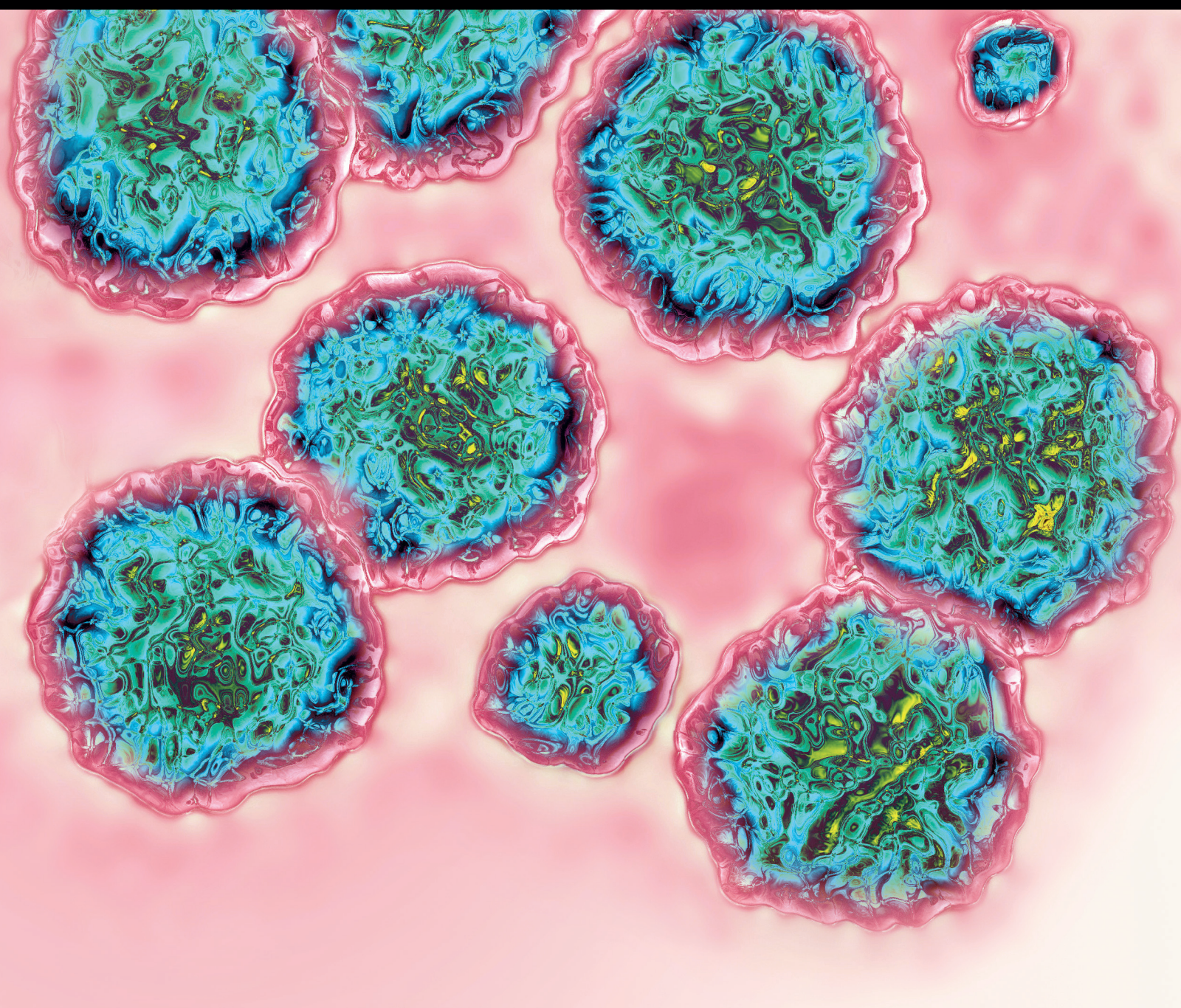
Canadian Journal of Gastroenterology and Hepatology

Gastroenterological Cancer and Immunotherapy

Lead Guest Editor: Xiaoping Wang
Guest Editors: Yixin E. Yang, Lintao Jia, and Qi Chen



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Editorial

Gastroenterological Cancer and Immunotherapy

Xiaoping Wang,[1] **Yixin Eric Yang**,[2] **Lintao Jia**,[3] **and Qi Chen**[4]

[1]*Department of Pathology, Shaanxi University of Chinese Medicine, Xianyang, Shaanxi, China*
[2]*School of Natural Sciences, College of Natural, Applied and Health Sciences, Kean University, Union, NJ, USA*
[3]*Department of Molecular Biology, Fourth Military Medical University, Xi'an, Shaanxi, China*
[4]*Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, KS, USA*

Correspondence should be addressed to Xiaoping Wang; wxpphd@aliyun.com

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Gastroenterological cancers are the most common malignant tumors with the highest mortality in the world [1]. With the recent improvement in immunology, novel potential therapeutic molecules and immune cells for gastroenterological cancers have been recognized [2, 3]. However, there remains a lot to be learned about the function of immune molecules or cells for cancers. In the last few decades, immunotherapy has become a well-established strategy and found several applications in clinics or under clinical trials [4, 5]. Now, numerous forms of immunotherapeutic approaches are being explored for gastroenterological cancers. It is beneficial to exert the effects of the potential therapeutic molecules or immunological molecules or cells for gastroenterological cancers and try to avoid their side effects in the body.

Recently, new insights into the mechanisms involved in the immunotherapy for gastroenterological cancers have been explored. Novel biological effects of known therapeutic immunological molecules or cells against gastroenterological cancers have attracted much attention [6–10].

Immune checkpoints are involved in regulation of antigen recognition of T-cell receptor by costimulatory or inhibitory signaling transduction in the immune system. Immune checkpoint blockade therapy achieves great success in treating many types of cancers [6–8]. It targets T-cell regulatory pathways to enhance anticancer immune response. In recent years, cytotoxic T lymphocyte protein-4 (CTLA-4) and programmed cell death protein-1 (PD-1) have shown promise as novel therapeutic targets in some cancers [7–10]. Cytotoxic T lymphocyte protein-4 (CTLA-4) and programmed cell death protein-1 (PD-1) are immune checkpoints that inhibit the T-cell response, which provide the escape mechanism of the tumor cells to T-cell antitumor activity [8, 9]. The B7-H1, also known as PD-L1, in positive tumors interacts with its receptor PD-1, which leads to the inhibition of T-cells migration, proliferation, resulting in an antiapoptotic signal, preventing overactivation of the immune system, and escaping from destruction [8–10]. CTLA-4 is an immune checkpoint receptor expressed on regulatory T (Treg) cells and recently activated conventional T-cells [11, 12]. It is a negative regulator of T-cell activation. The anti-CTLA-4 antibody can blockade CTLA-4 interaction with B7 and prevents the inhibitory signal [12]. Targeting CTLA-4 with a human anti-CTLA-4 antibody has demonstrated therapeutically success in the treatment of melanoma [12, 13]. Then blockade of CTLA-4 may be a promising new approach to cancer therapy and constitutes a novel approach to induce host responses against tumors. It could downregulate the immune system and produce durable anticancer responses.

The better understanding of T-cell biology and genetic engineering allows us to modify T-cells by associating a synthetic molecule and infusing them into tumor tissue to enhance the immune response against malignant lesion [14]. Genetically engineered T-cells can specifically target cancer cells to eradicate tumor burden through a T-cell receptor or chimeric antigen receptors (CARs). CARs, also known as chimeric immunoreceptors, are engineered recombinant receptors with an intracellular signaling domain consisting of T-cell receptor-CD3-ξ domain and an extracellular single-chain variable antibody fragment [15]. CARs can directly bind to tumor-associated antigens, carbohydrates, or glycolipids.

The antigens overexpressed on solid tumor cells but with limited or no expression on normal cells can be promising targets for CAR T-cell therapy.

Ongoing and future research will probably provide more efficient immunological molecules or cells for preventing and treating gastroenterological cancers.

This special issue encompasses cutting-edge research and review articles focusing on the role of the potential therapeutic and immunological molecules or cells in gastroenterological cancers. It includes 4 novel research articles and 3 reviews describing the advance of immunotherapy for gastric, colonic, and pancreatic cancers, summarized as follows.

(1) Immunotherapy for Gastric Cancer

In the review article titled "Immunotherapy in Advanced Gastric Cancer: An Overview of the Emerging Strategies", H. Magalhães et al. summarized that the recent molecular characterization in gastric cancer will help us to better select patients who might benefit from immune checkpoint inhibitors and other agents.

There are encouraging results with agents that target programmed death 1 (PD-1) and its ligands in gastric cancer; however, more trials are needed to identify predictive and prognostic biomarkers to select patients most appropriate for this treatment. In this review, the authors explore the current evidence supporting the use of immunotherapy in advanced GC.

