

# Expression and regulation of tumor necrosis factor $\alpha$ in normal and malignant ovarian epithelium

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## Abstract

Epidemiologic studies implicate inflammatory stimuli in the development of ovarian cancer. The proinflammatory cytokine tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and both its receptors (TNFRI and TNFRII) are expressed in biopsies of this malignancy. Here, we tested the hypothesis that TNF- $\alpha$  is a regulator of the proinflammatory microenvironment of ovarian cancer. A cancer profiling array showed higher expression of TNF- $\alpha$  in ovarian tumors compared with normal ovarian tissue, and cultured ovarian cancer cells expressed up to 1,000 times more TNF- $\alpha$  mRNA than cultured normal ovarian surface epithelial cells; TNF- $\alpha$  protein was only detected in the supernatant of tumor cell cultures. Treatment with TNF- $\alpha$  induced TNF- $\alpha$  mRNA via TNFRI in both malignant and normal cells with evidence for enhanced TNF- $\alpha$  mRNA stability in tumor cells. TNF- $\alpha$  induced TNF- $\alpha$  protein in an autocrine fashion in tumor but not in normal ovarian surface epithelial cells. The TNF- $\alpha$  neutralizing antibody infliximab reduced the constitutive levels of TNF- $\alpha$  mRNA in tumor cell lines capable of autocrine TNF- $\alpha$  production. Apart from TNF- $\alpha$  mRNA expression, several other proinflammatory cytokines were constitutively expressed in malignant and normal ovarian surface epithelial cells, including interleukin (IL)-1 $\alpha$ , IL-6, CCL2, CXCL8, and M-CSF. TNF- $\alpha$  treatment further induced these cytokines with *de novo* transcription of IL-6 mRNA contrasting with the increased stability of CCL2 mRNA. RNA interference

directed against TNF- $\alpha$  was highly effective in abolishing constitutive IL-6 production by ovarian tumor cells. In summary, we show that TNF- $\alpha$  is differentially regulated in ovarian cancer cells compared with untransformed cells and modulates production of several cytokines that may promote ovarian tumorigenesis. Infliximab treatment may have a role in suppressing the TNF- $\alpha$ -driven inflammatory response associated with ovarian cancer. [Mol Cancer Ther 2006;5(2):382–90]

## Introduction

Several inflammatory conditions, such as pelvic inflammatory disease and endometriosis, are associated with an increased risk of ovarian cancer (1). The links between inflammation and cancer are increasingly recognized; various mechanisms involving an array of mediators, including cytokines, chemokines, growth factors, and free radicals, have been described (2, 3).

*In situ* hybridization studies correlated expression of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) by the tumor epithelium and infiltrating macrophages, with increasing tumor grade in serous ovarian carcinoma (4). Others have shown that TNF- $\alpha$  mRNA is expressed by ovarian carcinoma cells and may be induced in normal ovarian surface epithelial cells, promoting growth in both cell types (5, 6). Previous studies have also indicated that ovarian surface epithelial cells can secrete cytokines such as interleukin (IL)-1, IL-6, and M-CSF, with a tendency for greater production *in vitro* compared with malignant cells (7). However, this contrasts with the frequently elevated levels of these cytokines in the serum and ascites of patients with ovarian cancer (8–12), suggesting that inflammatory cells and/or chronic activation of the tumor cells may be clinically of greater significance.

Here, we explored the hypothesis that TNF- $\alpha$  may be an important regulator of the proinflammatory milieu in ovarian cancer. We assessed the autocrine regulation of TNF- $\alpha$  in malignant (both ovarian carcinoma cell lines and primary ovarian tumor cells) and normal ovarian surface epithelial cells and its ability to induce a variety of cytokines that are present in ovarian epithelium. Our studies suggest that TNF- $\alpha$  within ovarian tumor epithelium is subject to complex regulation and that blocking its activity may, at least in part, suppress the inflammatory cytokines associated with this disease.

## Materials and Methods

### Cell Lines and Cell Culture

Ovarian carcinoma cell lines [IGROV-1 indirectly from Bernard et al. (13); OVCAR-3 from Hamilton et al. (14); PEO1 from Langdon et al. (15); and SKOV-3 from the American Tissue Culture Collection (Rockville, MD)] were

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grown in a humidified atmosphere at 37°C and 5% CO<sub>2</sub> in either endotoxin-free RPMI medium (IGROV-1, OVCAR-3, and PEO1 cell lines) or endotoxin-free DMEM medium (SKOV-3 cell line) supplemented with 10% fetal bovine serum (Sigma-Aldrich Ltd., Poole, United Kingdom). Insulin (2.5 µg/mL) was added to the medium of OVCAR-3 and PEO1 cell lines. TNF-α (20 ng/mL) was added to 70% confluent cells and analysis was done at 0, 0.5, 1, 3, 6, and 24 hours after addition of the cytokine. The TNF-α concentration was selected from dose-response studies for TNF-α mRNA induction (data not shown). Unless indicated, TNF-α refers specifically to human TNF-α whereas mTNF-α is used to denote the murine form (also used at 20 ng/mL). Actinomycin D experiments were done using a dose of 5 µg/mL. Infliximab (10 µg/mL) and the immunoglobulin G1 isotype control (10 µg/mL) were a kind gift from Centocor (Malvern, PA).

#### Transfection of IGROV-1 Cells

IGROV-1 cells were transfected with SUPER RNAi plasmids for TNF-α and isolated according to the protocols described (16). Four micrograms of plasmid DNA in 250 µL serum-free Opti-MEM medium (Life Technologies, Inc., Gaithersburg, MD) were mixed with 10 µL Lipofectamine 2000 (Invitrogen, Paisley, United Kingdom) in 250 µL Opti-MEM and incubated for 20 minutes at room temperature. Serum-free medium (1.5 mL) was mixed with the Lipofectamine 2000/DNA solution and added to the IGROV-1 cells, which were then incubated for 4 hours at 37°C before the medium was replaced with fresh medium supplemented with 10% FCS. Antibiotic selection for stable cell lines started after 48 to 72 hours; cells were selected for SUPER RNAi plasmid expressing cells in 4 µg/mL puromycin (Sigma) for 30 days.

