World Journal of Gastroenterology

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DOI: 10.3748/wjg.v21.i14.4216

World J Gastroenterol 2015 April 14; 21(14): 4216-4224
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Basic Study

Th22 cell accumulation is associated with colorectal cancer development

Yong-Hong Huang, Yun-Fei Cao, Zhi-Yuan Jiang, Sen Zhang, Feng Gao

Yong-Hong Huang, Yun-Fei Cao, Zhi-Yuan Jiang, Sen Zhang, Feng Gao, Department of Colorectal and Anal Surgery, First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China

Author contributions: Huang YH and Cao YF contributed equally to this work; Gao F designed the research; Zhang S and Cao YF contributed new reagents; Jiang ZY and Huang YH performed research; Cao YF and Huang YH analyzed data; and Huang YH wrote the paper.

Supported by National Natural Science Foundation of China, No. 81260316 and No. 81260335.

Ethics approval: This study was reviewed and approved by the First Affiliated Hospital of Guangxi Medical University Institutional Review Board.

Institutional animal care and use committee: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Guangxi Medical University (IACUC No: 201402068).

Conflict-of-interest: The authors report no conflicts of interest in this work.

Data sharing: The technical appendix, statistical code, and dataset are available from the corresponding author at doctorgao0771@hotmail.com. All participants gave informed consent for data sharing.

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Correspondence to: Feng Gao, Professor, Department of Colorectal and Anal Surgery, First Affiliated Hospital of Guangxi Medical University, No. 6 Shuangyong Road, Nanning 530021, Guangxi Zhuang Autonomous Region, China. doctorgao0771@hotmail.com
Telephone: +86-771-5356529
Fax: +86-771-5351442

Received: October 26, 2014
Peer-review started: October 27, 2014
First decision: November 14, 2014
Revised: November 27, 2014
Accepted: January 16, 2015
Article in press: January 16, 2015
Published online: April 14, 2015

Abstract

AIM: To investigate the expression of Th22 cells and related cytokines in colorectal cancer (CRC) tissues, and the probably mechanism.

METHODS: CRC tumor and paratumor tissues were collected to detect the expression levels of Th22 cells and of related cytokines by immunohistochemistry, flow cytometry and real-time quantitative polymerase chain reaction (RT-qPCR). Interleukin (IL)-22 alone or with a STAT3 inhibitor was co-cultured with RKO cells *in vitro* to study the effects of IL-22 on colon cancer cells. IL-22 alone or with a STAT3 inhibitor was injected into a BALB/c nude mouse model with subcutaneously transplanted RKO cells to study the effects of IL-22 on colon cancer growth.

RESULTS: The percentage of Th22 cells in the $CD4^+$ T subset was significantly higher in tumor tissues compared with that in paratumor tissues (1.47% ± 0.083% *vs* 1.23% ± 0.077%, $P < 0.05$) as determined by flow cytometry. RT-qPCR analysis revealed that the mRNA expression levels of IL-22, aryl hydrocarbon receptor, CCL20 and CCL22 were significantly higher in tumor tissues compared with those in paratumor tissues. CCL27 mRNA also displayed a higher expression level in tumor tissues compared with that in paratumor tissues; however, these levels were not significantly different (2.58 ± 0.93 *vs* 2.30 ± 0.78, $P > 0.05$). IL-22 enhanced colon cancer cell proliferation *in vitro* and displayed anti-apoptotic effects; these effects were blocked by adding a STAT3 inhibitor. IL-22 promoted tumor growth in BALB/c nude mice; however, this effect was reversed by adding a STAT3 inhibitor.

CONCLUSION: Th22 cells that accumulate in CRC may be associated with the chemotactic effect of the tumor microenvironment. IL-22 is associated with CRC development, most likely *via* STAT3 activation.

Key words: Th22 cells; Interleukin-22; STAT3; Colorectal cancer; Tumor microenvironment

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Core tip: Although the functional characteristics of Th22 cells in inflammatory and autoimmune diseases have been extensively studied, their role in colorectal cancer (CRC) remains unclear. This study demonstrated the differences in the expression of Th22 cells and their related cytokines between colorectal tumor and paratumor tissues and the accumulation of Th22 cells in CRC may be associated with the functions of chemotactic factors that are secreted by the tumor microenvironment. Interleukin-22 was found to be the functional factor of Th22 cells that is associated with CRC development in both *in vitro* and *in vivo* experiments, most likely *via* STAT3 pathway activation.

