MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2017; 23: 780-789
DOI: 10.12659/MSM.901880

Received: 2016.10.08
Accepted: 2016.11.07
Published: 2017.02.12

Oxaliplatin and Infliximab Combination Synergizes in Inducing Colon Cancer Regression

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Funds Collection G

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Source of support: This work was supported by the National Natural Science Fund from the National Natural Science Foundation of China (grant No. 81672427)

Background: Colon cancer is one of the most common malignant cancers and causes millions of deaths each year. There are still no effective treatments for colon cancer patients who are at advanced stage. Tumor necrosis factor-alpha (TNF-α) might be a good therapy target due to its widely-accepted roles in regulating multiple important biological processes, especially in promoting inflammation.

Material/Methods: We evaluated the expression of TNF-α in 108 human colon cancer tissue samples and 2 colon cancer cell lines (CT26 and HCT116), and analyzed its prognostic values. Further, we explored the roles and mechanism of anti-TNF-α treatment in combination with chemotherapy *in vitro* and *in vivo*.

Results: We found that TNF-α was highly expressed in colon cancer cell lines. The survival analysis and Cox regression analysis indicated that high TNF-α was an independent adverse prognosticator of colon cancer. In addition, anti-TNF-α treatment enhanced the effects of chemotherapy in the xenograft mouse model through inducing ADCC and CDC effects.

Conclusions: We conclude that TNF-α is an independent adverse prognosticator of colon cancer, and anti-TNF-α might benefit colon cancer patients.

MeSH Keywords: Antineoplastic Agents • Colorectal Neoplasms • Prognosis • Survival Analysis • Tumor Necrosis Factor-alpha

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/901880

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Background

Tumor necrosis factor-α (TNF-α), a 233-amino acid transmembrane protein made primarily by macrophages, was initially recognized as an endotoxin-inducible molecule that was cytotoxic to mouse fibrosarcoma L929 cells and caused necrosis of tumors [1]. There are 2 forms of TNF-α: a homotrimeric form on the cell membrane and a soluble protein form [2]. In past decades, the ability of TNF-α to regulate inflammation and to control tumor progression were widely proved [3–6]. It was also found to mediate systemic tissue injury and shock when administrated to rats [7]. Importantly, mounting evidence has shown that TNF-α can promote the survival, proliferation, and migration of cancers [8,9].

The functions of TNF-α are mediated by its 2 known transmembrane receptors – TNF receptor 1 (TNFR1) and TNFR2 – which interact with identical or different downstream signaling molecules. TNFR1 is widely expressed in various cells while TNFR2 is restricted to specific cells, such as immune cells [10,11]. TNFR1 can be activated by both the soluble TNF-α and membrane TNF-α, but TNFR2 only responds to membrane TNF-α. Many essential signaling pathways have been shown to be regulated by TNF-α [9,12], such as the activation of extracellular signal-regulated kinase (ERK) signaling axis, MAPK pathways, and NF-κB [9,12,13]. Therefore, TNF-α is widely involved in regulating vital biological processes such as inflammation, cell survival, proliferation, and apoptosis.

Colon cancer is one of the most common malignant cancers worldwide and causes about half a million deaths each year [14,15]. Surgical strategies are still the leading treatment for colon cancer patients. However, for advanced colon cancer, surgery is not an effective choice because distant metastasis and recurrence often occur [15,16]. Although adjuvant chemotherapy and radiotherapy are usually chosen for advanced colon cancer patients, not all patients benefit, and many advanced colon cancer patients do not respond to these adjuvant therapies, such as the patients with stage II colon cancer or distant metastasis [17–20]. During recent decades, intensive basic and clinical studies have been performed, but drug resistance still limits the application and long-term effects of colon cancer chemotherapy [19,21]. Thus, combining multiple treatments might be a way to meet these challenges. As TNF-α is a very active regulator of inflammation and is closely related to the development of malignant cancers, it might be a good target for exploring new colon cancer treatments.

Material and Methods

Patients and samples

This study was approved by the Medical Ethics Committee of the Cancer Hospital of China Medical University (Liaoning Cancer Hospital & Institute). Our study included 118 colon cancer patients, 10 colon adenoma patients, and 10 healthy volunteers after obtaining informed consent signed by them or their legal representatives. The formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples were collected from 108 colon cancer patients during their surgery, and follow-up data were obtained from the archives of the Cancer Hospital of China Medical University. These patients were diagnosed from 2005 to 2010 and none of them accepted chemotherapy or radiotherapy before the surgery. World Health Organization (WHO) criteria for differentiation and the AJCC TNM Staging Manual of Colon Cancer were used to evaluate each patient. Follow-up started on the day of diagnosis and ended in 2014, with an average length of 35 months. The overall survival (OS) was defined as the interval between the date of diagnosis and date of death. Patients who died due to other causes were excluded from this study. In addition, we also collected the blood of 10 colon cancer patients and 10 gastric adenoma patients to evaluate their serum TNF-α levels. The adjacent tumor tissue of these 10 colon cancer patients and adenoma tissue of the 10 colon adenoma patients were also collected to evaluate TNF-α expression.