#### Primary Cell Isolation, Culture, and TNF-α Stimulation

The study was approved by the East London and City Health Authority research ethics committee and informed consent was obtained from patients attending the gynecologic oncology unit at St. Bartholomew's Hospital (London, United Kingdom). Normal ovarian surface epithelial cells were obtained by scraping the ovarian surface at the time of laparotomy and cultured in a humidified atmosphere at 37°C and 5% CO<sub>2</sub> in endotoxin-free MCDB105/M199 medium (Sigma) supplemented with 15% fetal bovine serum (17). Primary ovarian cancer cells from ascites were obtained from patients undergoing surgery for ovarian cancer and cultured in RPMI medium (as above). Malignant and normal ovarian surface epithelial cells were identified on the basis of microscopic appearance, positive staining for cytokeratin and negative staining for factor VIII. TNF-α (or mTNF-α) was added to 70% confluent cells and analysis was done at intervals of 0, 1, 3, 6, and 24 hours after addition of the cytokine.

#### Reverse Transcription-PCR

DNase-treated total RNA was reverse transcribed using the Ready-To-Go kit (Amersham Pharmacia Biotech, Piscataway, NJ). cDNA (1 µL) was amplified by PCR using primers for TNF-α, TNFRI, and TNFRII (Clontech, Cowley, Oxford, United Kingdom) with β-actin primer as a positive control.

#### Quantitative Real-time Reverse Transcription-PCR

DNase-treated RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Southampton, United Kingdom) according to the instructions of the manufacturer. Multiplex real-time reverse transcription-PCR (RT-PCR) analyses were done using TNF-α, IL-1α, IL-6, CCL2, CXCL8, M-CSF (all FAM), and 18S rRNA (VIC) primers and probes with the ABI Prism 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Warrington, United Kingdom). Two microliters of cDNA were used per 25 µL reaction with cycling conditions as follows: 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Each sample was analyzed in duplicate or triplicate and normalized ( $\Delta C_t$ ) to 18S by removing the cycle threshold ( $C_t$ ) value of 18S from the  $C_t$  value of the gene under investigation. The  $\Delta C_t$  for the control was subtracted from the  $\Delta C_t$  for TNF-α stimulation (i.e.,  $\Delta\Delta C_t$ ) and the fold difference was calculated as  $2^{-\Delta\Delta C_t}$ .

#### Northern Blot Analysis

[ $\alpha$ -<sup>32</sup>P]dCTP-labeled TNF-α and β-actin cDNA probes were obtained from Dr. S.C. Robinson (Translational Oncology, QMUL, London, United Kingdom; TNF-α and β-actin), prepared and used for Northern blot analysis as previously described (18).

#### Cancer Profiling Array

The Cancer profiling array II (Clontech) comprises cDNA from tumor and corresponding whole normal ovarian tissue from individual patients spotted on a nylon membrane. The array was hybridized with a specific radio-labeled TNF-α probe according to the instructions of the manufacturer. Hybridization signals were detected by phosphorimaging and analyzed using ImageQuant Version 1.11. The membrane was stripped and reprobed with a ubiquitin probe to normalize each signal.

#### ELISAs

R&D ELISA kits (TNF-α, TNFRI, TNFRII, IL-1α, IL-6, CCL2, and M-CSF) and Biosource ELISA kits (Biosource Europe, Nivelles, Belgium; CXCL8 and TNF-α) were used to measure the cytokine content of cell supernatants. ELISAs were carried out according to the instructions of the manufacturer.

#### Flow Cytometry and Immunofluorescence

Monoclonal antibodies against TNFRI and TNFRII (R&D Systems, Abingdon, United Kingdom) and isotype-matched control were used. Cells were resuspended in PBS supplemented with 1% heat-inactivated FCS and 0.01% NaN<sub>3</sub>. Antibodies diluted in this buffer were used between 2 and 20 µg/mL and incubated for 30 minutes on ice. Cells were washed and 10,000 cells were analyzed by flow cytometry on a FACScan flow cytometer using CellQuest software (BD Pharmingen, Oxford, United Kingdom).

#### Data Analysis and Statistics

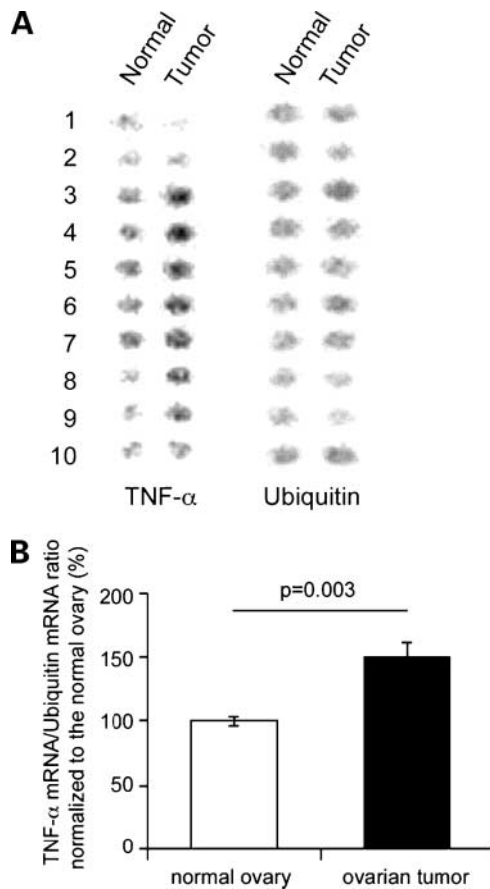
NIH Image v.1.61 software was used to analyze Western blots by densitometry and ImageQuant software (Molecular dynamics) v1.11 was used to analyze phosphorimaging

data (Storm scanner) obtained from the cancer profiling array. InStat v2.01 software was used to test results for statistical significance (Student's *t* test and Bonferroni test).

