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M196R-TNFR2 and membrane TNFα down-regulate soluble TNFα-induced pro-metastatic functions in triple-negative breast cancer cells

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Abstract

Tumor necrosis factor α (TNFα) was connected to disease progression in breast cancer, through the activation of TNFR1 and, to some extent also of TNFR2, in cancer cells and/or in immune cells. We found that in triple-negative breast cancer (TNBC) patients, TNFR2 expression by the cancer cells was significantly connected to improved survival. By setting up an 8-cell system including TNBC cells infected to express combinations of TNFR1 with WT-TNFR2/TNFR2 variants, we determined cell responses to soluble TNFα (sTNFα), presented as recombinant human TNFα. We found that sTNFα-induced activation of TNFR1 gave rise to p65/p38 activation, elevated spontaneous tumor cell migration, greater directed invasion, and increased expression of pro-metastatic molecules (CXCL8, ICAM-1); lower levels of these functions were induced by sTNFα in WT-TNFR2-expressing cells. In TNFR1 knocked-out cells, the above sTNFα-induced functions were reduced by a M196R-SNP-TNFR2 variant (termed SNP-TNFR2). Also, the membrane form of TNFα (mTNFα) gave rise to a pronounced inhibition of sTNFα-induced p65 activation, tumor cell invasion, CXCL8 and ICAM1 expression in cells expressing TNFR1 + TNFR2 (WT-TNFR2/SNP-TNFR2), only TNFR2 (WT-TNFR2/SNP-TNFR2) and only TNFR1. In cells expressing only TNFR1, ERK1/2 activation was negatively correlated with sTNFα-induced p65 activation, and mTNFα expression has given rise to down-regulation of TNF receptor-associated factor 2 (TRAF2) expression alongside down-regulation of p65 activation. These findings provide novel insights into the roles of TNFR2 and mTNFα in cancer, suggesting that their potentially protective roles against tumor progression need be taken into account when TNFα-TNFR-directed therapies are considered in TNBC and in other malignancies as well.

Keywords Breast cancer, Membrane TNFα, M196R-SNP-TNFR2, Soluble TNFα, TNFR1, WT-TNFR2

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Introduction

Chronic presence of the pro-inflammatory cytokine tumor necrosis factor α (TNFα) in tumors was connected to poor prognosis in many malignancies; studies in animal models have demonstrated causative roles for TNFα in increasing cancer aggressiveness, and the cytokine was found to induce many pro-metastatic functions in culture [1–4].

As a major inducer of chronic inflammation at the tumor microenvironment (TME), TNFα is also known as a key pro-metastatic player in breast cancer, including the most aggressive subtype of triple-negative breast cancer (TNBC) [1, 4, 5]. TNBC lacks targetable receptors for biological therapies, and currently the main treatments are chemotherapy and immunotherapy [6, 7]. Hence, there is a crucial need to better understand the involvement of additional key regulators, such as TNFα in TNBC progression.

Published studies have suggested that TNFα acts through its two receptors - TNFR1 and TNFR2 - to elevate the tumor-promoting functions of the tumor cells and immune cells at the TME [2, 4, 8, 9]. TNFR1 is ubiquitously expressed by almost all cell types, leading under specific conditions to cytotoxic effects but in others playing key roles in inducing chronic inflammation *via* activation of the canonical NF-κB (p65) pathway [2, 10]. In contrast, TNFR2 is devoid of apoptotic functions, its activation is mostly connected to cell proliferation and its expression is noted mainly in hematopoietic cells and endothelial cells [2, 10]. The two receptors also differ in their preference towards the soluble form of TNFα (sTNFα) and its membrane form (mTNFα), whose cleavage by TNFα-converting enzyme (TACE/ADAM17) generates sTNFα. Studies in different systems demonstrated that TNFR1 is the prime receptor activated by sTNFα, but it can also be activated by mTNFα. In contrast, TNFR2 responds best to mTNFα, with relatively low response levels to sTNFα [2, 10, 11]. However, these aspects have not been addressed in depth in TNBC.

sTNFα, mTNFα, TNFR1 and TNFR2 are expressed in breast cancer patients of different subtypes [12–21], with TNFR1-mediated functions being strongly associated with TNFα-induced tumor progression in breast cancer, including in TNBC [13, 22–27]. The connection of TNFR2 to malignancy was not investigated as much in breast cancer. Several observations have connected TNFR2 expression in tumor cells of breast cancer patients with poor prognosis, but they have not differentiated between the different subtypes of disease [14, 15]. In contrast, a MMTV-Wnt1 system in mice demonstrated that the lack of one TNFR2 allele has led to increased metastasis [28].

TNFR2 expression is highly connected to a subset of potent immune-suppressive $CD4^+$ $FOXP3^+$ T regulatory cells (Tregs) that contribute to tumor progression, leading to the general dogma that TNFR2 should be considered a preferential target option in cancer therapy [9, 29]. In contrast to these reports, our recent study - which was the first to investigate TNFR2+ tumor-infiltrating lymphocytes (TILs) specifically in TNBC patients - showed that high contents of TNFR2+ TILs in TNBC patient biopsies were significantly associated with improved survival [30]. Our follow up study in a mouse TNBC model indicated that chemotherapy leads to preferential presence of $CD8^+$ TNFR2+ TILs that have anti-tumor activities in the tumors, thus setting TNFR2 as a protective entity in this subtype of breast cancer [31].

In parallel, the data presented in our current study demonstrate a significant association between TNFR2 expression in cancer cells in TNBC patient biopsies, and better patient prognosis. These findings further support the possibility that TNFR2 may have beneficial roles in TNBC patients and thus should not be considered as a therapeutic target in this disease subtype.

To determine the mechanisms that may stand in the basis of our latter observations in TNBC patients, in this study we determined the roles of tumor cell-expressed TNFR2 in regulating cancer-supporting activities of TNBC cells. We included in the study cells expressing wild type (WT) TNFR2 and cells expressing the M196R-SNP variant of TNFR2 that exists in ~30% of breast cancer patients [32, 33]. This M196R-SNP-TNFR2 was detected in breast cancer patients in studies that did not divide the patients by subtypes, and was generally associated with improved survival [32, 33]; the M196R-SNP-TNFR2 was also connected to improved survival in non-small cell lung cancer patients [34]. Moreover, M196R-SNP-TNFR2 had a lower ability to induce TNFR2-mediated p65 signaling (p65 together with p50 constitute the canonical NF-κB pathway strongly activated by TNFα), and reduced ability to induce the release of the pro-inflammatory chemokine CXCL8, compared to WT-TNFR2 [35].

Based on these observations, we hypothesized that if TNFR2 expression in TNBC cells has protective roles against tumor progression, its activities may be even stronger when it exists in its M196R-SNP form. TNFR1 is expressed in tumors of all breast cancer patients and TNFR2 is detected in many patient tumors [12–15]. Therefore, we wished to determine the roles of TNFR2 - in its WT or SNP forms - on TNFα-induced pro-metastatic functions of TNBC cells, when it is expressed alone or in the context of TNFR1. To this end, we established a research system consisting of different combinations between TNFR1 and WT-TNFR2/SNP-TNFR2. Moreover, In view of the high prevalence of mTNFα in TNBC patients (84%) [19] and the different preferences of TNFR1/TNFR2 towards sTNFα/mTNFα [2, 10], the

cancer-promoting functions of the cells were determined following stimulation by sTNFα, in cells expressing mTNFα or lacking its expression.

The findings of this study indicate that in TNBC cells, M196R-SNP-TNFR2 reduces the sTNFα-induced tumor-promoting activities of TNBC cells, particularly when TNFR1 is not active. Moreover, we demonstrate that mTNFα strongly inhibits the ability of TNFR1 and TNFR2 to respond to sTNFα-induced signals, including p65 activation and pro-metastatic functions of TNBC cells. Additional studies demonstrated that in cells expressing only TNFR1, sTNFα-induced p65 activation was correlated with reduced ERK1/2 activation, and that mTNFα-induced p65 inhibition was connected to down-regulation of TNF receptor-associated factor 2 (TRAF2).

Thus, our observations provide novel findings on the roles of M196R-SNP-TNFR2 and mTNFα as negative regulators of sTNFα-induced pro-metastatic activities in cancer. Taken together with our findings on the potential protective roles of $CD8^+$ $TNFR2^+$ TILs, these observations suggest that TNFR2 is not an optimal target for therapy in TNBC. Thus, the protective roles of TNFR2 and mTNFα should be taken into account when TNFα-inhibiting modalities are considered in TNBC and possibly in other tumor types as well.

Materials and methods

Immunohistochemistry (IHC) and determination of TNFR2 expression patterns

The study included a retrospective cohort of 69 female adjuvant-treated patients (age 29–85), with inclusion criteria of TNBC disease (patient number adhered to power calculation); the same cohort was used in our previous publication on TNFR2-expressing TILs [30], where the clinicopathological characteristics of the patients were detailed; For readers' convenience, information on patients included in the current study is provided in Table S1 (in the current study, one patient was omitted from the cohort, due to lack of information on TNFR2 expression in the cancer cells).

As indicated in our published study [30], TNBC status was determined by board-certified pathologists based on ASCO/CAP guidelines. In parallel to hematoxylin and eosin (H&E) staining of formalin-fixed paraffin-embedded tumor sections (4 μm), the expression levels of HER2, estrogen receptors and progesterone receptors by tumor cells were determined by antibodies used in routine diagnosis hospital tests. To determine TNFR2 expression, Novus Biological antibodies having high specificity to TNFR2 in protein arrays (Company's data; #NBP1-88139; Littleton, CO) were used; at the study setup stage, the antibodies were validated compared to a non-relevant isotype-matched control antibodies (data not shown). Positive controls for TNFR2 staining included tonsil tissues. Heat-induced antigen retrieval was performed by CC1 solution (Ventana Medical Systems, Tucson, AZ) in alkaline conditions, followed by counterstaining with hematoxylin solution. DAB detection system was used to detect staining patterns (Ventana Medical Systems).

At Sheba Medical Center, H&E staining was used to validate the presence of tumor cells in biopsy sections. TNFR2 expression by the cancer cells was determined by certified breast pathologists who were accompanied by research coordinators from the Cancer Research Center, in a blind manner.

To determine survival outcomes, patients were allocated based on TNFR2 expression; Kaplan-Meier analyses were used, and groups were compared by LogRank statistics (performed in Matlab, using LogRank package by Cardillo G). $p \le 0.05$ was considered significant. Overall survival was defined as the time from diagnosis to death of any cause.

The study was approved (Approval No. SMC-8736-11) by the Institutional Review Board of Sheba Medical Center, with full exemption for consent form for anonymized samples. All samples were anonymized as defined in the study protocol. The research was also approved by the Institutional Review Board of Tel Aviv University.

Analyses of patient datasets

The METABRIC patient dataset was used to determine the associations between TNFR2 (*TNFRSF1B*) expression and percent survival in basal-like (total $n = 331$) and luminal-A (total $n = 721$) breast cancer patients. For each subtype, patients were divided into quartiles based on the expression levels of *TNFRSF1B*. The highest and lowest quartiles were compared. P values were calculated by Log-rank Mantel Cox test.

The Cancer Genomic Atlas (TCGA) breast cancer (BRCA) database of basal-like patients (total $n = 96$) was used to determine correlations between *TNFRSF1A*/*TNFRSF1B* transcriptome data and patient survival. Here, patients expressing high/low *TNFRSF1A* or high/low *TNFRSF1B* levels were determined based on median values of expression. Analyses were performed with the UCSC Xena platform, providing p values, as necessary.

Cell cultures

Cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). Human TNBC MDA-MB-231 cells (MDA; CRM-HTB-26) were maintained in Growth Medium, consisting of DMEM supplemented by 10% fetal bovine serum (FBS), 2 mM L-glutamine, 122 Units/ml penicillin, 122 μg/ml streptomycin and 250 ng/ml amphotericin (all materials were from Biological Industries, Beit Ha'Emek, Israel or from Sigma-Aldrich, Saint Louis, MO). Experimental Medium was used in

different assays, containing the same materials, but with 0.5% FBS only. Human TNBC BT-549 cells (BT; CRM-HTB-122) were grown in RPMI 1640 medium (Biological Industries, Beit Ha'Emek, Israel). Media were supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin-streptomycin solution (Biological Industries); for BT-549 cells, insulin (10 mg/ml; #I9278; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added to the medium.

