

# Tumor Necrosis Factor- $\alpha$ Differentially Modulates CD44 Expression in Ovarian Cancer Cells

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## Abstract

Chronic inflammation is implicated in the pathophysiology of ovarian cancer. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a major inflammatory cytokine, is abundant in the ovarian cancer microenvironment. TNF- $\alpha$  modulates the expression of CD44 in normal T lymphocytes and CD44 is implicated in ovarian carcinogenesis and metastases. However, little is known about the role of TNF- $\alpha$  in CD44 expression of cancer cells. Recent clinical work using TNF- $\alpha$  inhibitors for the treatment of ovarian cancer makes the study of TNF- $\alpha$  interactions with CD44 crucial to determining treatment a success or a failure. We studied the effect of TNF- $\alpha$  on ovarian cancer cells viability, CD44 expression, and *in vitro* migration/invasion. Our results revealed that TNF- $\alpha$  differentially modulates the expression of CD44 in TNF- $\alpha$ -resistant ovarian cancer cells, affecting their *in vitro* migration, invasion, and binding to hyaluronic acid. TNF- $\alpha$  up-regulation of CD44 expression was dependent on the activation of c-Jun NH<sub>2</sub>-terminal kinase (JNK) and this activation was accompanied by an increase in their invasive phenotype. On the contrary, if TNF- $\alpha$  failed to induce JNK phosphorylation, the end result was down-regulation of both CD44 expression and the invasive phenotype. These results were confirmed by the use of JNK inhibitors and a TNF receptor competitive inhibitor. (Mol Cancer Res 2006;4(8):511–20)

## Introduction

There is compelling evidence supporting a link between inflammation and the development of ovarian cancer (1-3). Various inflammatory cytokines, particularly tumor necrosis

factor- $\alpha$  (TNF- $\alpha$ ), are present in the ovarian microenvironment. TNF- $\alpha$  is a pleiotropic cytokine and may play a role in regulating the biology of ovarian cancer. For example, macrophages expressing TNF- $\alpha$  are often found surrounding ovarian tumors; ovarian cancer cells produce macrophage colony-stimulating factor, which is a potent chemoattractant for monocytes to secrete TNF- $\alpha$  (4); TNF- $\alpha$  is produced by ovarian tumors and is abundant in malignant ascites of patients with ovarian cancer. On the other hand, TNF- $\alpha$  has cytolytic activity against some but not all ovarian tumors (5-7). However, the fate of those ovarian cancer cells that escape TNF- $\alpha$  cytotoxicity or how a TNF- $\alpha$ -rich microenvironment affects these TNF- $\alpha$ -resistant cancer cells is not known.

Adhesion molecules play an important role in these tumor-environment interactions. CD44 is a multifunctional membrane receptor involved in cell adhesion, motility, and metastases (8). The gene that encodes CD44 contains 19 exons and it is alternatively spliced giving rise to many CD44 isoforms that probably impart the receptor its many functions (9). When the central 10 exons are spliced out, the CD44 standard isoform (CD44s) is expressed. CD44s is a  $M_r$  ~ 85,000 glycoprotein and is a major receptor for hyaluronic acid (10, 11). Hyaluronic acid is the principal glycosaminoglycan found in extracellular matrices and is a major component of the peritoneum, the most important site for ovarian cancer metastases (10, 12).

CD44 is a useful tumor marker for progression and metastasis in certain types of cancers. Our laboratory, as well as others, has shown that CD44 is not usually expressed in the normal ovary (13-16). However, CD44 is expressed in 40% to 60% of the primary ovarian tumors. CD44 expression may be specific to certain histologies. For example, CD44 is expressed in 86% of clear cell tumors of the ovary, one of the most aggressive histologic types, and is only expressed in 9% of endometrioid tumors that carry a better prognosis (15-18). Our laboratory, as well as others, has evidence implicating CD44 and its interactions with the tumor stroma and the tumor microenvironment as relevant to ovarian cancer metastatic growth (16, 19). In T cells, TNF- $\alpha$  affects cell migration by regulating CD44 expression through the activation of mitogen-activated protein kinases (MAPK). Given a recent report using TNF- $\alpha$  inhibitors to treat women with ovarian cancer, it becomes clinically relevant to determine whether TNF- $\alpha$  affects ovarian cancer similarly (20). The present work investigates whether TNF- $\alpha$  modulates CD44 expression and function in ovarian cancer cells that escape TNF- $\alpha$  cytotoxicity.

Received 11/11/05; revised 6/2/06; accepted 6/26/06.

**Grant support:** The Cancer Institute of New Jersey; the following shared resource: DNA Sequencing and Synthesis Core Facility and the Robert Black Charitable Foundation Grant and USPHS, National Cancer Institute grant CA66077.

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doi:10.1158/1541-7786.MCR-05-0232

## Results

### *TNF- $\alpha$ Differentially Modulates Viability and Proliferation of Ovarian Cancer Cells*

The cytotoxic activity of TNF- $\alpha$  in ovarian cancer cells is well known. However, some cells circumvent TNF- $\alpha$ -mediated toxicity (5). We first determined TNF- $\alpha$  cytotoxicity on four different ovarian cancer cell lines. Figure 1A depicts the effect of different concentrations of TNF- $\alpha$  (0-100 ng/mL) on ovarian cancer cell growth at different time points. We found that TNF- $\alpha$  decreased cell growth and was cytotoxic to MLS and SAU cell lines. However, SKA and SKOV3 cell lines were resistant to TNF- $\alpha$  cytotoxic effects. The results of [ $^3$ H]thymidine incorporation experiments and a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay corroborated these findings (Fig. 1B and C).