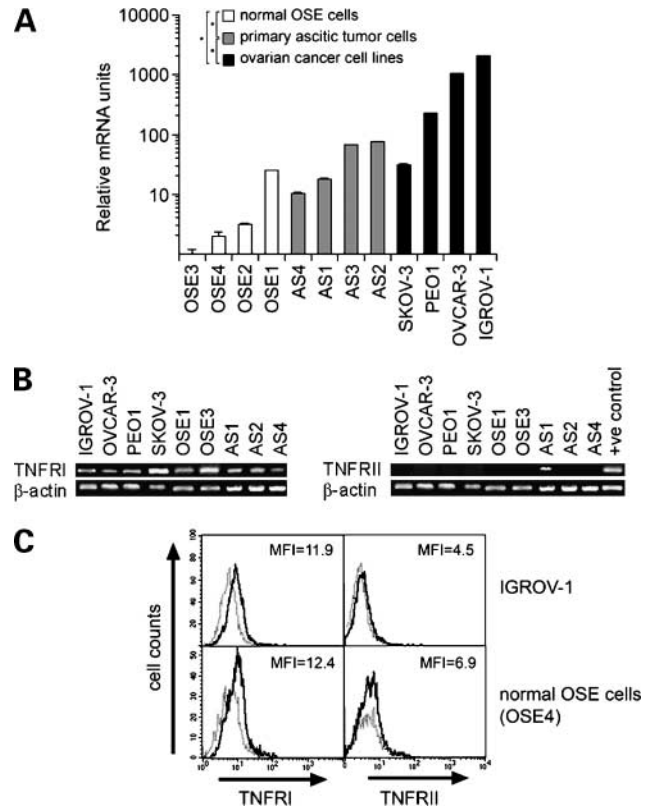
## Results

### Increased Expression of TNF- $\alpha$ in Ovarian Cancer versus Normal Ovarian Tissue

We screened for the expression of TNF- $\alpha$  in matched normal whole ovary and ovarian tumor tissue by hybridizing a radiolabeled TNF- $\alpha$  probe to a cDNA expression array. Levels of TNF- $\alpha$  mRNA were higher in malignant ovarian tumors compared with the corresponding normal tissue (Fig. 1). Increased TNF- $\alpha$  mRNA



**Figure 1.** TNF- $\alpha$  expression in matched normal and tumor ovarian tissue. The Cancer Profiling Array II (Clontech) was hybridized with a radiolabeled cDNA probe for TNF- $\alpha$ . **A**, differential expression of TNF- $\alpha$  was observed between ovarian tumor and corresponding normal ovarian tissue. The nylon membrane was stripped and reprobed with ubiquitin, an internal control to normalize each signal. Numbers indicate tissue source: 1, papillary serous cystadenoma, borderline malignancy; 2, leiomyoma; 3, adenocarcinoma; 4, clear cell adenocarcinoma; 5, adenocarcinoma; 6, leiomyoma; 7, mucinous cystadenocarcinoma; 8, adenocarcinoma; 9, serous surface papillary carcinoma; and 10, papillary serous cystadenoma, borderline malignancy. **B**, a graph comparing levels of TNF- $\alpha$  mRNA in normal ovarian tissue with corresponding ovarian tumors.



**Figure 2.** Constitutive expression of TNF- $\alpha$  and TNFRs in malignant and normal ovarian surface epithelial (OSE) cells. **A**, quantitative real-time RT-PCR detected increased TNF- $\alpha$  mRNA in malignant compared with normal ovarian surface epithelial cells (\*,  $P < 0.001$ ). RT-PCR (**B**) and fluorescence-activated cell sorting (**C**) data confirm the presence of TNFR I but absence of TNFR II in normal and malignant cells. Mean fluorescence intensity (MFI) is shown (control values for TNFR I: 7.9, IGROV-1 cancer cell line; 7.3, normal ovarian surface epithelial cells; and control values for TNFR II: 4.2, IGROV-1 cancer cell line; 6.3, normal ovarian surface epithelial cells).

was present in a variety of tumor subtypes, including adenocarcinoma (samples 3, 5, 7, and 8), clear cell adenocarcinoma (sample 4), and serous papillary carcinoma (sample 9).

### TNF- $\alpha$ mRNA Expression Is Increased in Malignant versus Normal Ovarian Surface Epithelial Cells

We determined the contribution of the ovarian epithelium to increased TNF- $\alpha$  mRNA expression by comparing the levels of TNF- $\alpha$  mRNA in unstimulated ovarian carcinoma cell lines, primary ascitic ovarian tumor cells, and normal ovarian surface epithelial cells. TNF- $\alpha$  mRNA expression was detected by conventional RT-PCR in four ovarian cancer cell lines but not in normal ovarian surface epithelial cells (data not shown). However, the more sensitive technique of quantitative real-time RT-PCR confirmed expression of very low levels of TNF- $\alpha$  mRNA in normal ovarian surface epithelial cells with up to 1,000-fold more TNF- $\alpha$  mRNA in the ovarian carcinoma cell lines (Fig. 2A). Intermediate levels of TNF- $\alpha$  mRNA expression were observed in unstimulated primary ascitic

ovarian tumor cells [i.e., ovarian carcinoma cell lines (IGROV-1 > OVCAR-3 > PEO1 > SKOV-3) > primary ovarian tumor cells > normal ovarian surface epithelial cells ( $P < 0.001$ ).

#### Detection of TNFR1 in Normal and Malignant Ovarian Surface Epithelial Cells

Conventional RT-PCR revealed expression of TNFR1, but not TNFR2, in the ovarian carcinoma cell lines and normal ovarian surface epithelial cells, indicating that TNF- $\alpha$  signals via the former receptor (Fig. 2B). Similarly, TNFR1, but not TNFR2, was expressed in the primary ascitic tumor cells; the only exception was AS1, which derived from a patient with a mixed mesodermal tumor and expressed both receptors subtypes. Fluorescence-activated cell sorting analysis of the ovarian cancer cell line IGROV-1 and normal ovarian surface epithelial cells confirmed the presence of TNFR1, but not TNFR2, at the cell surface (Fig. 2C). Similarly, only soluble TNFR1 was detected in the cell supernatant of IGROV-1 ovarian cancer cells by ELISA (range, 47–161 pg/mL).