Cell culture

Murine colon cancer cell line CT26.WT (ATCC® CRL-2638, referred to as CT26) and human colon cancer cell line HCT116 (ATCC® CCL-247) were cultured with RPMI-1640 and McCoy's 5A medium, respectively, supplemented with 2 mM L-glutamine, 100 unit/ml penicillin, and 10% FBS. Cells were cultured in a humidified incubator with 5% CO_2 at 37°C. Subculturing was conducted when the cells were 80% confluent.

Cell viability assay

The CCK-8 kit (Sigma-Aldrich) was used to evaluate cell viability. All procedures were performed according to the manufacturer's instructions. Briefly, cells (6×10^3/well) were plated to a 96-well plate with 100 µl culture medium. Gradient concentrations of 10 nM to 10^4 nM oxaliplatin (Oxa) and anti-TNF-α treatment (infliximab) were added to the medium for culturing for 48 h. After culturing with the CCK-8 solution (10 µl per well) for 30 min at 37°C, the absorbance at 450 nm was read with a MRX microplate reader (Dynex Technologies, USA). The cell viability of each group was determined by their absorbance percentage of the control group.

Immunostaining

The expressions of TNF-α and level of activated caspase 3 in colon tumor tissues were measured by standard immunohistochemistry (IHC). Antibodies were purchased from Abcam (CA, USA). First, the FFPE tissue sections were deparaffinized with xylene. Endogenous peroxidase was blocked by incubation with

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3% H_2O_2 in methanol. Then, sections were rehydrated with ethanol and heated in a microwave oven for antigen retrieval using the Reveal Decloaker device (Biocare Medical, CA, USA), then we added 5% bovine serum albumin buffer for incubation for 10 min. Primary antibodies (1: 100 dilution) were added for incubation overnight at 4°C. HRP-conjugated secondary antibody was added for incubation for 1 h at room temperature. DAB was also added to visualize the stain. Finally, all slides were observed under a microscope. The scoring of each slide was performed by 2 independent researchers without knowledge of the background information of the patients and mice. The score of each slide was determined by the number of positive cells: 0 for <20%, 2 for 20–40%, 3 for 40–70%, and 4 for more than 70%.

Animal model

The animal experiment was approved by the Experimental Animal Care and Use Committee of the Cancer Hospital of China Medical University. BALB/c mice were used to create a xenograft mouse model of the CT26 colon cancer cell line. All mice were 5-week-old females weighing 19–21 g (Animal Center of the Medical College of Beijing University, Beijing, China). All mice were maintained in a specific pathogen-free environment with free access to clean water and food. A total 5×10^6 CT26 cells were injected into the flank of each mouse subcutaneously. One week later, mice were assigned to 3 groups (10 per group) to accept different treatment: Oxa, Oxa plus infliximab, or saline. Tumor volume was measured every week and survival status was recorded every day. Tumor volume was calculated following the formula: tumor volume=width$^2\times$length$\times\pi/6$.

Complement-dependent cytotoxicity assay and antibody-dependent cellular cytotoxicity

Macrophages were purified from the peritoneal cavity flushing fluid of BALB/c mice using CD14 microbeads. Target cells, which were the colon cancer cell lines HCT116 and CT26, were plated to 96-well plates (1×10^5 in 100 µl per well). Then, infliximab (10 µg/ml) and/or guinea pig serum with active complements was added for incubation for 5 h at 37°C with 5% CO_2 to conduct the complement-dependent cytotoxicity (CDC) assay. For antibody-dependent cellular cytotoxicity (ADCC) assay, after 1 h of Oxa treatment, the effector cells (macrophages) were added for incubating for 48 h at 37°C with 5% CO_2. The cells were then subjected to cell viability assay. The cell cytotoxicity was calculated by following the method described by Yu [22].

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed to evaluate the levels of multiple cytokines, including TNF-α, IL-2, IL-4, and IL-6. The standard procedure of ELISA was followed according to the manufacturer's recommendation (ThermoFisher Scientific, CA, USA). The colon cancer tissue from the mouse model was immediately minced and dissociated into single-cell suspension and lysed by RIPA buffer containing the protease inhibitor.

Fluorescence-activated cell sorter analysis

Fluorescence-activated cell sorter (FACS) analysis was performed to measure the level of TNF-α expression in colon cancer cell lines HCT116 and CT26. TNF-α primary antibody was added to incubate the cells for 25 min at room temperature. We washed the cells 3 times with PBS, then fluorescence-labeled secondary antibody was added to incubate the cells for 15 min at room temperature. Cells were then washed with PBS 3 times. Finally, the cells were analyzed using a FACSCanto II machine (Becton Dickinson, NJ, USA). Data visualization was performed by Flow Jo software.