In the research article titled "Establishment of a Model of Microencapsulated SGC7901 Human Gastric Carcinoma Cells Cocultured with Tumor-Associated Macrophages", the authors established a model of microencapsulated SGC7901 human gastric cancer cells and evaluated the effects of coculturing spheres with tumor-associated macrophages (TAMs). SGC7901 cells were encapsulated in alginate-poly lysine-sodium alginate (APA) microcapsules using an electrostatic droplet generator. MTT assays showed that the numbers of microencapsulated cells were highest after culturing for 14 days. Metabolic curves showed consumption of glucose and production of lactic acid by day 20. Immunocytochemistry confirmed that Proliferating Cell Nuclear Antigen (PCNA) and Vascular Endothelial Growth Factor (VEGF) were expressed in microencapsulated SGC7901 cells on days 7 and 14. The expression of PCNA was observed outside of spheroids; however, VEGF was found in the entire spheroids. PCNA and VEGF were increased after being cocultured with TAMs. Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) expressions were detected in the supernatant of microencapsulated cells cocultured with TAMs but not in microencapsulated cells. The study confirms that coculturing of the microencapsulated GC cells with TAMs can promote PCNA, VEGF, MMP-2, and MMP-9 expressions of the GC cells.

(2) Anesthesia on Immune Function in Colorectal Cancer Patients

According to the review article titled "The Effect of Anesthesia on the Immune System in Colorectal Cancer Patients", colorectal cancer (CRC) is the key leading cause of high morbidity and mortality worldwide. Surgery excision is the most effective treatment for CRC. However, stress caused by surgery response can destroy the body's immunity and increase the likelihood of cancer dissemination and metastasis. Anesthesia is an effective way to control the stress response, and recent basic and clinical research has shown that anesthesia and related drugs can directly or indirectly affect the immune system of colorectal cancer patients during the perioperative period. Thus, these drugs may affect the prognosis of CRC surgery patients.

This review is intended to summarize currently available data regarding the effects of anesthetics and related drugs on perioperative immune function and postoperative recurrence and metastasis in CRC patients. Determining the most suitable anesthesia for patients with CRC is of the utmost importance.

The editors expect this special issue to be of interest to the readers of recent advances in immunotherapy for gastroenterological cancers and anticipate that it will help researchers in making further progress in the understanding and development of immunotherapy for gastroenterological cancers.

(3) Immunotherapy and Diagnostic Approaches for Pancreatic Cancer

According to the review article titled "Combination Immunotherapy Approaches for Pancreatic Cancer Treatment", immunotherapies have been evaluated in clinical trials and received great success in many types of cancers in last decades. However, they have very limited success in treating pancreatic cancer. As pancreatic cancer poorly responds to many single immunotherapeutic agents, combination immunotherapy was introduced to improve efficacy. The combination therapies hold great promise for enhancing immune responses to achieve better therapeutic effects. This review summarizes the existing and potential combination immunotherapies for the treatment of pancreatic cancer.

The research article titled "A Comparison of Endoscopic Ultrasound-Guided Fine-Needle Aspiration and Fine-Needle Biopsy in the Diagnosis of Solid Pancreatic Lesions" indicated that endoscopic ultrasound (EUS) guided fine needle aspiration (FNA) is the method of choice for sampling pancreatic lesions. This study compares the diagnostic accuracy and safety of fine needle biopsy (FNB) using a novel core needle to FNA in solid pancreatic lesions. A retrospective review of patients in whom EUS FNA or FNB was performed for solid pancreatic lesions was conducted. Diagnostic performance was calculated based upon a dual classification system. The results indicated that FNA and FNB have comparable sensitivity and diagnostic accuracy. FNB required fewer passes.

(4) Immune Balance and Ulcerative Colitis

The research article titled "EGCG Maintains Th1/Th2 Balance and Mitigates Ulcerative Colitis Induced by Dextran Sulfate Sodium through TLR4/MyD88/NFκB Signaling Pathway in Rats" aimed to observe the protective effect of epigallocatechin gallate (EGCG) on dextran sulfate sodium-(DSS) induced ulcerative colitis in rats and to explore the roles of TLR4/MyD88/NFκB signaling pathway in the protective effect of EGCG. Rat models of ulcerative colitis were

established by giving DSS. EGCG was given to assess disease activity index. The results showed that EGCG improved the intestinal mucosal injury in rats with ulcerative colitis, inhibited production of inflammatory factors, maintained the balance of Th1/Th2, and reduced the expression of TLR4, MyD88, and NFκB. After TLR4 antagonism, the protective effect of EGCG on intestinal mucosal injury was weakened in rats with ulcerative colitis, and the expressions of various inflammatory factors were upregulated. Therefore, EGCG can inhibit the intestinal inflammatory response by reducing the severity of ulcerative colitis and maintaining the Th1/Th2 balance through the TLR4/MyD88/NFκB signaling pathway, which provides theoretical basis for development of target therapy for ulcerative colitis.

The research article titled "Decreased Breg/Th17 Ratio Improved the Prognosis of Patients with Ulcerative Colitis" by X. Bing et al. aimed to investigate the effects of regulatory B (Breg) cells and T helper 17 (Th17) cells on pathogenesis of ulcerative colitis, explore the clinical significance of Breg/Th17 ratio on the prognosis of ulcerative colitis, and provide the theoretical basis for the targeted therapy, diagnosis, and prognosis of the disease. Peripheral blood and colonic mucosa were collected from patients with ulcerative colitis. The colonic mucosa of ulcerative colitis patients presented massive inflammatory cell infiltration and hemorrhagic necrosis. The number of Breg cells and Th 17 cells, the gene expressions of IL-10 and RORγT, and serum levels of IL-10 and IL-17 all increased in peripheral blood. Compared with nonremission group, the remission group showed that the percentage of Breg cells reduced, the percentage of Th 17 cells increased, and thus the B10/Th17 ratio was significantly decreased in peripheral blood. In addition, serum IL-10 levels diminished, IL-17 levels increased, and thus IL-10/IL-17 ratio was remarkably reduced in remission group. B10/Th17 ratio and IL-10/IL-17 ratio were positively correlated with the severity of disease. Therefore, Breg and Th17 cells participate in the occurrence and development of ulcerative colitis. B10/Th17 ratio and IL-10/IL-17 ratio can be used as prognostic markers for ulcerative colitis. This provides a theoretical basis for design of targeted treatment and prognosis assessment of the disease.

Xiaoping Wang
Yixin Eric Yang
Lintao Jia
Qi Chen

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Review Article

Immunotherapy in Advanced Gastric Cancer: An Overview of the Emerging Strategies

Helena Magalhães, Mário Fontes-Sousa, and Manuela Machado

Medical Oncology Department, Portuguese Institute of Oncology of Porto (IPO Porto), Porto, Portugal

Correspondence should be addressed to Helena Magalhães; hmmagalhaes88@gmail.com,
Mário Fontes-Sousa; mario_fontes_sousa@hotmail.com, and Manuela Machado; m.machado.fn@gmail.com

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Gastric cancer (GC) remains a public health problem, being the fifth most common cancer worldwide. In the western countries, the majority of patients present with advanced disease. Additionally, 65 to 75% of patients treated with curative intent will relapse and develop systemic disease. In metastatic disease, systemic treatment still represents the state of the art, with less than a year of median overall survival. The new molecular classification of GC was published in 2014, identifying four distinct major subtypes of gastric cancer, and has encouraged the investigation of new and more personalized treatment strategies. This paper will review the current evidence of immunotherapy in advanced gastric cancer.

1. Introduction

GC is the 5th most common cancer diagnosed worldwide, and it represents one of the major causes of malignant disease morbidity and mortality, with almost 107,000 deaths in Europe in 2012 [1, 2].

The majority of the patients are diagnosed with locally advanced disease not suitable for surgery or metastatic disease. For these patients, chemotherapy is the standard of care in patients with clinical conditions, with median overall survival (OS) of less than 12 months. When compared to best supportive care (BSC), systemic treatment showed a clear advantage in OS [3, 4].

Currently, a combination of a platinum and fluoropyrimidine doublet is the mainstay of chemotherapy. The addition of a taxane or an anthracycline to this combination in human epidermal growth factor receptor 2 (HER-2) negative population increases response rate and survival outcomes but also generally implies higher toxicity, so the risks versus benefits should be well balanced. In the phase III ToGA trial, the addition of trastuzumab to cisplatin and fluoropyrimidine backbone improved median overall survival (OS), progression free survival (PFS), and response rate (RR) in Her-2 positive advanced or metastatic gastric cancer and established this regimen as standard of care in those patients [5, 6].

Second line chemotherapy, is an option for patients with good performance status. Docetaxel, irinotecan, and paclitaxel have all demonstrated improved survival compared to BSC in this setting. Additionally, ramucirumab, a vascular endothelial growth factor receptor (VEGFR-2) antibody, was the first biological treatment given as a single drug or in combination with paclitaxel in patients with advanced gastric or gastroesophageal junction (GEJ) adenocarcinoma progressing after first-line chemotherapy that demonstrated survival benefits in two randomized trials [7–11].

Despite these treatment options, the prognosis of advanced and metastatic GC is still poor and novel treatment strategies and patient selection tools are needed.

