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IL-9 Exerts Antitumor Effects in Colon Cancer and Transforms the Tumor Microenvironment *In Vivo*

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Abstract

As a newly discovered cytokine, interleukin 9 was initially considered a T-lymphocyte growth factor. Interleukin 9 affects target cells by binding to a member of the γc-family of receptors and is involved in inflammation, autoimmune diseases, and other ailments. In recent years, mounting evidence reveals that interleukin 9 exerts antitumor effects, which has attracted considerable attention. Many previous studies were performed *in vivo* by establishing a mouse model of melanoma. Here, interleukin 9 protein and messenger RNA expression levels were both low in colon carcinoma tissue specimens, as assessed by immunohistochemistry and quantitative real-time polymerase chain reaction. In addition, interleukin 9 expression in these samples was correlated with TNM staging, Dukes staging, lymph node metastasis, and good prognosis, but not with gender, age, tumor size, tumor differentiation, and hepatic metastasis. *In vivo*, by establishing a mouse subcutaneous allograft model, we found that interleukin 9 overexpression inhibited tumor growth and resulted in longer survival time. Then, antitumor immune responses were increased by interleukin 9 as demonstrated by flow cytometry. Furthermore, interleukin 9 was shown to exert antitumor effects by regulating T-cell function and killing tumor cells in the tumor microenvironment. Overall, this study revealed that interleukin 9 exerts robust antitumor effects in colon cancer and transforms the tumor microenvironment *in vivo*.

Keywords

colon cancer, IL-9, anti-tumor effect, tumor microenvironment, cytokine

Abbreviations

CTL, cytotoxic T lymphocytes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemical; IL-9, interleukin 9; LNM, lymph node metastasis; MFIs, mean fluorescence intensities; mRNA, messenger RNA; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time polymerase chain reaction; TIL, tumor infiltrating leukocytes; TME, tumor microenvironment; Treg, regulatory T cells

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Introduction

Colon carcinoma represents a major malignancy of the alimentary canal and ranks third among malignant tumors around the world in terms of incidence.[1,2] The morbidity and mortality rates of colon cancer show an upward trend year after year, due to diet and lifestyle changes.[3,4] Although therapeutic regimens for colon cancer, including surgery, chemotherapy, radiotherapy, and immunotherapy, have been greatly improved, the 5-year survival rate only approximates 50%.[5] Invasion and recurrence are the leading causes of high mortality in patients with colon cancer.[6] The tumor microenvironment (TME) refers to the environment for tumor cell growth and consists of cancer cells as well as the stroma composed of fibroblasts, immune cells, cytokines, secreted proteins, and blood vessels.[7-9] The TME is critical to the occurrence and development of tumors and participates in carcinogenetic processes, including malignant transformation, tumor growth, metastasis, and drug resistance.[10] To design effective therapeutic strategies for colon cancer, novel molecules presenting antitumor effects by transforming the TME are urgently required.

Interleukin 9 was firstly discovered from Th2 cell lines by Uyttenhove *et al* in 1988 and initially considered a T-lymphocyte growth factor.[11] Actually, IL-9 belongs to the γc-family of cytokines, which are mainly obtained from activated Th9 cells but can also be secreted by mast cells, helper T cell subsets, regulatory T cells (Tregs), natural killer T (NKT) cells, eosinophils, and neutrophils.[12,13] Interleukin 9 receptors consist of α (IL-9Rα) and γ (IL-9Rγ) chains.[14] Upon receptor binding by IL-9, JAK1, and JAK3, cross-phosphorylation is stimulated, followed by STAT1, STAT3, and STAT5 forming homo/heterodimers and playing biological roles by entering the nucleus to induce the transcription of related genes as well as protein synthesis.[15-17] Interleukin 9 functions are multiple as its effector cells vary and include mast cells, T cells, antigen-presenting cells, and dendritic cells (DCs).[18] Previous studies have demonstrated that IL-9 affects different inflammatory and histiocytic cells to regulate inflammatory responses and this was shown to be associated with autoimmune colitis, bronchial asthma, and atopic dermatitis.[19-22] In addition, IL-9 is closely associated with autoimmune ailments such as systemic lupus erythematosus, systemic sclerosis, and experimental autoimmune encephalitis.[23] On the contrary, IL-9 exerts inhibitory effects in parasitic infections.[24] Recent studies have gradually discovered the antitumor effects of IL-9, attracting widespread attention.[25] Huang *et al* showed IL-9 has low amounts in tissue and plasma specimens from individuals with colon carcinoma, and these low levels are associated with tumor progression.[26] However, the associations of IL-9 expression with clinicopathological parameters and survival remain largely undefined. More importantly, the antitumor effects of IL-9 on colon carcinoma *in vivo* and the underlying mechanisms have not been previously assessed.

In this study, we evaluated the protein and messenger RNA (mRNA) amounts of IL-9 in colon carcinoma and adjacent normal tissue specimens by immunohistochemical (IHC) staining and quantitative real-time polymerase chain reaction (qRT-PCR) and assessed the associations of IL-9 expression with clinicopathological parameters and survival. Furthermore, we determined the antitumor effects of IL-9 on colon cancer *in vivo* by establishing subcutaneous allotransplanted tumors in mice and analyzed the transformations occurring in the TME by flow cytometry to explore the potential underlying mechanisms.

Materials and Methods

Patients With Colon Cancer and Clinical Tissue Specimens

The clinical specimens used in this study were obtained from 92 patients with colon cancer who were surgically treated from January to December 2010 in the Department of General Surgery, The First Affiliated Hospital of Soochow University, Suzhou, China. The diagnosis of colon carcinoma was confirmed by postoperative pathology. The patients had no history of autoimmune disorder and infectious diseases, including tuberculosis, hepatitis, and AIDS. No patients were treated by chemotherapy, radiotherapy, molecular-targeted therapy, and immunotherapy before the operation. In this study, colon cancer cases included 53 men and 39 women, aged between 46 and 91 years (mean, 70.1 years). There were 44 and 48 stage I-II and III-IV cases according to the seventh edition of TNM staging criteria, respectively; 45 and 6 cases had lymph node metastasis (LNM) and hepatic metastasis, respectively.

Colon carcinoma and paired adjacent noncancerous (resected from colon tissues at over 5 cm away from the tumor edge and confirmed as normal tissues without tumor invasion by postoperative pathology) tissue specimens were simultaneously resected. A portion of each tissue specimen immediately underwent fixation with 10% neutral formalin and paraffin embedding for IHC staining. Another portion of the tissue specimen was stored at −80°C for qRT-PCR. Written informed consent was obtained from all patients and the present study had approval from the ethics committee of First Affiliated Hospital of Soochow University. All experiments were performed in accordance with relevant guidelines and regulations.

Survival Analysis of Patients With Colon Cancer

Survival time was the period elapsed between initial colon cancer surgery and colon carcinoma-associated death. The clinical follow-up lasted 6 to 72 months until December 1, 2016; data were obtained from telephone calls or return visits. After the primary surgery, follow-up was carried out every 3 months in the first 2 years, every 6 months in years 2 to 5, and once yearly over 5 years.