Statistical analysis

The differences between groups were analyzed by one-way ANOVA or *t* test, as appropriate. The ROC curve was used to determine the cutoff point of TNF-α expression for outcome of colon cancer patients. Kaplan-Meier method was used for survival analysis and a log-rank test was used to evaluate the differences in survival curves. Cox regression originated hazard ratio (HR), 95% confidence interval (CI), and P value were used to evaluated the independent prognostic value of TNF-α for colon cancer patients' outcomes. For all statistical tests, P values less than 0.05 were recognized as statistically significant. All statistical analysis and data visualization were performed using GraphPad (CA, USA) or SPSS17.0 (IL, USA) software.

Results

TNF-α is highly expressed on CC

To explore the roles of TNF-α in colon cancer development, we measured the TNF-α expression level in colon cancer cell lines, patient serum, and human colon cancer tissues. We found that TNF-α was highly expressed in the colon cancer cell lines HCT116 and CT26 compared with the isotype level (Figure 1A–1D). The serum TNF-α level in colon cancer patients was much higher than that of healthy people (Figure 1E). Notably, the TNF-α expression in colon cancer tissue was also higher than that of the adenoma tissue and normal tissue (Figure 1F).

High expression of TNF-α predicts poor survival in CC patients

Aiming to further prove the important role of TNF-α in colon cancer development, we collected tumor tissue samples

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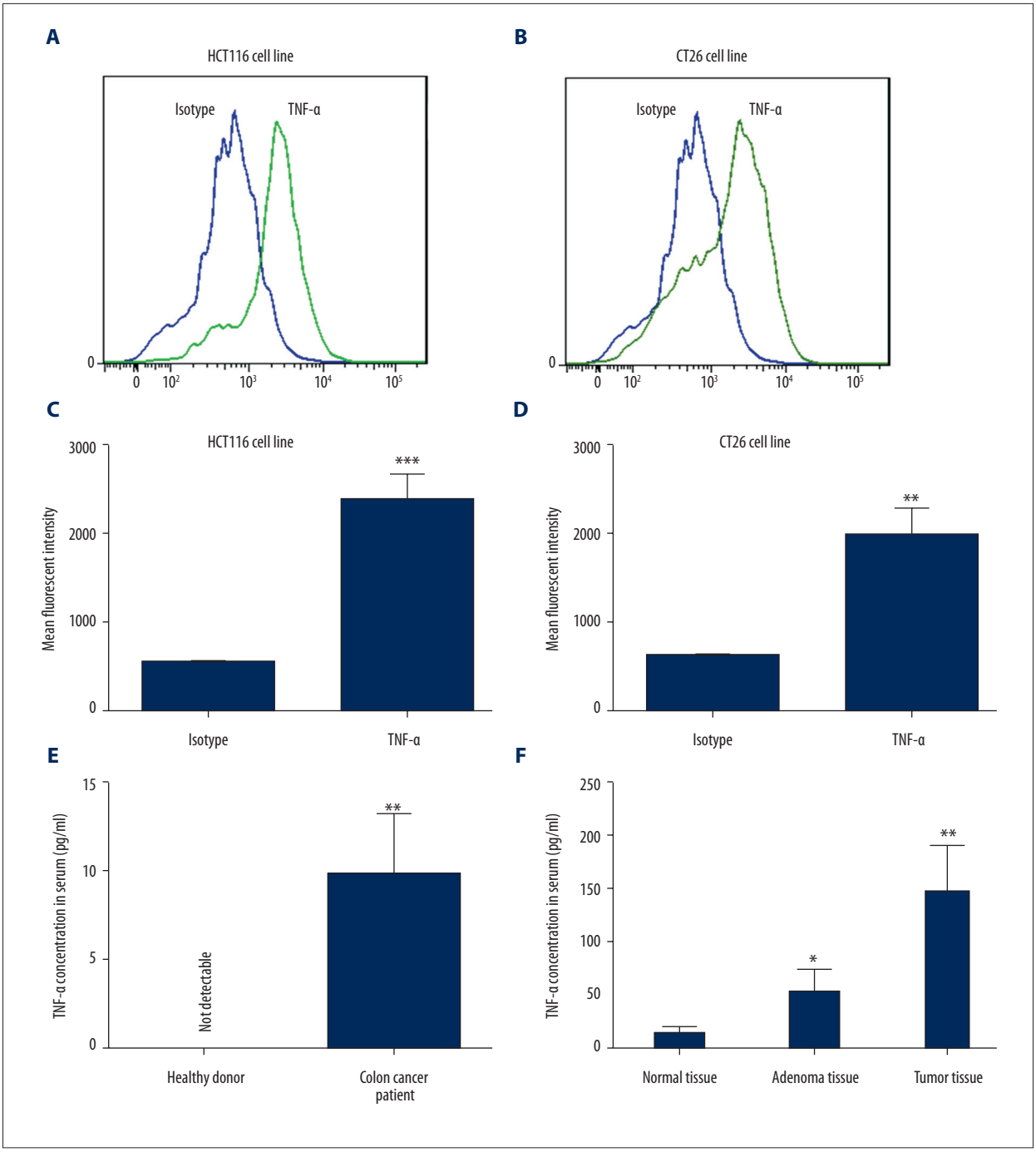