In the "era of the revolution" in cancer management with immunotherapy, it appears that a new hope is also arising for patients with advanced GC, as it has in other malignancies where this class of drugs demonstrated benefit.

Evidence and rationale for the use of immunotherapy in gastric cancer GC is a heterogeneous disease which can be divided into 4 major subtypes based on molecular signature according to Cancer Genome Atlas Research Network (TCGA): Epstein Barr virus (EBV) positive, microsatellite

unstable (MSI), and genomically stable (GS) and chromosomal instability (CIN) tumours [12].

Two subtypes, EBV positive and MSI GC, are considered to be most potentially responsive to immunotherapy drugs.

The EBV positive GC that represents 9% of all GC is more prevalent in younger patients, in males (a twofold ratio in male/female), with no difference between intestinal and diffuse histology. EBV positive GC is associated with programmed death-ligand 1 (PD-L1) gene amplification, which suggests higher immunogenicity, and might therefore be more likely to respond to immune checkpoint inhibition. It is known that PD-L1 is highly predictive in lung cancer, but yet controversial in gastric cancer.

MSI tumours seem to occur in 15–30% of GC and are related more commonly with female gender, older patients, and intestinal histology and tumours arising from the distal stomach. This category of gastric cancer is characterized by increased lymphocytic infiltrate, which may reflects activation of T-cells against tumour antigens and genomic changes in tumour cells that are linked to PD-L1 expression, indicating a potential role for immunotherapy [13].

Both MSI and EBV positive GCs have a high somatic mutational burden which also is a feature that has been associated with response to immunotherapy.

2. Checkpoint Inhibition

Given the success of checkpoint inhibitors in melanoma, non-small-cell lung cancer, renal cell cancer, urothelial carcinoma, and head and neck cancer it seemed logical to investigate the role of these agents in gastric cancer.

Cytotoxic T lymphocyte protein 4 (CTLA-4) and programmed cell death protein-1 (PD-1) are immune checkpoints that inhibit the T-cell response, which provide the escape mechanism of the tumour cells to T-cell antitumour activity [14].

The B7-H1, also known as PD-L1, in positive tumours interacts with its receptor PD-1 and this consequently leads to inhibition of the T-cells migration, proliferation, resulting in an antiapoptotic signal, preventing overactivation of the immune system, escaping from destruction [15].

In GC, some studies evaluate the expression and clinical significance of PD-1/PD-L1 pathway. Wu et al. found that PD-L1 was expressed in 42,2% of GC tissues and was not found in normal tissue. The immunodetection of PD- L1 was significantly associated with tumour size, invasion, lymph node metastasis, and survival time of patients [16].

In another study, Hou et al. found the expression of PD-L1 in 63% of the 111 GC patients analyzed and that its overexpression was linked to lymph node metastasis, an advanced clinicopathological stage, and lower overall survival rate [17].

Therefore, immunologic checkpoint blockade with antibodies that target CTLA-4 and PD-1/PD-L1 seemed promising strategies that could improve the outcomes in GC and deserved more specific studies (Figure 1).

We tried to summarize the relevant clinical data about specific immune checkpoints agents and the possible future applications in treatment of advanced gastric cancer.

3. Anti-CTLA4

Ipilimumab and tremelimumab are two anti-CTLA4 antibodies that were evaluated in GC.

A phase II trial evaluated the efficacy of ipilimumab immediately following 1st line chemotherapy in unresectable or metastatic adenocarcinoma of the gastric and GEJ compared with BSC. From 143 patients screened, 57 were randomized to each arm, and in an interim analysis, no differences were seen in PFS between groups, and the study ended early. At study closeout (8 months after interim analysis), the median OS was 12.7 months in BSC versus 12.1 months for the arm with ipilimumab [18].

Tremelimumab was investigated in a phase II trial as 2nd line treatment for patients with metastatic gastric and oesophageal adenocarcinomas. The response rate was only 5%, but there was a clinical benefit with evidence of stable disease in 4 of the 18 patients, and one patient showed a durable response, receiving 32.7 months of treatment after trial enrollment [19].

4. Anti-PD-1

Nivolumab is a PD-1 blocking antibody approved for the treatment of advanced melanoma, advanced non-small-cell lung cancer (NSCLC), advanced renal cell carcinoma, advanced squamous cell carcinoma of the head and neck (SCCHN), and urothelial carcinoma.

Two randomized trials showed efficacy and safety for nivolumab alone in both Asian and western populations in gastric cancer.

The phase I/II CHECKMATE 032 trial, a multicohort study, included patients with metastatic gastric or GEJ cancer, treated with nivolumab in monotherapy (3 mg/kg IV every 2 weeks) or in combination with ipilimumab, irrespective of PD-L1 status [20].

In the single-arm (the cohort with 59 patients), the objective response rate, defined as the proportion of patients who achieved a complete response or a partial response (ORR), with nivolumab was 14% (including 1 complete response and 7 partial responses). Moreover, the stable disease rate was 19%, for a total disease control rate of 32%. The median time to response was 1.6 months and the median duration of response was 7.1 months. The median OS was 5.0 months with nivolumab (95% CI, 3.4–12.4). The 12-month OS rate was 36%. The median PFS was 1.36 months (95% CI, 1.3–1.5) and the 6-month PFS rate was 18%. In the subgroup with PD-L1 expression on ≥1% of cells ($n = 15$), the ORR was 27% with nivolumab. In those with PD-L1 expression on <1% ($n = 25$), the ORR was 12%.

The combination of nivolumab with ipilimumab was also evaluated in this trial, with two separate dose levels: nivolumab 1 mg/kg and ipilimumab 3 mg/kg ($n = 49$) or nivolumab 3 mg/kg plus ipilimumab 1 mg/kg ($n = 52$). The ORR was 26% for the first arm and 10% for the second. Six-month PFS was 24% and 9%, respectively. The 12-month OS was 34% in first cohort and not available in the second. Grade 3 or greater adverse effects (AEs) were seen in 27 and 45%

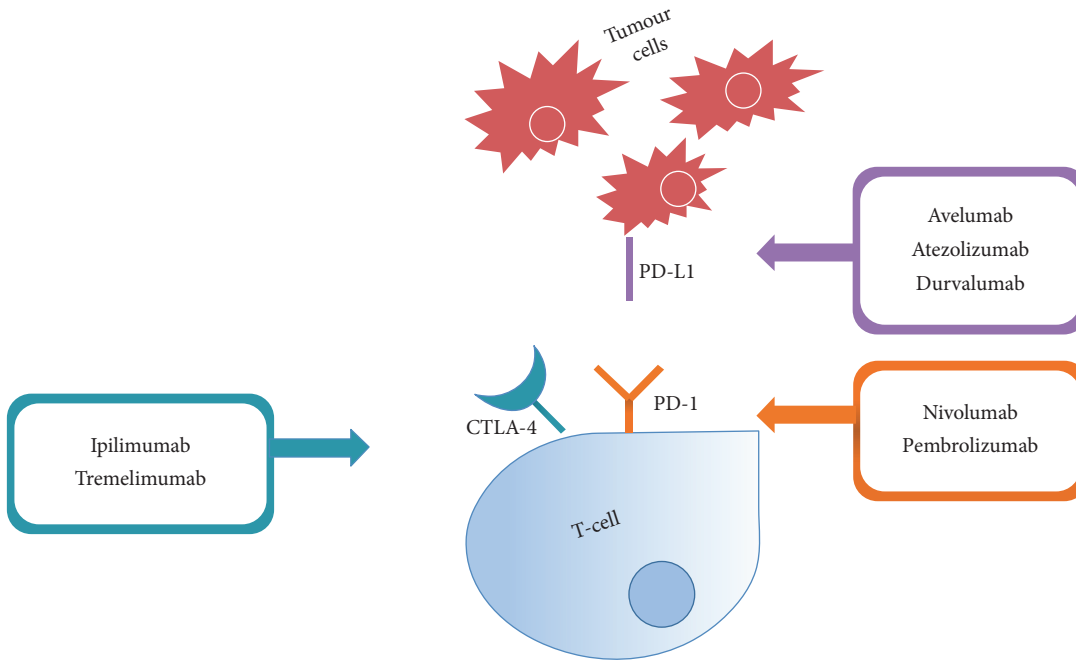

Figure 1: Immune checkpoint blockade with different monoclonal antibodies.

of the patients, respectively, which was higher than in the nivolumab alone arm (17%).

The ONO-4538-12 ATTRACTION-2 trial evaluated the efficacy and safety of nivolumab in Asian patients with unresectable advanced or recurrent gastric cancer (including GEJ) who progressed after two or more chemotherapy lines of treatment [21].

Median OS was 5.26 months (95% CI = 4.60–6.37) for patients treated with nivolumab, compared to 4.14 months (95% CI = 3.42–4.86) for those treated with placebo.