Immunohistochemical Staining

Paraffin-embedded tissues were sectioned at 4 μm on a microtome for IHC staining. The IHC staining was implemented by the ChemMate Envision/HRP method as instructed by the

manufacturer. After deparaffinization with xylene and dehydration in graded ethanol series, sodium citrate solution was used to retrieve antigens under high pressure for 30 minutes. Then, 3% H_2O_2 solution was used to inactivate the endogenous peroxidase for 15 minutes away from light and 3% bovine serum albumin was used for blocking. The slides were sequentially incubated with anti-IL-9 primary antibodies (1:100 dilution; Abcam, Shanghai, China) at 4°C overnight and secondary antibodies (1:200 dilution; Abcam) at 37°C for 30 minutes. After 5 phosphate-buffered saline (PBS) washes, the sections were reacted with diaminobenzene for visualization; hematoxylin was used for counterstaining. Finally, the sections were dehydrated by ethanol and mounted with neutral resin for further assessment.

Interleukin 9 was predominantly found in the cytoplasm of normal cells, but not tumor cells, and positive staining was reflected by faint yellow, yellowish brown, or brown signals. Ten high-power fields under a microscope were randomly selected for IL-9 expression evaluation. According to the percentage of positive staining cells, the score was set as follows: <5%, 0; 5% to 30%, 1; 31% to 60%, 2; and >60%, 3. Based on the signal intensity, the score was set as follows: no staining, 0; faint yellow, 1; yellowish brown, 2; and brown, 3. Both scores were multiplied to obtain the final grade: <2, negative IL-9 expression and ≥2, positive IL-9 expression. The IHC staining of IL-9 was independently evaluated by 2 observers absolutely blinded to the patients' clinicopathological data.

Quantitative Real-Time PCR

The 92 paired colon cancer tissue specimens which are stored at −80°C as mentioned above were prepared for qRT-PCR. Total RNA extraction was carried out from colon carcinoma and adjacent normal tissues with the RNeasy Mini kit (Qiagen, Duesseldorf, Germany) as directed by the manufacturer, and RNA amounts were quantified on an ultraviolet spectrophotometer (Bio Drop, Cambridge, England). First strand complementary DNA was synthetized with the Prime Script RT Master Reagent Kit (Takara, Beijing, China). Real-time PCR assessed IL-9 mRNA expression with the SYBR Premix Ex Taq Mix reagent (Takara), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. Relative mRNA levels were obtained by the $2^{-\Delta\Delta Ct}$method. Triplicate experiments were carried out, and values were averaged. The primer sequences were as follows: IL-9, 5′-CTCTGTTTGGGCATTCCCTCT-3′ (sense) and 5′-GGGTATCTTGTTTGCATGGTGG-3′ (antisense); GAPDH, 5′-TGTGGGCATCAATGGATTTGG-3′ (sense) and 5′-ACACCATGTATTCCGGGTCAAT-3′ (antisense).

Cells and Culture

Mouse colon cancer CT-26 cells were provided by the Institute of Clinical Immunology of Jiangsu Province (Suzhou, China). The cells were cultured in RPMI-1640 medium (Hyclone, Utah, USA) containing fetal bovine serum (Sijiqing Biological Manufacturer Co, Ltd, Hangzhou, China) and 1% penicillin–streptomycin (Institute of Clinical Immunology of Jiangsu Province, Suzhou, China), in a humidified environment containing 5% CO_2 at 37°C. The growth status of cells was observed daily, and the medium was replaced at regular intervals. Confluent CT-26 cells were passaged.

Lentiviral Transfection of Mouse Colon Cancer CT-26 Cells

The lentivirus stably overexpressing IL-9 and the corresponding negative control lentivirus were provided by Shanghai GeneChem Co, Ltd (Shanghai, China). The plasmid vector of the lentivirus carried the green fluorescent protein (GFP) sequence and could appear green under a fluorescent microscope. The sequences of primers designed to obtain the target gene were as follows: 5′-AGGTCGACTCTAGAGGATCCCGCCACCATGGCCTTACCAGTGACCGCCTTG-3′ (sense) and 5′-TCCTTGTAGTCCATACCTGGTCGGCTTTTCTGCCTTTGC-3′ (antisense).

CT-26 cells prepared for transfection were in the logarithmic growth phase, and a cell suspension containing 5×10^4 cells was seeded in a 6-well cell culture plate. Then, 20 μL of lentiviral particles (multiplicity of infection [MOI] = 100; concentration, 1×10^8 TU/mL) was added to transfect CT-26 cells at a confluency of 30%. Three cell groups were obtained: blank control group (CT26-WT), not transfected by lentivirus; negative control group (CT26-NC), transfected by the control lentivirus without IL-9 expression; experimental group (CT26-IL-9), transfected by the lentivirus stably overexpressing IL-9. After confirming that the cell growth was not inhibited by lentiviruses 12 hours after transfection, the culture medium was replaced by the normal culture medium, followed by incubation in a medium containing puromycin (5 μg/mL) for selecting stably transfected cells. The fluorescence microscopy was carried out to evaluate GFP expression 3 days after transfection. The IL-9 expression in transfected cells was assessed flow cytometrically.

At the same time, 24 and 48 hours after lentiviral transfection, cell-free supernatants were harvested and assayed for IL-9 production levels using a murine IL-9 enzyme-linked immunosorbent assay (ELISA) kit, respectively (Invitrogen, California, USA).

Establishment of a Mouse Subcutaneous Allograft Model

The laboratory mice used in the current study were 6-week-old female BALB/c mice (Suzhou Supusi Biological Technology Co, Ltd, Suzhou, China). They were housed in the Laboratory Animal Center of Soochow University. The animals were divided into 3 groups, including CT26-WT, CT26-NC, and CT26-IL-9 groups (n = 6). The transfected CT-26 cells were resuspended in PBS to obtain a density of 2×10^7 cells per mL. Next, 50 μL of the above cell suspension containing a total of 1×10^6 cells were subcutaneously injected into the right flank of each mouse to establish the mouse subcutaneous allograft model. The mice were observed daily for general fitness. The subcutaneous tumors were measured every 2 days from the