Huang YH, Cao YF, Jiang ZY, Zhang S, Gao F. Th22 cell accumulation is associated with colorectal cancer development. *World J Gastroenterol* 2015; 21(14): 4216-4224 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i14/4216.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i14.4216

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly occurring cancer in males and the second most commonly occurring cancer in females[1]. An increased overall survival rate has been observed in patients with CRC due to the detection of early stage CRC and to the improvement of therapeutic strategies[2]. However, over 1 million people develop CRC every year worldwide, and more than 500000 patients die, particularly those patients with advanced cancer[1,3]. Currently, the incidence rates of CRC are increasing in developing countries, including China[4].

Understanding the molecular pathways involved in CRC will help to improve cancer prevention and treatment[5]. Increasing evidence has shown that the dysregulation of different $CD4^+$ T lymphocyte subpopulations and cytokine networks is involved in the pathogenesis and progression of CRC[6-8]. *In situ* analysis of tumor-infiltrating immune cells may be a valuable prognostic tool in the treatment of CRC and possibly of other malignant tumors[9,10].

Traditionally, $CD4^+$ T helper cells (Th cells) include Th1, Th2, Th7, and regulatory T cells according to their cytokine milieu. Interleukin (IL)-22, which is a member of the IL-10 cytokine family, is regarded as a cytokine produced by Th1 cells and Th17 cells. Recently, two studies have shed new light on the unique features of this cytokine. IL-22-producing CD4+ T cells (Th22 cells), which are a new T helper cell subset, differ from Th1, Th2, or Th17 cells because this population only produces IL-22 and has low or undetectable expression of the Th17 and Th1 transcription factors ROR-γ and T-bet. Th22 cells express the chemokine receptors CCR4, CCR6 and CCR10 in human skin, and the transcription factor aryl hydrocarbon receptor (AHR) is required for IL-22 production[11,12]. The functional characteristics of Th22 cells in inflammatory and autoimmune diseases have been extensively studied[13]. Nevertheless, knowledge regarding the role of Th22 cells in malignant tumor immunity is limited; further research elucidating the pathogenesis of and therapy for carcinoma will be of interest. In the current study, we investigated the expression of Th22 cells and their related cytokines in colorectal tumor and paratumor tissues and determined their effects on colorectal cancer using *in vivo* and *in vitro* experiments.

MATERIALS AND METHODS

Ethics statement

All patients enrolled in this study provided written informed consent. This study protocol conformed to the ethical guidelines of the Declaration of Helsinki (Fortaleza, Brazil, October 2013) and was approved by the ethical committees and institutional Review Board of the First Affiliated Hospital of Guangxi Medical University, PRC.

Research subjects and samples

Fifty patients diagnosed with CRC who received surgical resection at The First Affiliated Hospital of Guangxi Medical University from April 2013 to March 2014 were enrolled in this study. None of the patients had received radiotherapy or chemotherapy before sampling. Individuals with an autoimmune disease, infectious disease, or multiple primary cancers were excluded. The basic data regarding the study population are shown in Table 1. The tumor and paratumor tissues (at least 5 cm away from the tumor site) were collected immediately after surgical resection and stored in liquid nitrogen for polymerase chain reaction (PCR), fixed with 4% paraformaldehyde for immunohistochemistry (IHC) or immediately isolated for flow cytometry.

IHC

Fresh tumor and paratumor tissues were fixed in 4% paraformaldehyde, embedded with paraffin and sectioned at 4-μm thickness. IHC was performed as previously described[14]; the sectioned slides were stained using IL-22 antibody, which was purchased from Bioss Company (Beijing, China).

Table 1 Basic data of the study population

Characteristics	Value
Sex	
Male	33
Female	17
Age (yr), mean (range)	60 (38-81)
Colon	22
Rectum	28
TNM stage	
Stage Ⅰ-Ⅱ	23
Stage Ⅲ-Ⅳ	27

Table 2 Primer sequences for polymerase chain reaction

Gene	Sequence (5′ to 3′)	Product (bp)	Tm (℃)
IL-22	F: GTTCTCCTTCCCCAGTCACCA R: AGCTGCTCCTCCCTGTACCAA	145	60
AHR	F: ACATCACCTACGCCAGTCG R: CGCTTGGAAGGATTTGACTTGA	94	60
CCL20	F: ATCCAAAACAGACTTGGGTGAA R: TCCATTCCAGAAAAGCCACA	89	60
CCL22	F: ATTACGTCCGTTACCGTCTGC R: TCCCTGAAGGTTAGCAACACC	100	60
CCL27	F: TCCTGAGCCCAGACCCTACA R: CGTTGAGCCAGGTGAAGCA	175	60
β-actin	F: TGACGTGGACATCCGCAAAG R: CTGGAAGGTGGACAGCGAGG	205	60