In addition, the 12-month OS in the nivolumab group was 26.2% (95% CI = 20.7–32.0) versus 10.9% (95% CI = 6.2–17.0) in the placebo group. Patients treated with nivolumab had an ORR of 11.2% (95% CI 7.7–15.6) compared to 0% (95% CI 0.0–2.8) with placebo. Patients with confirmed response to nivolumab had a median duration of response of 9.53 months (95% CI 6.14–9.82). Grade 3 or greater AEs occurred in 10% of nivolumab arm and 4% of placebo arm.

There were divergent results according to tumour negative PD-L1 expression versus ≥1%. In tumour with negative PD-L1 expression, median OS was 6.05 months in nivolumab arm (versus 4.19 months in the placebo arm; hazard ratio 0.72); in patients with PD-L1 expression ≥1%, median OS was 5.22 months in the arm of nivolumab (versus 3.83 months in the placebo arm; hazard ratio 0.51).

Currently, an important milestone marked the oncology community: pembrolizumab, a humanized IgG4 monoclonal anti-PD1 antibody, had accelerated approval by FDA (Food and Drug Administration) for the treatment of adult patients with unresectable or metastatic solid tumours that have been identified as having a biomarker referred to as microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR). This indication includes patients that have progressed following prior treatment and who have no satisfactory alternative treatment options.

Results on safety and efficacy from pembrolizumab specifically in gastric cancer were first presented at ESMO Congress 2014 by Muro et al. (KEYNOTE-012) and published in 2016 [22, 23]. Of the 39 patients included in gastric cancer cohort, the ORR was 22% (95% CI 10–39) by central review, all partial responses. Median time to response was 8 weeks (range 7–16), with median response duration of 24 weeks. The 6-month PFS rate was 26% (95% CI 13–41) and OS rate was 66% (95% CI 49–78) and 42% (95% CI 25–59) at 6 and 12 months, respectively. The toxicity was manageable, with only 5 patients experiencing grade 3 or greater adverse effects.

KEYNOTE-059 is a phase II trial multicohort study in advanced gastric or GEJ adenocarcinoma. In cohort 1, patients who have received at least two prior therapies received pembrolizumab as monotherapy. In cohort 2 patients who have not received any previous therapy for their disease received pembrolizumab in combination with cisplatin and 5-FU (in Japan capecitabine could be used instead of 5 FU). In Cohort 3, participants who did not received any previous therapy and who had PD-L1 positive tumours received monotherapy with pembrolizumab.

The results of cohort 1 were presented at ASCO 2017 and the updated data was also presented at ESMO 2017 [24, 25]. From 259 patients in cohort 1, 76.4% were male, and median age was 62.0 years, with patients from United States (47.9%), East Asia (13.1%), and the rest of the world (39.0%); 51.7% and 29% of the patients received pembrolizumab as 3rd line (3L) and 4th line therapy, respectively.

PD-L1 positive patients had expression in ≥1% tumour or stromal cells using immunohistochemistry (IHC). In this cohort 57.1% had PD-L1 positive tumours.

The ORR with pembrolizumab in all patients was 11.6% (95% CI 8.0–16.1). In PD-L1-positive ORR was 15.5% (95% CI 10.1–22.4) and in PD-L1 negative tumours ORR was 6.4% (95% CI 2.6–12.8). The median duration of response (DOR) in all patients was 8.4 months. The median DOR in the PD-L1–positive group was 16.3 versus 6.9 months in those with PD-L1–negative disease.

In the 7 patients with MSI-H tumours, ORR was 57.1%; in comparison with 167 patients with non-MSI-H tumours, ORR was 9.0%.

The median PFS was 2.0 months and the median OS was 5.6 months. Treatment was well tolerated, but 2 treatment-related grade 5 AEs were reported (acute kidney injury and pleural effusion).

In 3rd line the ORR was 16.4% (95% CI 10.6–23.8), with 3% of CR and 13.4% of PR; in 4th line the ORR was 6.4% (95% CI 2.8–12.2).

The cohort 2 was presented in ASCO 2017 and the updated data was also presented at ESMO 2017 [25, 26]. From 25 enrolled patients, 64% were men, median age was 64 years, 68% were Asian, and 64% had PD-L1 positive tumours. In PD-L1-expressing patients, ORR was 68.8% versus 37.5% in PD-L1-negative patients. Median duration of response was 4.6 months in overall population, 4.6 months in PD-L1-positive patients, and 5.4 months in PD-L1-negative patients. Investigators observed grade 3/4 AEs in 76% of patients.

The cohort 3 was presented at ESMO congress in September 2017 [25]. In the 31 patients included, with a median follow-up of 17.5 months, the ORR was 26% and the DCR 36%. The median PFS was 3.3 months and the median OS 20.7 months.

Several randomized clinical trials are currently ongoing to evaluate pembrolizumab and nivolumab in earlier lines of therapy in monotherapy and in combination with chemotherapy regimens or biologic agents for patients with advanced gastric/gastroesophageal cancer (Table 1).

5. Anti-PD-L1

Avelumab is a fully human anti-PD-L1 IgG1 antibody, and its efficacy and safety were first investigated in a phase 1b trial, in patients with advanced gastric or GEJ in first line as maintenance and in second line (2L) of treatment. Patients received avelumab at 10 mg/kg IV every 2 weeks until progression, unacceptable toxicity, or withdrawal [27].

The ORR, until now unconfirmed, in maintenance and 2L was 7.3% (with 1 complete response, 3 partial responses) and 15%, respectively. The disease control rate (DCR) was 54.5% and 50%, and median PFS was 14,1 and 11,6 weeks in two arms (maintenance and 2L respectively). A trend towards longer PFS was observed in patients with PD-L1-positive tumours. Grade ≥ 3 AEs were documented in 9.9% patients, which included fatigue, asthenia, increased gamma-glutamyl transferase (GGT), thrombocytopenia, and anaemia. There was 1 treatment-related death (hepatic failure/autoimmune hepatitis).

With these encouraging results, two randomized trials with avelumab were envisaged: JAVELIN Gastric 300 (NCT02625623) that will compare avelumab plus BSC in third line treatment versus physician's choice of chemotherapy plus BSC and JAVELIN Gastric 100 (NCT02625610), a phase 3 trial, whose purpose is to demonstrate the superiority of treatment with avelumab as maintenance versus continuation of first-line chemotherapy with oxaliplatin-fluoropyrimidine doublet.

Durvalumab is a humanized IgG-1κ monoclonal antibody that blocks PD-L1.

Segal et al. reported durvalumab clinical activity in an expansion study in multiple cancer types, including NSCLC, melanoma (cutaneous and ocular), gastroesophageal, hepatocellular carcinoma, pancreatic, SCCHN, and triple negative breast cancer. Durvalumab was administered as 10 mg/kg IV every 2 weeks for 12 months. This agent showed clinical activity in gastric cancer with an ORR of 25% (4 partial responses). Treatment-related AEs occurred in one-third of the patients, with ≥Grade 3 AEs in 7% and none led to discontinuation of study drug [28].

Durvalumab, as maintenance, as in combination with a variety of immunomodulators and targeted agents is ongoing in gastric cancer field (Table 2).

Atezolizumab is another human monoclonal antibody that contains an engineered Fc-domain that targets PD-L1, blocking PD-L1 from binding to PD-1 and B7.1, and demonstrated clinical activity in locally advanced and metastatic cancers. In a phase I trial, atezolizumab was administered as a single agent to patients with locally advanced or metastatic solid tumours or hematologic malignancies, and 175 patients were evaluated by RECIST v1.1 and confirmed that complete and partial responses were observed in 18% of patients with all tumour types, 21% in NSCLC, 26% in melanoma, 13% in renal cell carcinoma, and 13% of patients with other tumours including colorectal cancer, gastric cancer (only one patient), and head and neck squamous cell carcinoma. A statistical association between tumours expressing high levels of PD-L1 was observed, especially PD-L1 expressed by tumour-infiltrating immune cells and response to atezolizumab treatment [29, 30].

6. Discussion

After a long time of stagnation in GC treatment, with only two molecular target agents providing modest results in OS and PFS (trastuzumab and ramucirumab), maybe a new paradigm shift in oncology is arising: instead of targeting cancer cells, we can target immune cells, thus stimulating the host immune system against its own cancer cells [31].

Gastric cancer is a heterogeneous condition stratified in 4 molecular subtypes, based on genomic changes [12]. The molecular classification improved our knowledge about the biologic behavior of this disease and offered potential actionable oncogenic drivers. With this deep understanding, we will maximize treatment efficacy.

Certainly, MSI and EBV subtype are of particular interest, deriving from their high immunogenicity and potential greater response with immunotherapy agents.

Table 1: Ongoing trials with anti-PD1 in advanced gastric cancer.