Generating cells with different TNFR1/TNFR2 combinations, as part of the "8-cell system"

The system has been generated by using MDA cells that expressed endogenously TNFR1 and TNFR2 (Figs. S1A, B). The first stage in establishing the 8-cell system was to select for cell clones having no/minor levels of TNFR2 expression (the clones were originally produced during a TNFR2 knock-out procedure that was not productive). To screen the clones for lack of TNFR2 expression, we used a sensitive approach that we developed [36], based on detection of sTNFR2 in cell supernatants, following cell stimulation by recombinant human (rh) cytokines: TNFα, that will be termed herein sTNFα (50 ng/ml; Cat# 300–01A; PeproTech, Rocky Hill, NJ) + interleukin 1β (IL-1β; 500 pg/ml; #200-01B, PeproTech) (Fig. S2A1). At the end of this stage, two clones that demonstrated very low TNFR2 levels in cell supernatants (clones 5 and 14) were pooled together and were termed TNFR2- cells, lacking TNFR2 expression as validated by minimal sTNFR2 expression (Fig. S2A2).

At this point, the TNFR2- cells were treated by CRISPR-Cas9 to knock-out the expression of endogenous TNFR1 in the cells, in parallel to cells treated by control sgGFP. After two rounds of selection, based on the levels of soluble TNFR1 (sTNFR1) released by the cells (data not shown), two clones were selected for further research (clones 60 and 62). In these two clones, cDNA sequencing revealed the existence of an early stop codon, validating that the TNFR1 gene was indeed silenced (data not shown). These two clones did not respond well to stimulation by sTNFα in terms of CXCL8 production and p65 activation, compared to cells that expressed TNFR1 and responded well to this stimulation (Fig. S2B); these two clones were pooled, generating TNFR1-/TNFR2- cells. These TNFR1-/TNFR2- cells and their $TNFR1^{+}$/TNFR2- counterparts (cells that did not express TNFR2 and were infected with control sgGFP) were used in the follow up process in which WT-TNFR2 and TNFR2 variants were expressed in the cells (described below).

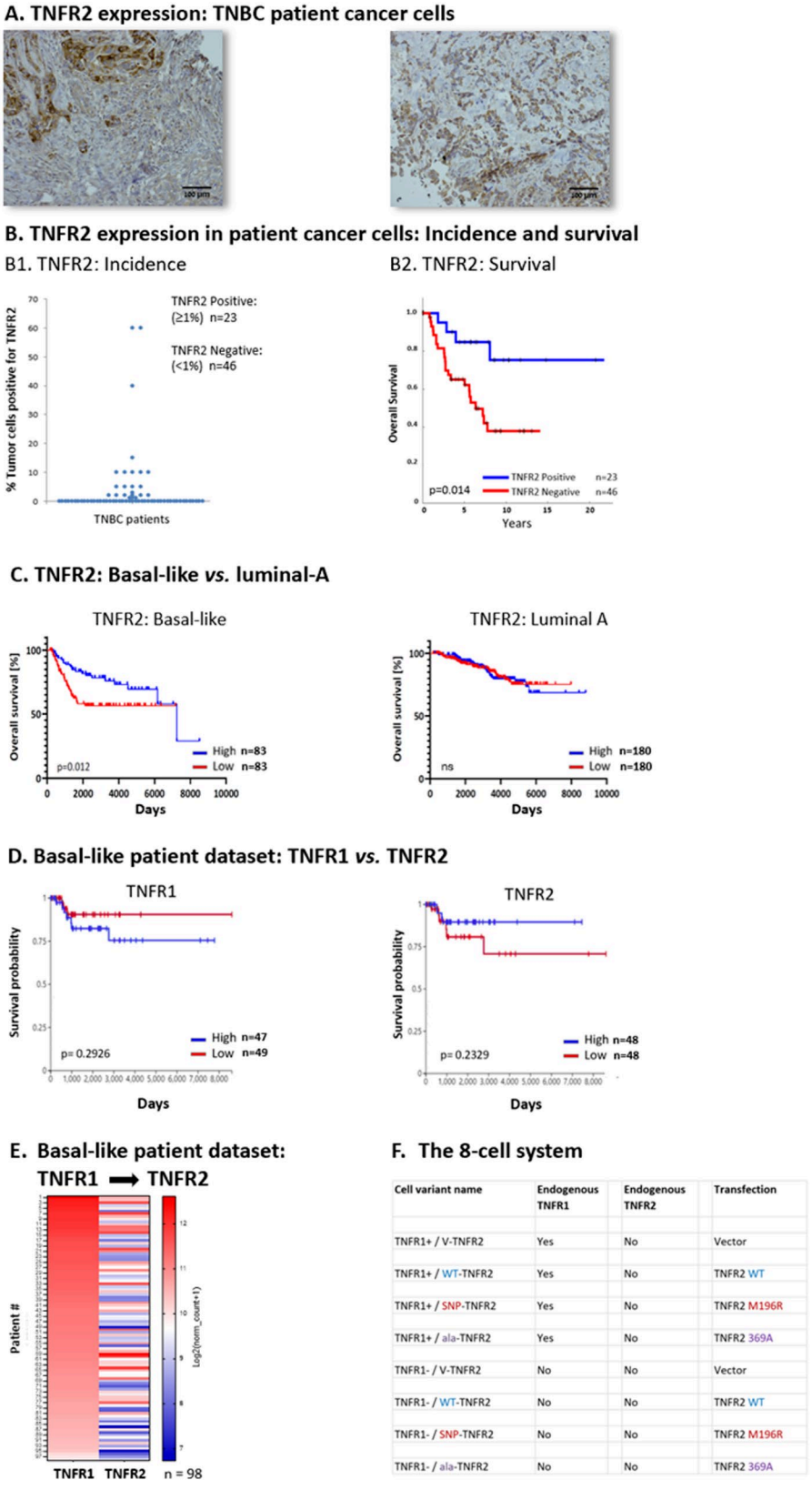
Next, we generated a vector coding for human WT-TNFR2 by performing RT-PCR reaction (using the primers detailed in Table S2) on sTNFα + IL-1β-stimulated MDA cells (as described above, to enhance TNFR2 expression). Following cloning, the cloned segment was sequenced, confirming the presence of WT-TNFR2. In addition to the WT form, two naturally-occurring variants of TNFR2 were identified in MDA cells during quantitative real-time PCR analyses. The first was M196R-SNP-TNFR2, and the second was a variant in which an alanine insertion was noted at position 369, referred to as +369A-TNFR2.

This step was followed by infection of the above-mentioned TNFR1+/TNFR2- and TNFR1-/TNFR2- cells with plasmids coding for WT-TNFR2, M196R-SNP-TNFR2, +369A-TNFR2 or empty vector control (V-TNFR2) (cell names and their contents are provided in Fig. 1F). This procedure has given rise to four types of TNFR1+ cells: cells expressing WT-TNFR2 (termed TNFR1+/WT-TNFR2), cells expressing M196R-SNP-TNFR2 (TNFR1+/SNP-TNFR2), cells expressing +369A-TNFR2 (TNFR1+/ala-TNFR2) and cells infected by the control vector of TNFR2 (TNFR1+/V-TNFR2). In parallel, four different types of TNFR1- cells were generated by this procedure (Fig. 1F): cells infected by WT-TNFR2 (TNFR1-/WT-TNFR2), cells infected by M196R-SNP-TNFR2 (TNFR1-/SNP-TNFR2), cells infected by +369A-TNFR2 (TNFR1-/ala-TNFR2) and cells infected by empty vector that corresponds to the TNFR2-coding plasmid (TNFR1-/V-TNFR2 cells).

Overall, an 8-cell system was produced, with different combinations of TNFR1 and TNFR2 expression (Fig. 1F), as validated by flow cytometry (Fig. S3). Here, it is necessary to indicate that although TNFR1 and TNFR2 are expressed by breast tumor cell lines, their detection by antibodies is not optimal in flow cytometry, as previously reported by others [15, 26]. Although the detection levels of TNFR1 and TNFR2 by flow cytometry were not very high, the analyses demonstrated that all TNFR1+ cells expressed TNFR1 at the cell surface, and that TNFR2 was expressed at similar levels in the different cell types that were infected to express WT-TNFR2, M196R-SNP-TNFR2 and +369A-TNR2 (Fig. S3). Moreover, as seen further below, cells that expressed TNFR1 responded strongly to stimulation by sTNFα, as indicated by p65 and p38 activation; in contrast, cells that expressed only TNFR2 responded less than cells expressing only TNFR1 to stimulation by sTNFα (as described in other cell types), thus supporting the expected nature of the cells.

Stimulation by sTNFα

Cells of the 8-cell system were stimulated with sTNFα (namely rhTNFα) or vehicle (0.1% BSA in double-distilled water [DDW]), diluted in Experimental Medium. Following titration and kinetics analyses performed in our lab in the different analyses (data not shown), stimulation by sTNFα was introduced at concentrations and conditions indicated in Figure legends.



Cell variant name	Endogenous TNFR1	Endogenous TNFR2	Transfection
TNFR1+ / V-TNFR2	Yes	No	Vector
TNFR1+ / WT-TNFR2	Yes	No	TNFR2 WT
TNFR1+ / SNP-TNFR2	Yes	No	TNFR2 M196R
TNFR1+ / ala-TNFR2	Yes	No	TNFR2 369A
TNFR1- / V-TNFR2	No	No	Vector
TNFR1- / WT-TNFR2	No	No	TNFR2 WT
TNFR1- / SNP-TNFR2	No	No	TNFR2 M196R
TNFR1- / ala-TNFR2	No	No	TNFR2 369A

Fig. 1 (See legend on next page.)

(See figure on previous page.)

Fig. 1 TNFR2 expression is connected to good prognosis in TNBC/basal-like breast cancer patients. (**A**, **B**) TNFR2 expression was determined in TNBC biopsies, and was correlated with patient survival. TNFR2 expression was determined by IHC in a 69-patient cohort of adjuvant-treated TNBC patients. The antibodies specifically bound TNFR2, as indicated by Novus Biological and by negative biopsy staining by a non-relevant isotype-matched control ("Materials and methods"). (**A**) TNFR2 expression in the cancer cells is demonstrated in biopsies of two different patients. (**B1**) The proportion of % TNFR2-expressing tumor cells in each patient (dot symbol) is demonstrated. (**B2**) The Figure presents Kaplan-Meier analyses of overall survival, performed on the patient cohort. TNFR2 positive tumors are those in which ≥1% of the cancer cells expressed TNFR2. +, Censored. (**C**) METABRIC analyses of basal-like and luminal-A breast cancer patients are demonstrated. Patients were divided to quartiles based on the expression levels of *TNFRSF1B*; high and low quartiles were compared. ns, Not significant. (**D**, **E**) TCGA analyses of basal-like patients. (**D**) Analyses of correlation between *TNFRSF1B* and *TNFRSF1A* expression levels in basal-like patients, and patient survival (based on median values of receptor expression. The association were not statistically significant because of relatively small numbers of patients in the two groups). (**E**) Associations between *TNFRSF1A* and *TNFRSF1B* expression levels in basal-like patients, demonstrating the expression levels of *TNFRSF1A* in each patient, and then the expression of *TNFRSF1*B in the same patient. Red = High; Blue = Low. **(F)** Establishment of the 8-cell system. The Table describes the different MDA cell types that were generated and their names, to be used herein in the manuscript

Western blot analyses

Cells of the 8-cell system were stimulated with sTNFα or vehicle (time points and concentrations are indicated in Figure legends), in Experimental Medium. Following lysis in RIPA buffer, conventional Western blot (WB) and transfer procedures were performed, using antibodies from Cell Signaling Technology (CST, Danvers, MA), unless otherwise indicated: Total (T)-p65: #8242; Phosphorylated (P)-p65: #3033; T-p38: #8690P; P-p38: #4511T; T-JNK2: #9258; P-JNK: #4668; T-ERK: #4695; P-ERK: #4370; TRAF2: #7346 (Santa Cruz Biotechnology, Denver, TX). GAPDH (#ab9485; Abcam, Cambridge, UK) or β Tubulin (#2146; CST) served as loading controls. The membranes were incubated with streptavidin-horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (#111-035-003; Jackson ImmunoResearch Laboratories, West Grove, PA) or goat anti-mouse IgG (#115-035-003; Jackson ImmunoResearch Laboratories), and then were subjected to enhanced chemiluminescence analysis (#WBLUR0500; Merck, Darmstadt, Germany) and visualized with Amersham ImageQuant 800 (GE Healthcare, Little Chalfont, UK).