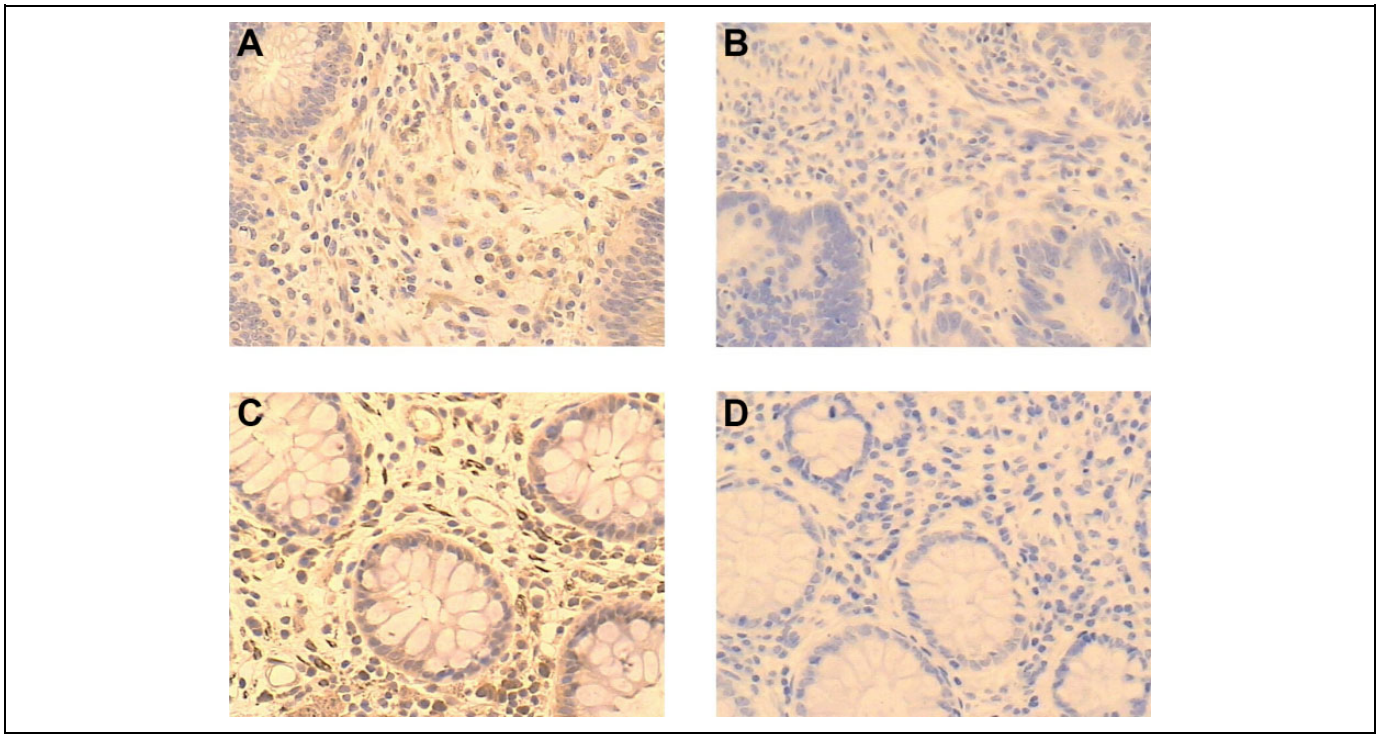

Figure 1. Protein amounts of interleukin 9 (IL-9) in colon carcinoma and adjacent noncancerous tissue samples assessed by immunohistochemical (IHC) staining. Positive (A) and no (B) IL-9 staining signals in colon carcinoma tissues. Positive (C) and no (D) IL-9 staining signals in adjacent normal specimens (magnification: ×200).

fifth day after injection, and tumor diameters were calculated by the formula $D = (L + W)/2$ (mm). The experiment was repeated, and the survival time for each mouse was recorded. The animal studies were carried out at Laboratory Animal Center of Soochow University and performed according to a protocol approved by the Animal Care and Use Committee of Soochow University.

Flow Cytometry

Subcutaneous tumors from mice were immediately harvested after sacrifice by cervical dislocation and placed in RPMI-1640 without serum. Sheared tumors were digested in TL digestive juice (Sigma Aldrich Trading Co, Ltd, Shanghai, China) at 37°C for 25 minutes, filtered, and resuspended in 0.5 mL Hanks with 1% PBS. Then the cell suspensions were adjusted to 2×10^6 cells per 100 μL on ice and centrifuged to prepare for antibody staining. To stain proteins on the cell membrane, antibodies (eBioscience, Beijing, China) were added to the cell suspension (diluted by 1:200) and incubated at 4°C overnight in the dark. To label intracellular proteins, the cells were fixed with 100 μL of 1× Perm Wash Buffer (diluted by 1:100) and incubated with antibodies (eBioscience) at 4°C overnight in the dark. To label nuclear proteins, the cells were fixed with 100 μL True-Nuclear 1× Perm Wash Buffer (diluted by 1:150) and incubated with antibodies (eBioscience) at room temperature in the dark for 30 minutes. Then the collected cells were resuspended and assessed flow cytometrically (Becton-Dickinson, California, USA). Quantitation was performed by the Flowjo V10.0 software (FlowJo software v10; New Jersey, USA).

Statistical Analysis

All data were statistically assessed by GraphPad Prism 7.0 (GraphPad Software Inc, California, USA). Enumeration data were presented as rate and compared by the χ^2 test. Measurement data were presented as mean Pad Prism 7.0 (Graph [SD]) and assessed by Student t test. The Kaplan-Meier method was used for survival analysis, with log-rank test used for comparisons. $P < .05$ indicated statistical significance.

Results

Interleukin 9 Levels in Colon Carcinoma and Adjacent Noncancerous Tissues

Interleukin 9 protein amounts were determined by immunohistochemistry, and representative IHC staining images are shown in Figure 1. Positive IL-9 IHC signals were predominantly found in the intercellular space of normal colon tissues. Interleukin 9 was weakly expressed in colon carcinoma samples, in comparison with adjacent noncancerous specimens (Figure 1). Detailed results of IL-9 expression are shown in Table 1. The positive expression rate of IL-9 in colon carcinoma tissues was 37.0% (34/92), including 58 negative and 34 positive cases; meanwhile, 77.2% (71/92) was found in adjacent normal

Table 1. Expression Levels of IL-9 in Colon Carcinoma and Adjacent Noncancerous Tissue Samples.

Group	n	IL-9 Expression (%)		χ^2	*P*
		Negative	Positive		
Tumor tissues	92	58 (63.0)	34 (37.0)	30.367	.000
Noncancerous tissues	92	21 (22.8)	71 (77.2)		

Abbreviation: IL-9, interleukin 9.

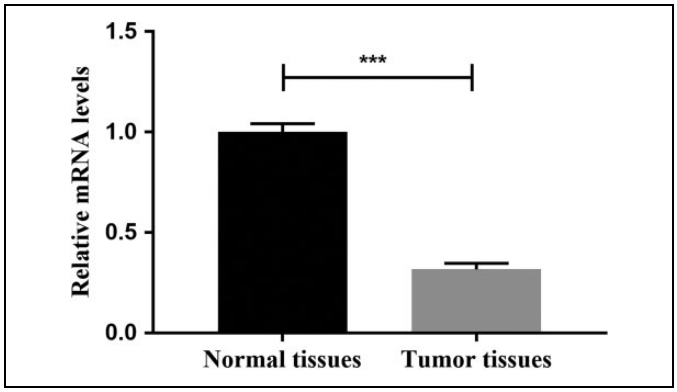

Figure 2. Relative messenger RNA (mRNA) amounts of interleukin 9 (IL-9) in colon carcinoma and adjacent noncancerous tissue specimens determined by quantitative real-time polymerase chain reaction (qRT-PCR). Ninety-two paired colon cancer tissue specimens were taken into the qRT-PCR. The mean value of IL-9 mRNA amounts in adjacent normal tissues was set as "1." (***$P < .001$).

samples, including 21 negative and 71 positive cases. These findings suggested that IL-9 was less expressed in colon carcinoma.

Interleukin 9 mRNA levels were detected by qRT-PCR (Figure 2). The mean value of IL-9 mRNA expression levels in adjacent normal tissues was set as "1." Relative *IL-9* gene expression levels were therefore 1.00 $\pm$ 0.13 in adjacent normal tissues and 0.32 $\pm$ 0.09 in colon cancer specimens, indicating IL-9 downregulation in colon carcinoma ($P < .001$). Taken together, IL-9 protein and mRNA amounts were both markedly elevated in adjacent normal tissue samples compared with colon cancer specimens, suggesting that IL-9 may exert antitumor effects in colon cancer.

Associations of IL-9 Levels With Clinicopathological Parameters

We found that the positive expression rate of IL-9 (50.0%) in TNM stage I to II disease was higher than 25.0% obtained in stage III to IV samples ($P = .013$; Table 2). Similarly, the positive expression rate of IL-9 (47.1%) in Dukes stage A to B was higher than that of stage C to D cases (24.4%; $P = .025$; Table 2). Moreover, patients without LNM distinctly showed higher IL-9 expression (51.1%) compared with those with LNM (22.2%; $P = .004$; Table 2). As shown above, IL-9 expression in colon carcinoma was correlated with TNM staging, Dukes staging, and LNM ($P < .05$; Table 2). However, IL-9 expression in colon cancer was not correlated with gender, age, tumor size, tumor differentiation, and hepatic metastasis ($P > .05$; Table 2). The above data suggested that IL-9 may inhibit colon cancer progression and metastasis.