Study ID	Study phase	Treatment	Population	Status
NCT02901301	Ib/II	Pembrolizumab + trastuzumab + cisplatin + capecitabine	HER2 positive advanced gastric cancer	Recruiting
CP-MGAH22-05 (NCT02689284)	Ib/II	Margetuximab in combination with pembrolizumab	Relapsed/refractory advanced HER2+ GEJ or gastric cancer	Recruiting
NCT02318901	Ib/II	Pembrolizumab and monoclonal antibody therapy	Patients with advanced cancer (one cohort for patients with unresectable HER2 overexpressing gastric or GEJ cancers)	Active, not recruiting
NCT03095781	Ib	Pembrolizumab and XL888	Patients with stage IV or locally advanced unresectable gastrointestinal adenocarcinomas who have failed at least one prior therapy	Recruiting
NCT02178722	I/II	Pembrolizumab in combination with epacadostat	Patients with selected cancers (including gastric cancer)	Recruiting
NCT03342937	II	Pembrolizumab + oxaliplatin and capecitabine	First-line treatment of patients with gastroesophageal cancer	Not yet recruiting
NCT02954536	II	Pembrolizumab in combination with trastuzumab, capecitabine/cisplatin	First-line stage IV HER2-positive metastatic esophagogastric (EG) cancer	Recruiting
NCT03196232	II	Epacadostat and pembrolizumab	Metastatic or unresectable GEJ or gastric cancer that progressed at least first line of prior therapy	Recruiting
KEYNOTE KN-463 (NCT03122548)	II	CRS-207 and pembrolizumab	Recurrent or metastatic gastric, GEJ, or esophageal cancer who have received 2 prior systemic chemotherapy treatment	Recruiting
KEYNOTE-063 (NCT03019588)	III	Pembrolizumab versus paclitaxel	Asian subjects with advanced gastric or GEJ adenocarcinoma who progressed after first-line therapy with platinum and fluoropyrimidine	Recruiting
KEYNOTE-062 (NCT02494583)	III	Pembrolizumab as monotherapy and in combination with cisplatin + 5-fluorouracil versus placebo + cisplatin + 5-fluorouracil	As first-line treatment in subjects with advanced gastric or GEJ adenocarcinoma	Active, not recruiting
KEYNOTE-061 (NCT02370498)	III	Pembrolizumab versus paclitaxel	Advanced gastric or GEJ adenocarcinoma who progressed after first-line therapy with platinum and fluoropyrimidine	Active, not recruiting
ONO4538 (NCT02267343)	III	Nivolumab versus placebo	Unresectable advanced or recurrent gastric cancer (including esophagogastric junction cancer) refractory to or intolerant of standard therapy	Active, not recruiting
CA209-929 (NCT03342417)	II	Combination of nivolumab and ipilimumab in breast, ovarian, and gastric cancer patients	In gastric cancer arm: advanced gastric cancer patients who are recurrent/refractory to a prior therapy not involving herceptin	Recruiting
ONO-4538-37 (NCT02746796)	II/III	Nivolumab and chemotherapy versus placebo and chemotherapy	Unresectable advanced or recurrent gastric cancer (including esophagogastric junction cancer) not previously treated with the first-line therapy	Recruiting
CheckMate 649 (NCT02872116)	III	Nivolumab plus ipilimumab or nivolumab in combination with oxaliplatin plus fluoropyrimidine versus oxaliplatin plus fluoropyrimidine	Patients with previously untreated advanced or metastatic gastric or gastroesophageal junction cancer	Recruiting
FRACTION-GC (NCT02935634)	II	Nivolumab plus ipilimumab versus nivolumab plus relatlimab versus nivolumab and BMS-986205	Patients with advanced gastric cancer	Recruiting
NCCH-1611 NCT02999295	I/II	Ramucirumab plus nivolumab	Second-line therapy in Participants with gastric or GEJ cancer	Recruiting

Table 1: Continued.

Study ID	Study phase	Treatment	Population	Status
AIO-STO-0217 (NCT03409848)	II	Ipilimumab or FOLFOX in combination with nivolumab and trastuzumab	Previously untreated HER2 positive locally advanced or metastatic esophagogastric adenocarcinoma	Not yet recruiting
INCAGN 1876-201 (NCT03126110)	I/II	INCAGN01876 combined with nivolumab versus INCAGN01876 combined with ipilimumab versus INCAGN01876 combined with nivolumab and ipilimumab	Subjects with advanced or metastatic malignancies	Recruiting

Table 2: Ongoing trials with anti-PD-L1 in advanced gastric cancer.

Study ID	Study phase	Treatment	Population	Status
YO39609 (NCT03281369)	I/II	Multiple immunotherapy-based treatment combinations, including atezolizumab as immunotherapeutic agent	Patients with locally advanced unresectable or metastatic gastric or gastroesophageal junction cancer	Recruiting
JAVELIN Gastric 300 (NCT02625623)	III	Avelumab + best supportive care (BSC) versus physician's choice chemotherapy + BSC or BSC alone	Unresectable, recurrent, locally advanced, or metastatic gastric or gastroesophageal junction adenocarcinoma gastric cancer third line	Active, not recruiting
JAVELIN Gastric 100 (NCT02625610)	III	Avelumab (MSB0010718C) versus continuation of first-line chemotherapy	Unresectable, locally advanced, or metastatic adenocarcinoma of the stomach or of the gastroesophageal junction	Active, not recruiting
JAVELIN MEDLEY (NCT02554812)	Ib/II	Avelumab (MSB0010718C) in combination with other cancer immunotherapies	Patients with locally advanced or metastatic solid tumors	Recruiting
MEDIOLA (NCT02734004)	I/II	MEDI4736 in combination with olaparib	Patients with advanced solid tumors, selected based on a rationale for response to olaparib	Active, not recruiting
I4T-MC-JVDJ (NCT02572687)	I	Ramucirumab plus MEDI4736	Participants with locally advanced and unresectable or metastatic gastrointestinal or thoracic malignancies including gastric or gastroesophageal junction (GEJ) adenocarcinoma, non-small-cell lung cancer (NSCLC) or hepatocellular carcinoma (HCC)	Active, not recruiting
PLATFORM (NCT02678182)	II	Maintenance therapies following completion of standard first-line chemotherapy: placebo versus capecitabine versus durvalumab versus trastuzumab versus rucaparib	Patients with locally advanced or metastatic HER-2 positive or HER-2 negative oesophagogastric adenocarcinomas	Recruiting
D419SC00001 (NCT02658214)	Ib	Durvalumab and tremelimumab in combination with first-line chemotherapy	Patients with advanced solid tumors	Recruiting

MEDI4736 also known as durvalumab.

As detailed before, immune checkpoint blockade with antibodies targeting CTLA-4, PD-1, and PD-L1 has revealed clinical activity in gastric cancer. While anti-CTLA4 showed only slight activity in gastric cancer, and PD-1 and PD-L1 inhibitors showed promising results and will probably take place in gastric cancer management in the near future.

We would like to highlight the phase III KEYNOTE-059 trial, as it showed antitumour activity and durable responses in patients with advanced gastric/GEJ cancer progression after more than 2 lines of therapy. Until now there was no evidence for 3rd and 4th lines in gastric cancer, and based on the cohort 1 results, pembrolizumab was approved by the FDA recently [24].

In the cohort 2, patients received pembrolizumab and chemotherapy with cisplatin and 5- fluorouracil, with favourable clinical activity and manageable toxicity, though more data is needed to draw conclusions [25, 26].

Also, a question to consider is if the results of nivolumab in Asian patients will be reproduced in western patients? [20, 21]

Results from phase I/II CheckMate 032 trial, which included heavily pretreated European and North American population, revealed long-term overall survival and responses with nivolumab. These findings suggest a possible benefit with nivolumab in Asian and western patients, although we need more studies to make a definitive conclusion.

This and much more questions remain to be answered: which gastric cancer subpopulation does benefit more from immune checkpoints inhibitors? In which stage of the disease should we use immunotherapy, in earlier lines or after progression of more than 2 lines of therapy?

We look forward at the ongoing phase III trials and wait with hope for their results. Besides, more studies are needed to validate predictive and prognostic biomarkers to immunotherapy agents in gastric cancer.

Additionally, integration of immune checkpoints combined with targeted agents, chemotherapy, or radiotherapy appears to be exciting multimodal approaches and randomized trials are also ongoing.

In conclusion, some progress has been reached in the treatment of advanced gastric cancer in the last years.With the recent biologic and molecular knowledge, we have recognized that gastric cancer is a group of distinct molecular entities rather than a single disease. This molecular characterization will allow achieving a better selection of patients that can benefit from a treatment strategy.

The field is unquestionably moving towards a more precise medicine, and the progressing accomplishments will transform the clinical practice in the management of advanced gastric cancer in the near future.

Additional Points

Core Tip. GC is a highly heterogeneous disease and the recent molecular characterization will help us to better select patients who might benefit from immune checkpoint inhibitors and other agents. There are encouraging results with agents that target programmed death 1 (PD-1) and its ligands in gastric cancer; however more trials are needed to identify predictive and prognostic biomarkers to select patients most appropriately for this treatment. In this review, we explore the current evidence supporting the use of immunotherapy in advanced GC.

Conflicts of Interest

Helena Magalhães and Mário Fontes-Sousa have no conflicts of interest to disclose. Manuela Machado has participated as consultant and/or in advising boards from Roche, Amgen, Merck Serono, Bayer, Celgene, Lilly, Servier, Angelini, and Grunenthal.