### *TNF- $\alpha$ Modulates CD44 Expression in Ovarian Cancer Cells*

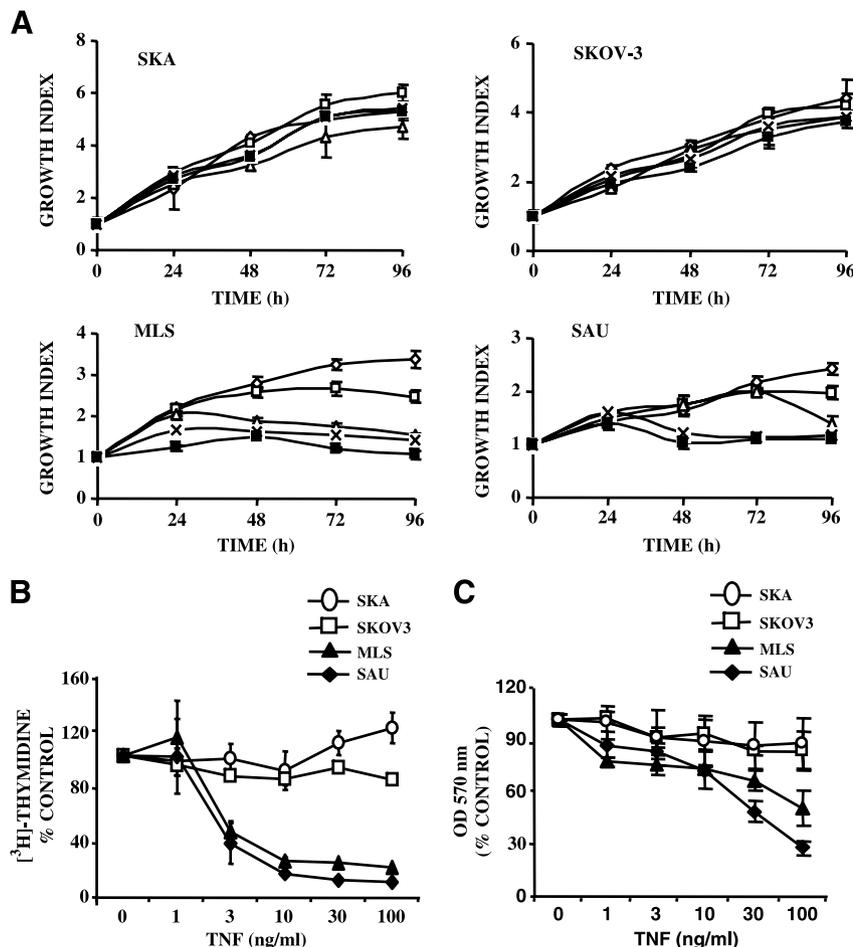
TNF- $\alpha$  is known to up-regulate CD44 expression in monocytes and astrocytes (21, 22). We therefore investigated whether TNF- $\alpha$  had a similar effect in ovarian cancer cells. We chose SKA and SKOV3 cells for the following experiments because they are resistant to TNF- $\alpha$  cytotoxic effects. As illustrated on Fig. 2A, TNF- $\alpha$  treatment reduced the level of CD44s protein expression by 40% in SKA cells. In contrast,

SKOV3 cells showed instead an increase in CD44s expression of 150% to 200%.

We tested the effect of TNF- $\alpha$  on the expression of other CD44 isoforms for which reliable antibodies are available. In SKA cells, TNF- $\alpha$  treatment also resulted in the reduced expression of some CD44 isoforms. CD44-v5 expression decreased by 60% and CD44-v10 expression decreased by 30%. SKOV3 cells do not have a baseline expression of either isoform and remained, as expected, without expression of these isoforms after TNF- $\alpha$  exposure (Fig. 2A). TNF- $\alpha$  did not have an effect on the expression level of other isoforms tested (CD44-v3, CD44-v4, CD44-v6, and CD44-v7; data not shown) on either cell line. We also determined whether TNF- $\alpha$  affected the expression of CD44 at the mRNA level. SKA and SKOV3 cells were treated with TNF- $\alpha$  for 48 hours. Expression of mRNA was measured using semiquantitative reverse transcription-PCR (RT-PCR). TNF- $\alpha$  decreased the mRNA expression of *CD44s* and *CD44-v5* by 90% and 40%, respectively, in SKA cells and had no effect on *CD44-v10* mRNA levels. In contrast, TNF- $\alpha$  induced the expression of *CD44s* in SKOV3 cells by 30% to 50% (Fig. 2B).

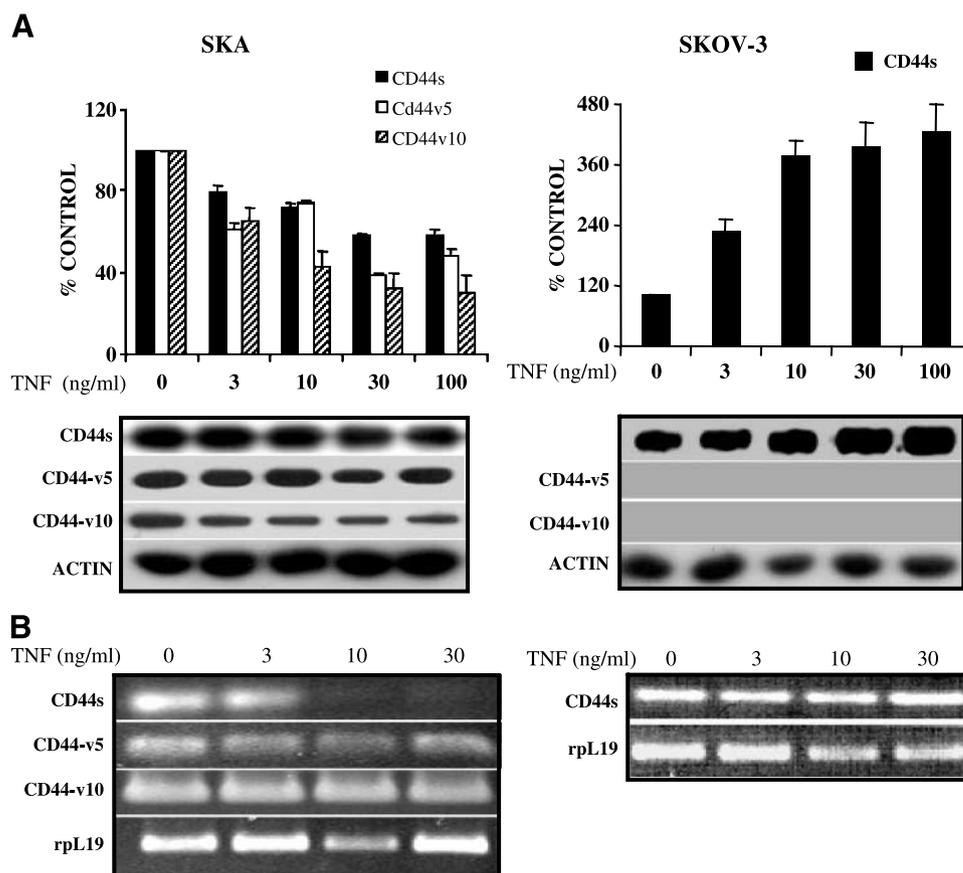
### *TNF- $\alpha$ Affects In vitro Invasion on Matrigel*

Because the CD44 receptor is involved in cell migration and tumor invasion, we examined whether the change in



**FIGURE 1.** TNF- $\alpha$  differentially modulates viability and proliferation of ovarian cancer cells. **A.** TNF- $\alpha$  differentially modulates cell viability. Four different ovarian carcinoma cells (SKA, SKOV3, MLS, and SAU) were incubated in the presence of TNF- $\alpha$  [0 ( $\circ$ ), 3 ( $\square$ ), 10 ( $\Delta$ ), 30 ( $\times$ ), and 100 ( $\blacksquare$ ) ng/mL] for different time periods (0-96 hours) and the cell number was measured by trypan blue exclusion (see Materials and Methods). The cell count at time point 0 was taken as 1 and compared with TNF- $\alpha$ -treated cells. Representative of three independent experiments. Points, mean of triplicates; bars, SE. **B.** TNF- $\alpha$  differentially modulates cell proliferation as measured by [ $^3$ H]thymidine incorporation. Ovarian carcinoma cells (60% confluence) were incubated for 48 hours with different concentrations of TNF- $\alpha$  in complete medium and pulsed with 1  $\mu$ Ci [ $^3$ H]thymidine for 18 hours. Representative of four independent experiments. Points, mean of eight replicates; bars, SE. **C.** MTT assay corroborates the above findings.