Table 2. Associations of IL-9 Levels With Clinicopathological Parameters in Colon Carcinoma.[a]

Clinicopathological Parameters	n	IL-9 Expression (%)		χ^2	*P*
		Negative	Positive		
Gender				1.279	.258
Male	53	36 (67.9)	17 (32.1)		
Female	39	22 (56.4)	17 (43.6)		
Age (years)				1.596	.206
≤65	29	21 (72.4)	8 (27.6)		
≤65	63	37 (58.7)	26 (41.3)		
Tumor size				2.287	.130
≤3 cm	17	8 (47.1)	9 (52.9)		
≤3 cm	75	50 (66.7)	25 (33.3)		
Differentiation				3.048	.081
Poor or intermediate	69	47 (68.1)	22 (31.9)		
Well	23	11 (47.8)	12 (52.2)		
TNM staging				6.158	**.013**
I-II	44	22 (50.0)	22 (50.0)		
III-IV	48	36 (75.0)	12 (25.0)		
Dukes staging				5.013	**.025**
A-B	51	27 (52.9)	24 (47.1)		
C-D	41	31 (75.6)	10 (24.4)		
LNM				8.208	**.004**
Without	47	23 (48.9)	24 (51.1)		
With	45	35 (77.8)	10 (22.2)		
Hepatic metastasis				3.763	.052
With	6	6 (100.0)	0 (0.00)		
Without	86	52 (60.5)	34 (39.5)		

Abbreviation: IL-9, interleukin 9; LNM, lymph node metastasis.
[a]Values in bold are referred to as statistical significance at $P < .05$.

Associations of IL-9 Expression With Patient Survival

The patients were followed up for an average of 68 months, with a follow-up rate of 93.3%. Overall 5-year survival of all 92 colon cancer cases was 57.6% (53/92). One-, 3-, and 5-year survival rates in cases with no IL-9 expression in colon cancer tissues (58 cases) were 94.8%, 74.1%, and 53.4%, respectively. Meanwhile, 1-, 3-, and 5-year survival rates in those with positive IL-9 signals in colon cancer tissues (34 cases) were 100.0%, 91.2%, and 76.5%, respectively. Univariate survival analysis revealed that patients with positive IL-9 expression possessed longer survival time compared with those not expressing the protein, suggesting that IL-9 may result in good prognosis ($P < .05$; Figure 3).

Successful Lentiviral Transfection of Mouse Colon Cancer CT26 Cells for Stable IL-9 Overexpression

The expression levels of GFP in the 3 cell groups were evaluated under a fluorescence microscope 3 days after lentiviral

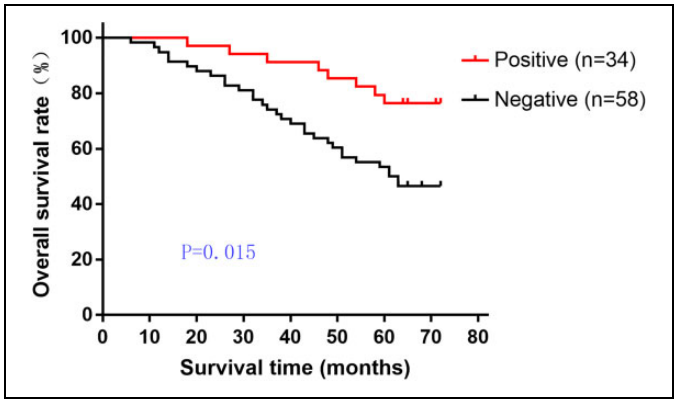

Figure 3. Survival of colon cancer cases based on interleukin 9 (IL-9) levels in colon carcinoma tissue samples.

transfection of CT26 cells. GFP expression was overtly higher in the CT26-NC and CT26-IL-9 groups compared with the CT26-WT group, suggesting that the lentiviral vector was successfully transfected to CT-26 cells (Figure 4A). Then, IL-9 expression in transfected cells was assessed by flow cytometry. The rates of GFP expression were over 90% in the CT26-NC and CT26-IL-9 groups, but 0% in the CT26-WT group (Figure 4B). Moreover, IL-9 expression was markedly higher in the CT26-IL-9 group in comparison with the CT26-NC group ($P < .01$; Figure 4C). Also, ELISA was used to evaluate the production levels of secreted IL-9. Interleukin 9 production was significantly higher in the CT26-IL-9 group compared with the CT26-WT and CT26-NC groups at 24 and 48 hours, suggesting successful establishment of a CT26 cell line stably overexpressing IL-9 ($P < .001$; Figure 4D).

Tumoral Expression of IL-9 Inhibits Tumor Growth and Prolongs Survival in Mice

To further assess whether IL-9 shows antitumor effects, a mouse subcutaneous allograft model was established for *in vivo* experiments. First, tumors were resected from mice in the 3 groups at 25 days after cell injection, and tumor images were acquired (Figure 5A). It turned out that tumor sizes in mice were smaller in the CT26-IL-9 group compared with the CT26-NC group (Figure 5A). The growth rate of tumors was lower and final tumor sizes were reduced in the CT26-IL-9 group compared with the CT26-NC group ($P < .05$; Figure 5B). However, no overt differences were found between the CT26-WT and CT26-NC groups ($P > .05$; Figure 5B). In another experiment, the survival time of each mouse was recorded and assessed by the log-rank test. Mice in CT26-IL-9 group were more active and had a longer survival time compared with the CT26-NC group ($P = .0018$; Figure 5C). However, the CT26-WT and CT26-NC groups showed comparable values ($P > .05$; Figure 5C). The above data showed that tumor IL-9 greatly reduced subcutaneous tumor growth and prolonged survival time in mice, suggesting that IL-9 may exert antitumor effects in colon cancer *in vivo*.

Tumoral Expression of IL-9 Increases Anticancer Immune Reactions in the TME

To further assess and confirm the antitumor effects of IL-9 in colon carcinoma, we detected tumor infiltrating leukocytes (TILs) in tumor samples by flow cytometry to explore the potential mechanisms. First, the percentages of $CD45^+$ TILs were assessed, and an obvious increase in CT26-IL-9 tumors was found compared with the CT26-WT or CT26-NC group (both $P < .01$; Figure 6A); however, the CT26-WT and CT26-NC groups showed comparable values ($P > .05$; Figure 6A), suggesting that antitumor responses were increased in CT26-IL-9 tumors. Next, we respectively detected $CD4^+$ T cells and $CD8^+$ T cells among $CD45^+$ TILs. The results showed that $CD4^+$ T-cell percentages among $CD45^+$ TILs were not significantly different between CT26-IL-9 and CT26-NC tumors ($P > .05$; Figure 6B). However, as a result of excessive $CD45^+$ TILs in CT26-IL-9 tumors, the total $CD4^+$ T cell amounts were elevated in CT26-IL-9 tumors compared with CT26-WT or CT26-NC tumors ($P < .001$, $P < .01$; Figure 6C); the CT26-WT and CT26-NC groups were comparable ($P > .05$; Figure 6C). Similarly, although $CD8^+$ T-cell percentages among $CD45^+$ TILs were comparable in CT26-IL-9 and CT26-NC tumors ($P > .05$; Figure 6B), the total amounts of $CD8^+$ T cells were elevated in the CT26-IL-9 group in comparison with CT26-NC or CT26-NC tumors ($P < .01$, $P < .01$; Figure 6C). CT26-WT and CT26-NC tumors showed similar values ($P > .05$; Figure 6C). This may be due to $CD8^+$ T-cell recruitment and local infiltration. These data suggested that IL-9 increased antitumor immune responses in the TME.