#### Evidence for Differential Regulation of TNF- $\alpha$ mRNA in Malignant and Normal Ovarian Surface Epithelial Cells

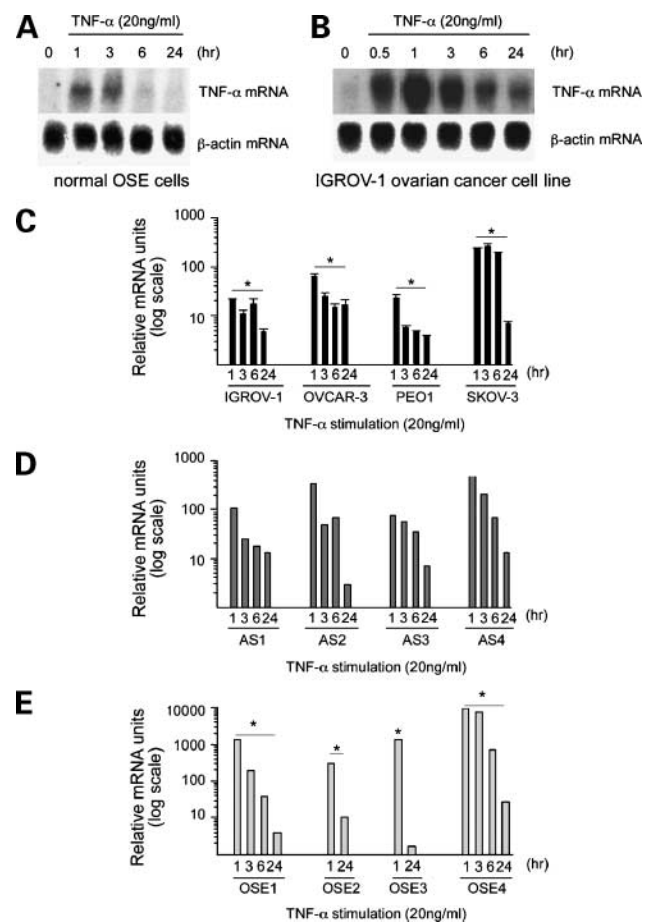
Based on the elevated levels of TNF- $\alpha$  mRNA in malignant epithelium and the presence of TNFR1 in tumor and normal ovarian surface epithelial cells, we proceeded to compare the expression of TNF- $\alpha$  mRNA in both cell types following stimulation with 20 ng/mL of TNF- $\alpha$  (a dose selected on the basis of efficient induction of TNF- $\alpha$  mRNA at 1 and 24 hours in the IGROV-1 cell line; data not shown). Experiments were initially done using Northern blot analysis (Fig. 3A and B) followed by more extensive studies with quantitative real-time RT-PCR (Fig. 3C–E). Both malignant and normal ovarian surface epithelial cells displayed maximal up-regulation of TNF- $\alpha$  mRNA by 1 hour. TNF- $\alpha$  mRNA expression remained elevated over the 24-hour period of TNF- $\alpha$  stimulation (compared with control) and declined slowly in the majority of the cancer cell lines (Fig. 3C). The SKOV-3 cell line was an exception, revealing a rapid decline to baseline of the mRNA transcript, showing similar TNF- $\alpha$  mRNA kinetics to the normal ovarian surface epithelial cells between 6 and 24 hours (Fig. 3E). The limited number of experiments done in the primary tumor cells (Fig. 3D) revealed mRNA expression kinetics halfway between the cell lines and normal ovarian surface epithelial cells.

#### TNF- $\alpha$ Induces TNF- $\alpha$ mRNA Stability in Malignant Ovarian Epithelium

The differential TNF- $\alpha$  mRNA expression profile observed in the tumor cells may be due to enhanced mRNA stability. Actinomycin D chase experiments were done comparing the stability of TNF- $\alpha$  mRNA in IGROV-1 and OVCAR-3 cell lines with that in the SKOV-3 cell line. Cells were treated with 20 ng/mL of TNF- $\alpha$  for either 1 or 24 hours before the addition of actinomycin D. IGROV-1 and, to a lesser extent, OVCAR-3 displayed significantly increased TNF- $\alpha$  mRNA stability after 24 hours compared with 1 hour of TNF- $\alpha$  treatment (Fig. 4A and B). In

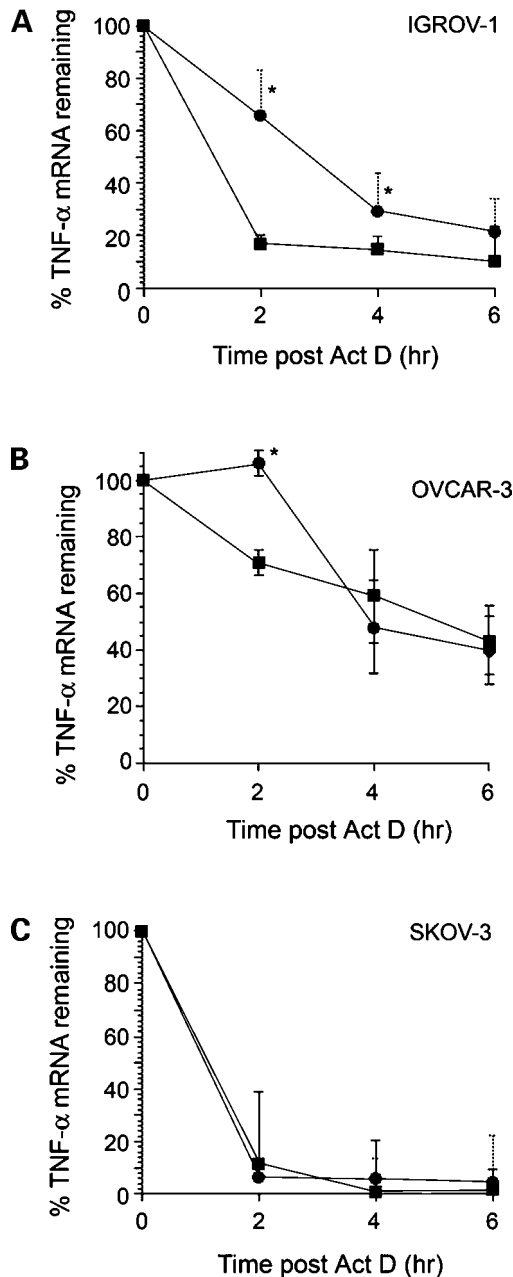
contrast, there was no evidence for TNF- $\alpha$  mRNA stability in the SKOV-3 cell line (Fig. 4C), consistent with the abrupt decline in TNF- $\alpha$  mRNA expression observed between 6 and 24 hours. Thus, the regulation of TNF- $\alpha$  mRNA expression in ovarian tumor cells by TNF- $\alpha$  occurs both at the transcriptional and posttranscriptional levels.