**FIGURE 2.** TNF- $\alpha$  differentially affects the expression of CD44 at protein (**A**) and mRNA (**B**) levels in SKA and SKOV3 cells. **A.** Representative Western blot of SKA and SKOV3 cell lysates immunoblotted with CD44s, CD44-v5, and CD44-v10 antibodies after treatment with TNF- $\alpha$  for 48 hours. The CD44-specific intensity of the band was normalized for actin. Values are % control. The experiment was repeated thrice. **B.** Total RNA was extracted after TNF- $\alpha$  treatment, reverse transcribed, and amplified by RT-PCR as described in Materials and Methods. CD44s, CD44-v5, and CD44-v10 mRNA expression in TNF- $\alpha$ -treated SKA and SKOV3. The equivalence of sample loading was determined via expression of the ribosomal protein rpL19 mRNA transcript. The experiment was repeated twice.



CD44 expression induced by TNF- $\alpha$  had an effect on *in vitro* invasion. SKA and SKOV3 cells were treated with TNF- $\alpha$  for 48 hours and their *in vitro* invasive potential was tested in a Matrigel system. TNF- $\alpha$  reduced *in vitro* invasion of SKA cells by  $40 \pm 10\%$  at concentrations ranging from 30 to 100 ng/mL. In contrast, similar TNF- $\alpha$  treatment of SKOV3 cells resulted in a dose-dependent increase on *in vitro* invasion up to 200% (Fig. 3A). We corroborated the role of CD44 on *in vitro* invasion using transient transfection of CD44 on SKOV3 cells. Figure 3B illustrates that over-expression of CD44 in SKOV3 cells increased *in vitro* invasion potential ( $P < 0.05$ ). We also did knockdown experiments of CD44 in SKA cells that have high baseline expression of CD44 (Fig. 3C). Down-regulation of CD44 expression at the protein level was obtained using two different small hairpin RNA sequences. As expected, *in vitro* invasion decreased when CD44 was knocked down ( $P < 0.05$ ; Fig. 3C). There was neither down-regulation of CD44 nor changes on *in vitro* invasion with transfected empty vector or a negative control small hairpin RNA sequence.

#### TNF- $\alpha$ Differentially Modulates Hyaluronic Acid Binding of Treated Cells

The biological functions of CD44 are attributed to the generation of the functionally active, hyaluronic acid–adhesive form of this molecule (23). Additionally, TNF- $\alpha$  is responsible for the induction of hyaluronic acid–binding CD44 forms in

human monocytes. Therefore, we tested whether TNF- $\alpha$  also had an effect on CD44 binding to hyaluronic acid in ovarian cancer cells. We found that TNF- $\alpha$  lowered hyaluronic acid binding by 40% in SKA cells and increased hyaluronic acid binding of SKOV3 cells by 35% (Fig. 3D).

#### MAPKs Are Differentially Modulated by TNF- $\alpha$ and Regulate CD44 Expression

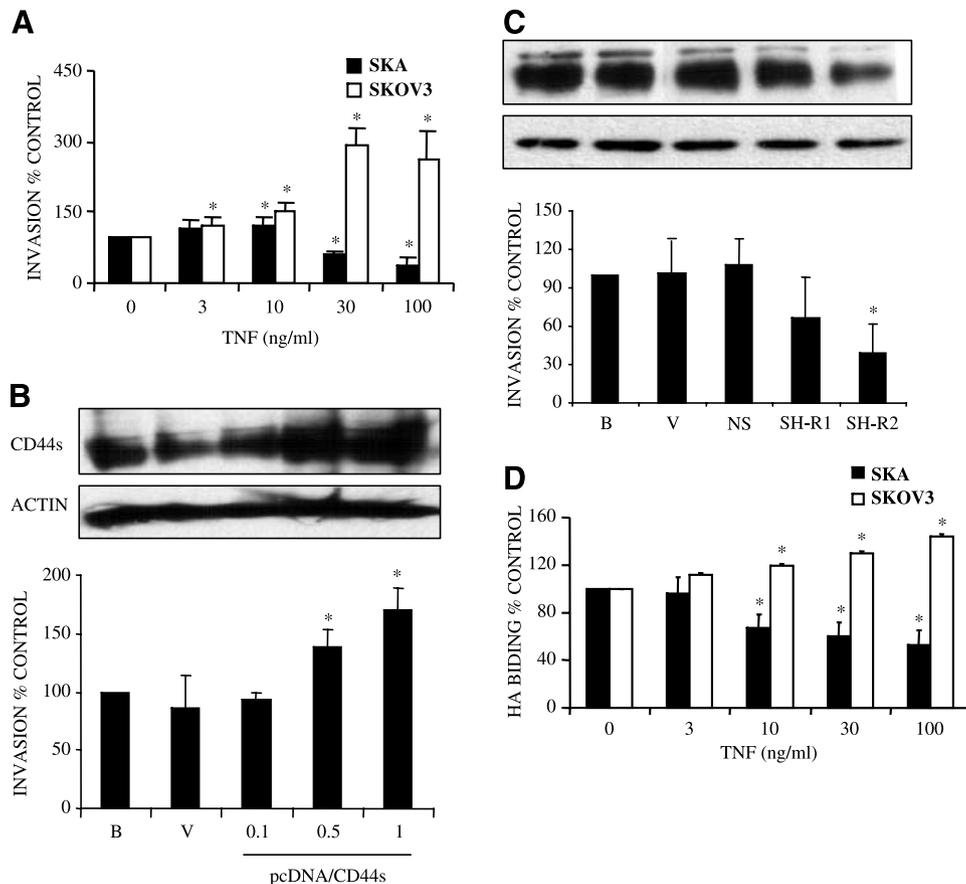
The MAPK pathway that comprise extracellular signal-regulated kinases (ERK), p38 MAPK, and c-Jun NH<sub>2</sub>-terminal kinase (JNK) plays a major role in cytokine-mediated induction of several surface molecules, including the induction of CD44 by TNF- $\alpha$  in monocytes. Therefore, we investigated whether MAPKs were involved in the TNF- $\alpha$ -induced modulation of CD44 observed in the ovarian cancer cells. We investigated the involvement of ERK, p38, and JNK by examining their activation following TNF- $\alpha$  treatment of SKA and SKOV3 cells. We found that the level of phosphorylated ERK (p-ERK) and phosphorylated p38 MAPK increased in both cell lines after TNF- $\alpha$  treatment. To corroborate the effect of TNF- $\alpha$  on ERK and p38 MAPK activation, we used specific MAPKs inhibitors before exposing cells to TNF- $\alpha$ . As expected, the specific inhibitors blocked TNF- $\alpha$ -induced activation of ERK and p38 MAPKs (Fig. 4A and B).