Tumoral Expression of IL-9 Promotes Antitumor Effects in the TME Through Specific Mechanisms

In order to further assess the changes in the TME, we next detected other immune indexes of TILs. As shown in Figure 7A, both $CD4^+CD44^+$ and $CD8^+CD44^+$ T cells had higher mean fluorescence intensities (MFIs) in CT26-IL-9 tumors compared with the CT26-WT and CT26-NC groups ($P < .05$, $P < .05$; $P < .05$, $P < .05$). Similarly, $CD4^+$IL-$7Ra^+$ and $CD8^+$IL-$7Ra^+$ T cells showed increased MFIs in CT26-IL-9 tumors in comparison with CT26-WT and CT26-NC tumors ($P < .05$, $P < .05$; $P < .01$, $P < .01$; Figure 7B). In addition, the MFI of $CD8^+$ granzyme B^+ T cells was starkly higher in CT26-IL-9 tumors in comparison with CT26-WT or CT26-NC tumors ($P < .05$, $P < .05$; Figure 7C). Moreover, the $CD4^+$ $Foxp3^+$ T-cell percentage was higher in CT26-IL-9 tumors, indicating increased amounts of Treg in CT26-IL-9 tumors in comparison with CT26-WT and CT26-NC tumors ($P < .05$; Figure 7D). However, CT26-WT and CT26-NC tumors showed similar values for the above immune indexes (all $P > .05$; Figure 7A-7D). The above observations suggested that IL-9 promoted anticancer immune reactions by elevating the rates of $CD4^+$/$CD8^+CD44^+$, $CD4^+$/$CD8^+$IL-$7Ra^+$, and $CD8^+$ granzyme B^+ T cells in the TME, with immune responses regulated by Tregs.

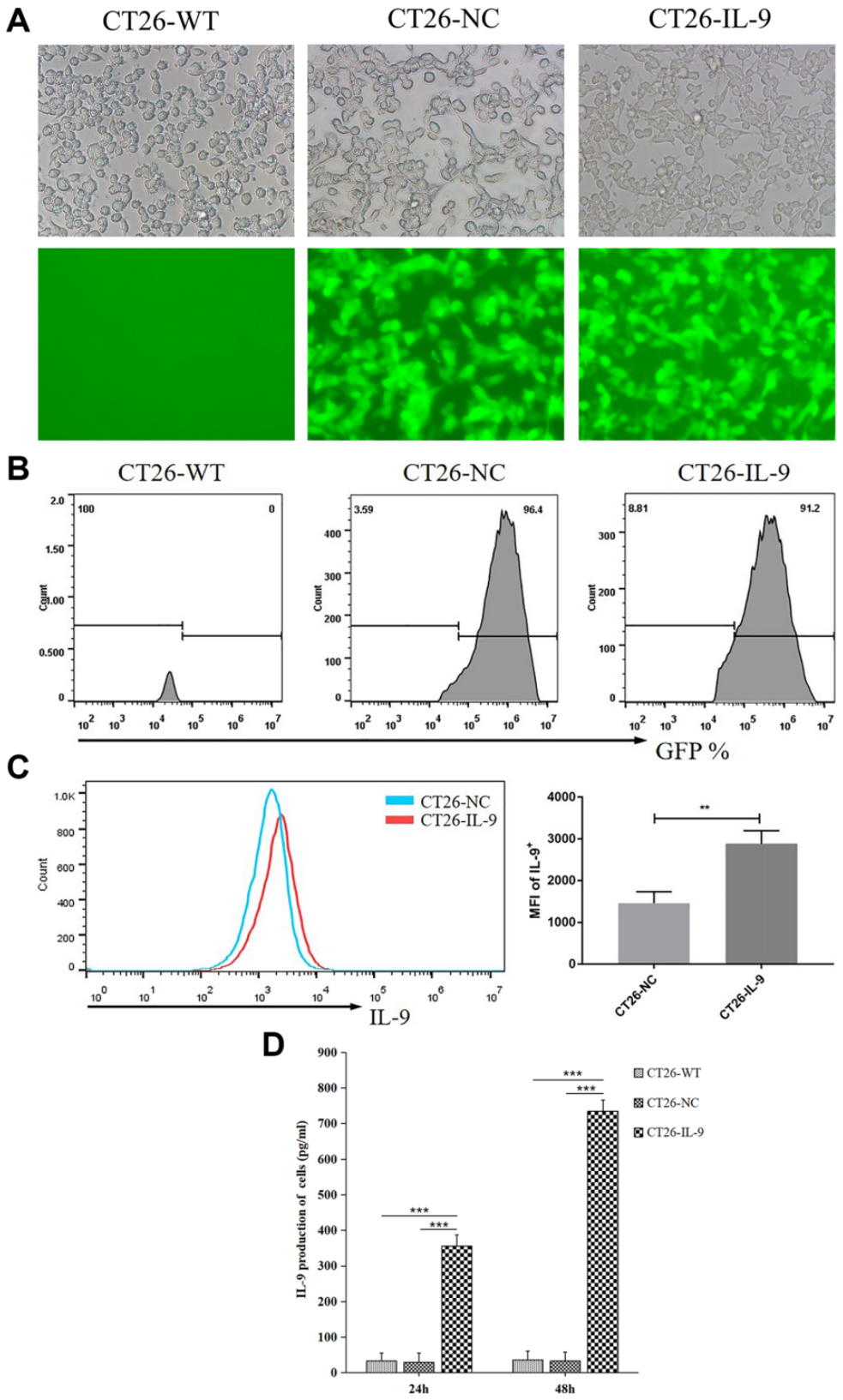

Figure 4. Successful establishment of a CT26 cell line stably overexpressing interleukin 9 (IL-9) by lentiviral transfection. A, Micrographs of CT26 cells under a fluorescence microscope 72 hours after lentiviral transfection in the 3 indicated groups (magnification: ×200). B, Positive rates of green fluorescent protein (GFP) in the 3 groups. C, Representative flow cytograms and mean fluorescence intensities (MFIs) of the CT26-NC and CT26-IL-9 groups. D, The production level of IL-9 at 24 and 48 hours were measured by enzyme-linked immunosorbent assay (ELISA) (*$P < .05$, **$P < .01$, ***$P < .001$).