Real-time quantitative PCR

Fresh tumor and paratumor tissue samples for determining cytokine expression were stored at -80 ℃ until analysis. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was immediately reverse transcribed from the extracted total RNA using the SuperScript Ⅲ First-Strand Synthesis System (Invitrogen). Real-time quantitative PCR (RT-qPCR) was performed using a SYBR Green PCR kit (Roche). Amplification was performed using standard conditions and was normalized to transcripts of the housekeeping gene β-actin. The primer sequences for PCR are shown in Table 2. Relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method as described by Livak *et al*[15] and adjusted by the level of β-actin mRNA for each sample.

Cell isolation

Tumor and paratumor tissues were washed three times in RPMI 1640 before being cut into small pieces (1 mm tissue samples). Then, the specimens were collected in RPMI 1640 containing 1 mg/mL collagenase Ⅳ, 30 μg/mL DNase I and 0.1 mg/mL hyaluronidase, and then a magnetic stirrer was used for stirring the digestion mixture for 3 h. Next, the dissociated cell suspensions were filtered through 150-μm and 70-μm cell strainers to obtain cell suspensions, which were centrifuged in a discontinuous Percoll gradient (75% and 40%). The cells at the interface were harvested and resuspended at 1 × 10^6 cells/mL in RPMI 1640 containing 10% fetal calf serum. Cell viability was determined by trypan blue exclusion.

Flow cytometry

The cell suspensions were stimulated in culture for 4 h with 50 ng/mL PMA, 1 μg/mL ionomycin and 0.7 μL/mL GolgiStop reagent at 37 ℃ in a CO_2 incubator (5% CO_2 in humidified air). The cultured cell suspensions were stained with surface and intracellular anti-human-specific antibodies, which were conjugated with PE, PE-Cy5 or APC. These human antibodies included anti-CD4, IL-22 and IL-17, which were purchased from BD Biosciences (Franklin Lakes, NJ, United States) or eBioscience (San Diego, CA, United States). Then, the cells were resuspended and analyzed using a FACSCaliburflow cytometer (BD Bioscience). The data were analyzed using FlowJo software (TreeStar, Ashland, OR, United States). Cellular debris was eliminated from the analysis using a gate set at forward and side scatter.

Cell co-culture in vitro

The human colon cancer cell line RKO was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Recombinant human IL-22 was purchased from PeproTech Company, United States. STAT3 inhibitor (S3I-201) was purchased from Selleck Chemicals, United States. RKO cells were cultured in complete DMEM medium supplemented with 10% FBS and 1% antibiotic/antimycotic in a humidified incubator at 37 ℃ in an atmosphere of 95% air and 5% CO_2 for 24 h. Then, IL-22 (50 ng/mL) or S3I-201 (50 μmol/L) was added to the experimental medium for co-culture. After 24 h, the RKO cells were trypsinized and then stained with intracellular Ki-67 (BD Bioscience) to detect cell proliferation or stained with Annexin V and 7-amino-actinomycin (7-AAD) (BD Bioscience) to detect apoptosis.

Animal experiments in vivo

BALB/c nude mice (6-8 wk of age) were obtained from Guangxi Medical University Animal Experiment Center, and all animal experiments conformed to the National Guidelines of the Animal Care Committee. Twenty-one BALB/c nude mice were injected subcutaneously with RKO cells; each mouse was injected with 5 × 10^6 cells in 300 μL of saline solution. Tumor growth was monitored every two days. Tumor volume was calculated by the following formula: (major circumference×minor circumference^2)/2. After the tumor volumes reached 60 mm^3, the 21 mice were divided into 3 groups. The IL-22 group was injected intraperitoneally with IL-22 (1 μg/100 μL) and DMSO (100 μL) every other day, the IL-22 + S3I-201 group was injected with IL-22 (1 μg/100 μL) and S3I-201 (100 μg/100 μL), and the control group was injected with saline solution (100 μL) and DMSO (100 μL) simultaneously, each group for a total of 5 times. The