Figure 4C shows that phosphorylated JNK (p-JNK) was also activated after TNF- $\alpha$  exposure but only in SKOV3 cells. SKA cells show a low basal level of p-JNK that

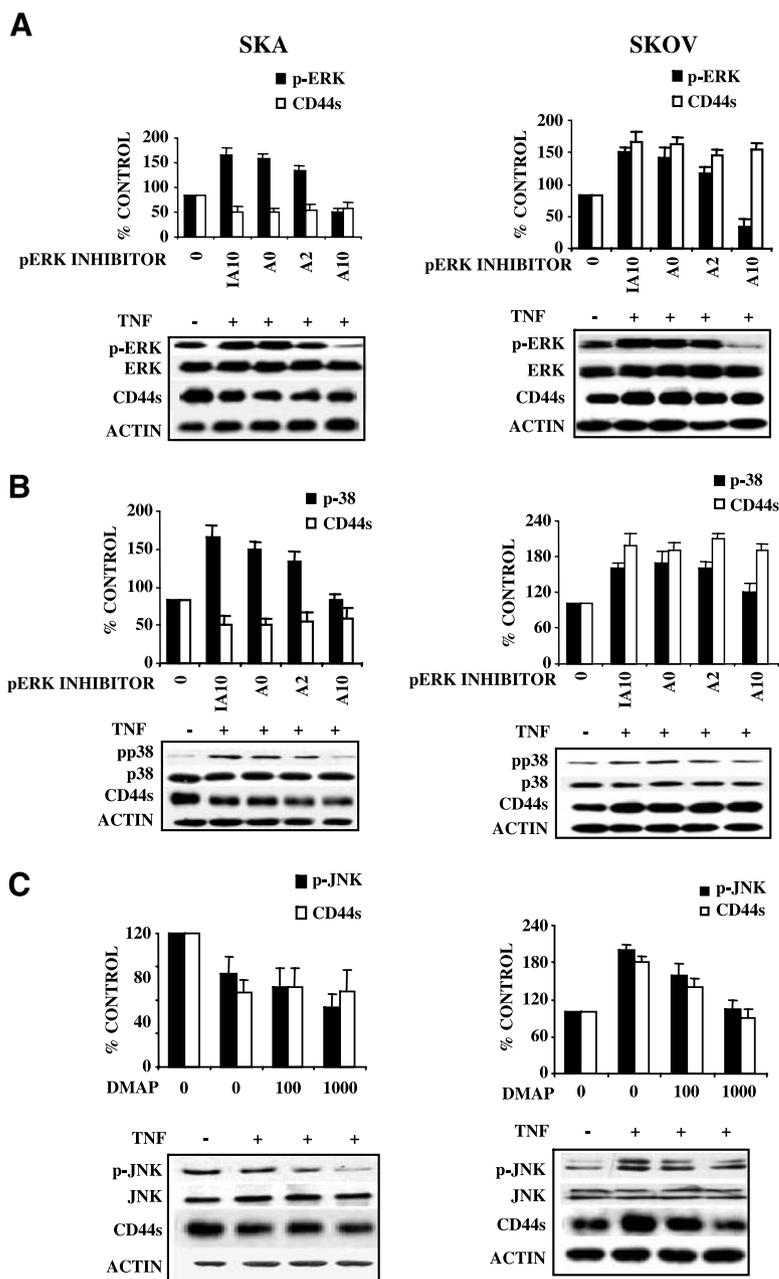
diminished even further when the cells were exposed to TNF- $\alpha$  (Fig. 4C).

The finding that both ERK and p38 MAPKs were activated by TNF- $\alpha$  in both ovarian cancer cell lines, but JNK was activated only in SKOV3, prompted us to examine the possibility of the involvement of JNK in the TNF- $\alpha$ -mediated differential modulation of CD44 expression in these two cell lines. Figure 4A to C shows that although TNF- $\alpha$  induced activation of ERK and p38 MAPKs, when their phosphorylation was inhibited, the level of CD44 expression did not change. On the other hand, when JNK activation was inhibited, CD44 expression decreased to baseline levels in SKOV3 cells. Interestingly, in the SKA cell line where TNF- $\alpha$  down-regulated CD44 expression, we observed a concomitant reduction of JNK phosphorylation after TNF- $\alpha$  treatment. When the constitutively low expression of p-JNK was further decreased with a specific inhibitor, SKA cells underwent a further reduction of CD44 expression. We tested the effect of

JNK phosphorylation inhibition on *in vitro* invasion (Fig. 5A and B). We showed that in SKOV3 cells, when the activation of JNK is inhibited, the TNF- $\alpha$  effect of increasing *in vitro* invasion was inhibited. In SKA cells, where TNF- $\alpha$  already decreases the baseline level of activated JNK, additional inhibition of JNK phosphorylation resulted in further inhibition of *in vitro* invasion. Because TNF- $\alpha$  inhibitors in the form of soluble TNF- $\alpha$  receptor are presently being evaluated for use in the treatment of ovarian cancer, we tested the effect of a soluble TNF- $\alpha$  receptor in CD44 expression, JNK activation, and *in vitro* invasion (Fig. 6). We found that when we used a TNF- $\alpha$  competitive inhibitor in the form of TNF- $\alpha$  soluble receptor, CD44 expression returns to baseline expression, JNK phosphorylation also returns to baseline expression, and, consequently, the invasive phenotype returns to baseline. These findings identify a role for the JNK pathway in the TNF- $\alpha$  regulation of CD44 expression in ovarian cancer cells that are resistant to TNF- $\alpha$  cytotoxicity and provide a mechanism to



**FIGURE 3.** TNF- $\alpha$  treatment differentially modulates *in vitro* Matrigel invasion and hyaluronic acid binding. **A.** TNF- $\alpha$  decreased *in vitro* invasion in SKA cells and increased *in vitro* invasion in SKOV3 cells. Cells were treated with TNF- $\alpha$  (0–100 ng/mL) for 48 hours. Invasion was measured after 18 hours. \*,  $P < 0.05$ , significant differences from control (Student's  $t$  test;  $n = 4$ ). **B.** Overexpression of CD44 increases *in vitro* invasion. *In vitro* invasion was tested on SKOV3 cells transiently transfected with different amount of CD44-expressing plasmid (pcDNA/CD44s). The CD44 protein expression is shown by Western blots. **C.** Down-regulation of CD44 decreases *in vitro* invasion. \*,  $P < 0.05$ , significant differences from control (Student's  $t$  test;  $n = 3$ ). SKA cells were transiently transfected with small hairpin RNA-expressing constructs targeting two different CD44 sequences (*SH-R1* and *SH-R2*). \*,  $P < 0.05$ , significant differences from control (Student's  $t$  test;  $n = 3$ ). Down-regulation of CD44 protein expression is shown by Western blot. **D.** TNF- $\alpha$  treatment differentially modulates hyaluronic acid binding. Cells were exposed to TNF- $\alpha$  for 48 hours, labeled with [ $^3$ H]thymidine, harvested, and seeded into hyaluronic acid-coated wells as described in Materials and Methods. The amount of hyaluronic acid-bound cells was determined by measuring  $^3$ H activity. Values of hyaluronic acid binding in SKA (■) and SKOV3 (□) cells are % untreated control. \*,  $P < 0.05$ , significant differences from control (Student's  $t$  test;  $n = 8$ ). B, baseline; V, vector; NS, negative control.