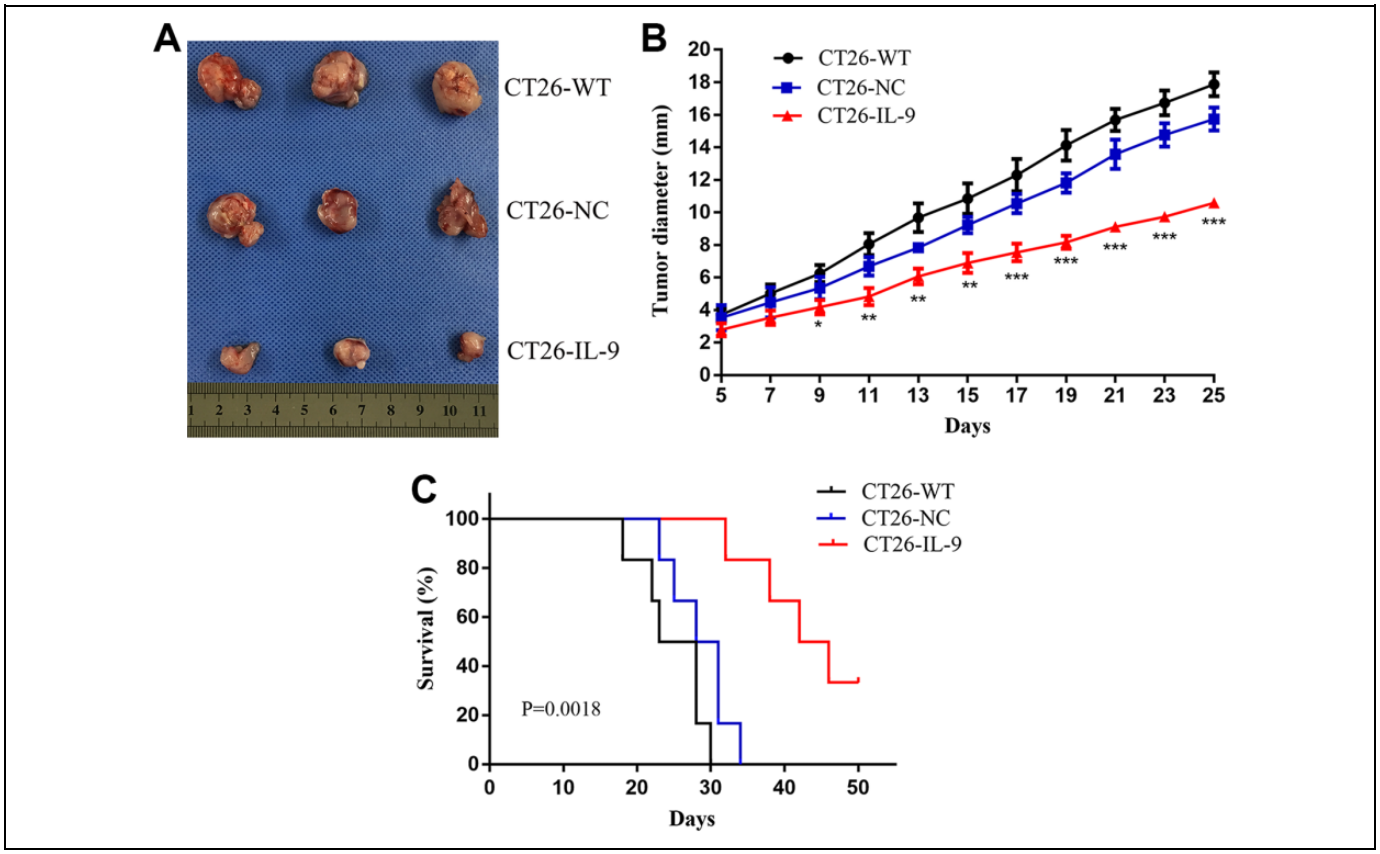

Figure 5. Tumor interleukin 9 (IL-9) exerts antitumor effects *in vivo* in the subcutaneous allograft model in BALB/C mice. A, Subcutaneously transplanted tumors in the indicated mouse groups were resected 25 days after subcutaneous injection of lentivirus-transfected CT-26 cells. B, Tumor diameters in the 3 groups were measured and recorded every 2 days. C, Each group had 6 mice, and animal survival in the 3 groups was monitored (*$P < .05$, **$P < .01$, ***$P < .001$).

Discussion

Tumor immunity has been considered a leading research topic in recent years. The general process of tumor immunity includes 3 stages: immune clearance, balance, and escape.[27] Tumor cells can escape immune surveillance, recognition, and attack by the immune system through multiple mechanisms so that the body cannot produce effective antitumor immune responses.[9] Tumor immunity mainly relies on the interactions between cancer cells and multiple immune cells, including T cells, DCs, and macrophages in the TME.[28,29] Recently, immunotherapy has become the most promising method to cure cancer after surgery, radiotherapy, chemotherapy, and molecular targeted therapy and constitutes an important field of tumor therapy research. Indeed, the Nobel Prize in Physiology or Medicine of 2018 went to pioneers of cancer immunotherapy. Tumor immunotherapy aims to stimulate and enhance the body immune function in order to control and kill tumor cells.[29] T lymphocytes, especially cytotoxic T lymphocytes (CTLs) are major effectors in antitumor immune responses in the TME.[30] However, there are still many immune cells, including Tregs, regulatory DCs, myeloid suppressor cells, and tumor-associated macrophages,[30-32] as well as negative co-stimulatory molecules such as PD-L1, that inhibit antitumor effects.[33] Currently, there are few immune molecules with proven antitumor effects. Therefore, finding novel immune molecular targets is significant for the progress of antitumor immunotherapy.

In recent years, the antitumor effects of IL-9 have been gradually discovered by multiple studies, attracting attention from researchers. Previous studies showed that IL-9 inhibits subcutaneous melanoma and prolongs survival time in mice[34] and reduces the number of lung metastases in mice.[35,36] Besides, IL-9 stimulates the function of DCs to enhance antitumor immunity in tumor-specific CTLs.[37] Moreover, IL-9 inhibits squamous cell carcinoma in mice.[38] However, although these reports revealed antitumor effects for IL-9, they all used *in vivo* mouse xenograft models and did not assess IL-9 expression in human tumors, especially colon cancer; in addition, these studies did not explore the mechanisms underlying IL-9's antitumor effects through changes in the TME. Although Huang *et al* found reduced IL-9 levels in colon carcinoma tissues and an association with colon cancer progression,[26] they only evaluated tissue IL-9 expression by immunohistochemistry, a semi-quantitative technique, and did not evaluate the association of IL-9 expression with survival or performed *in vivo* experiments to explore the possible mechanisms.