Where indicated, the TNFα-induced nuclear localization of p65 was determined in the cells by using a cell fractionation kit (#78833; Thermo Fisher Scientific, Waltham, MA), according to manufacturer's instructions; loading controls included KDM2A for the nuclear fraction (using antibodies #AB191387; Abcam) and calnexin for whole cell lysates (antibodies #2679S; CST). Also, in specific experiments the cells were treated by the MEK1/2 inhibitor U0126 (5μM, #222395; Santa Cruz Biotechnology) or its vehicle (DMSO) for 2, 24, 48 h. At the end of the 48-hour growth, the cells were stimulated by sTNFα (50 ng/ml) or exposed to its vehicle, for 15 min, followed by determination of ERK and p65 activation and expression, as described above. Parallel analyses were performed to determine the activation of p38 (antibodies as above) and c-Jun, using the following antibodies: T-Jun: #610326 (BD Transduction Laboratories, San Diego, CA); P-c-Jun: #9261 (CST), followed by secondary antibodies, as above. In the case of c-Jun, calnexin was used as loading control (as above).

Migration and invasion

Migration in wound-closure assays was determined by the IncuCyte® Live-Cell Imaging system. Cells of the 8-cell system were plated in IncuCyte® ImageLock 96-well plates (#4379; Essen BioScience, Ann Arbor, MI). The cells were plated in Growth Medium and were exposed to sTNFα (concentrations as described in Figure legends) or vehicle for 24 h. Then, a scratch was made in each well, and kinetics analyses of tumor cell migration, giving rise to wound-closure, were performed for 24 h, in the presence of the same sTNFα stimulation (or vehicle).

In data presenting the average of cell migration, a comparison was made between a certain cell type (called herein "test cell type") and cells expressing WT-TNFR2. To this end, the extent of wound confluency (presented in %) in the test cell line and in WT-TNFR2-expressing cells was determined in several wells, and an average of % cell confluency that was obtained for each of the two cell types was calculated. Average wound confluency in the test cell type at the 20-hour time point was subtracted from the average wound confluency obtained for WT-TNFR2 cells at the same time point and at the same experiment. This approach has yielded a value of net wound confluency for each test cell type (%). The respective graphs demonstrate the average net values of several experiments, each presented as a dot.

Cell invasion was assessed with transwell permeable supports (#CLS3422;, Sigma-Aldrich). The two compartments were separated by an 8-μm polycarbonate PVPF filter, coated with 20 μg/ml matrigel (#354234;, Corning, Corning, NY). Cells of the 8-cell system were exposed before the invasion assay to sTNFα or vehicle for 24 h in Experimental Medium. Then, the cells were plated in the upper part of the transwells, in the presence of the same sTNFα stimulation, or its vehicle. The bottom wells of the transwells contained 10% FBS-containing medium. Based on kinetics assays (data not shown), cell invasion analyses were performed for 11 h. Then, the filter was removed, and cells were fixed and stained with Hemacolor staining set (#1.11661; Merck, Darmstadt, Germany). Cells that migrated to the underside of the filter were counted by

light microscopy in high-power fields (HPFs), and data are presented as "Cell number/HPF".

When indicated, in each transwell experiment performed on cells of the 8-cell system, a comparison was made between a certain cell type (test cell type) and cells expressing WT-TNFR2. In each experiment, the average number of WT-TNFR2-expressing cells that successfully invaded was divided by the mean number of the invading test cell type, generating the value of "Invasion (fold)". The respective graphs demonstrate the average invasion capacity values of several experiments, each presented as a dot.

Transwell experiments were also performed with cells infected to express Δ12-TNFα or its vector (described below). Generally, the above-mentioned experimental procedures were used, except for the suspension of the cancer cells in serum-free medium lacking sTNFα at the time of invasion in transwells.

ELISA analyses

Centrifugation-cleared cell supernatants were obtained following stimulation by sTNFα (concentrations are indicated in Figure legends) or its vehicle (in Experimental Medium) for 24 h. Then, CXCL8 expression levels were determined by ELISA assays, performed at the linear range of absorbance using a standard curve generated with rhCXCL8 (#200-08; Peprotech). The following materials were used (all from PeproTech): Coating antibodies to CXCL8 (#500-P28); Detection antibodies to CXCL8 (#500-P28BT).

TNFα expression levels were determined in centrifugation-cleared cell supernatants collected from cells cultured for 24 h in Experimental Medium. TNFα expression levels were determined by ELISA assays, performed at the linear range of absorbance using a standard curve generated with rhTNFα (#300-01A; Peprotech). The following materials were used (all from Peprotech): Coating antibodies to TNFα (#500-M26); Detection antibodies to TNFα (#500-P31ABT).

Following the addition of HRP-conjugated streptavidin (#016-030-084; Jackson ImmunoResearch Laboratories) and substrate TMB/E solution (#TM4500;, ScyTek Laboratories, UT), the reaction was stopped by the addition of 0.18 M H_2SO_4. Absorbance was measured at 450 nm and the findings are presented following adjustment to cell numbers at the time of supernatant collection.

Generating TNFα-expressing cells

Selected cells of the 8-cell system (as indicated in the "Results" section) have been infected with plasmids coding for WT-TNFα, Δ12-TNFα (primers are indicated in Table S2), or a control vector. The expression of TNFα at the cell membrane was detected by flow cytometry, as indicated below. The expression of sTNFα in cell supernatants was determined by ELISA assays, described above.

Flow cytometry analyses

Membrane expression of TNFR1 and TNFR2 was determined by specific antibodies (#MAB225 and #MAB226, respectively; R&D Systems, Minneapolis, MN), using non-relevant isotype-matched controls to determine baseline staining (#400102 and #401502, respectively; Biolegend, San Diego, CA). Secondary antibodies included FITC-conjugated goat antibodies (#115-095-003; Jackson ImmunoResearch Laboratories). The cell surface expression of ICAM-1 was determined in cells treated by sTNFα/vehicle by flow cytometry, using APC-conjugated ICAM-1 antibodies (#130-121-342; Miltenyi Biotec , Bergisch Gladbach, Germany). Baseline staining was determined by a non-relevant isotype-matched REA control antibody (#130-113-446; Miltenyi Biotec). The cell surface expression of TNFα (mTNFα) was determined using FITC-conjugated antibodies directed to mTNFα (#FAB210F; R&D Systems). Baseline staining was determined by a non-relevant isotype-matched control antibody (#400109; BioLegend). The expression of these proteins was determined using S1000EXi flow cytometer (Stratedigm, San Jose, CA). Staining patterns and analyses were performed using the Flow-Jo software.

Statistical analyses

In vitro experiments were performed in $n \geq 3$ independent experimental repeats, demonstrating similar findings. Results of all assays were analyzed by two-tailed unpaired Student's *t*-tests to calculate p-values (two-tailed, unpaired). $p \leq 0.05$ was considered statistically significant.

Results

TNFR2 expression in cancer cells is significantly associated with better prognosis in TNBC patients

This study is the first to specifically determine the associations between TNFR2 expression in breast cancer cells and survival in TNBC patients, using immunohistochemistry (IHC) staining analyses; in parallel, the METABRIC and TCGA patient datasets were used to determine parameters of TNFR2 (and TNFR1 when relevant) expression levels in basal-like breast cancer patients (basal-like is the closest subtype to TNBC in patient datasets). Data on genomic mutations - and thus on the M196R SNP of TNFR2 - was not available for any of the cohorts; however, cBioPortal information of basal-like patients (including data from the METABRIC and TCGA datasets) did not report on somatic mutations of TNFR1, and only two cases of deletions were noted in TNFR2.

The patient cohort used in IHC studies included archived tumor biopsies that were collected from primary tumors prior to chemotherapy, excluding the

possibility that TNFR2 expression was influenced by treatment (patient characteristics are provided in Table S1). Patient biopsies in which ≥ 1% of the cancer cells expressed TNFR2 were considered positive (Fig. 1A and B). TNFR2 expression by the tumor cells was significantly associated with improved overall survival ($p = 0.014$). The positive correlation between TNFR2 expression and better prognosis is emphasized by the fact that TNFR2+ tumor cells were noted in only a small fraction of the patients (33%), with a relatively low incidence of TNFR2+ cancer cells in many of them.

Analyses using the METABRIC dataset of basal-like breast cancer patients demonstrated that TNFR2 mRNA expression was significantly associated with improved survival in these patients ($p = 0.012$), but not in luminal-A breast cancer patients (Fig. 1C), suggesting that the connection of TNFR2 expression to good prognosis is subtype-dependent. High levels of TNFR2 expression demonstrated a trend towards association with improved survival in basal-like patients also when the TCGA dataset was used (Fig. 1D; The association was not statistically significant due to the relatively small number of patients in each of the two groups of patients that were included in the dataset). Importantly, the mRNA expression data in the patient dataset represents bulk expression from all cell types, including both cancer cells and immune cells. Along these lines, in our previous study, we found that TNFR2+ TILs were also associated with good prognosis in TNBC patients [30]. Thus, the bulk expression data captured by the current dataset analyses agree and support our IHC findings in TNBC patient study, demonstrating the correlation of TNFR2+ cancer cells as well as TNFR2+ TILs with good prognosis in TNBC patients.

These observations suggest that in TNBC, TNFR2 expression by the cancer cells may have protective roles against TNFα-induced disease progression. Unlike TNFR2, high TNFR1 expression was linked to reduced survival in basal-like patients (Fig. 1D), being in line with the metastasis-promoting activities attributed to this receptor. Moreover, TNFR2 expression levels in the patients were not well-correlated with the expression levels of TNFR1 (Fig. 1E). These findings suggest that TNFR1 and TNFR2 have divergent roles in regulating TNBC progression.

M196R-SNP-TNFR2 reduces sTNFα-induced intracellular signaling in TNBC cells lacking TNFR1 expression

The possibility that TNFR1 activities are different from TNFR2 functions in TNBC progression emphasize the need to identify the way they act - each individually and together - when TNBC cells are stimulated by TNFα, be it in its soluble form or in its membrane form. In this context, it is also important to determine whether M196R-SNP-TNFR2 acts similarly or differently to WT-TNFR2.

To address these questions, it was necessary to identify human TNBC cells that endogenously express TNFR1 and TNFR2, thus having the molecular intracellular machinery required to respond to TNFα. The data of Figures S1A and B indicate that both MDA-MB-231 (MDA) and BT-549 (BT) human TNBC cells expressed TNFR1 and TNFR2 endogenously. To connect our findings more conclusively with other reports in the field, we chose to continue our research with MDA cells: MDA cells serve the cells of choice in many studies analyzing TNBC cell responses to sTNFα; accordingly, we demonstrated in our research that p65 is strongly activated by sTNFα (Fig. S1A1 and other figures of the study) and sTNFα increased the nuclear localization of activated p65 (Fig. S1C). Moreover, MDA cells did not express endogenous mTNFα (Fig. S4), enabling us to exclude background signals when the cells are exposed to sTNFα, in the systems that we were about to establish (described below).