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Research Article

A Comparison of Endoscopic Ultrasound-Guided Fine-Needle Aspiration and Fine-Needle Biopsy in the Diagnosis of Solid Pancreatic Lesions

Lachlan R. Ayres,[1] **Elizabeth K. Kmiotek**,[2] **Eric Lam**,[3] **and Jennifer J. Telford**[3]

[1]*Poole Hospital NHS Foundation Trust, Poole, UK*
[2]*Jagiellonian University Medical College, Krakow, Poland*
[3]*Division of Gastroenterology, St. Paul's Hospital, University of British Columbia, Vancouver, BC, Canada*

Correspondence should be addressed to Lachlan R. Ayres; lachlanayres@hotmail.com

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Background and Aims. Endoscopic ultrasound (EUS) guided fine-needle aspiration (FNA) is the method of choice for sampling pancreatic lesions. This study compares the diagnostic accuracy and safety of FNB using a novel core needle to FNA in solid pancreatic lesions. *Methods*. A retrospective review of patients in whom EUS FNA or FNB was performed for solid pancreatic lesions was conducted. Diagnostic performance was calculated based upon a dual classification system: classification 1, only malignant pathology considered a true positive, versus classification 2, atypical, suspicious, and malignant pathology considered a true positive. *Results*. 43 patients underwent FNB compared with 51 FNA. Using classification 1, sensitivity was 74.0% versus 80.0%, specificity 100% versus 100%, and diagnostic accuracy 77.0% versus 80.0% for FNB versus FNA, respectively (all $p > 0.05$). Using classification 2, sensitivity was 97% versus 94.0%, specificity 100% versus 100%, and diagnostic accuracy 98.0% versus 94.0% for FNB versus FNA, respectively (all $p > 0.05$). FNB required significantly fewer needle passes (median = 2) compared to FNA (median = 3; $p < 0.001$). Adverse events occurred in two (4.5%) FNB patients compared with none in the FNA group ($p > 0.05$). *Conclusion*. FNA and FNB have comparable sensitivity and diagnostic accuracy. FNB required fewer passes.

1. Introduction

Endoscopic ultrasound (EUS) guided fine-needle aspiration (FNA) is the method of choice for evaluating and sampling solid pancreatic lesions [1–3]. EUS can detect cancers less than 10 mm in size and is more sensitive than computed tomography (CT) [4]. It has a safe, cost-effective, and highly accurate method to diagnose solid pancreatic mass lesions [2, 4–7].

There is uncertainty relating to the optimal needle gauge, number of needle passes, presence of an on-site pathologist, and more recently whether the ability to procure core samples using fine-needle biopsy (FNB) is advantageous [3, 8–11]. Several core biopsy needles are available which have the potential to preserve tissue architecture and morphology which is helpful for the characterization of some lesions such as stromal tumors and lymphomas [12–14]. Generally, FNB is comparable to FNA in terms of diagnostic accuracy for solid pancreatic lesions [13–17].

The recently developed SharkCore™ FNB needle is designed with six cutting edge surfaces and an opposing bevel to trap core tissue which preserves architecture and limits tissue fracturing in addition to including a passively activated safety sheath to prevent needle stick injuries. A recently published pilot study demonstrated comparable diagnostic performance compared to FNA [18].

The aim of this study was to compare the sensitivity, specificity, and safety of SharkCore FNB to conventional FNA in evaluating solid pancreatic masses.

2. Methods

A retrospective review was performed on consecutive patients who underwent index EUS guided FNB of solid

Table 1: Patient demographics.

	FNA ($n = 51$)	FNB ($n = 43$)	p
Sex n (%)			0.147
Female	29 (56.9)	18 (41.9)	
Male	22 (43.1)	25 (58.1)	
Age			0.756
Median (IQR)	66.0 (55.0–75.0)	66.0 (56.0–75.0)	
Mean (SD)	64.8 (12.2)	65.9 (12.7)	
Range	33.0–83.0	36.0–88.0	

FNA = fine needle aspiration; FNB = fine needle biopsy; IQR = interquartile range; SD = standard deviation.

pancreatic lesions by two experienced endosonographers at St. Paul's Hospital, Vancouver, BC, using the Covidien Shark-Core platform (Shark Core®, Covidien, Dublin, Leinster, Ireland) using 19 G, 22 G, or 25 G needles. When the study was conceived 50 FNB had been performed on solid pancreatic lesions (November 2014 to July 2015). Thus a similar number of consecutive patients undergoing FNA using a 22 G or 25 G needle (Expect, Boston Scientific, Natick, MA, USA) of solid pancreatic lesions were taken for comparison (from October 2013 to October 2014). There was no on-site pathologist present for either cohort. The number of needle passes and needle throws was not standardized and was at the discretion of the endosonographer. Assessment of an adequate specimen was also at the discretion of the endosonographer generally using a crude visual assessment of the material expressed from the needle.

Patients were excluded when there was a predominantly cystic component to the mass or if adequate follow-up was not available: either surgical pathology or six months' clinical follow-up. The study was approved by the University of British Columbia Ethics Board.

Electronic medical records were interrogated and demographic data recorded. The size of the lesion was documented based on the largest dimension reported in millimeters. The location of the lesion was categorized as falling within the head, uncinate, genu, body, or tail of pancreas. Technical failures, number of needle passes, and needle gauge were recorded.

The pathological diagnosis by EUS was categorized as nondiagnostic, benign, atypical, suspicious, or malignant (i.e., classes 1–5, resp.). Diagnostic accuracy, positive predictive value, negative predictive value, sensitivity, and specificity were calculated using a dual classification system employed in a meta-analysis of EUS FNA by Hewitt et al. 2012 [2]. Under this approach malignancy status was established as follows:

(i) Classification 1: nondiagnostic, benign, atypical, and suspicious are considered negative for malignancy. Only the designation "malignant," that is, class 5, is considered a true positive.

(ii) Classification 2: nondiagnostic and benign are negative for malignancy. Atypical and suspicious are also considered positive for malignancy.

Diagnostic accuracy was compared to gold standard surgical pathology, subsequent EUS FNA/FNB, or six-month clinico-radiological follow-up. It was computed as the ratio between the sum of true positive and true negative values divided by the total number of lesions. Neuroendocrine tumors which have a range of malignant potential were all regarded as a "positive" diagnosis and grouped with adenocarcinoma and lymphoma for the purposes of the analysis. Adverse events, as determined by interrogating electronic medical records, were also recorded and compared.

2.1. Statistical Analysis. Statistical analysis was performed using the chi-square test, Fisher's exact test, or Wilcoxon rank sum test as appropriate using SAS 9.4 and R 3.2.0. A p value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Patient Demographics. Patients ranged from 33 to 88 years of age (median = 66 years of age for both groups). There were 22 (43.1%) versus 25 (58.1%) males in the FNA and FNB groups, respectively. Demographic data of the study population is reported in Table 1.

3.2. Lesion Characteristics. There was no difference in location, size, or pathological class (all $p > 0.05$) between the two groups. Lesion characteristics are shown in Table 2. Most lesions were located in the head of pancreas (61.7%) followed by the body (13.8%), tail (10.6%), uncinate (7.4%), and genu (6.4%). The mean lesion size was 27 mm ($\pm$12.2 (SD)), 72.3% were malignant, 10.6% suspicious, 6.4% atypical, 8.5% benign, and 2.1% nondiagnostic. The majority of lesions (68%) were pancreatic ductal adenocarcinoma with neuroendocrine lesions the second most common. The two groups differed significantly in terms of the final diagnosis ($p = 0.018$) in that there were more neuroendocrine tumors (NET) in the FNA group and more inflammatory lesions and lymphomas in the FNB group.

3.3. Diagnostic Accuracy: Classification 1. The diagnostic performance of FNA and FNB is presented in Table 3. In the FNA group 39/51 (77%) specimens were positive for malignancy compared to 29/43 (67%) in the FNB group. Of 12 samples negative for malignancy in the FNA group, ten were false negatives (14 and ten for FNB). This translates to a

Table 2: Lesion characteristics.

	FNA ($n = 51$)	FNB ($n = 43$)	p
Location of lesion in pancreas, n (%)			0.275
Head	27 (52.9)	31 (72.1)	
Uncinate	5 (9.8)	2 (4.7)	
Genu	3 (5.9)	3 (7.0)	
Body	8 (15.7)	5 (11.6)	
Tail	8 (15.7)	2 (4.7)	
Lesion size (largest dimension, mm)			0.787
Missing, *n* (%)	1 (2.0)	5 (11.6)	
Median (IQR)	26.0 (18.0, 34.0)	26.0 (18.0, 36.0)	
Mean (SD)	26.8 (12.8)	27.4 (11.5)	
Range	(5.0, 70.0)	(9.0, 55.0)	
Cytologic/histologic diagnosis			0.468
Nondiagnostic	2 (3.9)	0 (0.0)	
Benign	3 (5.9)	5 (11.6)	
Atypical	2 (3.9)	4 (9.3)	
Suspicious	5 (9.8)	5 (11.6)	
Malignant	39 (76.5)	29 (67.4)	
Final diagnosis			0.018
Pancreatic adenocarcinoma	37 (72.5)	31 (72.1)	
Neuroendocrine tumour	12 (23.5)	4 (9.3)	
Inflammatory	0 (0.0)	4 (9.3)	
Lymphoma	0 (0.0)	1 (2.3)	
Benign/normal	2 (3.9)	1 (2.3)	
Other	0 (0.0)	2 (4.7)	

FNA = fine needle aspiration; FNB = fine needle biopsy; IQR = interquartile range; SD = standard deviation.