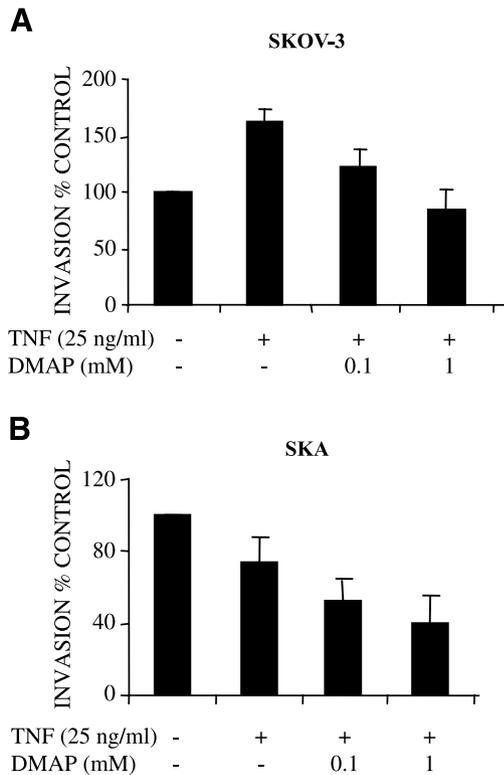
**FIGURE 4.** Effect of TNF- $\alpha$  on ERK (A), p38 MAPK (B), and JNK (C) specific MAPK activity and CD44s expression. **A.** Representative Western blot of p-ERK and CD44s expression of cells treated with either U-0126 (a p-ERK MAPK inhibitor) at 2 and 10  $\mu$ mol/L or U-0124 (an inactive MAPK inhibitor) before treatment with TNF- $\alpha$ . Lane 1, control untreated cells (0). The p-ERK-specific intensity of the band was normalized to unphosphorylated ERK. The CD44-specific intensity of the band was normalized to actin. Values are % control. **B.** Representative Western blot of phosphorylated p38 and CD44s expression in cells treated with either SB-203580 (a p38 MAPK inhibitor) at 2 and 10  $\mu$ mol/L or SB-202474 (an inactive p38 MAPK inhibitor) before treatment with TNF- $\alpha$ . Lane 1, control untreated cells. The phosphorylated p38-specific intensity of the band was normalized to unphosphorylated p38. The CD44-specific intensity of the band was normalized to actin. Values are % control. **C.** Representative Western blot of p-JNK and CD44s expression in cells treated with DMAP (JNK inhibitor) at 100 or 1,000  $\mu$ mol/L before treatment with TNF- $\alpha$ . Lane 1, control untreated cells. The p-JNK-specific intensity of the band was normalized to unphosphorylated JNK. The CD44-specific intensity of the band was normalized to actin. Values are % control. A, active inhibitor; IA, inactive inhibitor.

explain the differential modulation of CD44 expression by TNF- $\alpha$  in ovarian cancer cells.

## Discussion

The interaction of the tumor with its microenvironment determines in part the ability of the tumor to proliferate, migrate, and invade other organs. Hyaluronic acid-rich peritoneal surface is the key metastatic site of ovarian cancer whose disruption may affect peritoneal tumor spread. TNF- $\alpha$  alters the morphology of the peritoneal mesothelium exposing the submesothelial matrix and it also down-regulates the expression of peritoneal CD44 (19). We have shown previously

that CD44 in the ovarian cancer microenvironment, as determined by CD44 stromal expression, correlates with improved survival in ovarian cancer patients (16). Our present work links TNF- $\alpha$  in the ovarian microenvironment with the regulation of CD44 expression and invasive potential in ovarian cancer cells. We show that TNF- $\alpha$  regulates the expression of CD44 in TNF- $\alpha$ -resistant ovarian cancer cells through JNK activation. Several types of experiments support this conclusion. First, as shown in Fig. 2, CD44 expression changes at both protein and mRNA levels when ovarian cancer cells are exposed to TNF- $\alpha$ . Second, Fig. 3A shows that TNF- $\alpha$  treatment changes the *in vitro* invasion potential of ovarian cancer cell lines. Interestingly, in one cell line (SKA), TNF- $\alpha$



**FIGURE 5.** Effect of JNK phosphorylation inhibitor on *in vitro* invasion. SKOV3 (A) and SKA (B) cells were treated with TNF- $\alpha$  and/or DMAP.

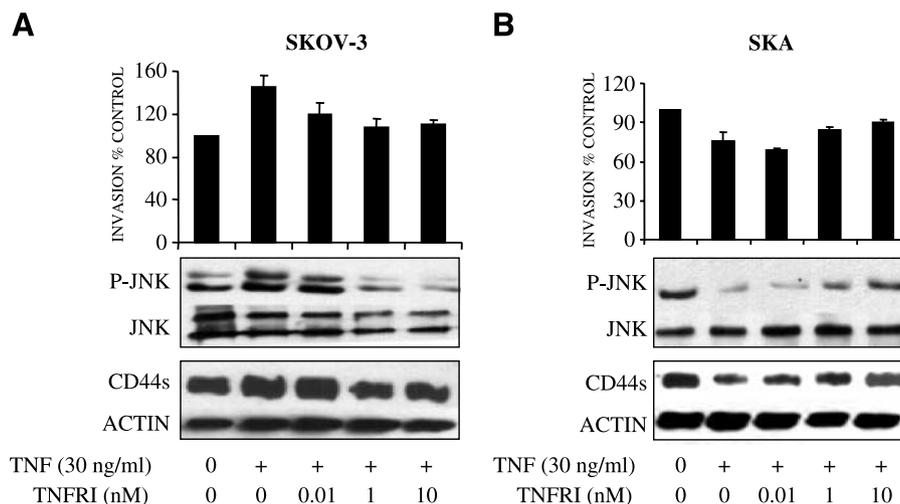
exposure decreased invasion, and in the other cell line (SKOV3), the effect of TNF- $\alpha$  was a dramatic increase of *in vitro* invasion. Third, the change in CD44 expression directly corresponds to the changes of *in vitro* invasion and the ability of the cells to bind hyaluronic acid (Fig. 3D), indicating that the CD44 expressed is functional. Fourth, Fig. 4A to C shows that TNF- $\alpha$  activates JNK in SKOV3 cells and this activation corresponds to an increase in CD44 expression that is inhibited by a JNK kinase inhibitor. On the other hand, TNF- $\alpha$  treatment resulted in a decrease of activated JNK levels in SKA cells.

When JNK inhibitors were added to TNF- $\alpha$ -treated SKA cells, JNK phosphorylation decreased even further; in turn, CD44 expression decreased and this is accompanied by a decrease on *in vitro* invasion below baseline (Fig. 5A and B). The same results were obtained when a TNF- $\alpha$  inhibitor was used in the form of soluble TNF- $\alpha$  receptor (Fig. 6B and C). These experiments show that the effect of TNF- $\alpha$  in CD44 expression is dependent on JNK phosphorylation. Therefore, if TNF- $\alpha$  induces JNK phosphorylation, the end result is increased CD44 expression that in turn results in increased *in vitro* invasion and hyaluronic acid binding. However, when TNF- $\alpha$  is not able to induce JNK phosphorylation, as observed in SKA cells, CD44 expression decreases with the corresponding phenotype of lower *in vitro* invasion and hyaluronic acid binding. The mechanism of this reduction is yet to be determined.