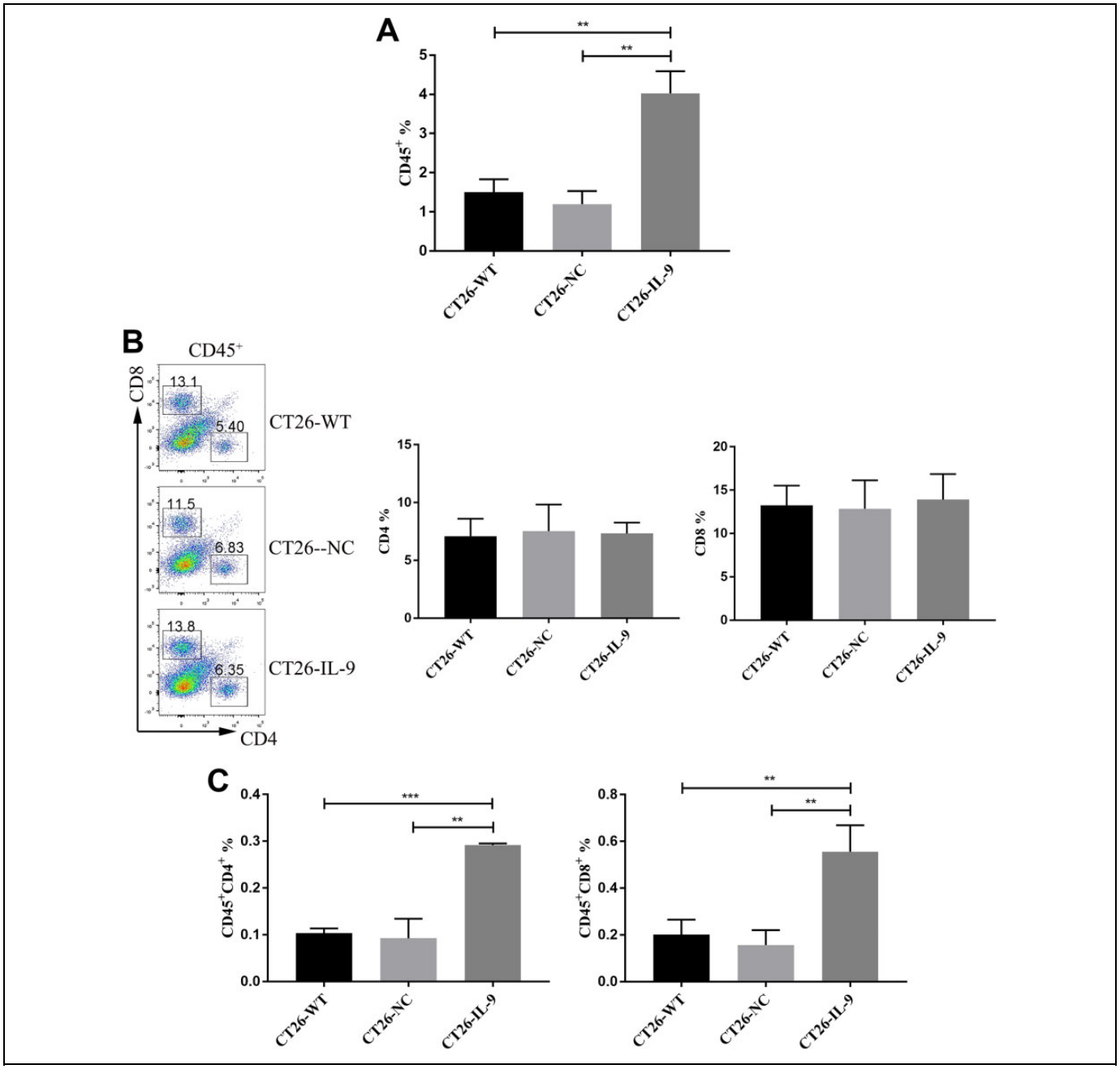

Figure 6. Tumoral expression of interleukin 9 (IL-9) increases antitumor immune responses in immunogenic tumor microenvironment (TME). A, $CD45^+$ tumor infiltrating leukocytes (TIL) percentages in tumor cell suspensions. B, Representative flow cytograms and $CD4^+$ T and $CD8^+$ T-cell percentages among $CD45^+$TILs. C, $CD45^+CD4^+$ T and $CD45^+CD8^+$ T-cell percentages in tumor cell suspensions (*$P < .05$, **$P < .01$, ***$P < .001$).

Therefore, we carried out this study to further assess the antitumor effects of IL-9.

As shown above, IL-9 was less expressed in colon carcinoma tissues. In addition, IL-9 levels were correlated with TNM staging, Dukes staging, LNM, and patient survival, while no associations were found with gender, age, tumor size, tumor differentiation, and hepatic metastasis. First, IHC staining and qRT-PCR were employed to evaluate IL-9 levels in colon cancer specimens. Of the 92 paired clinical specimens, IL-9 protein and mRNA levels showed markedly decreased amounts in colon cancer samples compared with adjacent noncancerous specimens. The reason may be that the intestinal tract is an important immune screen for the human body; in addition, there are many immune cells as well as cytokines in normal colonic tissues, but immune cells are in an immunosuppressive state in colon cancer tissues, which causes less immune cells to infiltrate. The above results suggested that IL-9 may exert antitumor effects in colon cancer. Second, we analyzed the

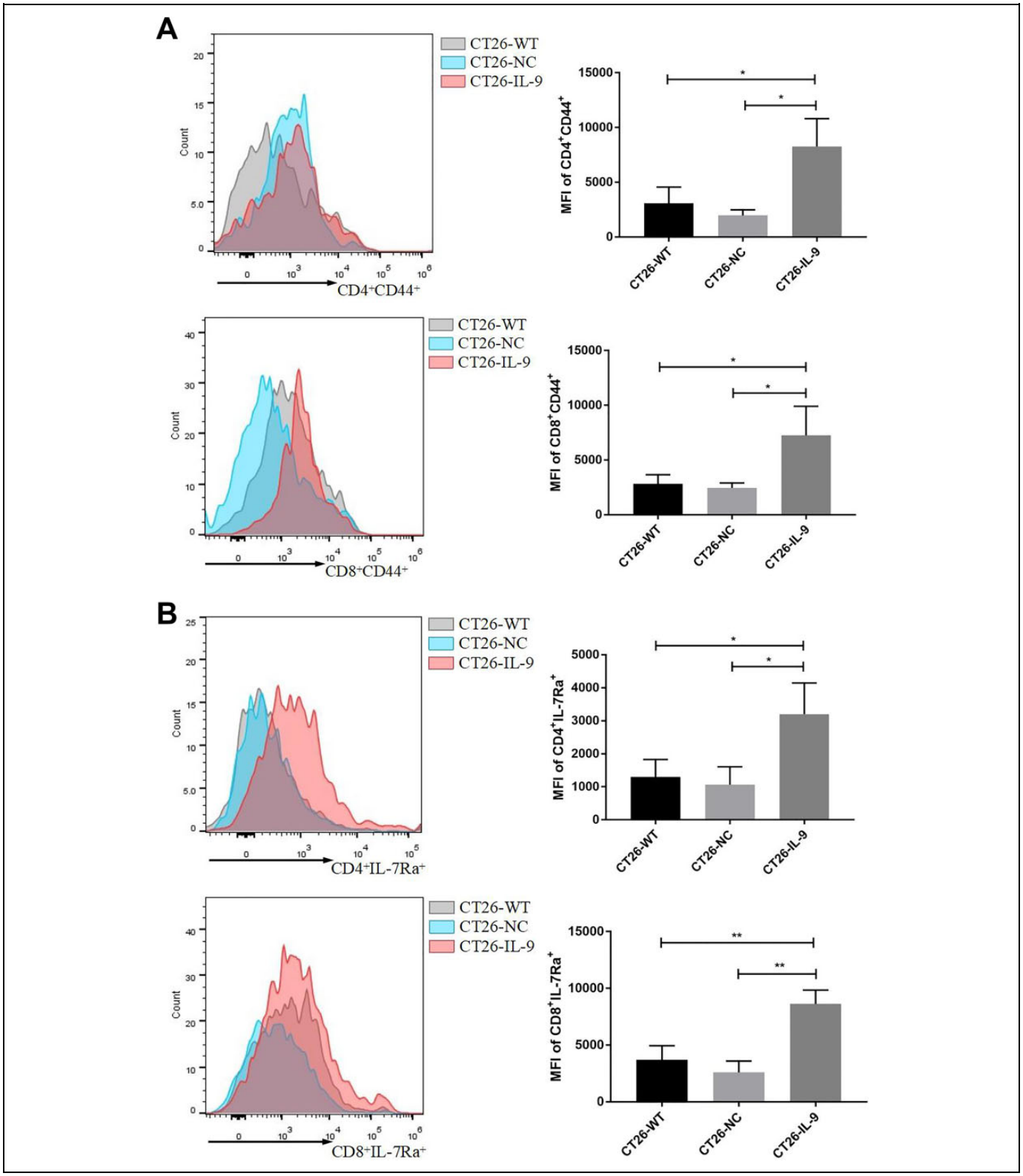

Figure 7. Tumor interleukin 9 (IL-9) promotes antitumor effects in immunogenic tumor microenvironment (TME). A, Representative flow cytograms and mean fluorescence intensities (MFIs) of $CD4^+CD44^+$ and $CD8^+CD44^+$ T cells in cancer cell suspensions. B, Representative cytograms and MFIs of $CD4^+IL\text{-}7Ra^+$ and $CD8^+IL\text{-}7Ra^+$ T cells in cancer cell suspensions. C, Representative cytograms and MFIs of $CD8^+$ granzyme B^+ T cells in cancer cell suspensions. D, Percentages of $CD4^+Foxp3^+$ T cells in cancer cell suspensions (*$P < .05$, **$P < .01$, ***$P < .001$).