Table 3: Diagnostic performance: classification 1.

	FNA	FNB	p
Sensitivity	0.80 (0.66, 0.90)	0.74 (0.58, 0.87)	0.615
Specificity	1.00 (0.09, 1.00)	1.00 (0.28, 1.00)	1.000
PPV	1.00 (0.87, 1.00)	1.00 (0.83, 1.00)	1.000
NPV	0.17 (0.02, 0.48)	0.29 (0.08, 0.58)	0.652
Accuracy	0.80 (0.67, 0.90)	0.77 (0.61, 0.88)	0.801

Values in brackets are 95% confidence interval. p value is based on Fisher's exact test; FNA = fine needle aspiration; FNB = fine needle biopsy.

sensitivity of 80% versus 74%, specificity of 100% versus 100%, positive predictive value (PPV) of 100% versus 100%, negative predictive value (NPV) of 17% versus 29%, and an accuracy of 80% versus 77% for FNA and FNB, respectively (all $p > 0.05$).

3.4. Diagnostic Accuracy: Classification 2. Using the less stringent classification 2 (see Table 4), the sensitivity, NPV, and accuracy all increased as compared to classification 1, but there was still no statistically significant difference between FNA and FNB in any of these measures. In the FNA group 46/51 (90%) were malignant versus 38/43 (88%) in the FNB group. Of five samples negative for malignancy three were false negatives for FNA (compared to five and one for FNB). Thus sensitivity was 94% versus 97%, specificity 100% versus 100%, PPV 100% versus 100%, NPV 40% versus 80%, and accuracy 94% versus 98% for FNA and FNB, respectively.

3.5. Technical Outcomes. In the FNB group, 35 (81%) lesions were sampled using a 25 G needle, six (14%) lesions were sampled using 22 G, and one (2%) lesion was sampled using 19 G (needle gauge not reported in one case). Technical outcomes are reported in Table 5. Fewer needle passes were performed in the FNB group: median two (mean 2.1) compared to a median of three (mean 3.2) in the FNA group ($p < 0.001$). In the FNA group five (9.8%) patients required a repeat EUS compared to eight (18.6%) in the FNB group ($p = 0.218$). Two adverse events were reported (one gastrointestinal bleed, no blood transfusion or endoscopic therapy required, and one self-limiting episode of mild acute pancreatitis) in the FNB group compared to none in the FNA group $p > 0.05$. No technical failures were reported in either group.

In the FNA group four (7.8%) specimens were paucicellular/inadequate, three of which required repeat EUS versus

Table 4: Diagnostic performance: classification 2.

	FNA	FNB	p
Sensitivity	0.94 (0.83, 0.99)	0.97 (0.87, 1.00)	0.626
Specificity	1.00 (0.09, 1.00)	1.00 (0.28, 1.00)	1.000
PPV	1.00 (0.89, 1.00)	1.00 (0.87, 1.00)	1.000
NPV	0.40 (0.05, 0.85)	0.80 (0.28, 0.99)	0.524
Accuracy	0.94 (0.84, 0.99)	0.98 (0.88, 1.00)	0.623

Values in brackets are 95% confidence interval. p value is based on Fisher's exact test; FNA = fine needle aspiration; FNB = fine needle biopsy

Table 5: Technical outcomes.

	FNA (n = 51)	FNB (n = 43)	p
Passes			<0.001
Unknown	1	0	
1	1 (2.0)	0 (0.0)	
2	5 (10.0)	36 (83.7)	
3	27 (54.0)	6 (14.0)	
4	16 (32.0)	1 (2.3)	
5	1 (2.0)	0 (0.0)	
Required a repeat EUS/FNA, n (%)			0.218
No	46 (90.2)	35 (81.4)	
Yes	5 (9.8)	8 (18.6)	
Adverse events, n (%)	0 (0)	2 (4.65)	0.207

FNA = fine needle aspiration; FNB = fine needle biopsy; EUS = endoscopic ultrasound.

three (7.0%) specimens in the FNB group of which two required repeat EUS (p = 1.00).

4. Discussion

In this study comparing FNA to FNB, both needles demonstrate similar diagnostic performance, with FNB requiring significantly fewer needle passes to obtain sufficient diagnostic material regardless of the classification system used. To our knowledge this is the largest study comparing SharkCore FNB to conventional FNA in the diagnosis of solid pancreatic masses.

The dual classification system used in this investigation was adapted from Hewitt et al. 2012 who conducted a meta-analysis of EUS FNA in the diagnosis of pancreatic masses. Their pooled findings for sensitivity, specificity, PPV, and NPV were 85%, 98%, 99%, and 64% under classification I and 91%, 94%, 98%, and 72% under classification 2 [2]. These data are comparable to the findings presented here.

Our experience with SharkCore is similar to the findings described in a North American multicenter study using SharkCore for the diagnosis of various solid lesions. Of 250 lesions sampled, 88% were diagnostic with a median of two passes. Subgroup analysis showed that for pancreatic masses 86% were diagnostic [19]. They found a trend towards superior pathologic yield when compared to cytologic yield (87% versus 68%) but this was not statistically significant. Similarly, the Newcastle group (UK) found that SharkCore had a 90.1% sensitivity compared to ProCore™ 71.1% in a cohort of 201 patients with solid pancreatic lesions [20].

Various studies demonstrate that ProCore FNB requires fewer passes to obtain a pathologic diagnosis [13, 15–17, 19, 21]. Similarly, a recent pilot study by Adler et al. found that the SharkCore FNB required fewer passes compared to standard FNA (1.5 passes versus three passes, resp.) in a 30-patient cohort [18]. This finding supports the results of this study where a median number of two passes was needed for FNB versus three for FNA.

Of the studies investigating ProCore FNB, most did not identify a significant difference in diagnostic sensitivity between FNA and FNB for solid pancreatic masses [13–16, 19, 22]. Furthermore, when other lesions were analyzed in conjunction with pancreatic masses such as lymph nodes and gastrointestinal mass lesions, both needles still exhibited comparable levels of accuracy [17, 21, 23]. FNA sensitivity ranged within 72–92% versus 90–97.8% for FNB, and specificity ranged within 80%–100% versus 100%, respectively [13, 16, 17, 21]. This is similar to our own findings where FNA sensitivity was 80%–94% compared to 74%–97% for FNB in classification systems 1 and 2, respectively. Two studies found FNB (ProCore) to be inferior to FNA in the diagnosis of pancreatic masses [24, 25]. However both studies were small and one used a different number of passes for FNA and FNB.

Accuracy ranged within 90–94.8% for FNA and 84.6–98.3% for FNB in previously published comparative studies [15–17, 21, 24]. Not all investigations examined in this paper specified the criteria establishing malignancy; thus stringent comparisons using the two classification systems are not possible. As with most studies of EUS FNA/FNB false

positives are rare and none occurred in either cohort in our study yielding specificities of 100% for both FNA and FNB under both classification systems.

Despite reports of FNB providing improved tumor type discrimination, histopathological quality, and preservation of architecture [13, 14], other studies, including our own, have not demonstrated that it increases sensitivity, although we speculate that this could be borne out if the number of needle passes was increased for FNB.

Interestingly we found that with FNA five of ten false negatives (using classification I) were neuroendocrine tumors. For FNB, seven of ten false negatives (using classification I) were pancreatic adenocarcinoma and none were eventually proven to be neuroendocrine tumors. The number of cases is too small to draw strong conclusions but this may indicate that FNB is advantageous in neuroendocrine tumors which have a spectrum of malignant potential and where a core specimen may be more important.

Although we retrospectively applied two classifications to the data, it is interesting to observe how cases were managed in reality. In the FNB group, eight patients (18.2%) required a second EUS (six pancreatic adenocarcinomas, one hemangioendothelioma, and one benign). This compares to five (9%) in the FNA group (three pancreatic adenocarcinomas and two neuroendocrine tumors). This trend is not statistically significant.

It is perhaps intuitive that, for a needle designed to obtain a core, the trade-off for obtaining more tissue comes at the expense of more adverse events. The only adverse events that occurred in the study population (a gastrointestinal bleed and an episode of pancreatitis) were in the FNB group although this was not statistically significant. To date, studies using other FNB needles have reported a similar safety profile to FNA [22] and many reported no adverse events at all [13, 14, 19, 25]. Studies with larger sample sizes are required to assess the safety profile of the SharkCore FNB.