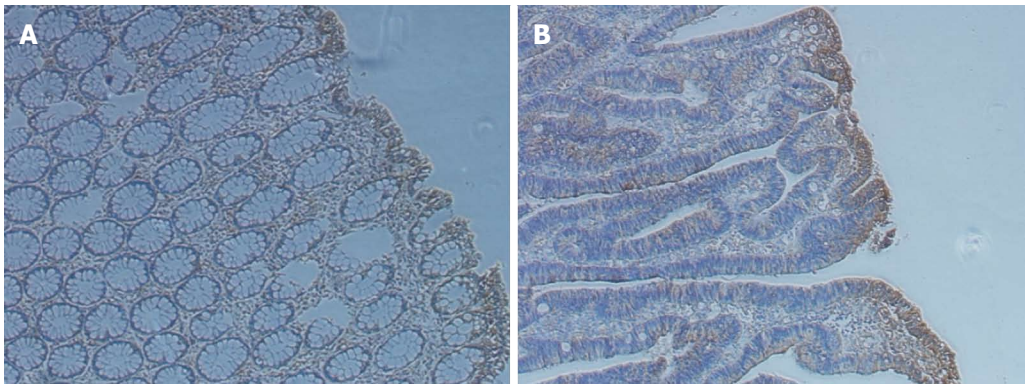

Figure 1 Immunohistochemistry staining of tissues. A: Immunohistochemistry (IHC) staining of normal colon tissues; B: IHC staining of colon cancer tissues.

mice were sacrificed at 48 h after the last intervention.

Statistical analysis

The data are expressed as the mean ± SE. Data comparisons between the different groups were performed using Student's *t*-test, a paired *t*-test or one-way ANOVA. Analysis was completed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, United States), and *P* values that were less than 0.05 were considered statistically significant.

RESULTS

Th22 cells are enriched in CRC tumor tissues

IL-22 is a functional cytokine that is primarily produced by Th22 cells. By IHC, we observed that IL-22 was present in both tumor and paratumor tissues and was particularly enriched in tumor tissues (Figure 1). To further understand the roles of Th22 cells in the tumor microenvironment in patients with CRC, the proportion of Th22 cells in tumor and paratumor tissue was detected by flow cytometry (Figure 2A). As shown in Figure 2B, the prevalence of Th22 cells in the $CD4^+$ T subset was higher in tumor tissues compared with that in paratumor tissues ($P < 0.05$).

Expression of Th22 cells and related cytokines in the CRC microenvironment

The relative expression levels of IL-22, AHR, CCL20, CCL22 and CCL27 in colorectal tumor and paratumor tissues were measured by RT-qPCR. CCL20, CCL22 and CCL27 are common chemokines that have been identified as attractants of different types of leukocytes to sites of tumors and of inflammation. As shown in Figure 2C, the mRNA expression levels of IL-22, AHR, CCL20 and CCL22 were significantly higher in tumor tissues compared with those in paratumor tissues ($P < 0.05$). CCL27 mRNA also displayed a higher expression level in tumor tissues compared with that in paratumor tissues; however, these levels were not significantly different ($P > 0.05$).

Effects of IL-22 on colon cancer cells in vitro

The effects of IL-22 on colon cancer cells were assessed by co-culturing with RKO cells *in vitro*. As shown in Figure 3A and C, compared with control medium, the proliferation of RKO cells was significantly promoted by IL-22 treatment ($P < 0.05$). This enhanced proliferation was blocked when S3I-201 was added to the RKO cell culture in the presence of IL-22. In contrast, the apoptosis of RKO cells was significantly inhibited by IL-22 treatment ($P < 0.05$) compared with the control medium. The inhibition of STAT3 signaling by S3I-201 completely abrogated this suppression of apoptosis (Figure 3B and 3D).

Effects of IL-22 on colon cancer in vivo

BALB/c nude mice transplanted subcutaneously with RKO cells were used to investigate the effects of IL-22 on colon cancer growth *in vivo*. As shown in Figure 4, the tumor growth of nude mice was significantly promoted ($P < 0.05$) after intraperitoneal injection with IL-22 every other day compared with that of the control mice. However, this promoting effect induced by IL-22 treatment could be completely reversed by S3I-201 treatment.