To perform a structured study of the functions of TNFR1 and TNFR2 in MDA cells, we established a cell system in which TNFR1+ and TNFR1- cells were infected to express WT-TNFR2 or two TNFR2 variants (Figs. 1F, S2, S3; Detailed in the "Materials and methods"): (1) M196R-SNP-TNFR2 - termed in brief SNP-TNFR2 in Figures and in cell names; (2) A variant that was identified in the course of TNFR2 sequencing in MDA cells, in which an alanine insertion was noted at position 369 (+369A-TNFR2), called herein ala-TNFR2. In parallel, TNFR1+ and TNFR1- cells were infected with the empty vector (V-TNFR2), as control. Overall, we have generated eight different cell types (Fig. 1F), including: (1) Four TNFR1+ cells: TNFR1+/***V***-TNFR2, TNFR1+/***WT***-TNFR2, TNFR1+/***SNP***-TNFR2, TNFR1+/***ala***-TNFR2; (2) Four TNFR1- cells: TNFR1-/***V***-TNFR2, TNFR1-/***WT***-TNFR2, TNFR1-/***SNP***-TNFR2, TNFR1-/***ala***-TNFR2.

At this stage, we validated that following infection with the relevant constructs, the pattern of TNFR1 and TNFR2 expression that was denoted in the cells by flow cytometry agreed with their expected phenotype (Fig. S3) (in line with other studies, their expression is not optimally detected by antibodies in flow cytometry) [15, 26]). Of note, in all TNFR1+ cells - independently of TNFR2 expression - sTNFα has strongly induced the activation of its key targets, p65 and p38 (Figs. 2A1, A2; Densitometry: Figs. S5A and B, respectively), as well as JNK (Fig. S6A; Densitometry: Fig. S6B). Our findings also indicated that sTNFα-induced activation through TNFR1 was not reproducibly affected by any of the forms of TNFR2: WT-TNFR2, M196R-SNP-TNFR2 and +369A-TNFR2 (Fig. 2A; Densitometry: Figs. S5, S6). Then, in analyses of TNFR1- cells expressing WT-TNFR2 (TNFR1-/WT-TNFR2), the activation of p65, p38 and JNK was lower than in TNFR1+ cells (Fig. 2A; Densitometry: Figs. S5, S6). Of note, the sTNFα-induced activation of p65, p38

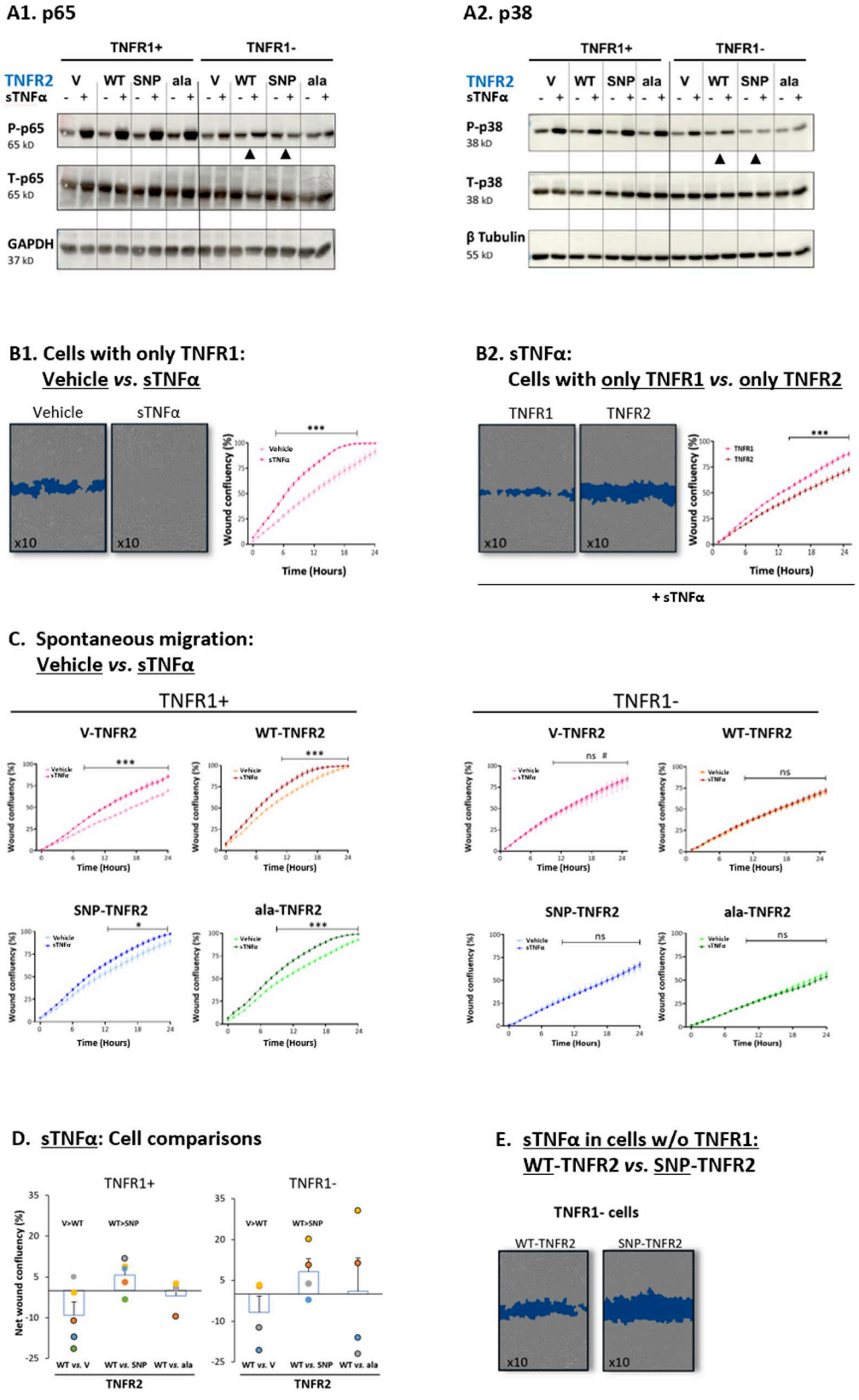


Fig. 2 (See legend on next page.)

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Fig. 2 M196R-SNP-TNFR2 down-regulates sTNFα-induced signaling and spontaneous migration in TNBC cells. MDA cells that express TNFR1 or do not express TNFR1 were infected to express WT-TNFR2, M196R-SNP-TNFR2, ala-TNFR2 or control vector (V) (names are provided in Fig. 1F; procedures of cell preparation are described in "Materials and methods", alongside data demonstrated in Figs. S2, S3). (**A**) The cells were stimulated with sTNFα (10 ng/ml) or its vehicle for 10 min, followed by determination of p65 activation (**A1**) or p38 activation (**A2**). GAPDH or β Tubulin were used as loading controls. Densitometry analyses are demonstrated in Figures S5A and S5B, respectively. π;points at the difference between TNFR1-/WT-TNFR2 and TNFR1-/SNP-TNFR2 cells. (**B**-**E**) Spontaneous migration in scratch assays (no gradient included). The cells were stimulated with sTNFα (10 ng/ml) or vehicle for 24 h before scratch, and during the migration assay (performed for 24 h). After validating that the different cell types did not differ in their proliferation potential (Fig. S7), their migration was determined in 1-hour intervals in IncuCyte® live-cell imaging system; the results are demonstrated in kinetics graphs, in which "Wound confluency (%)" indicates the proportion of the original wound area that has become covered by migrating cells at each time point. (**B**) Photos and migration curves are demonstrated for: (**B1**) Cells expressing only TNFR1 (TNFR1+/V-TNFR2), exposed to sTNFα (10 ng/ml) or vehicle. (**B2**) Cells expressing only TNFR1 or only WT-TNFR2 (TNFR1+/V-TNFR2 and TNFR1-/WT-TNFR2, respectively), stimulated similarly by sTNFα. (**C**) Representative experiments, demonstrating the migration of cells of the 8-cell system, exposed to sTNFα (10 ng/ml) or vehicle. #indicates that in this case, the results were not fully consistent. (**D**) Averages ± SD of values obtained in several experiments included in the same comparison (bars), and values of specific experiments (each dot represents a particular experiment), comparing between different cell types. Dots surrounded by black circles (O) indicate experiments in which the difference between migration values of the two cell types that were compared was $p \leq 0.05$. The values of migration at the 20-hour time point were used for analysis (selected based on the kinetics studies performed during the research). Value of 0 = The migration of the two cells that were compared was equal. An average positive value obtained in different experiments belonging to each comparison suggests an advantage of WT-TNFR2 cells over a specific variant, and vice versa. Full description of the approach taken in this analysis is described in "Materials and methods." (**E**) Representative photos of the migration of cells that did not express TNFR1, but expressed WT-TNFR2 or M196R-SNP-TNFR2 (TNFR1-/WT-TNFR2 and TNFR1-/SNP-TNFR2, respectively). In all panels, the findings are representative of $n \geq 3$ independent experiments that have shown similar results (in Panel **D**: averages ± SD values are also shown). ***$p < 0.001$, *$p < 0.05$. ns, Not significant

and JNK was lower in TNFR1-/SNP-TNFR2 cells than in TNFR1-/WT-TNFR2 cells (Fig. 2A; Densitometry: Figs. S5, S6). The presence of ala-TNFR2 in TNFR1- cells generally did not lead to any notable effect, compared to TNFR1-/WT-TNFR2 cells (Fig. 2A; Densitometry: Figs. S5, S6).

Overall, these findings are the first to show that in TNBC cells, TNFR1 is the prime mediator of sTNFα-induced signals in TNBC cells, and that the high potency of TNFR1-induced responses is not affected by WT-TNFR2 or any of its variants. Yet, in TNFR1- cells, the activation generated by M196R-SNP-TNFR2 was lower than produced by WT-TNFR2, indicating that in this context, M196R-SNP-TNFR2 limits the effects of sTNFα on signaling.

WT-TNFR2, and more so M196R-SNP-TNFR2, reduce sTNFα-induced migration and invasion of TNBC cells

Next, we analyzed the spontaneous migration of the cells using wound-closure assays with IncuCyte®, in which no gradient was present. The cells were cultured with sTNFα for 24 h prior to the assay, and during its 24-hour course (in parallel to vehicle); the proliferation levels of all cells of the 8-cell system were similar (Fig. S7).

The data of Fig. 2B and C indicate that in line with their highly aggressive phenotype, MDA cells of all types demonstrated the ability to spontaneously migrate, prior to any stimulation by sTNFα; the migration of tumor cells expressing only TNFR1 (TNFR1+/V-TNFR2) was increased by sTNFα stimulation (photos and graphs are demonstrated in Fig. 2B1). Upon sTNFα stimulation, these TNFR1+/V-TNFR2 cells migrated significantly better than cells expressing only WT-TNFR2 (TNFR1-/WT-TNFR2) (Fig. 2B2). These findings indicate that TNFR1 rather than WT-TNFR2 is the main receptor mediating sTNFα-induced migration of TNBC cells. Moreover, our data demonstrate that only TNFR1+ cells responded to sTNFα stimulation by elevated levels of migration (Fig. 2C). Of note, the graphs of Fig. 2C do not compare the migratory capacities of the different cell types to each other, but rather identify the ability of sTNFα to promote their spontaneous cell migration.

Then, we compared the sTNFα-induced migratory potential of TNFR1+ cells that expressed the different variants of TNFR2 (WT-TNFR2, SNP-TNFR2, ala-TNFR2); in parallel, similar analyses were performed in cells not expressing TNFR1 (Fig. 2D). Here, the findings are presented as "Net wound confluency (%) (more details are provided in "Materials and methods"). The value obtained represents the level of contribution of WT-TNFR2 to the spontaneous migration in comparison to each TNFR2 variant or to cells not expressing TNFR2 at all: an average positive value obtained in the different experiments of each comparison suggests an advantage for WT-TNFR2 cells over a specific variant, and vice versa. The Figure also demonstrates the value obtained in each experiment (shown as a colored dot, surrounded by a black circle (O) when the differences were significant).