In monocytes, interleukin-10 also increases CD44 expression and monocyte migration through JNK activation. Interestingly, TNF- $\alpha$  induces CD44 expression in monocytes but through p38 and ERK pathways (24).

Our findings stress the importance of understanding the ovarian tumor interactions with its microenvironment. TNF- $\alpha$  is a pleiotropic cytokine and is abundant in the ovarian microenvironment. TNF- $\alpha$  induces the apoptotic pathway in ovarian cancer cells through mechanisms that are not yet clear but that may involve FLIP induction; however, some ovarian cancer cells escape TNF- $\alpha$ -induced apoptosis (5, 25). Two different cell lines that evade TNF- $\alpha$ -induced apoptosis were used in this work. TNF- $\alpha$  regulated CD44 expression differentially on the two cell lines studied depending on its ability to activate JNK. These findings suggest that JNK phosphorylation inhibitors may be effective in the treatment of specific ovarian cancers.

Madhusudan et al. reported on a clinical trial where a TNF- $\alpha$  inhibitor was used in women with recurrent ovarian cancer. They observed stabilization of disease in 20% of this group of heavily pretreated women (20). With the advent of the use of biologicals and targeted therapies for the treatment of cancer, understanding not only the function but also the inhibition of the molecules targeted becomes increasingly relevant.



**FIGURE 6.** Competition with soluble TNF- $\alpha$  receptor inhibits TNF- $\alpha$  effect on *in vitro* invasion, JNK phosphorylation, and CD44 expression. SKOV3 (A) and SKA (B) cells were treated with a soluble TNF receptor (TNFRI). *In vitro* invasion was tested. JNK phosphorylation and CD44 expression were examined by Western blot.

## Materials and Methods

### Cell Lines and Cell Culture

Human ovarian carcinoma cell lines SKA, SAU, and MLS were kindly provided by Dr. John Ludlow (University of Rochester, NY). SKOV3 cells were obtained from American Type Culture Collection (Manassas, VA). The cells, except SKOV3, were maintained in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% FCS (Life Technologies), 100  $\mu$ g/mL streptomycin, and 100 units/mL penicillin (complete medium). Cells were incubated at 37°C under 95% air/5% CO<sub>2</sub> in a standard humidified incubator. SKOV3 cells were maintained in RPMI 1640 (Life Technologies) supplemented with 10% FCS and antibiotics. Confluent monolayer cultures were trypsinized and counted in a hemocytometer by trypan blue exclusion method. The cultures were passaged with 0.05% trypsin/0.02% EDTA (Life Technologies) every 5 days.

### Cell Viability Assay

Viable cells ( $0.5 \times 10^6$ ) were seeded onto 100-mm tissue culture dishes in complete medium. After 24 hours of cell attachment, the cells were incubated in complete medium (7 mL/dish) containing indicated concentrations of TNF- $\alpha$  (0-100 ng/mL) for 24 to 96 hours. Fresh medium was added to the cells every 48 hours. Human TNF- $\alpha$  (210-TA; R&D Systems, Inc., Minneapolis, MN) was diluted from stock solution (concentration, 10  $\mu$ g/mL) in complete medium immediately before each experiment. At each time point, triplicate cell layers were trypsinized, stained with trypan blue, and counted in a hemocytometer. The cell number at time point 0 was taken as 1 and compared with treated cells.

### [<sup>3</sup>H]Thymidine Incorporation

Aliquots (50  $\mu$ L) of the cells ( $2 \times 10^5$ /mL) were added to each well of 96-well plates and allowed to attach for 24 hours. The cells were incubated with 200  $\mu$ L complete medium containing TNF- $\alpha$  at a final concentration of 0 to 100 ng/mL for 48 hours. [<sup>3</sup>H]thymidine (Perkin-Elmer, Boston, MA; 1  $\mu$ Ci/well) was added to the growth medium 18 hours before harvesting. The cells were harvested and the <sup>3</sup>H activity was quantitated. Cell survival is directly related to radioactive incorporation value (26).

### MTT Assay

Cytotoxicity and cell survival was determined by MTT assay (27). Briefly, cells were plated at 15,000 per well in 96-well plates. The next day, cells were treated (200  $\mu$ L/well) with TNF- $\alpha$  (0-100 ng/mL) and incubated at 37°C for 96 hours. At the end of incubation, 10  $\mu$ L MTT (5 mg/mL in PBS; Sigma, St. Louis, MO) was added and the plates were incubated at 37°C for an additional 3 hours. At the end of incubation, the formazan crystals were dissolved in 0.1 mL DMSO and the absorbance was read at 570 nm in a microplate reader (Victor<sup>3</sup>, Perkin-Elmer). The percentage of cell survival was defined as the relative absorbance of untreated versus treated cells. All the assays were done in triplicates and repeated thrice.

### Preparation of Cell Lysate and Western Blot Analysis

Cell lysates were prepared from cells exposed to TNF- $\alpha$  for 48 hours. The cells were washed twice with PBS and harvested and extracted ( $5 \times 10^6$  cells/mL) with (30 minutes at 4°C) lysis buffer as described in ref. 28 with the following modification [25 mmol/L Tris (pH 7.5), 0.15 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 5 mmol/L NaF, 20 mmol/L sodium pyrophosphate, 2 mmol/L sodium orthovanadate, 1% Triton X-100, 1  $\mu$ g/mL leupeptin (Sigma), 1  $\mu$ g/mL aprotinin (Sigma), 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride (Sigma)]. The extract was clarified using an Eppendorf microfuge (14,000 rpm for 10 minutes at 4°C). The concentration of total soluble protein in the supernatant was quantitated by the Bradford method (Bio-Rad, Hercules, CA). The extracts were employed for immunoblot analysis. Proteins (100  $\mu$ g/lane) were resolved by SDS-PAGE (10% polyacrylamide gel) under reducing conditions (50 mmol/L DTT final) and electroblotted onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membranes were incubated with 5% nonfat dry milk (at 4°C for 1 hour) and then probed with antibodies to CD44s (1:1,000 dilution; R&D Systems), CD44-v3 (1:1,000 dilution; R&D Systems), CD44-v4 (1:1,000 dilution; R&D Systems), CD44-v5 (1:1,000 dilution; Biosource, Camarillo, CA), CD44-v6 (1:1,000 dilution; Biosource), CD44-v7 (1:1,000 dilution; Biosource), and CD44-v10 (1:1,000 dilution; Biosource). Immune complexes were detected by enhanced chemiluminescence (Pierce, Rockford, IL). The blots were stripped with stripping buffer (Pierce) and equality in loading was checked by immunostaining the blot with 1:5,000 dilution of antibody for

**Table 1. Sequence and Location of Oligonucleotides Used for PCR and RT-PCR Amplifications of the CD44 Gene**

Designation	Sequence (5'-3')*	5' Coordinate †
v10-P1A	ATAggAATgATgTCACA	1785 (sense)
v10-P2	CTgATAAggAACgATTgACATTAg	1988 (antisense)
v5-P1A	ATgTAGACAgAAATggC	1215 (sense)
v5-P2	TTgTgCTTgTAGAATgTgggg	1331 (antisense)
v5-KA	TgATgCTCATggTgAATgAgg	1296 (antisense)
CD44s-For	CAGAACgAATCCTgAAgAC	661 (sense)
CD44s-Rev	<u>gTgTCTTggTCTCTggTAg</u>	2001 (antisense)
CD44s-pcDNA-FP	CCCAAgCTTATggACAAGTTTTggTggCACgCAG	179 (sense)
CD44s-pcDNA-RP	CTgAATTCCACCCCAATCTTCATgTCCACATTCTg	2404 (antisense)

\*Underlined and bold sequence in oligonucleotide CD44s-Rev corresponds to the 5' and 3' ends of CD44 exons 15 and 5, respectively.