Figure 1. TNF-α expression in colon cancer cell lines and tissues. (**A, B**) The expression level of TNF-α of 2 colon cancer cell lines, HCT116 and CT26 respectively, which were evaluated by FACS analysis; (**C, D**) The quantitative data of TNF-α level evaluated by FACS analysis in HCT116 and CT26 cells. (**E**) TNF-α expression in healthy colon tissue and colon cancer tissue. (**F**) TNF-α expression level in normal colon tissue, colon adenoma tissue, and colon cancer tissue. The concentrations were normalized to the same concentration of total protein extracted from the tissues.

from 108 colon cancer patients, evaluated the TNF-α expression, and analyzed its prognostic value. Interestingly, various expression levels of TNF-α were detected among these colon cancer patients showing the expression score ranging from 0 to 4 (Figure 2A, 2B). Interestingly, a significant difference in the TNF-α score was detected between the patients with advanced TNM stage and early TNM stage, as well as between the patients with advanced grade and lower grade (Figure 2C, 2D).

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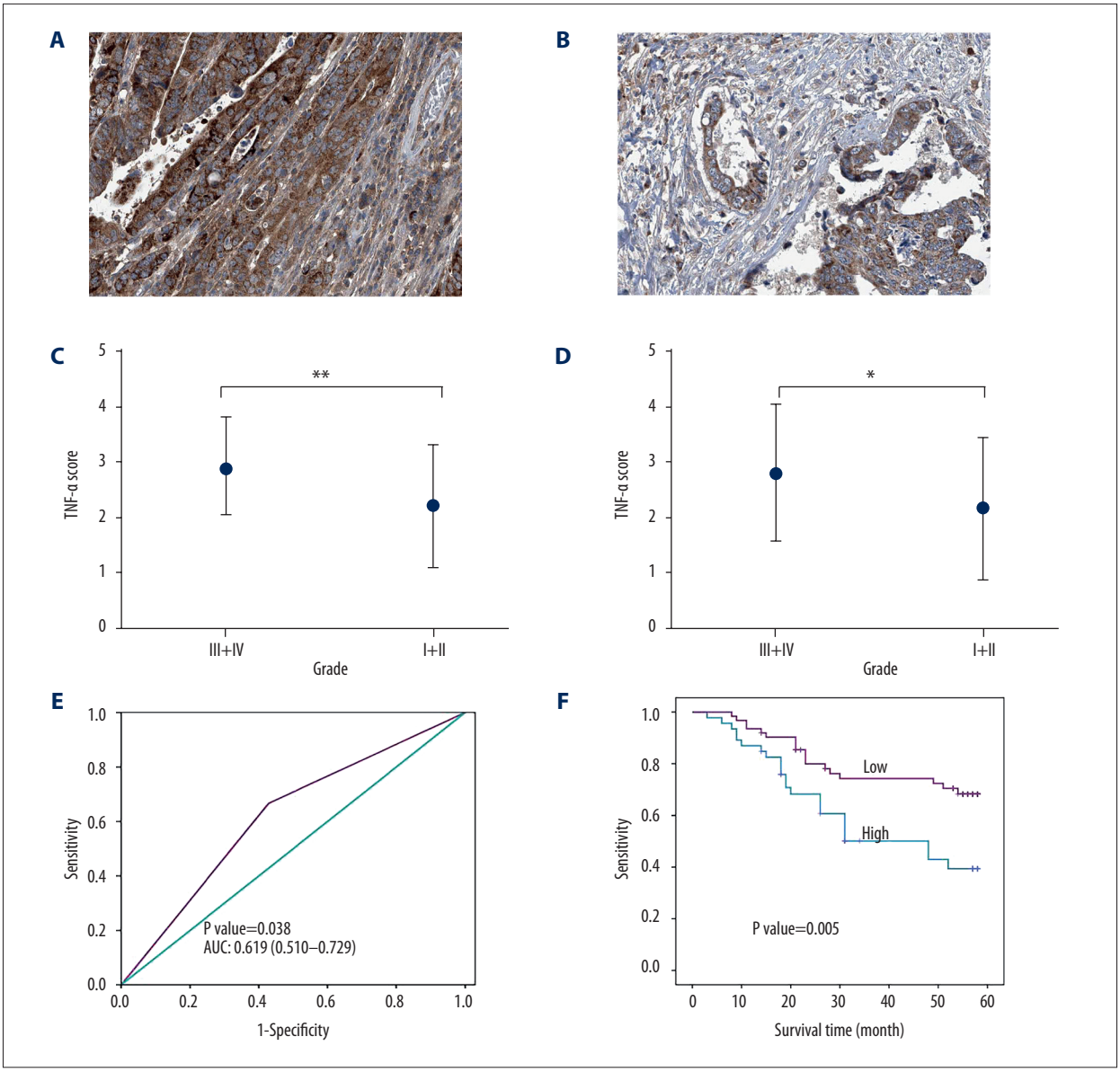