Figure 2D demonstrates that the spontaneous migration of sTNFα-stimulated TNFR1+ cells that also expressed WT-TNFR2 (TNFR1+/WT-TNFR2) was lower than of cells expressing TNFR1 without any form of TNFR2 (TNFR1+/V-TNFR2) (Fig. 2D). Similarly, cells that expressed only WT-TNFR2 (TNFR1-/WT-TNFR2) tended to have lower spontaneous migration compared to cells not expressing any of the receptors (TNFR1-/V-TNFR2). These findings suggest that sTNFα-induced signaling, through WT-TNFR2, leads to down-regulation of the spontaneous migration of the cells, independently

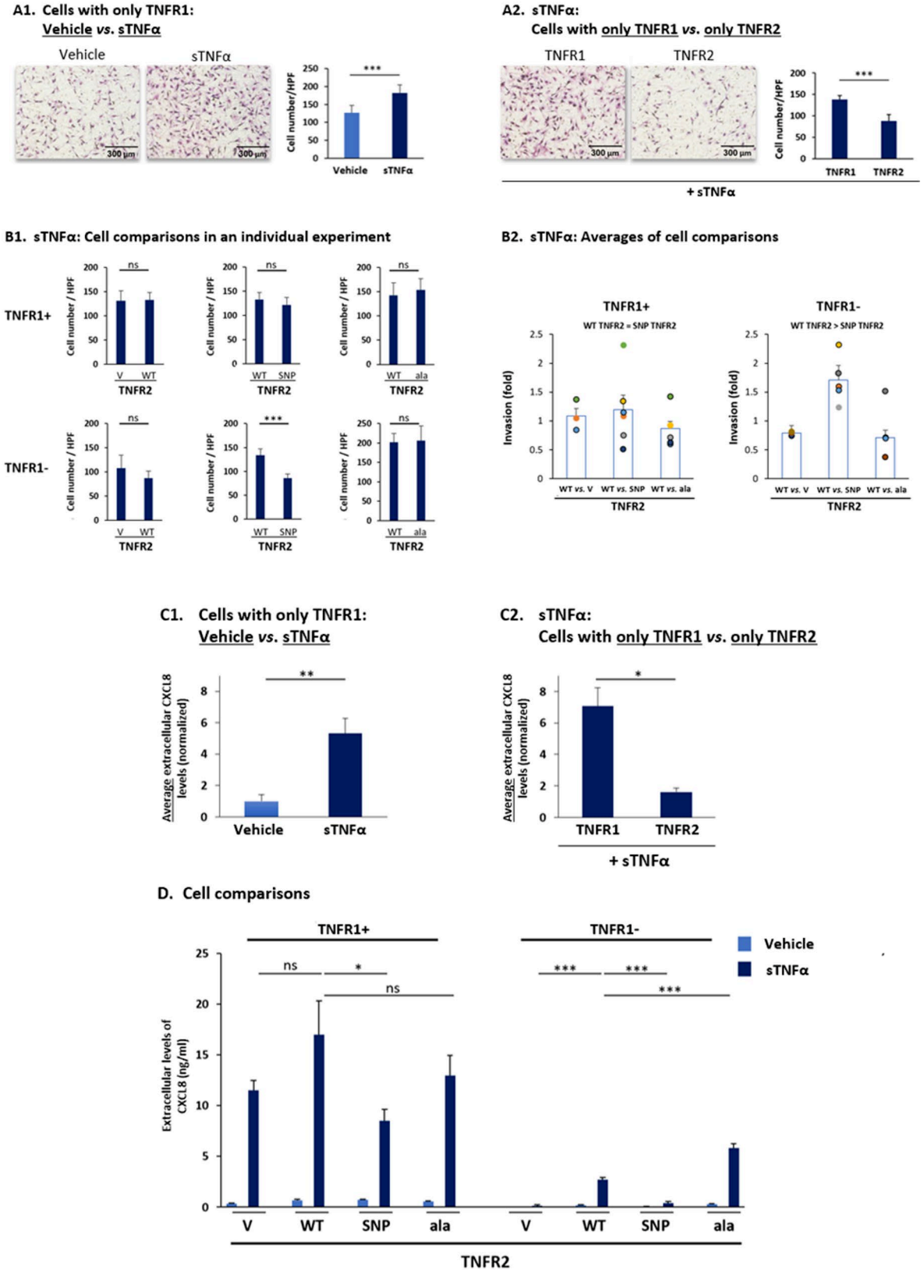


Fig. 3 (See legend on next page.)

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Fig. 3 M196R-SNP-TNFR2 reduces sTNFα-induced tumor cell invasion and CXCL8 production by TNBC cells. (**A**, **B**) MDA cells of the 8-cell system were stimulated with sTNFα (10 ng/ml) or vehicle for 24 h. Then, cell invasion was determined in matrigel-coated transwells in response to serum-containing medium in the bottom wells, for 11 h. Cells were fixed, stained, and counted by light microscopy in high-power fields (HPFs). (**A1**) Invasion photos and graphs of cells expressing only TNFR1 (TNFR1+/V-TNFR2), exposed to sTNFα or vehicle. (**A2**) Invasion photos and graphs of cells expressing only TNFR1 (TNFR1+/V-TNFR2) and of cells expressing only WT-TNFR2 (TNFR1-/WT-TNFR2), stimulated similarly by sTNFα. Both parts (**A1** and **A2**) demonstrate the results of a representative experiment of $n > 3$ independent experiments that have shown similar results. ***$p < 0.001$. (**B1**) Invasion graphs comparing between different cell types, all of them stimulated by sTNFα, as above. In each comparison, the findings demonstrate the results of a representative experiment of $n > 3$ independent experiments that have shown similar results. ***$p < 0.001$. ns, Not significant. (**B2**) Averages ± SD of fold values of invasion, obtained in several experiments included in the same comparison (bars), and values of specific experiments (each dot represents a particular experiment), comparing between different cell types. Dots surrounded by black circles (O) indicate experiments in which the difference between migration values of the two cell types that were compared was $p \leq 0.05$. (**C**, **D**) CXCL8 production. MDA cells of the 8-cell system were stimulated with sTNFα (10 ng/ml) for 24 h, followed by determination of CXCL8 levels in cell supernatants by ELISA. (**C**) The averages ± SD values of several experiments, where the vehicle-treated cells in each experiment were given the value of 1 are demonstrated. (**C1**) CXCL8 levels produced by cells expressing only TNFR1 (TNFR1+/V-TNFR2), stimulated or non-stimulated by sTNFα. (**C2**) CXCL8 levels of cells expressing only TNFR1 (TNFR1+/V-TNFR2) and of cells expressing only WT-TNFR2 (TNFR1-/WT-TNFR2), stimulated similarly by sTNFα. A different number of experiments was executed in Parts C1 and C2. (**D**) CXCL8 levels in all cells of the 8-cell system, with and without sTNFα stimulation, presented in ng/ml normalized per cell number. The findings are representative of $n > 3$ independent experiments that have shown similar results ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. ns, Not significant

of the expression of TNFR1, namely in cells expressing TNFR1 and in cells lacking TNFR1, alike.

Of major importance is the observation that in sTNFα-stimulated TNFR1+ cells and TNFR1- cells (TNFR1+/V-TNFR2 and TNFR1-/V-TNFR2, respectively), the migration of cells expressing WT-TNFR2 tended to be higher than of cells expressing M196R-SNP-TNFR2 (Fig. 2D and E). In TNFR1+ and TNFR1- cells, ala-TNFR2 did not show a notable or a reproducible effect on spontaneous cell migration compared to cells expressing WT-TNFR2 (Fig. 2D). Overall, these findings indicate that down-regulation of WT-TNFR2 signaling leads to reduced TNFα-induced migration of TNFR1+ and TNFR1- cells, and that signaling through M196R-SNP-TNFR2 down-regulates these migratory abilities even more.

At this stage, the directed invasion of the different cell types towards serum-containing medium was analyzed in transwells. Here, sTNFα has elevated the invasion of cells expressing only TNFR1 (TNFR1+/V-TNFR2), and these cells migrated more potently than cells expressing WT-TNFR2 without TNFR1 (TNFR1-/WT-TNFR2) (Figs. 3A1, A2). In parallel, in sTNFα-stimulated TNFR1-expressing cells, the presence of WT-TNFR2 did not affect the directed invasion of tumor cells, and the invasion of TNFR1+/SNP-TNFR2 and TNFR1+/ala-TNFR2 cells was similar to that of TNFR1+/WT-TNFR2 cells (Figs. 3B1, B2). Overall, these findings indicate that the ability of sTNFα to induce tumor cell directed invasion was fully dependent on TNFR1, without contribution of any form of TNFR2.

When similar analyses were continued in cells lacking TNFR1 expression, it was found that cells that expressed M196R-SNP-TNFR2 invaded significantly less than cells expressing WT-TNFR2 (Figs. 3B1, B2), indicating that in the absence of TNFR1, M196R-SNP-TNFR2 reduced the ability of the tumor cells to exert directed invasion. As was the case in TNFR1+ cells, the expression of ala-TNFR2 in cells not expressing TNFR1 did not affect the invasion properties of the cells (Figs. 3B1, B2).

Overall, our findings indicate that the migratory capacities of MDA cells that over-express TNFR1 - spontaneous and directed, alike - are intensified by sTNFα stimulation, much more than in cells expressing only WT-TNFR2. When the TNFR1+ and TNFR1- cells executed spontaneous migration (IncuCyte) in the presence of sTNFα, WT-TNFR2 has reduced the motility of the cells, and M196R-SNP-TNFR2 had even lower sTNFα-induced motility than WT-TNFR2-expressing cells. In similarity, M196R-SNP-TNFR2 also down-regulated the directed invasion of sTNFα-stimulated TNFR1- cells, when they were compared to TNFR1-/WT-TNFR2 cells. These observations suggest protective roles for the WT-TNFR2 and M196R-SNP forms of TNFR2 against metastasis-promoting functions in TNBC cells.

M196R-SNP-TNFR2 reduces sTNFα-induced expression of pro-metastatic molecules by TNBC cells

Next, we determined the roles of TNFR1 and different forms of TNFR2 in regulating sTNFα-induced release of the strong pro-metastatic chemokine CXCL8 [37]. First, we found that cells expressing TNFR1 only (TNFR1+/V-TNFR2) responded vigorously to sTNFα stimulation, and produced much higher levels of CXCL8 than cells that expressed only WT-TNFR2 (TNFR1-/WT-TNFR2) (Figs. 3C1, C2). These results again indicate that in response to sTNFα stimulation, it is mainly TNFR1 that signals towards increased production of CXCL8.

In parallel, we found that in sTNFα-stimulated TNFR1+ and TNFR1- cells expressing M196-SNP-TNFR2 (TNFR1+/SNP-TNFR2 and TNFR1-/SNP-TNFR2), the release of CXCL8 was lower than in TNFR1+ and TNFR1- cells that expressed WT-TNFR2. Here, a definite phenotype was also observed for ala-TNFR2, promoting the release of CXCL8 compared to cells expressing WT-TNFR2, in the absence of TNFR1 (Fig. 3D).

Then, we determined the response of the different cell types to sTNFα, in terms of membrane expression of ICAM-1, serving as an adhesion molecule that also triggers signaling responses potentially contributing to a metastatic phenotype [38, 39]. Like all other parameters analyzed so far following sTNFα stimulation, TNFR1 was the dominant receptor leading to increased ICAM-1 expression, compared to WT-TNFR2 (Figs. 4A1, A2). Then, we found that in TNFR1- cells stimulated by sTNFα, M196R-SNP-TNFR2 has led to a significant down-regulation of ICAM-1 expression, when compared to cells expressing WT-TNFR2 (Figs. 4B1, B2). Ala-TNFR2 did not affect the expression levels of ICAM-1 in TNFR1+ and TNFR1- cells stimulated by sTNFα, when it was compared to WT-TNFR2 (Fig. 4B1).