DISCUSSION

The roles of tumor antigen-specific $CD4^+$ T cells in cancer immunity have been extensively studied in recent years[16,17]. Th22 cells, which are a newly described subset of $CD4^+$ T cells, play important roles in a variety of carcinomas. The percentage of Th22 cells is significantly increased in both the peripheral blood and tumor tissues in patients with gastric cancer; this percentage correlates with gastric cancer progression and can predict poor patient survival[18,19]. The over-expression of Th22 cells is also present in hepatocellular carcinoma[20], pancreatic cancer[21] and malignant pleural effusion[22]. In the current study, we demonstrated that the proportion of Th22 cells was enriched in tumor tissues relative to paratumor

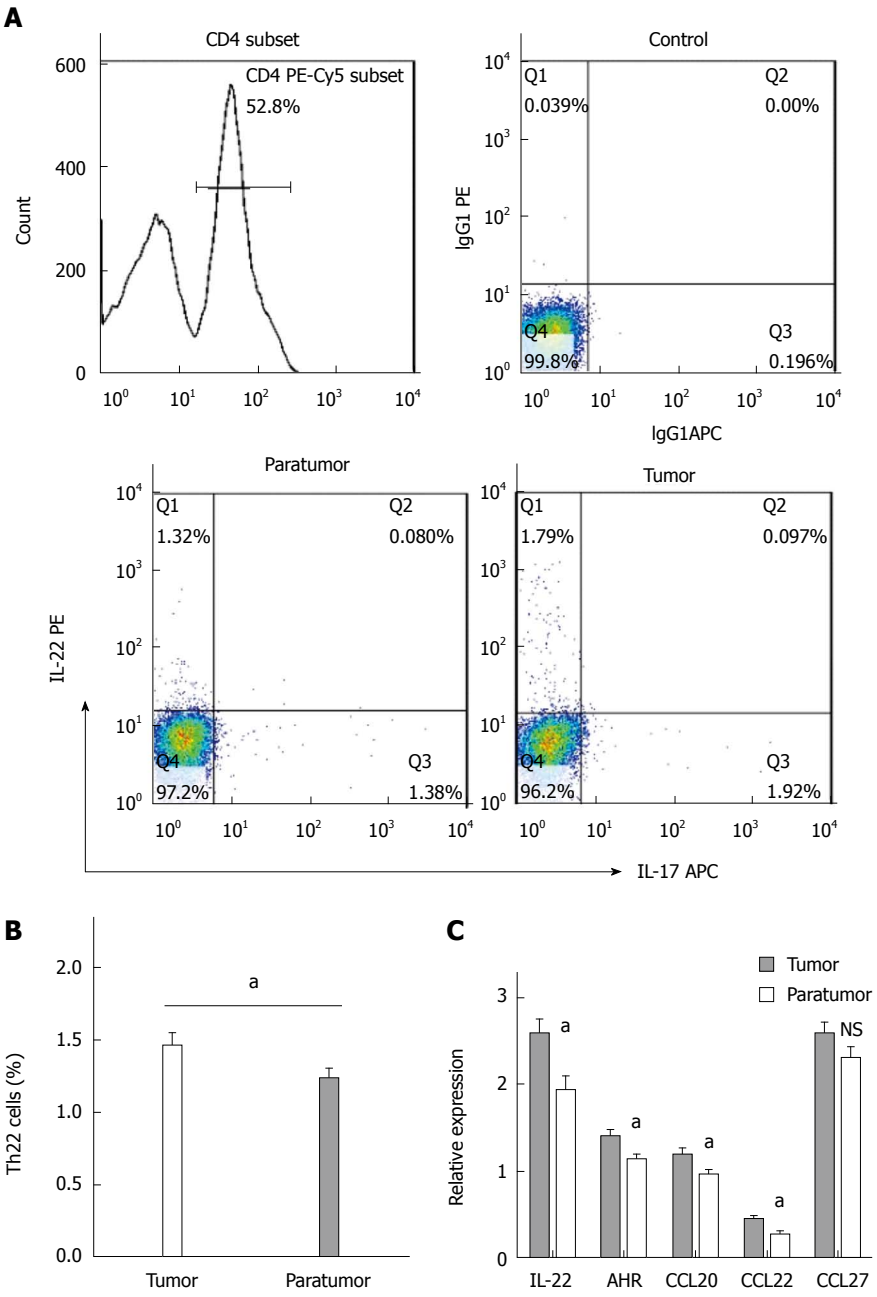

Figure 2 Expression of Th22 cells and related cytokines in colorectal cancer. A: Gated on FSC/SSC and $CD4^+$ subset, the proportion of Th22 cells in the $CD4^+$ subset is presented in quadrant Q1; B: Average proportion of Th22 cells in tumor and paratumor tissues; C: Expression levels of interleukin-22, AHR, CCL20, CCL22 and CCL27 in tumor and paratumor tissues were measured by RT-qPCR. The relative expression levels were normalized to the level of β-actin mRNA for each sample. Each bar represents the mean ± SE (n = 50), $^aP < 0.05$, tumor *vs* paratumor. NS: Not significant.