†For oligonucleotide CD44s-Rev, the 5' coordinate corresponds to a nucleotide (no. 13 from the 5' end) on exon 15.

$\beta$ -actin (Sigma). The blots were quantified by densitometric scanning followed by data analysis using Quantity One software (Bio-Rad). Cell lysates from transfected SKA or SKOV3 cells were prepared using the PARIS kit (Ambion, Austin, TX) cell disruption buffer as suggested by the manufacturer and analyzed as above.

#### Total RNA Preparation

Total RNA from TNF- $\alpha$ -treated and untreated SKA cells was extracted using the RNA extraction RNeasy kit (Qiagen, Valencia, CA) as suggested by the manufacturer. The quantity and purity of the RNA were determined using UV spectrophotometry ( $A_{260}$  and  $A_{260}/A_{280}$ , respectively) and each preparation was diluted to the same concentration (0.9  $\mu\text{g}/\mu\text{L}$ ) using DEPC-treated distilled water. The quality of the RNA was tested by agarose gel electrophoresis (1% agarose with 0.22 mol/L formaldehyde). The ethidium bromide-stained gel was visualized under UV lighting using the Gel Doc 1000 system hardware (Bio-Rad) and the 18S rRNA bands were quantitated by densitometry with the Quantity One software and used to normalized the experimental RT-PCR product bands.

#### Oligonucleotides Primers

The sequence and location of each oligonucleotide used for PCR and RT-PCR amplifications of the CD44 gene (PubMed accession no. AJ251595; ref. 9) are shown in Table 1.

The primer pairs were v10-P1A/v10-P2 for CD44-v10 (204-bp product), v5-P1A/v5-P2 for CD44-v5 (117-bp product), v5-P1/v5-KA for CD44-v5 seminested PCR (82-bp product), and CD44s-For/CD44s-Rev for CD44s (198-bp product).

#### Semiquantitative RT-PCR

Semiquantitative RT-PCR of each RNA template was carried out using the One-Step RT-PCR kit as suggested by the manufacturer (Qiagen). Briefly, reverse transcription was done by incubation at 50°C for 30 minutes followed by 15-minute incubation at 95°C to inactivate the reverse transcriptase. The resulting cDNA was PCR amplified as follows. For CD44-v10, the conditions were 5 cycles of 94°C for 30 seconds, 45°C for 1 minute, and 72°C for 30 seconds followed by 25 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. For CD44-v5, a Touchdown RT-PCR protocol was followed by a seminested PCR amplification. The conditions for the Touchdown PCR are 2 cycles at 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds; 10 cycles at 94°C for 30 seconds, 57°C for 30 seconds; decrease in temperature of 1°C/cycle and 72°C for 30 seconds; and 18 cycles at 94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 30 seconds. The conditions for the seminested PCR were 94°C for 1 minute; 5 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and 25 cycles of 94°C for 10 seconds, 52°C for 30 seconds, and 72°C for 20 seconds. For CD44s, the conditions were 5 cycles of 94°C for 20 seconds, 46°C for 30 seconds, and 72°C for 25 seconds followed by 25 cycles of 94°C for 20 seconds, 50°C for 30 seconds, and 72°C for 25 seconds. All reactions were followed by 5- to 10-minute incubation at 72°C. All cDNA PCR or seminested PCR products were resolved in

1.5% agarose gels. Ethidium bromide-stained gels were visualized under UV lighting in the Gel Doc 1000 system hardware and the bands were analyzed by densitometry using the Quantity One software.

#### Control RT-PCR and PCR Amplifications

To semiquantitatively analyze the changes in gene expression of the CD44 isoforms studied, dilutions from 1:10 to 1:10<sup>5</sup> of the RNA preparations were RT-PCR amplified as above. To control genomic DNA contamination of the RNA preparations, a PCR amplification of each RNA template was carried out using Platinum Taq DNA polymerase (Life Technologies) as suggested by the manufacturer. The conditions for the control PCRs were identical to their corresponding experimental RT-PCRs, with the exception of the steps involved in reverse transcription (30- and 15-minute incubation at 50°C and 95°C, respectively) that were replaced by a 1-minute incubation at 94°C.

#### Matrigel Invasion Assay

Viable cells ( $0.5 \times 10^6$ ) were seeded onto 100-mm tissue culture dishes in complete medium. After 24 hours of cell attachment, the cells were incubated in the complete medium (7 mL/dish) containing 0 to 100 ng/mL TNF- $\alpha$  (0-100) for 48 hours. Replicate cell layers were incubated in EDTA (0.2 solution; Sigma), harvested using a cell scraper, and diluted to  $1 \times 10^5/\text{mL}$  in serum-free growth medium. For *in vitro* invasion assay, Matrigel-coated Falcon Transwell chambers (24-well format, 8  $\mu\text{m}$  pore; BD Pharmingen, San Diego, CA) were used. The chambers were rinsed gently with serum-free growth medium before addition of cells. Serum-free growth medium (0.5 mL) containing  $5 \times 10^4$  cells were added to the upper chamber. The lower chamber contained growth medium. After overnight incubation at 37°C for 18 hours, cells on the upper surface of the filter were removed using a cotton wood swab (7). Migrated cells on the lower surface were stained using DiffQuick stain kit (Dade AG, Duding, Switzerland). For each Transwell (four per condition), the cells that migrated were counted in 10 different medium-power fields ( $\times 20$ ). The invaded cell score was expressed as a percentage of the untreated control.

To study the effect of 6-(Dimethylamino)purine (DMAP; Sigma) treatment on *in vitro* invasion, viable cells at 80% confluence in complete medium were used. Before DMAP inhibitor treatment, the cells were transferred to serum-free RPMI 1640 containing 0.1% bovine serum albumin (BSA) for 24 hours. SKA and SKOV3 cells were pretreated for 1 hour with DMAP (JNK inhibitor) at 0, 0.1, and 1.0 mmol/L amounts in serum-free RPMI 1640-0.1% BSA. The cells were detached and seeded on Matrigel with TNF- $\alpha$  (0 or 25 ng/mL) for 24 hours.