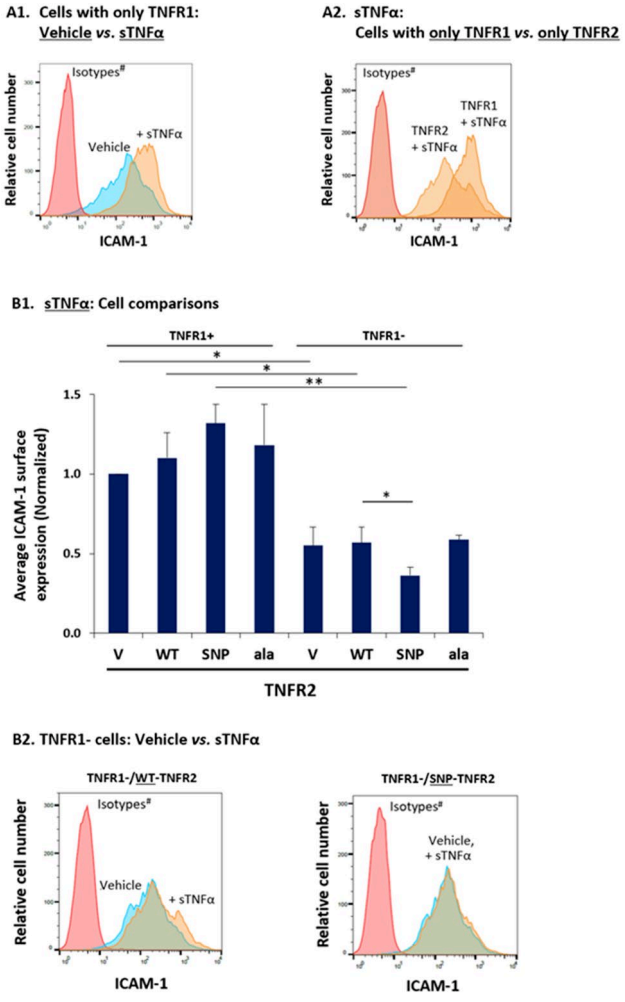


Fig. 4 M196R-SNP-TNFR2 reduces sTNFα-induced ICAM-1 expression by TNBC cells. (**A**, **B**) MDA cells of the 8-cell system were stimulated with sTNFα (25 ng/ml) or vehicle for 48 h, followed by determination of cell surface expression levels of ICAM-1 by flow cytometry. (**A1**) ICAM-1 expression levels in cells expressing only TNFR1 (TNFR1+/V-TNFR2), stimulated or non-stimulated by sTNFα. (**A2**) ICAM-1 expression levels in cells expressing only TNFR1 (TNFR1+/V-TNFR2) and of cells expressing only WT-TNFR2 (TNFR1-/WT-TNFR2), exposed similarly to sTNFα or vehicle. For both cell types, non-relevant isotype-matched antibodies were used as controls (#only one is shown). (**B1**) The average ± SD values of normalized ICAM-1 expression levels obtained by the cells of the 8-cell system is demonstrated (the value in TNFR1+/V-TNFR2 cells was given the value of 1), following sTNFα stimulation. (**B2**) ICAM-1 expression levels, with and without sTNFα stimulation, in cells expressing only WT-TNFR2 (TNFR1-/WT-TNFR2) or only M196R-SNP-TNFR2 (TNFR1-/SNP-TNFR2). For both cell types, non-relevant isotype-matched antibodies were used as controls (#only one is shown). Parts A and B2 demonstrate findings of a representative experiment of $n > 3$ independent experiments that have shown similar results. $^{**}p < 0.01$, $^{*}p < 0.05$

Together, these results indicate that in TNBC cells, TNFR1 is the major receptor mediating the responses to sTNFα in terms of expression of pro-metastatic molecules such as CXCL8 and ICAM-1. Of importance is the fact that independently of TNFR1 expression, M196R-SNP-TNFR2 suppressed sTNFα-induced CXCL8 production; M196R-SNP-TNFR2 also attenuated sTNFα-induced ICAM-1 expression, in cells lacking TNFR1 expression.

mTNFα inhibits the signaling and functions of TNFR1 and TNFR2 in response to sTNFα

Considering that the prime ligand for TNFR2 is mTNFα and that mTNFα is expressed in tumor cells in over 80% TNBC patients [19], we next analyzed the way mTNFα regulates the signaling and functions of the cells. To this end, we induced the expression of mTNFα in selected cells of the 8-cell system, by using a vector coding for a Δ12-TNFα variant lacking the cleavage site that enables the generation of sTNFα. The resulting cells expressed TNFα at the cell membrane and unlike WT-TNFα-infected cells, did not release any sTNFα to the cell culture medium (Fig. S8A-C). Of note, the sTNFα released by WT-TNFα-expressing tumor cells was functional in inducing p65 activation (Fig. S8D), providing evidence of the intact functionality of the extracellular domain of TNFα, which is a part of mTNFα too.

First, we analyzed TNFR1- cells that expressed WT-TNFR2 as well as mTNFα (TNFR1-/WT-TNFR2/***mTNFα);*** these cells did not produce sTNFα and were studied in parallel to control vector-infected cells that did not express mTNFα (TNFR1-/WT-TNFR2/***V***) (Figs. 5A1, S8A1). The ability of TNFR1-/WT-TNFR2/*V* cells to respond to sTNFα stimulation (relatively low, in line with the corresponding TNFR1-/WT-TNFR2 cells in Figs. 2, 3 and 4) was abrogated when mTNFα was expressed in the cells, at all levels assessed: p65 activation, CXCL8 production and ICAM-1 expression (Fig. 5B and C, D1; Densitometry: Fig. S9A).

Although TNFR1-/SNP-TNFR2 cells did not respond to stimulation by sTNFα at any level (Fig. 5B, C and D2, as also demonstrated before in Figs. 2, 3 and 4), the complexity of the TNFα-TNFR network has led us to determine the impact of mTNFα on the activities of these cells,

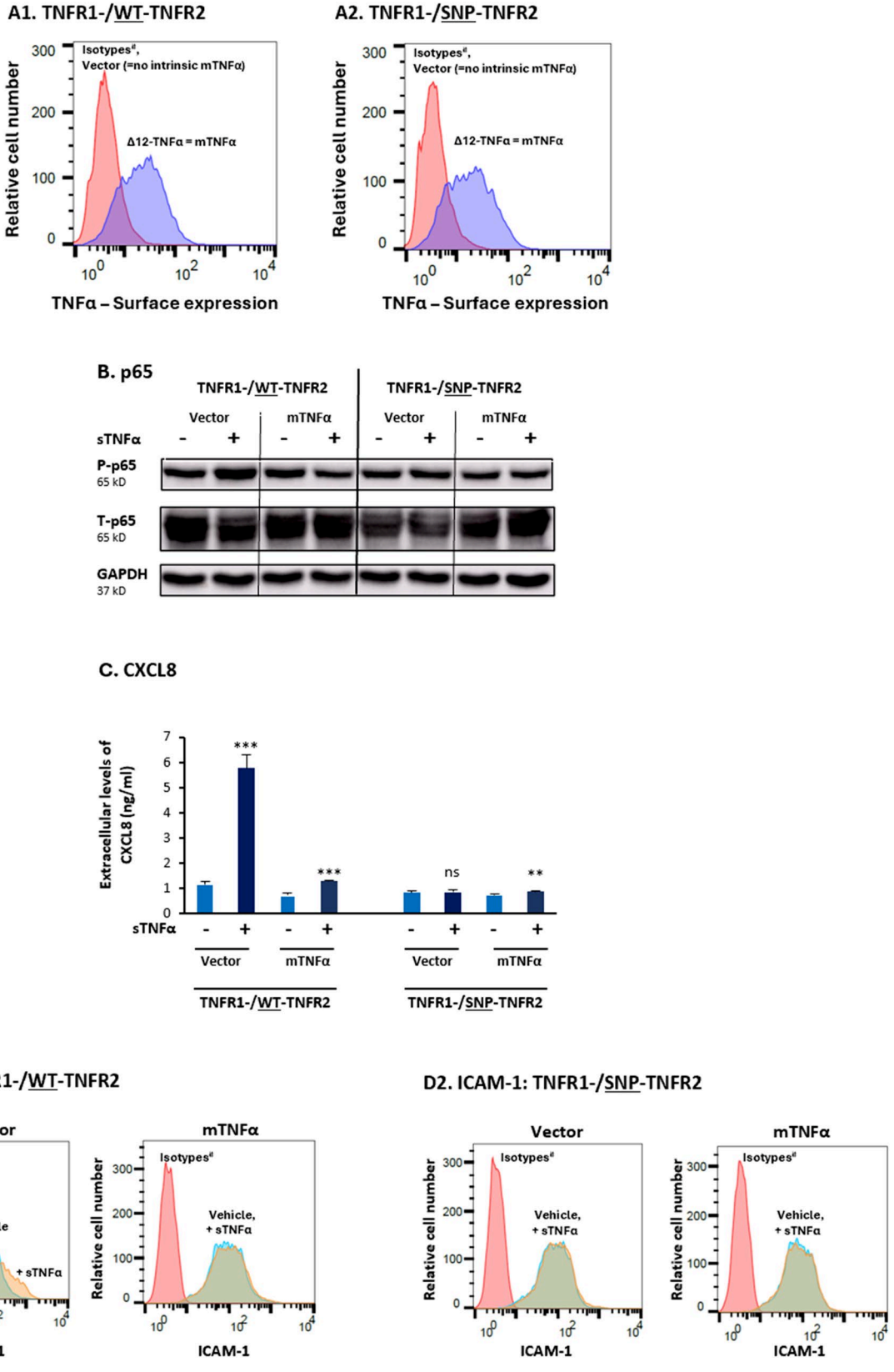


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Fig. 5 mTNFα inhibits sTNFα-induced signaling and functions in TNBC cells expressing TNFR2 only. (**A**) The expression of mTNFα was determined in TNFR1- MDA cells expressing WT-TNFR2 (**A1**) or M196R-SNP-TNFR2 (**A2**). To this end, TNFR1-/WT-TNFR2 cells (**A1**) and TNFR1-/SNP-TNFR2 cells (**A2**) were infected to express Δ12-TNFα, giving rise to mTNFα expression by the cells; vector-infected cells that did not express mTNFα were used in parallel. TNFα expression at the cell membrane was determined by flow cytometry, using non-relevant isotype-matched antibodies as controls (#only one is shown). The corresponding Figures S8A1 and A2 demonstrate that cells expressing mTNFα did not release sTNFα at all. (**B**-**D**) The cells generated in Part (**A**) were subjected to stimulation with sTNFα (50 ng/ml), and then to determination of p65 activation (**B**), CXCL8 production (**C**) and ICAM-1 expression (**D1**, **D2**), using the procedures described in Figs. 2, 3 and 4. Densitometry results of p65 activation are demonstrated in Figure S9A. In flow cytometry, non-relevant isotype-matched antibodies were used for both cell types, as controls (#only one is shown). All panels demonstrate findings of a representative experiment of $n > 3$ independent experiments that have shown similar results. ***$p < 0.001$. **$p < 0.01$. ns, Not significant

as well. We found that the expression of mTNFα in cells with only M196R-SNP-TNFR2 (Fig. S8A2) did not affect the responses of the resulting TNFR1-/SNP-TNFR2 cells, and they remained unresponsive to sTNFα stimulation (Fig. 5B, C and D2; Densitometry: Fig. S9A).

Next, we determined the effects of mTNFα on signaling induced by sTNFα stimulation in cells expressing both TNFR2 and TNFR1. To this end, mTNFα was expressed in TNFR1+/WT-TNFR2 and TNFR1+/SNP-TNFR2 cells, thus generating TNFR1+/WT-TNFR2/***mTNFα*** and TNFR1+/SNP-TNFR2/***mTNFα*** cells, respectively. These cells, which did not produce sTNFα (Figs. S8B1, B2), were compared to their corresponding vector controls that did not express mTNFα: TNFR1+/WT-TNFR2/***V*** and TNFR1+/SNP-TNFR2/***V*** cells (Figs. 6A1, A2; Figs. S8B1, B2).