tissues in patients with CRC. By IHC and RT-qPCR, we observed that the expression level of IL-22 was significantly higher in tumor tissues than in paratumor tissues. AHR is known as the key transcription factor of Th22 cells[11,12]; in the present study, AHR displayed a higher level of expression in tumor tissues compared with that in paratumor tissues. These results are similar to those of aforementioned reports that indicated that the accumulation and differentiation of Th22 cells are induced by the tumor microenvironment.

The phenotypic characteristics of Th22 cells have been described as $CCR4^+CCR6^+CCR10^+$, and the chemotactic factors CCL22, CCL20 and CCL27 are their corresponding ligands[12]. In this study, we observed that the colorectal tumor microenvironment expressed higher levels of CCL22, CCL20 and CCL27 compared with those of the paratumor tissues, suggesting that the accumulation of Th22 cells in tumor tissues may be mediated by the chemotactic cytokines that are secreted by the tumor microenvironment. This result is similar to that of a study of malignant pleural effusion[22].

Many studies have demonstrated the constitutive activation of STAT3 in a wide variety of human car-

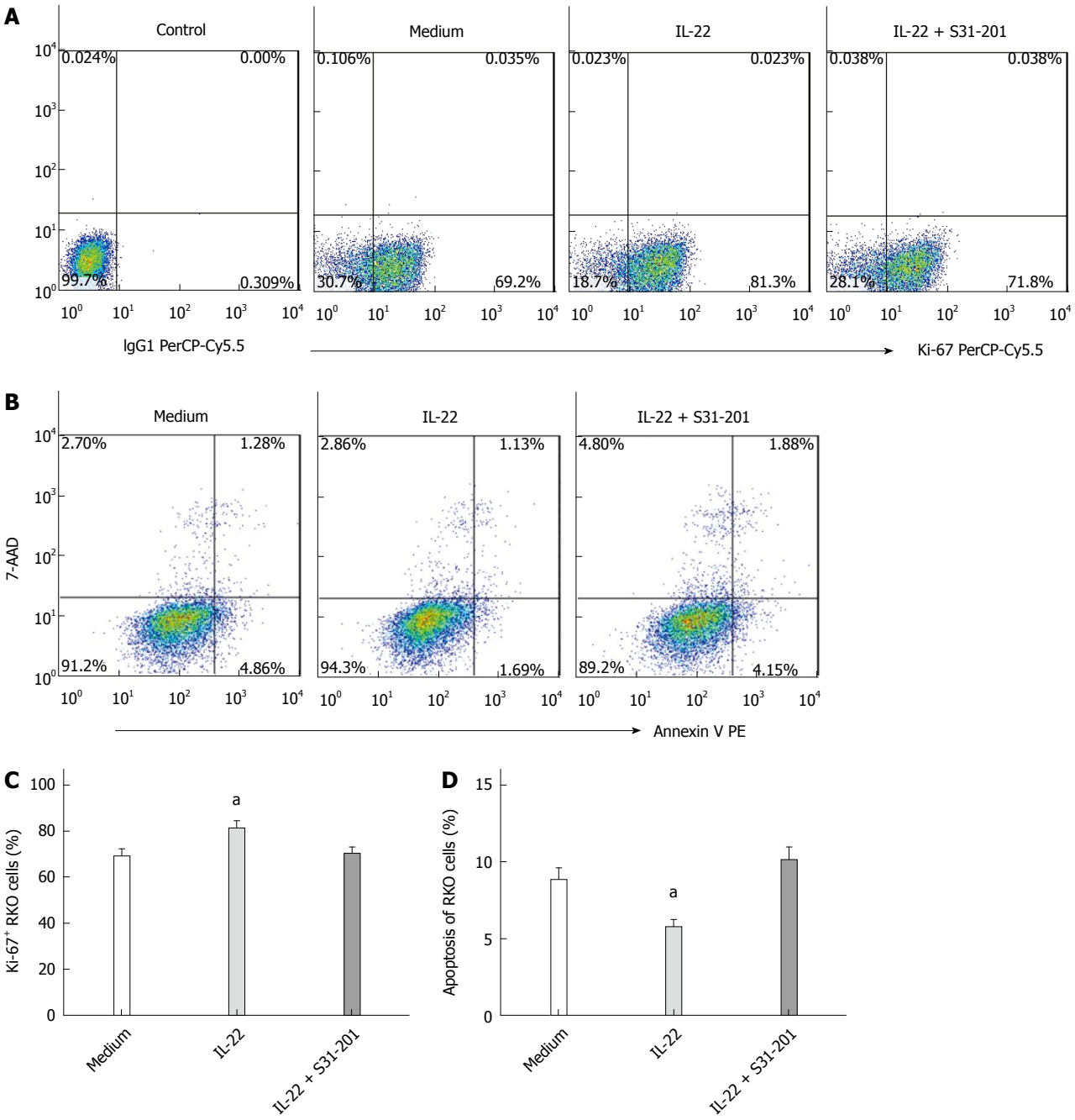

Figure 3 Effects of interleukin-22 on colon cancer cells. A: Flow cytometry to measure colon cancer cell proliferation in the presence of interleukin (IL)-22 or IL-22 + S3I-201; B: Flow cytometry for colon cancer cell apoptosis in the presence of