Figure 2. The association of TNF-α expression and pathological features of colon cancer patients. (**A, B**) The representative images of TNF-α expression in tumor tissue of colon cancer patients stained by IHC (high expression and low expression, respectively). (**C, D**) The graphs showed the mean and standard deviation (SD) of TNF-α expression scores for grade and TNF stage, respectively, of the colon cancer patients. The patients with high grade (III+IV) or high TNF stage (III+IV) had higher TNF-α expression scores than those with low grade (I+II) or low TNF stage (I+II). (**E**) The ROC curve showed the sensitivity and specificity of TNF-α expression level of colon cancer patient outcomes based on the cutoff point of 2 (original TNF-α score ≥2 was defined as "high" expression, original TNF-α score <2 was defined as "low" expression). P value was 0.038, and the area under the curve was 0.619 with 95% CI of 0.510–0.729. (**F**) Survival curve of colon cancer patients with different levels of TNF-α expression. The patients with high TNF-α expression tended to have lower overall survival rates than the patients with low TNF-α expression. The P value was 0.005. * Indicates P value less than 0.05; ** indicates P value less than 0.01.

Through ROC curve analysis, TNF-α expression was divided into "high" and "low" expression (Figure 2E). Notably, the TNF-α expression was highly associated with the survival of colon cancer patients (Figure 2F). The patients with high TNF-α expression tended to have lower survival rate and shorter survival time, while the patients with low TNF-α expression had higher survival rate and longer survival time (log-rank test P value: 0.005). Furthermore, Cox regression analysis indicated that TNF-α was an independent prognosticator of colon cancer, and high TNF-α predicted poor prognosis, with HR of 2.611 (95% confidence interval: 1.361–5.025, Table 1).

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Table 1. Cox regression analysis of overall survival of colon cancer patients regarding to their clinicopathological features.

Clinicopathological features	P-value	HR (95%CI)
Age (≥59 *vs.* <59)	0.559	0.801 (0.381–1.685)
Gender (Male *vs.* Female)	0.187	0.637 (0.327–1.244)
Grade (III+IV *vs.* I+II)	0.775	1.103 (0.563–2.164)
TNM stage (III–IV *vs.* I–II)	0.589	1.270 (0.534–3.022)
T stage (T3–T4 *vs.* T1–T2)	0.530	0.799 (0.397–1.609)
Lymph node (N3–N4 *vs.* N0–N2)	0.716	0.873 (0.421–1.812)
Metastasis (Yes *vs.* No)	0.631	0.851 (0.353–1.897)
TNF-α (High *vs.* Low)	0.004	2.557 (1.361–5.025)

Infliximab kills colon cancer cells *in vitro* via ADCC and CDC effects

The results of survival analysis and Cox regression analysis show the important roles of TNF-α in colon cancer, but the mechanism by which TNF-α affects the development of colon cancer is still unclear. As shown in Figure 3, anti-TNF-α treatment with infliximab alone had very little effect on inhibiting the survival of colon cancer cell lines HCT116 and CT26. However, in the presence of effector cells (macrophages) or active complements, infliximab treatment significantly suppressed the survival of the 2 colon cancer cell lines, especially at a relatively high concentration of infliximab, such as 32 μg/ml.

Infliximab enhances the anti-tumor effect of Oxaliplatin *in vitro* and *in vivo*

ADCC and CDC assays indicated that anti-TNF-α treatment might inhibit the survival of colon cancer cells, so we combined the anti-TNF-α treatment with chemotherapy using oxaliplatin (Oxa) in colon cancer cell lines and a xenograft mouse model. As shown in Figure 4A and 4B, HCT116 and CT26 cell lines showed a certain level of drug resistance to Oxa treatment. However, when combining the chemotherapy with anti-TNF-α treatment (infliximab) and complements, the suppression effects were significantly enhanced. Consistently, the CT26 xenograft model showed a sensitive response to the combination treatment, with significantly decreased tumor growth and extended survival time compared with the Oxa single-treatment group (Figure 4C, 4D).