The findings of Fig. 6 indicate that the expression of mTNFα has given rise to a profound down-regulation of the response to sTNFα in cells expressing both TNFR1 and TNFR2. Specifically, whereas TNFR1+/WT-TNFR2/*V* cells demonstrated a notable response to stimulation by sTNFα in terms of p65 and p38 activation, the responses of TNFR1+/WT-TNFR2/*mTNFα* cells to sTNFα were relatively lower (Figs. 6B1, B2; Densitometry: Figs. S9B1, B2). Likewise, TNFR1+/WT-TNFR2/*mTNFα* cells that were stimulated by sTNFα demonstrated reduced production of CXCL8, expression of ICAM-1 and invasion, compared to their vector controls (TNFR1+/WT-TNFR2/*V*) (Fig. 6C, D1 and E1). In parallel, the expression of mTNFα in TNFR1+/SNP-TNFR2 cells (TNFR1+/SNP-TNFR2/*mTNFα*) also has led to major down-regulation of sTNFα-induced p65 and p38 activation, CXCL8 production, ICAM-1 expression, and cell invasion (Fig. 6B, C, D2 and E2; Densitometry: Figs. S9B1, B2).

Together, these findings identified mTNFα as a negative regulator of sTNFα-induced signaling and functions in cells expressing TNFR2, either alone or in the presence of TNFR1, raising the possibility that in both cell types TNFR2 is the receptor that was inhibited by mTNFα. This possibility is supported by the fact that mTNFα is the favorable ligand of TNFR2. However, mTNFα also functions as a TNFR1 ligand [2, 10, 11], leading us to inquire whether TNFR1 can be also inhibited by mTNFα.

To this end, we generated TNFR1+ cells that expressed mTNFα without TNFR2 (TNFR1+/V-TNFR2/***mTNFα***) and their corresponding vector controls (TNFR1+/V-TNFR2/***V***) that did not express mTNFα (Figs. 7A, S8C); as seen in Fig. S8C, the mTNFα-expressing cells (as well as their vector control) did not produce sTNFα. In these TNFR1+/V-TNFR2/*mTNFα* cells, all signaling and functional responses to sTNFα were strongly inhibited when they were compared to TNFR1+/V-TNFR2/*V* cells: p65 and p38 activation (Figs. 7B1, B2; Densitometry: Figs. S9C1, C2), as well as c-Jun activation (Fig. S10); in parallel, the ability of sTNFα to induce CXCL8 production and elevation of ICAM-1 expression was much reduced in cells expressing mTNFα (Figs. 7C, D). sTNFα stimulation also could not elevate the invasive properties of the cells expressing mTNFα (TNFR1+/V-TNFR2/*mTNFα)*, compared to their corresponding control cells (TNFR1+/V-TNFR2/*V*). Of interest is the fact that in the lack of sTNFα stimulation, the basal invasion level of TNFR1+/V-TNFR2/*mTNFα* cells was higher than of TNFR1+/V-TNFR2/*V* control cells (Fig. 7E), suggesting that the interactions between mTNFα and TNFR1 on neighboring cells can activate invasive reactions in the absence of sTNFα stimulation.

Overall, our data point at a unique control mechanism in which mTNFα inhibits diverse metastasis-promoting activities induced by sTNFα in TNBC cells, through both TNFR1 and TNFR2.

TNFα-induced p65 activation in TNBC cells is correlated with reduced ERK1/2 activation, and in cells expressing mTNFα is associated with down-regulation of TRAF2 expression

Our data so far indicate that TNFR1 is the main receptor responding to sTNFα stimulation in TNBC cells, and that mTNFα down-regulates its activities. To identify the mechanisms regulating TNFR1 responses under these conditions, we searched for regulators of p65 activation in cells expressing TNFR1, with or without mTNFα. Here, we focused on the MEK/MAPK pathway by analyzing ERK1/ERK2, found in our study to be constitutively strongly activated in the cells, and during cell growth in culture for different time points (Figs. 7F1 and S11A). In this experiment, the cells were incubated with the MEK inhibitor U0126 for 2 h, 24 h and 48 h (in comparison to

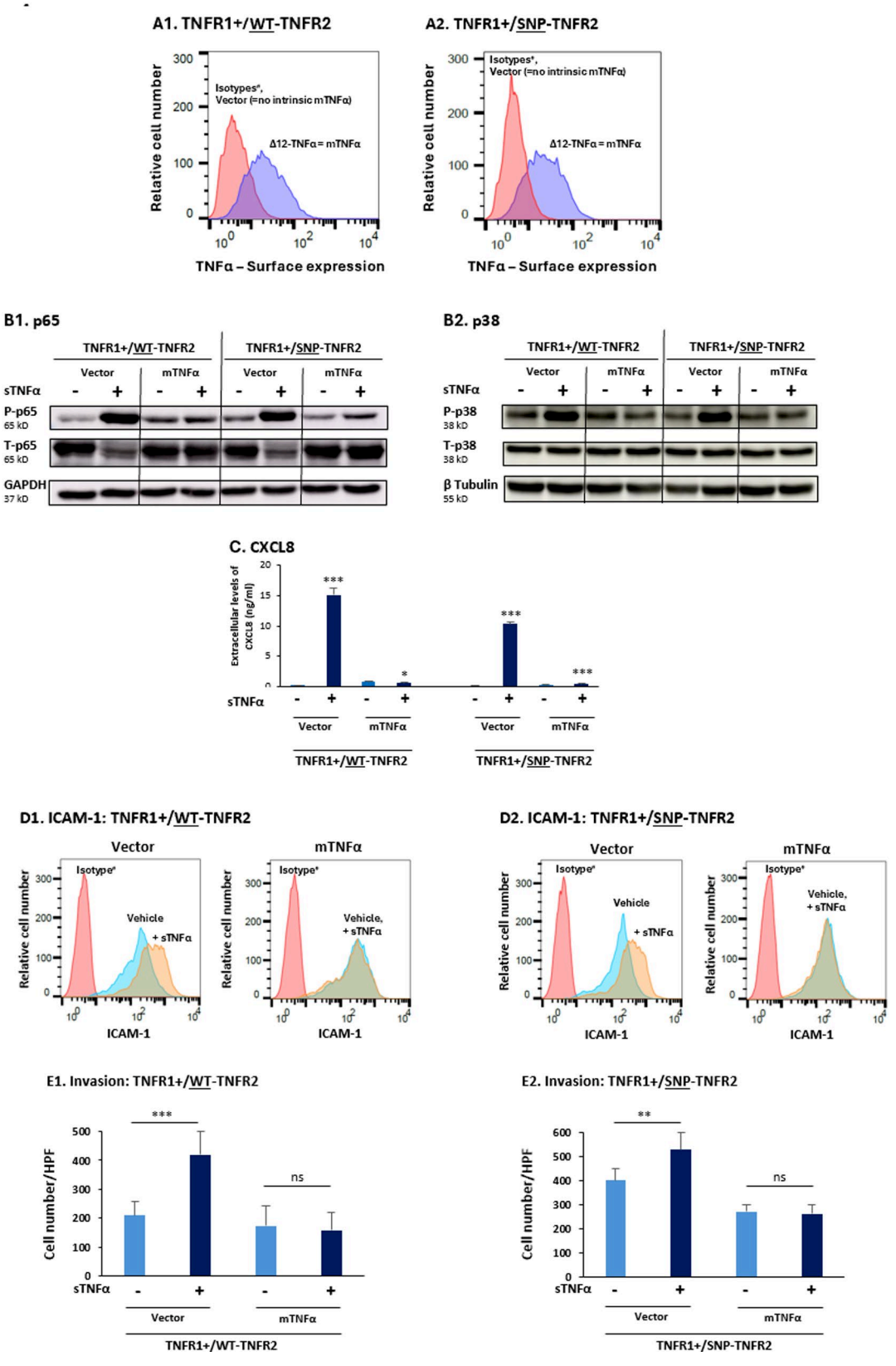


Fig. 6 (See legend on next page.)

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Fig. 6 mTNFα inhibits sTNFα-induced signaling and functions in TNBC cells expressing TNFR1 + TNFR2. (**A**) The expression of mTNFα was determined in MDA cells expressing TNFR1 + WT-TNFR2 (**A1**) or TNFR1 + M196R-SNP-TNFR2 (**A2**). To this end, TNFR1+/WT-TNFR2 and TNFR1+/SNP-TNFR2 cells were infected to express Δ12-TNFα, giving rise to mTNFα expression by the cells; vector-infected cells that did not express mTNFα were used in parallel. TNFα expression at the cell membrane was determined by flow cytometry, using non-relevant isotype-matched antibodies as controls (#only one is shown). The corresponding Figures S8B1 and B2 demonstrate that cells expressing mTNFα did not release sTNFα at all. (**B**-**E**) The cells generated in Part (**A**) were subjected to stimulation by sTNFα (50 ng/ml) and then to determination of p65 activation (**B1**), p38 activation (**B2**), CXCL8 production (**C**), ICAM-1 expression (**D1**, **D2**) and invasion (**E1**, **E2**), using the procedures described in Figs. 2, 3 and 4. Densitometry results of p65 and p38 activation are demonstrated in Figures S9B1 and B2, respectively. In flow cytometry, non-relevant isotype-matched antibodies were used for both cell types, as controls (#only one is shown). All panels demonstrate findings of a representative experiment of $n > 3$ independent experiments that have shown similar results. ***$p < 0.001$, **$p < 0.05$, *$p < 0.01$. ns, Not significant

its vehicle control (DMSO) that did not affect the basal high expression levels of ERK1/ERK2; Fig. S11A), and were then stimulated by sTNFα for 15 min or its vehicle (also not affecting the basal levels of ERK; Fig. S11B).

Figure 7F1 demonstrates that U0126 has led rapidly (after 2 h) to a potent down-regulation of ERK1/2 activation, with only minimal effects on p38 or c-Jun activation at the same time point (Fig. S10). In parallel, and in a time-dependent manner, p65 activation has been gradually increased in cells expressing TNFR1, independently of mTNFα expression (TNFR1+/V-TNFR2/*mTNFα*, TNFR1+/V-TNFR2/*V*). These findings suggest that a connection may exist, either directly or indirectly, between the MEK/MAPK and p65 pathways upon sTNFα stimulation, independently of mTNFα expression by the cells.

In parallel, we analyzed mTNFα-affected TNFR2-linked pathways that may be connected to reduced p65 activation following 15 min of stimulation by sTNFα. Our data indicate that p100 expression levels were not affected in control TNFR1+/V-TNFR2/*V* cells by sTNFα, and p100 was not processed to p52 (Fig. S10B). Moreover, p100 expression levels were not affected and the protein was not processed to p52 also in sTNFα-stimulated TNFR1+/V-TNFR2/*mTNFα* cells (Fig. S10B), suggesting that this is not a major route of TNFR2-induced downstream effects in the current system.

However, in the presence of mTNFα, the levels of TRAF2 - a key participant in sTNFα-induced activation of TNFR1 and TNFR2 [40] - were lower in TNFR1+/V-TNFR2/*mTNFα* cells than in vector-expressing cells (TNFR1+/V-TNFR2/*V* cells) (Fig. 7F2). In parallel, in the same experiments, p65 activation in mTNFα-expressing cells was reduced following sTNFα stimulation. These findings suggest that mTNFα expression per se leads to TRAF2 down-regulation and consequently to reduced sTNFα-induced signaling and thus also to its pro-metastatic responses.

Discussion and conclusions

Unlike other studies that have connected TNFR2 expression to poor prognosis, in this TNBC-focused research we demonstrated that TNFR2 expression by the tumor cells was significantly connected to improved patient survival. To follow up on these findings, we set to determine the roles of WT-TNFR2 and its variant forms in regulating the pro-metastatic functions of TNBC cells. These analyses were performed in the absence or presence of TNFR1. The functions of TNFR2 and TNFR1 - each alone and together - were determined in the context of sTNFα and mTNFα, thus accounting for the different preferences of the two receptors towards the two forms of ligand.

Our research has provided evidence of three novel molecular observations on the TNFα-TNFR axis, which apply to TNBC but may also have important implications to other malignancies as well:

(1) By dissociating the signals delivered by TNFR1 and WT-TNFR2, our study shows that TNFR1 is the key inducer of sTNFα-induced pro-metastatic effects in TNBC cells, while TNFR2 is less active. These findings reflect the preferred binding of sTNFα to TNFR1, reported in other systems [2, 10, 11]. Thus, the network of sTNFα interactions with TNFR1 and TNFR2 is kept intact in TNBC cells, enabling them to respond by elevated pro-metastatic functions when they are exposed to sTNFα which is present in the patients [21], and exploit the cytokine for their own benefit.