IL-22 or IL-22 + S3I-201; C: Average proportion of proliferating colon cancer cells in the presence of IL-22 or IL-22 + S3I-201; D: Average proportion of apoptotic colon cancer cells in the presence of IL-22 or IL-22 + S3I-201. Each bar represents the mean ± SE (n = 18). $^{a}P < 0.05$ *vs* the control medium.

cinomas, including hematological malignancies and diverse solid tumors[23]. Abundant evidence has suggested that the dysregulation of IL-22 is associated with aberrant STAT3 signaling in liver injury[24], ulcerative colitis[25], oral squamous cell carcinoma[26], and gastric cancer[27]. STAT3 activation in CRC correlates with adverse clinical results[28]. In this study, we co-cultured RKO cells with IL-22 *in vitro* to investigate the effects of IL-22 on colon cancer cells. We observed that IL-22 enhanced RKO cell proliferation and had anti-apoptotic effects; these effects were blocked by adding S3I-201, suggesting that IL-22 exerts its functions in CRC *via* STAT3 signaling. These results are similar to those found in studies of lung cancer cells[22] and of Hct-116 colon cancer cells[29]. Moreover, by activating the STAT3 pathway, IL-22 may act as a novel chemoresistance cytokine that prevents CRC patients from benefiting from FOLFOX chemotherapy[30], and promote CRC invasiveness and stemness[31]. Finally, we verified this effect in a subcutaneous tumor model. We observed that tumor growth in nude mice could be significantly promoted by IL-22 but completely reversed by adding