Infliximab enhances oxaliplatin effects via inducing apoptosis and suppressing inflammation

Because the combination of chemotherapy and anti-TNF-α treatment achieved significant benefits in the CT26 xenograft mouse model, we collected tumor tissues from mice and analyzed the changes in key cytokines of key pathways. As shown in Figure 5A, various levels of activated caspase 3 were detected within these colon cancer tissues from mice of different groups. The quantitative data showed that the average number of activated caspase-3-positive cells was much higher in

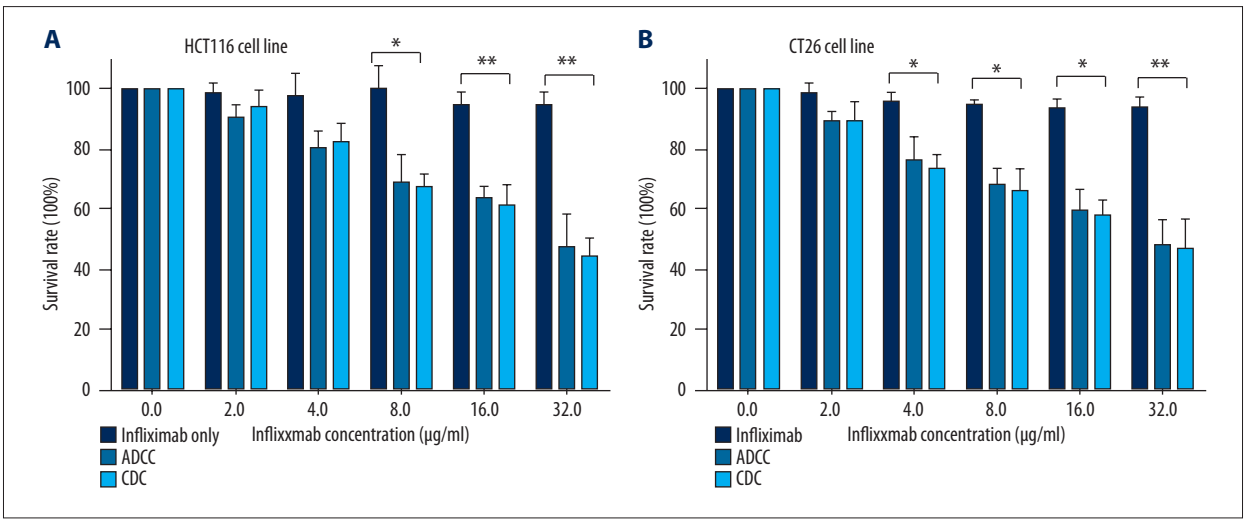

Figure 3. Anti-TNF-α treatment inhibited colon cancer cells survival via ADCC and CDC *in vitro*. (**A, B**) Infliximab treatment inhibited the survival of colon cancer cell lines HCT116 and CT26 in the presence of macrophages or active complement; however, infliximab single-treatment did not show any effects on the survival of these 2 cell lines.

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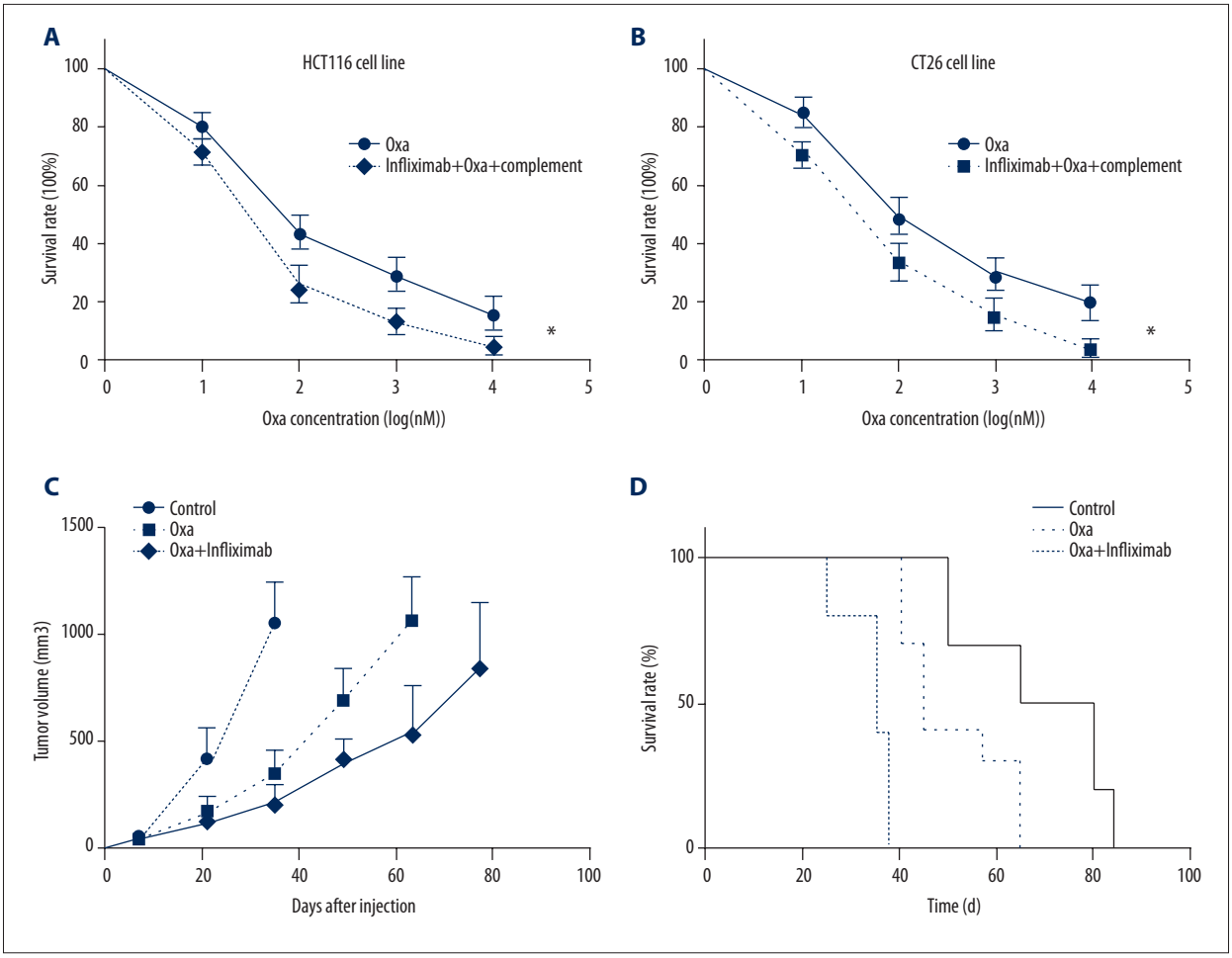

