcriptome related to the pro-adhesive phenotype induced by secreted factors from ZR75.30 breast cancer cells reveals inflammatory mediators and NFκB as essential regulators of this phenotype, and pharmacological inhibition of NFκB validated this prediction. Since all these changes occur in primary non-transformed human ECs, and this response is performed by a mixture of cytokines and growth factors like those secreted by tumor cells, interfering with dominant transcription pathways could be an alternative therapeutic strategy to interfere with metastasis.

Acknowledgements

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