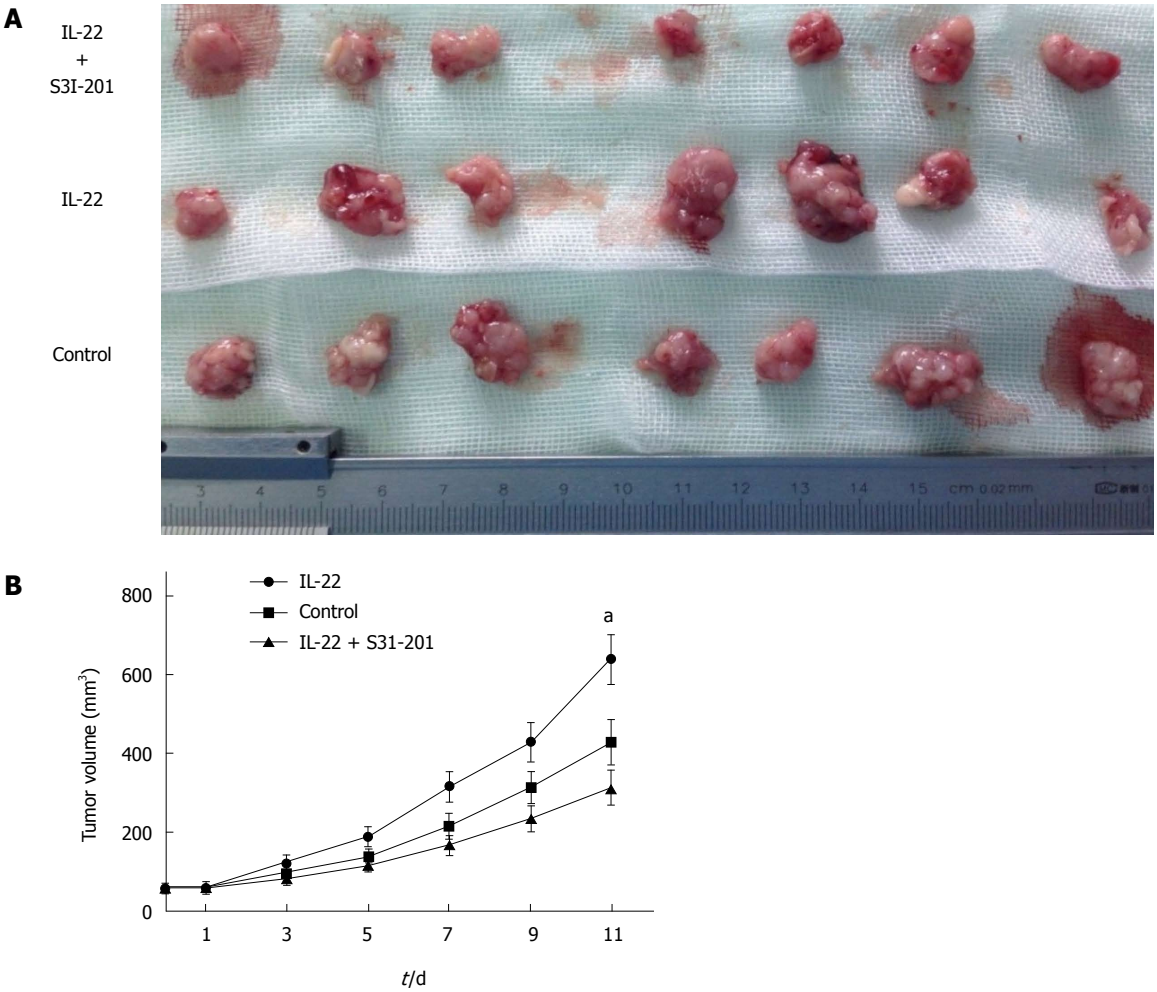

Figure 4 Effects of interleukin-22 on colon cancer *in vivo*. A: Tumor tissues were obtained from BALB/c nude mice; B: Tumor growth curves for interleukin (IL)-22, IL-22 + S3I-201 and control group mice. The tumor volume was calculated as follows: (major circumference × minor circumference^2)/2. Each plot represents the mean ± SE (n = 7). $^aP < 0.05$ *vs* the control.

a STAT3 inhibitor.

In conclusion, we measured the proportion of Th22 cells in the colorectal tumor microenvironment and found that the accumulation of Th22 cells in tumor sites may be related to the functions of chemotactic factors that are secreted by the tumor microenvironment. In addition, IL-22 was associated with CRC development in both *in vitro* and *in vivo* experiments, most likely by activating the STAT3 signaling pathway. The correlation between immunology and malignant tumors has become an important research area[32,33]. Further understanding the regulation and mechanism of Th22 cells in tumor microenvironments may provide new insights into immune therapeutic strategies for patients with CRC.

COMMENTS

Background

Colorectal cancer (CRC) is one of the most commonly occurring cancers worldwide. In recent years, tumor immunology has become a research hotspot, and understanding the molecular pathway involved in CRC will help to improve cancer prevention and treatment. Th22 cells were first introduced in 2009, and the functional characteristics of these cells in inflammatory and autoimmune diseases have been extensively studied. However, knowledge regarding their role in tumor immunity is relatively limited, particularly in CRC.

Research frontiers

Studies have shown that Th22 cells are involved in the progression of many digestive malignant tumors. However, the specific participation mechanism of these cells remains unclear.

Innovations and breakthroughs

The authors analyzed the relation between Th22 cells and the colorectal tumor microenvironment from a new perspective, verified their effects on CRC *via in vivo* and *in vitro* experiments, and attempted to demonstrate the specific signaling pathway by which Th22 cells participate in carcinogenesis.

Applications

The results of this study indicated that Th22 cells might be a prognostic factor and a potential therapeutic target for patients with CRC.

Terminology

Flow cytometry, which is a biophysical technology employed in cell counting, cell sorting and biomarker detection, widely used in basic research, clinical trials and blood cancer diagnosis.

Peer-review

This is a well conducted study on very timely topics. The authors can improve this paper with more thorough literature review in the context of tumor changes

and immunity.

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ISSN 1007-9327

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