**TNF- $\alpha$  Protein Production Is Confined to Malignant Ovarian Epithelial Cells.** We reasoned that TNF- $\alpha$  mRNA stability may correlate with the production of TNF- $\alpha$  protein. Previously such an assessment has proved difficult due to the excess TNF- $\alpha$  used for stimulation. It is known, however, that mTNF- $\alpha$  binds human TNFR1 with identical affinity to human TNF- $\alpha$  but does not cross-react in a specific TNF- $\alpha$  ELISA kit. Hence, we stimulated human

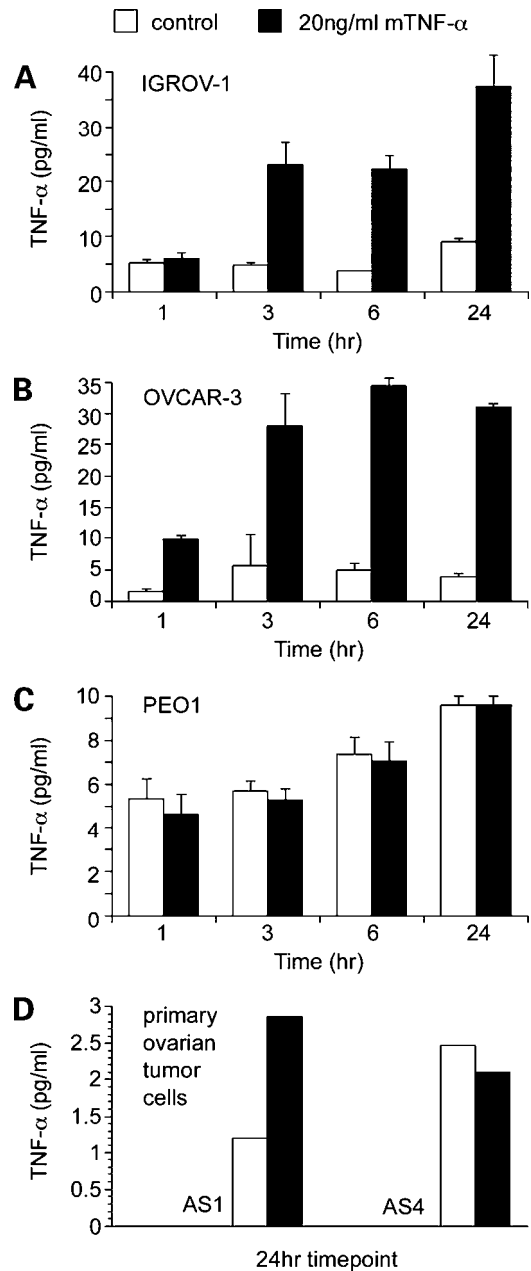


**Figure 3.** TNF- $\alpha$  mRNA expression is up-regulated in ovarian carcinoma cell lines in response to exogenous TNF- $\alpha$ . Representative Northern blot analyses of normal ovarian surface epithelial cells ( $n = 2$ ; **A**) and the IGROV-1 cell line ( $n = 9$ ; **B**) treated with 20 ng/mL of TNF- $\alpha$  over a 24-h period. A cDNA probe for  $\beta$ -actin was used to normalize the signal. Quantitative real-time RT-PCR analysis of TNF- $\alpha$  mRNA in ovarian carcinoma cell lines ( $n = 4$ ; **C**), primary ascitic ovarian cancer cells ( $n = 4$ ; **D**), and normal ovarian surface epithelial cells ( $n = 4$ ; **E**) stimulated with 20 ng/mL of TNF- $\alpha$ . Maximal induction of TNF- $\alpha$  mRNA was observed at 1 h in malignant and normal ovarian surface epithelial cells (\*,  $P < 0.001$ ). SE was derived from the  $\Delta C_t$  values. Relative mRNA units refers to the amount of TNF- $\alpha$  mRNA normalized to 18S rRNA.

cells with mTNF- $\alpha$  and measured human TNF- $\alpha$  production. Both IGROV-1 and OVCAR-3 ovarian cancer cells released human TNF- $\alpha$  into the cell supernatant in response to mTNF- $\alpha$ , with maximal levels detected by



**Figure 4.** Actinomycin D chase experiments in IGROV-1, OVCAR-3, and SKOV-3. The ovarian cancer cell lines IGROV-1 ( $n = 6$ ; **A**), OVCAR-3 ( $n = 8$ ; **B**), and SKOV-3 ( $n = 5$ ; **C**) were treated with TNF- $\alpha$  (20 ng/mL) for 1 (■) and 24 (●) h. Actinomycin D (Act D; 5  $\mu$ g/mL) was added at the same time and RNA was extracted at 0, 2, 4, and 6 h. RNA was converted to cDNA and TNF- $\alpha$  mRNA (normalized to 18S rRNA) was analyzed using quantitative real-time RT-PCR and compared with the control (–Act D) at each time point. Increased TNF- $\alpha$  mRNA stability was observed in IGROV-1 and OVCAR-3 cells following 24 h compared with 1 h of TNF- $\alpha$  treatment (\*,  $P < 0.05$ ). The amount of TNF- $\alpha$  mRNA is expressed as a percentage of the control. Representative of two or more experiments.



**Figure 5.** TNF- $\alpha$  protein production in malignant and normal ovarian surface epithelial cells. ELISAs for human TNF- $\alpha$  in cell supernatants following stimulation of IGROV-1 ( $n = 6$ ; **A**), OVCAR-3 ( $n = 6$ ; **B**), and PEO1 ( $n = 6$ ; **C**) cell lines with 20 ng/mL of mTNF- $\alpha$  over a 24-h period. **D**, two of three primary ovarian (ascitic) cancer samples were positive for human TNF- $\alpha$  protein in the cell supernatant. No TNF- $\alpha$  protein was detected in SKOV-3 ( $n = 6$ ) or ovarian surface epithelial cell ( $n = 6$ ) supernatants.

24 hours (Fig. 5A and B). PEO1 cells also made TNF- $\alpha$  constitutively (Fig. 5C) whereas no TNF- $\alpha$  was detected in SKOV-3 cell supernatant ( $n = 6$ ; data not shown; Fig. 5D). One of three primary ascitic tumor isolates (AS4) revealed constitutive TNF- $\alpha$  production. Induction of human TNF- $\alpha$  protein was only observed in one of three primary ascitic

tumor isolates (AS1) following mTNF- $\alpha$  stimulation. In contrast, TNF- $\alpha$  was not detected when cultures of normal ovarian surface epithelial cells were treated with mTNF- $\alpha$  ( $n = 6$ ; data not shown).