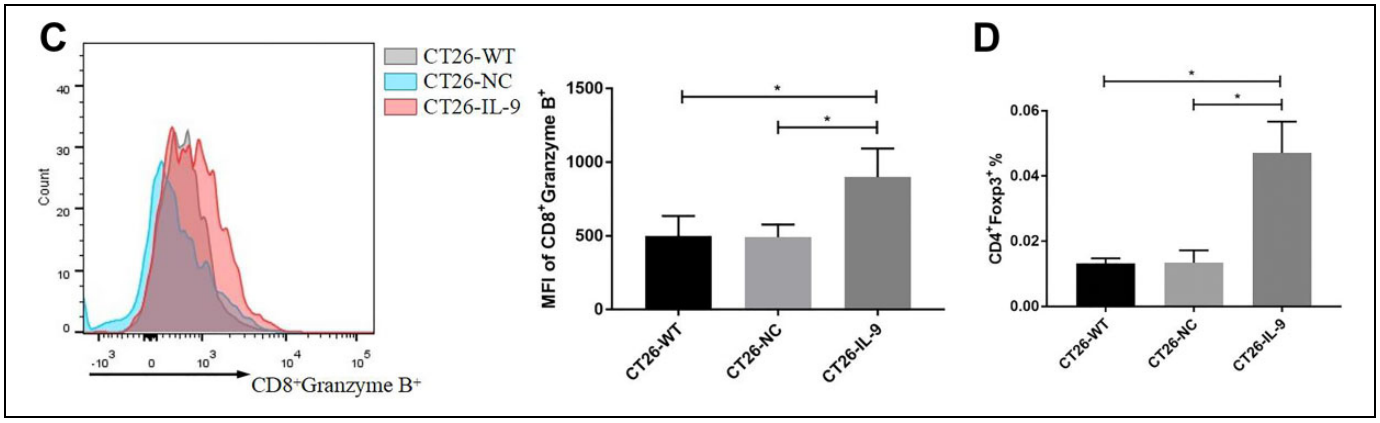

Figure 7. (continued).

associations of IL-9 levels with clinicopathological parameters and showed that IL-9 was highly expressed in TNM stage I to II samples, Dukes stage A to B specimens, and in patients without LNM. Huang *et al*[26] also found that IL-9 levels in TNM stage III to IV samples are reduced compared with those of stage I to II specimens, corroborating our findings. Furthermore, we found that survival time in colon carcinoma cases with positive IL-9 expression was markedly longer compared with that of cases not expressing IL-9. The above results suggest that IL-9 may exert strong antitumor effects in colon cancer; the body might accumulate antitumor immune cells in the TME and secrete IL-9 to produce antitumor immunity and inhibit local invasion as well as distant metastasis of tumor cells, which finally results in improved prognosis. Next, we established a mouse subcutaneous allograft model to further assess whether IL-9 has antitumor effects *in vivo*. We found that tumor growth was slower, and the sizes of resected tumors were reduced in the CT26-IL-9 group compared with the other 2 groups. Furthermore, the survival time in the CT26-IL-9 group was obviously longer compared with the other 2 groups. These results indicated that IL-9 inhibits tumor growth and prolongs animal survival.

Finally, we analyzed the changes in the TME of excised subcutaneous tumors from mice flow cytometrically. As shown above, $CD45^+$ TIL percentages were markedly elevated in CT26-IL-9 tumors in comparison with the other 2 groups, suggesting that IL-9 improves inflammatory responses. Besides, $CD4^+$ and $CD8^+$ T-cell amounts were also greatly elevated in CT26-IL-9 tumors, suggesting IL-9 improves antitumor immune responses, as tumor-specific $CD8^+$ T cells (tumor-specific CD8+ CTL) are the most important effector cells in antitumor immune responses.[39,40] In addition, $CD4^+$/$CD8^+CD44^+$, $CD4^+$/$CD8^+$IL-$7Ra^+$, $CD8^+$ granzyme B^+, and $CD4^+Foxp3^+$ T cells were markedly elevated in the CT26-IL-9 group in comparison with the other 2 groups. Increased $CD4^+$/$CD8^+CD44^+$ T cells, $CD4^+$/$CD8^+$IL-$7Ra^+$ T cells, $CD8^+$ granzyme B^+ T cells, and $CD4^+Foxp3^+$ T cells revealed that IL-9 promotes T-cell activation, self-duplication, and survival in T cells; tumor cell killing; and immune regulation by Tregs through a negative feedback mechanism, respectively. Taken together, these findings suggested IL-9 promotes anticancer effects by regulating T-cell function and killing tumor cells in the TME. Meanwhile, studies have explored other mechanisms by which IL-9 stimulates antitumor immune responses. For instance, IL-9 promotes apoptosis in melanoma cells by upregulating the antiproliferation molecule p21 as well as the apoptotic molecule TRAIL in mouse melanoma.[41] In addition, IL-9 may affect mast cells, stimulating innate immune responses and exerting antitumor effects.[25,34,42] Furthermore, IL-9 induces the production of CCL20-CCR6-dependent DCs and activates CTLs to exert antitumor effects in tumor tissues, simultaneously stimulating $CD8^+$ CTL-mediated antitumor immunity.[35,36] However, it is worth noting that Hoelzinger *et al*[43] put forward the different ideas. In their study, they found that the deficiency of IL-9 achieved rapid CD4+ and CD8+ T-cell sensitization, and IL-9 inhibited the activation of adaptive antitumor immunity. The difference may be that they used the different models and methods compared to us. But we should concern that the antitumor mechanisms of IL-9 are not fully understood and require further investigation.

Conclusion

In conclusion, this study found that IL-9 exerts antitumor effects in colon cancer and transforms the TME *in vivo*, suggesting that the cytokine has significant implications for immune therapy in colon cancer. Compared to previous studies, we included qRT-PCR to quantitatively determine IL-9 mRNA levels in human colon carcinoma and adjacent noncancerous tissue specimens and found that IL-9 expression in colon carcinoma is correlated with patient survival. Moreover, we carried out *in vivo* experiments by stably upregulating IL-9 in tumor cells and used flow cytometry to evaluate infiltrating immune cells in the TME. Overall, the current study may provide a new research direction and novel insights into tumor immunity. However, as a single-center study, the current findings should be further confirmed. In addition, the antitumor immune mechanisms of IL-9 in the TME of colon cancer also require further investigations using cellular immunology and molecular biology techniques.

Authors' Note

Jin Wang and Mingbing Sun have contributed equally to this work. Xin Zhao, Xinguo Zhu, and Xiaoqiang Dong proposed the research and are guarantors. Jin Wang wrote the manuscript. Jin Wang and Mingbing Sun contributed equally to the current work. All authors contributed to the study design and data interpretation and have reviewed the final version of the manuscript. This study was approved by the First Affiliated Hospital of Soochow University Ethical Committee (approval no. 2010633). All patients provided written informed consent prior to enrollment in the study. The Soochow University Medical College Animal Care and Use Committee approved the experimental procedures (approval no. 2017286). All animal housing and experiments were conducted in strict accordance with the Institutional Guidelines for Care and Use of Laboratory Animals.

Declaration of Conflicting Interests

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