(2) Our findings indicate that WT-TNFR2 and more so M196R-SNP-TNFR2 can down-regulate the sTNFα-induced pro-metastatic functions in TNBC cells. Following stimulation with sTNFα, TNFR1+ and TNFR1- cells demonstrated lower spontaneous migration (IncuCyte) if WT-TNFR2 was expressed in them as well. Most importantly, sTNFα-induced tumor cell motility and CXCL8 production were even more reduced when TNFR1+ and TNFR1- cells expressed the M196R-SNP-TNFR2; sTNFα-induced ICAM-1 expression was also down-regulated in M196-SNP-TNFR2-expressing cells that lacked TNFR1. Likewise, in cells lacking TNFR1 expression, M196R-SNP-TNFR2 has down-regulated the ability of sTNFα to induce p65, p38 and JNK activation. These findings demonstrate that M196R-SNP-TNFR2 down-regulates the pro-inflammatory and pro-metastatic functions of TNBC cells, primarily in cells lacking TNFR1;

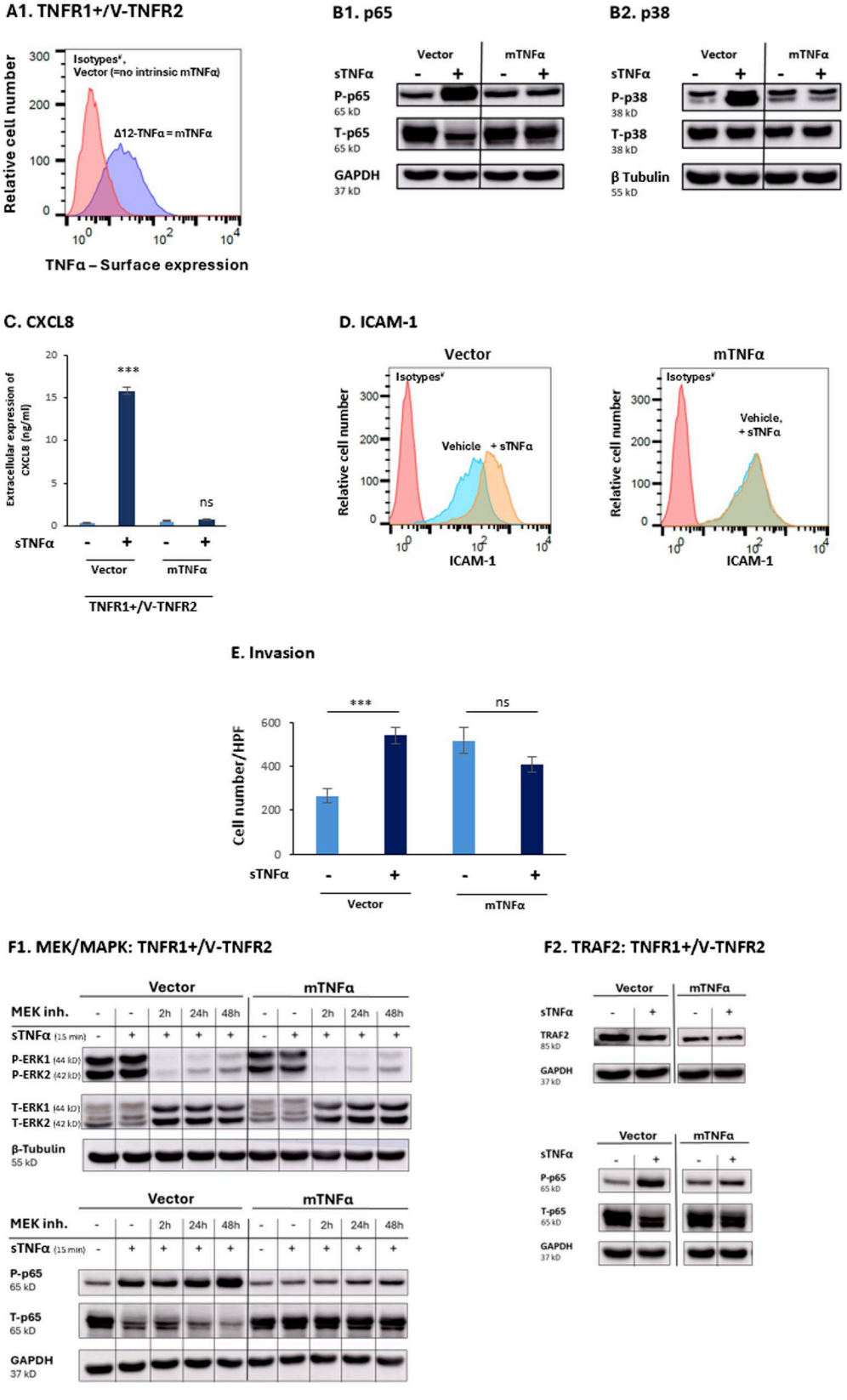


Fig. 7 (See legend on next page.)

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Fig. 7 mTNFα inhibits sTNFα-induced signaling and functions in breast tumor cells expressing TNFR1 only, accompanied by TRAF2 down-regulation. (**A**) The expression of mTNFα was determined in MDA cells expressing TNFR1 only. To this end, TNFR1+/V-TNFR2 cells were infected to express Δ12-TNFα giving rise to mTNFα expression by the cells; vector-infected cells that did not express mTNFα were used in parallel. TNFα expression at the cell membrane was determined by flow cytometry, using non-relevant isotype-matched antibodies as controls (#only one is shown). The corresponding Figure S8C demonstrates that cells expressing mTNFα did not release sTNFα at all. (**B**-**E**) The two cell types generated in Part (**A**) were subjected to stimulation by sTNFα (50 ng/ml) and then to determination of p65 activation (**B1**), p38 activation (**B2**), CXCL8 production (**C**), ICAM-1 expression (**D**) and invasion (**E**), using the procedures described in Figs. 2, 3 and 4. Densitometry results of p65 and p38 activation are demonstrated in Figures S9C1 and S9C2, respectively. In flow cytometry, non-relevant isotype-matched antibodies were used for both cell types, as controls (#only one is shown). **(F)** Determining mechanisms involved in sTNFα and mTNFα activities in TNFR1+ cells. (**F1**) TNFR1+/V-TNFR2/*V* and TNFR1+/V-TNFR2/*mTNFα* cells were grown with the MEK inhibitor U0126 or its vehicle (DMSO) for 2 h, 24 h and 48 h and were then exposed to sTNFα (50 ng/ml) or its vehicle for 15 min. The data of Figure S11 demonstrate that DMSO or the solubilizer of TNFα (0.1% BSA in DDW) did not have any effects on ERK1/2 activation. ERK1/2 and p65 activation were determined by WB analyses. (**F2**) TNFR1+/V-TNFR2/*V* and TNFR1+/V-TNFR2/*mTNFα* cells were stimulated by sTNFα (50 ng/ml) or its vehicle for 15 min, followed by determination of TRAF2 expression and p65 activation. All panels demonstrate findings of a representative experiment of $n > 3$ independent experiments that have shown similar results. ***$p < 0.001$. ns, Not significant

our observations are also in line with other reports describing the ability of M196R-SNP-TNFR2 to turn-off pro-inflammatory responses or to improve the response of inflammatory diseases to treatment [35, 41].

TNFR1 is expressed by all breast cancer patient tumors, alongside TNFR2 that is detected in many breast tumors [12–15]. M196R-SNP-TNFR2 is found in ~30% of breast cancer patients, thus it is possible that in our TNBC patient cohort, the expression of this TNFR2 variant leads in some of the patients to limited tumor progression. Unfortunately, samples of our cohort patients are no longer available, thus this possibility cannot be determined. Yet, we suggest that in patients expressing M196R-SNP-TNFR2, treatments blocking TNFR2 are not desired.

(3) The membrane form of TNFα is highly potent in down-regulating the pro-metastatic effects induced by sTNFα in TNBC cells. This effect is mainly expected in cells expressing TNFR2, due to the preference of TNFR2 towards mTNFα binding [2, 10, 11]. However, our data show that mTNFα serves as a protective element against sTNFα-induced pro-metastatic effects also in cells that express TNFR1 only. Further analyses demonstrated that the interaction of TNFR1 with mTNFα has led to reduced expression of TRAF2, a key mediator of TNFR1 activities.

Thus, we have revealed a mechanism that is induced by mTNFα, which may impact the responses of TNBC patients to tumor cell/TME-derived sTNFα. To date, few papers have analyzed the regulation of pro-metastatic functions in cancer by mTNFα; some have demonstrated pro-tumor effects of mTNFα [18, 42, 43], and others suggested anti-tumor effects [44, 45]. Here, we used a designated system that could clearly identify the impact of mTNFα on sTNFα-induced pro-metastatic effects in an isolated manner, in TNBC cells. mTNFα is detected in the tumor cells of majority of breast cancer patients and in 84% of TNBC patients [18, 19]; thus, our in vitro data suggest that mTNFα may be protective and limit to a certain degree the aggressiveness of disease in these patients. This possibility calls for studies that would determine the association of mTNFα in TNBC patients with disease course and patient survival, and for the study of the impact of mTNFα on a broad array of pro-metastatic functions in TNBC, beyond those investigated in the current research.

The general dogma in the field considers TNFR2 as a tumor-supporting factor, acting by increasing the pro-metastatic functions of the tumor cells and the immune-suppressive functions of Tregs [9, 29]. In contrast to those studies, our investigations were the first to analyze the specific group of TNBC patients in a designated manner. Our previously-published reports demonstrated that in TNBC patients, TNFR2+ TILs were connected with improved survival, and that in TNBC mouse models TNFR2 + CD8+ TILs were elevated alongside reduced tumor growth [30, 31]. In parallel, in the current research, we demonstrated that TNFR2 expression by the cancer cells is also associated with better clinical outcome in TNBC patients and that its M196R variant has anti-metastatic effects.

Together, our findings suggest potential protective roles of TNFR2 in TNBC, particularly in its M196R-SNP form, implying that TNFR2 may not be a suitable therapeutic target in TNBC because its inhibition may diminish TNFR2-mediated beneficial effects in TILs and in cancer cells. Moreover, our findings on mTNFα-driven down-regulation of TRAF2 expression in TNFR1+ cells - observed even when the cells were not stimulated by sTNFα - suggest that mTNFα expression by the cancer cells may be advantageous for the patients. In this case, treating patients by modalities that inhibit TNFα may lead to inhibition of the pro-tumoral activities of sTNFα but also of the potentially protective functions of mTNFα.

Thus, it is possible that TNFR2 - particularly in its M196R-SNP form - as well as mTNFα, may not be the optimal targets when inhibition of the TNFα-TNFR axis is desired in TNBC. Instead, our findings suggest that TNFR1 may be a more suitable target for inhibition, as it is the main responder to sTNFα-induced pro-metastatic signals and effects in TNBC. Here, the preferred therapeutically-relevant approach would possibly be to use antibodies or small molecules that are specific to TNFR1, sparing the potentially protective roles of TNFR2 and mTNFα. The clinical implications of these findings are particularly relevant for TNBC, but may also apply for other malignancies and require detailed analysis in other tumor types.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12935-026-04388-y.

Supplementary Material 1.

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Author contributions
Conceptualization, A. B-B.; Methodology, A. B-B., S. S., M. P., T. B., T. M.; Software, M. P., C. K.; Validation, A. B-B., S. S., M. P.; Formal Analysis, S. S., M. P.; Investigation, S. S., M. P., T. M.; Resources, T. M.; Data Curation, S. S., M. P., T. M., T. B., M. D., N. E., A. A. R., T. H., M. S., Y. L., T. K.J., A. M., N. B-L., A. P., D. M-S., E. N. G-Y., I. B; Writing – Original Draft Preparation, A. B-B.; Writing – Review & Editing, A. B-B., S. S., M. P., Y. L.; Supervision, A. B-B.; Project Administration, A. B-B. and T. M.; Funding Acquisition, A. B-B.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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