Figure 4. The combination of anti-TNF-α treatment and chemotherapy suppressed the survival of colon cancer cells and reduced the drug resistance. (**A, B**) Colon cancer cell lines HCT116 and CT26 were treated by infliximab and/or gradient concentrations of Oxa. The survival rates of HCT116 and CT26 cells treated by Oxa plus infliximab were lower than those treated by Oxa alone. (**C**) Tumor growth in the CT26 xenograft mouse. Tumor growth of the mice treated by Oxa plus infliximab was much slower than that of the mice treated by Oxa alone or in the control group. (**D**) The survival curve of the CT26 xenograft mouse model. The mice treated by Oxa plus Infliximab had longer survival times and higher survival rates than those treated by Oxa alone and in the control group. * Indicates P value less than 0.05.

the Oxa plus infliximab-treated group than that in the Oxa single-treatment group. Interestingly, the TNF-α expression level was decreased by the combination treatment (Figure 5B). Many other inflammatory cytokines were also decreased by the combination treatment, including IL-2, IL-6, and IL-4 (Figure 5B).

Discussion

TNF-α is among the most pleiotropic cytokines due to its complex functions in regulating various biological processes, such as inflammation, apoptosis, and immune response [1,23,24]. The roles of TNF-α in cancer development are also widely studied. As TNF-α was originally recognized as a killer of tumor cells [1], recombinant TNF-α therapy was studied in many cancers, such as melanoma [5,25]. Although local administration of TNF-α achieved benefits for certain cancer patients [26], systemic toxicity and adverse effects limited further application [25,26]. Many anti-TNF-α agents have been proved to treat many inflammatory diseases, such as Crohn's disease and rheumatoid arthritis [32,27]. This allowed more exploration of anti-TNF-α treatment in various cancers, and promising effects were detected in various cancers, such as ovarian cancer, breast cancer, pancreatic tumors, and renal cell carcinoma [28–32]. Here, we studied the effects of anti-TNF-α in combination with chemotherapy *in vitro* and *in vivo*, which provided new evidence suggesting the potential effectiveness of anti-TNF-α treatment in colon cancer.