**Infliximab Down-Regulates TNF- $\alpha$  mRNA in Ovarian Cancer Cells.** The TNF- $\alpha$  neutralizing antibody infliximab has been shown to block TNF- $\alpha$  activity in several inflammatory conditions, advancing the clinical management of patients with rheumatoid arthritis and Crohn's disease. Several mechanisms of action have been postulated in addition to neutralization of soluble TNF- $\alpha$ , including antibody- and complement-dependent cellular cytotoxicity (reviewed in ref. 19). In view of the increased expression of TNF- $\alpha$  in ovarian cancer, we treated the two cell lines that release TNF- $\alpha$  in an autocrine fashion, IGROV-1 and OVCAR-3, with infliximab and assessed the effect on TNF- $\alpha$  mRNA. TNF- $\alpha$  mRNA decreased by 30% to 40% in IGROV-1 and OVCAR-3 following 3 and 6 hours of incubation, respectively, with 10  $\mu$ g/mL of infliximab (Fig. 6).

#### Modulation of the Ovarian Cancer Cytokine Network by TNF- $\alpha$

Next, we asked whether the abnormal expression and regulation of TNF- $\alpha$  in malignant cells was, in part, responsible for modulating the proinflammatory cytokine milieu in ovarian cancer. First, we evaluated the constitutive and TNF- $\alpha$ -inducible levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, CCL2, CXCL8, and M-CSF mRNA in the IGROV-1 cell line using quantitative real-time RT-PCR. All these cytokines were constitutively expressed and TNF- $\alpha$ -inducible in the IGROV-1 cell line (Fig. 7A). In particular, TNF- $\alpha$  induced a sustained increase in IL-6 and CCL2 mRNA with maximal levels by 24 hours, contrasting with a transient increase in the expression of the remaining cytokines (maximal by 1–3 hours). We then screened the remaining cell lines, primary tumor cells and normal ovarian surface epithelial cells, selecting the time point that showed maximal cytokine mRNA induction in the IGROV-1 cell line by TNF- $\alpha$ . All the cell types, except for the SKOV-3 cell line, expressed similar levels of constitutive cytokine mRNA with TNF- $\alpha$  inducing further up-regulation of proinflammatory cytokine mRNA (data not shown). Of note, TNF- $\alpha$  differentially modulated cytokine mRNA levels with *de novo* transcription required for IL-6 induction and enhanced mRNA stability, a feature of CCL2 induction in the IGROV-1 and OVCAR-3 cancer cell lines (Fig. 7B).

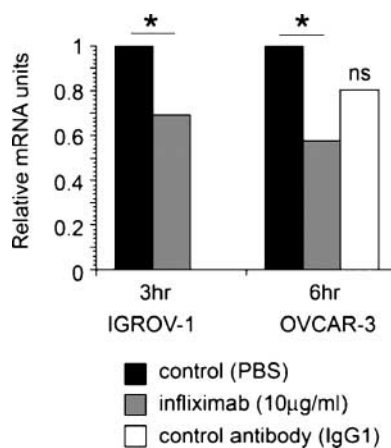
We confirmed both constitutive and TNF- $\alpha$ -inducible levels of IL-6, CXCL8, CCL2, and M-CSF proteins in the supernatant of malignant and normal ovarian surface epithelial cells (Table 1). No TNF- $\alpha$ -inducible IL-6 or CCL2 protein was detected in the SKOV-3 cell line, confirming the earlier findings of weak or absent mRNA induction in response to TNF- $\alpha$ . Constitutive IL-1 $\alpha$  was absent but could be induced by TNF- $\alpha$  in both malignant and normal ovarian surface epithelial cells. Although IL-1 $\beta$  protein was detected in the supernatant of normal ovarian surface epithelial cells, it was absent in the supernatant of malignant epithelial cells, consistent with published data (20).

#### Endogenous TNF- $\alpha$ Sustains Production of IL-6 and CCL2 by Malignant Ovarian Epithelium

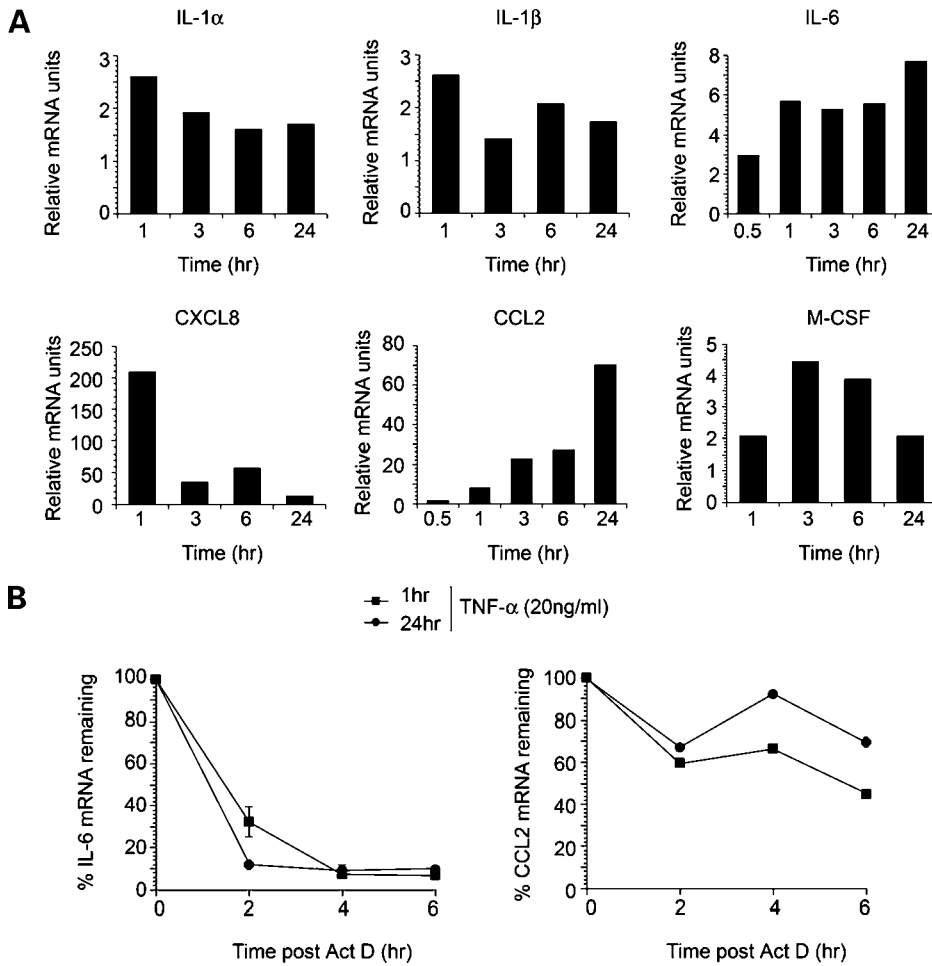
We sought to understand further the role of TNF- $\alpha$  in the regulation of the ovarian cancer cytokine network by treating IGROV-1 and OVCAR-3 cells with infliximab. We revealed a 10% to 15% reduction in constitutive CCL2 ( $P < 0.001$ ) and IL-6 ( $P < 0.01$ ) protein in the supernatant of IGROV-1 and OVCAR-3 cells exposed to infliximab for 24 hours (Fig. 8A and B). Infliximab had no effect on the levels of IL-1 $\alpha$ , CXCL8, or M-CSF (data not shown). On the other hand, RNA interference directed against TNF- $\alpha$  largely abrogated IL-6 production by IGROV-1 cells (Fig. 8C), suggesting a critical role for TNF- $\alpha$  in maintaining endogenous levels of this cytokine, known to be linked to poor prognosis in ovarian cancer (21).