To study the effect of a competitive inhibitor for TNF- $\alpha$  binding to its receptor on *in vitro* invasion, a recombinant soluble human TNF- $\alpha$  receptor 1 extracellular domain (TNFRI; R&D Systems) was used. For *in vitro* invasion, viable cells at 80% confluence were used. Before treatment, the cells were transferred to serum-free RPMI 1640-0.1% BSA for 24 hours. Before the start of the invasion experiment, aliquots of TNF- $\alpha$  (30 ng/mL) were preneutralized with TNFRI (final 0-10 nmol/L)

at 37°C for 30 minutes (premix). Cells were detached and seeded onto the top of the inserts together with the premix and the bottom chamber of the insert was filled with serum-free RPMI 1640-0.1% BSA. After overnight incubation at 37°C for 18 hours, the migrated cells were quantified as described previously. The data were analyzed with Student's *t* test and the statistical significance was set at  $P < 0.05$ .

#### *Hyaluronic Acid Adhesion Assay*

SKA or SKOV3 cells ( $0.5 \times 10^6$ ) were seeded onto 100-mm tissue culture dishes and after attachment for 24 hours left untreated or were exposed to TNF- $\alpha$  (0-100 ng/mL) in complete medium (7 mL) for 48 hours. [<sup>3</sup>H]thymidine (1  $\mu$ Ci/mL) was added to the growth medium 18 hours before harvesting. The cells were detached with 5 mmol/L EDTA (in PBS) at 37°C to obtain nonadherent single-cell suspension (29). The cells ( $1 \times 10^4$ ) in 60  $\mu$ L BSA (1% in PBS) were seeded (8 replicates) onto hyaluronic acid (Sigma)-coated wells (250  $\mu$ g/100  $\mu$ L PBS) in a 96-well plate (Costar, Corning, NY; ref. 30). After 30-minute incubation at 4°C, the nonadherent cells were removed by washing the wells with PBS. The bound and total counts/min was counted in Top Count NXT (Packard, Meriden, CT). A set of wells coated with BSA (1% in PBS) served as control (6 replicates). % Binding was calculated as follows: % binding = counts/min bound (hyaluronic acid – BSA)  $\times$  100/counts/min [total – mean (BSA)]. Data were analyzed with Student's *t* test and the statistical significance was set at  $P < 0.05$ .

#### *MAPK Inhibitor Treatment and Western Blot*

Viable cells at 80% confluence in complete medium were used. Before MAPK inhibitor treatment, the cells were transferred to serum-free RPMI 1640 for 24 hours. The MAPK inhibitors SB-203580 [4-(4-fluorophenyl)-2-(4-methylsulfanylphenyl)-5-(4'-pyridyl)-1H-imidazole], SB-202474 [4-ethyl-2-(*p*-methoxyphenyl)-5-(4'-pyridyl)-1H-imidazole], U-0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene], and U-0124 [1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene] were purchased from Calbiochem (La Jolla, CA). The inhibitors were dissolved in DMSO as 10 mmol/L stock and diluted appropriately. DMSO-alone vehicle (final 0.01%) was used as control. For ERK inhibitor study, the cells were treated for 1 hour with either U-0126 (a p-ERK MAPK inhibitor) at 2 and 10  $\mu$ mol/L or U-0124 (an inactive negative control for U-0126) at 10  $\mu$ mol/L before treatment with TNF- $\alpha$  (25 ng/mL) for 30 minutes (p-ERK determination) or 24 hours (CD44s determination). The Western blot for activated ERK determinations was probed with p-ERK-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1,000 dilution and reprobed with a rabbit antibody specific for unphosphorylated ERK (Santa Cruz Biotechnology) at 1:2,000 dilution.

For p38 MAPK inhibitor studies, SKA and SKOV3 cells were treated for 1 hour with either SB-203580 (a p38 MAPK inhibitor) at 2 and 10  $\mu$ mol/L or SB-202474 (an inactive negative control for SB-203580) at 10  $\mu$ mol/L before treatment with TNF- $\alpha$  (25 ng/mL) for 30 minutes (phosphorylated p38 MAPK determination) or 24 hours (CD44s determination). The Western blot for activated p38 MAPK determinations was

probed with phosphorylated p38 MAPK-specific antibody (Sigma) at 1:1,000 dilution and reprobed with a rabbit antibody specific for unphosphorylated p38 MAPK (Santa Cruz Biotechnology) at 1:2,000 dilution.

For p-JNK studies, SKA and SKOV3 cells were treated for 1 hour with DMAP at 100 or 1,000  $\mu$ mol/L before treatment with TNF- $\alpha$  (25 ng/mL) for 30 minutes (p-JNK determination) or 24 hours (CD44s determination). The Western blot for activated JNK determinations was probed with p-JNK-specific antibody (Sigma) at 1:1,000 dilution and reprobed with a rabbit polyclonal antibody specific for unphosphorylated JNK (Santa Cruz Biotechnology) at 1:2,000 dilution.

#### *Soluble TNFR1 Treatment and Western Blot*

Viable cells at 80% confluence in six-well clusters were used. Before treatment, the cells were transferred to serum-free RPMI 1640-0.1% BSA for 24 hours. Aliquots of TNF- $\alpha$  (30 ng/mL) were preincubated with TNFR1 (final 0-10 nmol/L) at 37°C for 30 minutes and added to the cells in triplicates. Cell lysates were prepared for Western blot following 30 minutes (p-JNK determination) or 24 hours (CD44s determination) after treatment and analyzed for p-JNK and CD44 as described elsewhere.

#### *Plasmid Construction and Transfections*

The retroviral expression vector pSIREN-RetroQ-DsRed-Express (Clontech, Mountain View, CA) was used to clone and express small hairpin RNA targeting CD44 expression. The two CD44 sequences used (31) were generated and ligated in the supplied linear vector as suggested by the manufacturer. The recombinant plasmids identity (named pSIREN/CD44 Seq 1 and Seq 2) were confirmed by restriction digestion with *Mlu*I. Transient transfections (48 hours) of pSIREN/CD44 Seq 1 or Seq 2 (1  $\mu$ g) into SKA cells seeded in six-well plates (100,000 cells per well) were carried out using the transfection reagent GeneJuice (Novagen, San Diego, CA) as suggested by the manufacturer. The expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA) was used to clone and express the human CD44s cDNA sequence in SKOV3 cells. Briefly, CD44s cDNA was PCR amplified from plasmid pAZ (a generous gift of U. Gunthert, Basel Institute, Basel, Switzerland) and restricted with *Hind*III and *Eco*RI (sequence added in the PCR primers CD44s-pcDNA-FP and CD44s-pcDNA-RP). The restricted DNA fragment was ligated in similarly restricted pcDNA3.1(+) vector, amplified in DH5 $\alpha$ -competent cells (Invitrogen), and transfected in SKOV3 cells as shown above. SKA and SKOV3 transfectants were analyzed for CD44s expression by Western blotting. After detaching the transfected cells with EDTA (0.2 solution), Matrigel invasion assays were carried out as shown above.

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*Mol Cancer Res* 2006;4:511-520.

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