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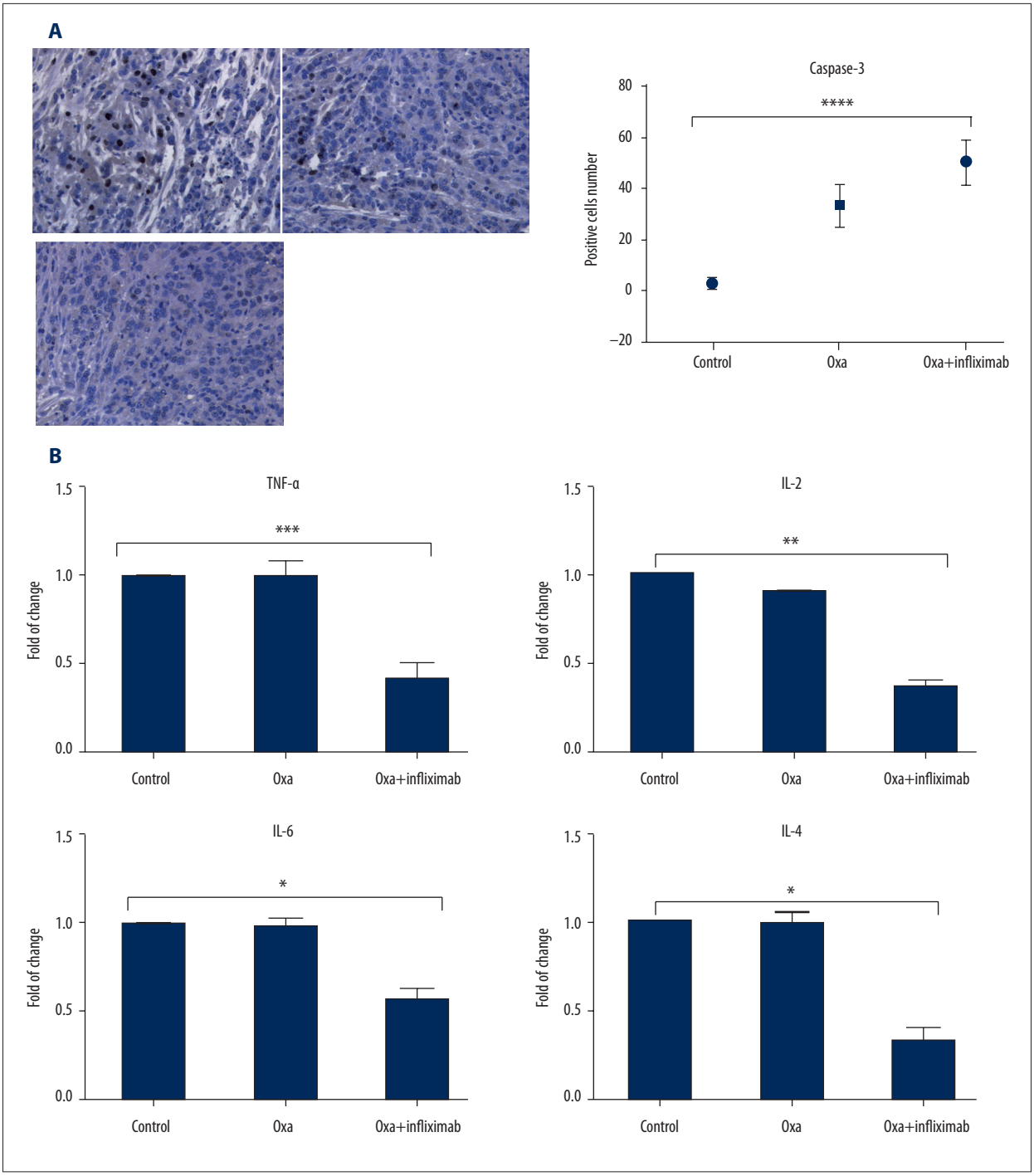

Figure 5. Anti-TNF-α treatment induced apoptosis and inhibited inflammation in the colon cancer xenograft mouse model. (**A**) Activated caspase 3 level in the colon cancer tissue from CT26 xenograft mouse model. Representative pictures of activated caspase 3 levels (IHC staining) are shown: the upper left panel is the control group, the upper right panel is the Oxa single-treatment group, and the lower left panel is the Oxa and infliximab combination treatment group. The lower right panel shows the quantitative data of the activated caspase 3 levels. The red arrows indicate positive cells. (**B**) Cytokines with expression altered due to the treatment. Expression of TNF-α, IL-2, IL-6, and IL-4 was decreased by Oxa plus infliximab combination treatment compared with the Oxa single-treatment group. * Indicates P value less than 0.05; ** indicates P value less than 0.01; *** indicates P value less than 0.001.

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Our results showed that TNF-α expression was increased in colon cancer cell lines and in the animal model. Previous studies have shown that TNF-α was ubiquitously expressed across different tissues under physiological condition, but could be altered in response to pathological conditions, such as inflammation and malignant conditions [9,33,34]. Our result was consistent with previous studies of ovarian cancer and renal cancer [23,33]. In our study, we also observed a significant association between TNF-α expression and TNF stage or grade of colon cancer patients, which suggests that TNF-α promotes colon cancer progression. Notably, the significant difference in survival curves between colon cancer patients with high TNF-α expression and low TNF-α expression and the increased HR value of high TNF-α expression further prove the prognostic value of TNF-α.

The combination of anti-TNF-α treatment and chemotherapy achieved apparent benefits in the colon cancer animal model, which confirmed the clinical application value of anti-TNF-α treatment for colon cancer. Importantly, the animal study also indicated that the anti-TNF-α further inhibited the tumor growth, even when the mice showed resistance to the chemotherapy. The NF-κB pathway has long been considered a prototypical pro-inflammatory signaling pathway. Previous studies also suggested that TNF-α could activate NF-κB pathway, which mediates drug resistance, invasion, and metastasis of tumor cells [9,35]. Thus, anti-TNF-α treatment might be useful way to reduce the drug resistance of chemotherapy in colon cancer.

Conclusions

In summary, based on our solid results, we conclude that high TNF-α expression is an independent prognosticator of poor survival in colon cancer patients. TNF-α might be a good target for colon cancer therapy. When combined with other chemotherapies, anti-TNF-α treatment might help decrease drug resistance in colon cancer.

Disclosure of conflict of interest

None.

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