#### Discussion

Chronic inflammation is recognized as an important component of the tumor-stromal interaction and is increasingly considered to be a valid target for modulation (2, 3). Persistent inflammation triggered by TNF- $\alpha$  may predispose to DNA damage, angiogenesis, invasion and metastasis, and local immunosuppression (22). Animal models investigating the link between inflammation and tumorigenesis have emphasized a central role for tumor epithelial TNF- $\alpha$  in this process. For instance, TNF- $\alpha$  is involved in the progression of chemically induced skin cancer with TNF- $\alpha^{-/-}$  mice displaying a 10-fold reduction in papilloma growth (23). Similarly, our laboratory has shown a modest antitumor effect of a TNF- $\alpha$  neutralizing antibody in models of skin and breast tumorigenesis (24). Pikarsky et al. (25) have recently shown that neutralizing stromal TNF- $\alpha$  is also



**Figure 6.** Effect of infliximab on TNF- $\alpha$  mRNA in ovarian cancer cell lines. IGROV-1 (A) and OVCAR-3 (B) cell lines were treated with 10  $\mu$ g/mL infliximab for 3 and 6 h, respectively. RNA was extracted at 3 and 6 h and converted to cDNA. TNF- $\alpha$  mRNA was analyzed using quantitative real-time RT-PCR and normalized to 18S rRNA ( $n = 8$ ). Infliximab reduced TNF- $\alpha$  mRNA in the IGROV-1 and OVCAR-3 cell lines, respectively, compared with PBS and immunoglobulin G1 (IgG1) isotype control (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). Representative of two experiments each done in quadruplicate.



**Figure 7.** Differential regulation by TNF- $\alpha$  of proinflammatory cytokines in malignant ovarian epithelial cells. The IGROV-1 cell line was treated with 20 ng/mL of TNF- $\alpha$  over a 24-h period and induction of various proinflammatory cytokines was confirmed using quantitative real-time RT-PCR: IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, CXCL8, CCL2, and M-CSF (A). Regulation of these cytokines was also revealed in the remaining cancer cell lines (see text), primary tumor cells, and normal ovarian surface epithelial cells (data not shown). B, actinomycin D chase experiments of TNF- $\alpha$  treated IGROV-1 ( $n = 6$ ) showed that IL-6 levels were dependent on *de novo* transcription contrasting with posttranscriptional regulation of CCL2 mRNA. Similar results were obtained with the OVCA9-3 cell line (data not shown).

protective in an animal model of inflammation-associated liver cancer. In the present study, we have compared the expression and regulation of TNF- $\alpha$  in normal and malignant ovarian epithelium based on previous work linking TNF- $\alpha$  expression with ovarian tumor progression (4). We confirmed that TNF- $\alpha$  is overexpressed in ovarian tumor epithelium and we also provide evidence for dysregulated TNF- $\alpha$  activity with the promotion of a proinflammatory tumor microenvironment.

Our results obtained using matched normal tumor tissue show that TNF- $\alpha$  mRNA is increased in ovarian cancer. This was attributed in part due to significantly increased levels of constitutive TNF- $\alpha$  mRNA within the ovarian tumor epithelium as compared with normal ovarian surface epithelial cells. Furthermore, our studies of TNF- $\alpha$  mRNA decay indicate that posttranscriptional regulation is an important mechanism underlying ovarian tumor cell TNF- $\alpha$  production. In renal cancer, the von Hippel-Lindau

**Table 1. Constitutive and TNF- $\alpha$ -inducible cytokines in malignant and normal ovarian surface epithelial**

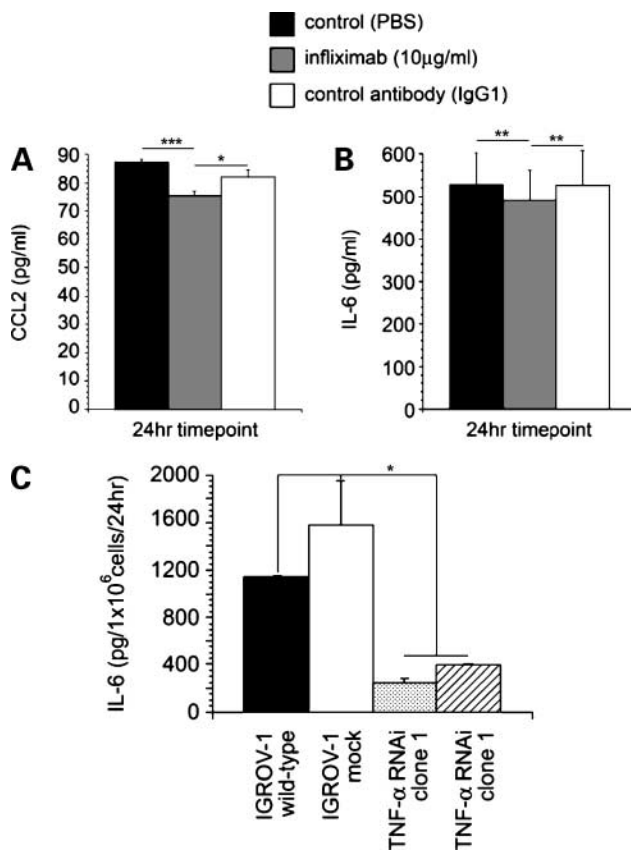
Cytokine (pg/mL)	Malignant ovarian cells*		Normal ovarian surface epithelial cells	
	Constitutive	Inducible	Constitutive	Inducible
IL-1 $\alpha$	0	0–1.7	0	0–2.7
IL-1 $\beta$	0	0	0	3.8–6.7
IL-6	0–1,500	0–7,800	100–5,100	300–12,700
CCL2	10–1,600	10–2,300	650–1,660	1,620–2,350
CXCL8	0–750	30–2,200	860–11,600	24,200–60,000
M-CSF	45–100	75–380	120–250	185–1,180

NOTE: Data are representative of  $\geq 6$  experiments for malignant cells and  $\geq 4$  experiments for normal ovarian surface epithelial cells.

\*Ovarian cancer cell lines and primary tumor cells.

mutation (also described in clear cell carcinoma of the ovary) is causally linked to increased TNF- $\alpha$  expression via derepression of mRNA translation (26, 27). TNF- $\alpha$  also regulated the stability of TNF- $\alpha$  mRNA as well as CXCL8 and vascular endothelial growth factor mRNA in glioma cells (28). The mechanism involved HuR, an RNA-binding protein that binds to the AU-rich sequence in TNF- $\alpha$  mRNA (29). As HuR is up-regulated in ovarian cancer (30, 31) and responds to stimulation by TNF- $\alpha$  (32), this pathway may be involved in stabilizing TNF- $\alpha$  mRNA in ovarian cancer.

The high levels of TNF- $\alpha$  mRNA observed in ovarian tumor cells were partly suppressed by infliximab. This may



**Figure 8.** TNF- $\alpha$  regulates endogenous levels of CCL2 and IL-6 in ovarian cancer cells. IGROV-1 and OVCAR-3 cells were incubated with PBS, infliximab (10  $\mu$ g/mL) or an immunoglobulin G1 isotype control antibody (10  $\mu$ g/mL) for 24 h. Supernatant was harvested and assessed for the production of several TNF- $\alpha$ -inducible cytokines using ELISA. Small but statistically significant reductions in CCL2 ( $n = 6$ ; **A**) and IL-6 ( $n = 6$ ; **B**) were observed in IGROV-1 cell supernatant (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ) with similar results in the OVCAR-3 cell line (data not shown). IGROV-1 cells were then transfected with SUPER RNAi plasmids for TNF- $\alpha$  and a 75% reduction in TNF- $\alpha$  protein was confirmed in two different clones for up to 48 h as compared with mock transfectants (data not shown). Endogenous IL-6 production was markedly down-regulated in both TNF- $\alpha$  RNAi clones compared with mock transfectants and wild-type IGROV-1 cells (\* $P < 0.001$ ; **C**). Representative of three experiments done in duplicate. Western blots of RNAi and control transfectant lysates and wild-type IGROV-1 cell lysates showed comparable levels of the constitutively expressed protein,  $\beta$ -actin (data not shown).

be explained either by a direct effect of infliximab on blocking the autocrine TNF- $\alpha$  loop (i.e., simple sequestration of soluble TNF- $\alpha$ ) or the triggering of inhibitory reverse signaling by binding to transmembrane TNF- $\alpha$  (33). The latter is supported by recent work showing that infliximab can bind to the surface of ovarian tumor cells.<sup>4</sup> In view of our findings of both transcriptional and post-transcriptional regulation of TNF- $\alpha$  in ovarian cancer cells, an analysis of intracellular signaling following infliximab is warranted.

Besides regulating TNF- $\alpha$  expression, TNF- $\alpha$  also stimulated the differential release of other proinflammatory cytokines in malignant and normal ovarian surface epithelial cells. IL-6 is increased in the serum and ascites of patients with malignant ovarian disease compared with patients with benign ovarian disease, and indicates a poor prognosis (21). The chemokine CCL2 has been implicated in the recruitment of tumor-associated macrophages in ovarian cancer (34). Infliximab produced a modest reduction of tumor-derived IL-6 and CCL2, with TNF- $\alpha$  RNAi indicating that the former is largely dependent on endogenous TNF- $\alpha$ . M-CSF, a potent macrophage chemoattractant was also induced by TNF- $\alpha$  and is aberrantly expression in gynecological tumors (12). Recent work in breast cancer has shown a critical requirement for M-CSF in the modulation of the macrophage content of developing tumors (35).

Currently, clinical trials aimed at inactivating TNF- $\alpha$  in solid tumors are under evaluation. A recent phase I study of the specific TNF- $\alpha$  antagonist etanercept (TNFR2:Fc fusion protein) in ovarian cancer provided biochemical (CA125) and radiological evidence for prolonged disease stabilization in 6/30 patients with advanced disease (36). Maisey et al. (37) documented a partial response in 3 of 19 patients with renal cell cancer treated with single agent infliximab although the mechanism at present is unclear. Studies of infliximab are also ongoing in patients with ovarian cancer and should enable delineation of the role of TNF- $\alpha$  within the ovarian cytokine network. Of note, the cancer profiling array also revealed increased TNF- $\alpha$  mRNA expression in lung, breast, cervix, melanoma, and thyroid tumors (data not shown). Lastly, in view of the "double-edged" nature of the inflammatory and immune response in cancer, it will be important to continue careful monitoring of patients enrolled on such studies. Although TNF- $\alpha$  may trigger a proinflammatory protumorigenic milieu, an acute inflammatory response such as that observed with immune manipulation (38) or with hyperinduction of TNF- $\alpha$  may produce an antitumor effect (39).

In conclusion, we have identified a role for TNF- $\alpha$  in the transcriptional and posttranscriptional regulation of endogenous TNF- $\alpha$  in ovarian cancer. Increased understanding of the posttranscriptional regulation of TNF- $\alpha$ , in particular, may lead to more potent drugs that target TNF- $\alpha$  expression. We also showed that TNF- $\alpha$  differentially

<sup>4</sup> Dr. Charles, personal communication.



regulates various proinflammatory cytokines expressed in ovarian tumor epithelium and propose that TNF- $\alpha$  may be considered a key regulator of this cytokine pattern *in vivo*. Finally, TNF- $\alpha$  antagonists may provide novel therapeutic approaches by targeting the inflammation associated with the tumor microenvironment of ovarian cancer.

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