This study was retrospective and so has inherent limitations. Other limitations are that tissue sampling was not standardized in terms of number of passes, number of needle throws, and use of suction or "slow-pull" technique. Secondly the FNA group differs significantly from the FNB group in terms of the final diagnosis; however the majority of lesions were pancreatic adenocarcinoma and neuroendocrine tumors for both groups and this is unlikely to have had a material effect on diagnostic performance. Thirdly pathological specimens were not always reported in a strict categorical format. Finally, the FNB cohort dates from when the SharkCore needle was introduced, whereas the FNA cohort represents data taken at a time when both endosonographers were very familiar with that needle. There is inevitably a learning curve with new equipment and it is possible that, with more experience and familiarity, performance could improve slightly for FNB. Lastly, the sample size reported here is relatively small.

5. Conclusions

In summary, the study found FNA and FNB to have comparable diagnostic accuracy and safety, with FNB requiring fewer passes. A prospective, randomized trial is warranted to establish whether FNB has an advantage over FNA.

Disclosure

This data was presented in abstract format at DDW 2016 (http://www.giejournal.org/article/S0016-5107(16)01175-5/fulltext).

Conflicts of Interest

Drs. Elizabeth K. Kmiotek, Lachlan R. Ayres, Eric Lam, and Jennifer J. Telford have no conflicts of interest or financial ties to disclose.

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Research Article

Establishment of a Model of Microencapsulated SGC7901 Human Gastric Carcinoma Cells Cocultured with Tumor-Associated Macrophages

Jin-Ming Zhu,[1,2] Xiu-Lian Quan,[1,2] Shi-Chao Han,[3] Xue-Jun Fan,[4] He-Ming Li,[1,2] Shan-Shan Liang,[1,2] Xi Chen,[1,2] Ruo-Yu Wang,[1,2] and Xue-Ning Ji[1,2]

[1]*Department of Oncology, Zhongshan Hospital, Dalian University, Dalian, Liaoning, China*
[2]*The Key Laboratory of Biomarker High Throughput Screening and Target Translation of Breast and Gastrointestinal Tumor, Liaoning Province, China*
[3]*Department of Gynaecology and Obstetrics, The Second Affiliated Hospital of Dalian Medical University, Dalian, Liaoning, China*
[4]*Department of Oncology, Zunyi Medical University, Zunyi, Guizhou, China*

Correspondence should be addressed to Xue-Ning Ji; xueningji@foxmail.com

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The important factors of poor survival of gastric cancer (GC) are relapse and metastasis. For further elucidation of the mechanism, a culture system mimicking the microenvironment of the tumor in humans was needed. We established a model of microencapsulated SGC7901 human GC cells and evaluated the effects of coculturing spheres with tumor-associated macrophages (TAMs). SGC7901 cells were encapsulated in alginate-polylysine-sodium alginate (APA) microcapsules using an electrostatic droplet generator. MTT assays showed that the numbers of microencapsulated cells were the highest after culturing for 14 days. Metabolic curves showed consumption of glucose and production of lactic acid by day 20. Immunocytochemistry confirmed that Proliferating Cell Nuclear Antigen (PCNA) and Vascular Endothelial Growth Factor (VEGF) were expressed in microencapsulated SGC7901 cells on days 7 and 14. The expression of PCNA was observed outside spheroids; however, VEGF was found in the entire spheroids. PCNA and VEGF were increased after being cocultured with TAMs. Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) expressions were detected in the supernatant of microencapsulated cells cocultured with TAMs but not in microencapsulated cells. Our study confirms the successful establishment of the microencapsulated GC cells. TAMs can promote PCNA, VEGF, MMP-2, and MMP-9 expressions of the GC cells.

1. Introduction

Gastric cancer is one of the most common malignancies and the second leading cause of cancer-related death worldwide [1]. Although many therapies are currently available for GC, the 5-year overall survival rate is only about 50% owing to tumor relapse and metastasis. Recent evidence suggests that the tumor microenvironment (TME) is critical for tumor progression and metastasis [2]. Tumor-associated macrophages (TAMs) are derived from circulating monocytes, which are the most abundant immune cells in the tumor microenvironment [3] and are subjected to an intense cross talk with tumor cells. Macrophages can be polarized by cytokines, chemokines, and growth factors which are produced by stromal and tumor cells [4]. Meanwhile, TAMs secrete lots of factors that induce the formation of a network in which tumor cells can benefit by receiving nutrients and migrating to other sites [5]. Thus, TAMs can facilitate cancer promotion, angiogenesis induction, and tumor cell migration and metastasis [6].

However, studies that performed *in vitro* culturing of tumor cells or TAMs have important limitations. Most tumor cells cultured *in vitro* are grown as monotypic cultures in two-dimensional (2D) conditions, which cannot simulate *in vivo* TME conditions [7]. In comparison, three-dimensional (3D) cell culture conditions enable tumor cells to establish cell-cell

and cell-extracellular interactions, which are important elements in tumor signaling and modulating tumor responses to therapeutic agents [8, 9].

Microcapsules are spherical, with diameters in the range of 200–1500 μm, and feature a biocompatible semipermeable membrane that permits the bidirectional diffusion of nutrients, secreted therapeutic products, oxygen, and waste but prevents the passage of high-molecular-weight substances into the microcapsule. So, microcapsules can be used as an immune-isolation device [10]. Microcapsules can provide contained environments in which tumor cells grow in a three-dimensional manner and become adjusted to the intravital environment of the host so that they can perform a cross talk with the extracellular matrix. The reaction between the extracellular matrix and alginate microcapsules enhanced cell proliferation by triggering a cascade of intracellular signaling events through cell-matrix interactions [11, 12].

The model of microencapsulated tumor cells is a short-time, simple, and relatively inexpensive assay. Currently, many studies on microencapsulated tumor cell models on breast cancer, pancreatic cancer, lymphoma, melanoma, and osteosarcoma cells have already been performed. However, the microencapsulated human GC cell model is still lacking.

Here, we established a microencapsulated human GC cell model, called the microencapsulated SGC7901 model, using alginate-polylysine-sodium alginate (APA). This model can be widely used for preclinical studies and it provides reliable evidence for clinical therapy in GC patients. We evaluated the biological characteristics of this model and the effects on the encapsulated cells upon cocultivation with TAMs.

2. Materials and Methods

2.1. Materials. The human GC SGC7901 cell line was purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Encapsulation device: a high-frequency pulse microdroplet generator was provided by Dalian Institute of Chemical Physics, Chinese Academy of Sciences.

2.2. Cell Culture. SGC7901 cells were cultured in a T flask (75 cm^2) in RPMI1640 medium (Gibco, Grand Island, NY, USA) supplemented with 1% (v/v) penicillin-streptomycin and 10% fetal bovine serum (FBS, Gibco) in an incubator at 37°C and 5% CO_2 at 100% relative humidity. The growth medium was replenished as necessary.

2.3. Differentiation of Macrophages from Peripheral Blood Mononuclear Cells. Blood samples (15 mL) were collected from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated according to a previous publication [13]. PBMCs were plated in three 6-well plates (1×10^6 cells/well) and incubated for 3 h in RPMI1640 (Gibco) supplemented with 1% (v/v) penicillin-streptomycin and 10% FBS in an incubator at 37°C and 5% CO_2 at 100% relative humidity. On the next day, Recombinant Rat Granulocyte Macrophage-Colony Stimulating Factor (rGM-CSF) (Rocky Hill, NJ) was added (1000 u/ml) in 2 mL of RPMI 1640 (Gibco) containing 10% FBS (Gibco). The cells were cultured for 7 days, and the medium was changed every 3 days. Macrophages were harvested on day 7.

2.4. Identification of Macrophages. The cell suspension was prepared when the macrophage was cultured for 7 days. The cells were plated in a 24-well plate and cultured for 72 h. Cells were then fixed with 4% paraformaldehyde for 30 min, incubated with 0.2% Triton X-100 (Gibco) for 20 min, and then blocked with 5% BSA for 30 min. Cells were washed three times with PBS for 5 min between the different steps above. The fixed cells were incubated with primary antibody against CD68 (ZSGB-BIO, Beijing, China) (1 : 100) at 4°C overnight and then incubated for 30 min with secondary antibody (Santa Cruz, Dallas, TX, USA) at room temperature in the dark. Cells incubated with PBS as a primary antibody served as the negative control. Stained cells were sealed with glycerol and observed with a confocal microscope (Olympus Optical Co., Ltd., Japan).

2.5. Cell Microencapsulation. The process for encapsulation of human tumor cells was based on the encapsulation technology provided by Dalian Institute of Chemical Physics. The encapsulation preparation was performed according to a previous publication [14]. SGC7901 cells were suspended at a final concentration of 4×10^9 cells/L in sodium alginate. The suspension was then passed through a jet head droplet-forming device yielding spherical microdroplets (diameter: 600 μm), with about 600–800 cells in each droplet. These microdroplets were formed into discrete water-insoluble gel spheres by contact with a lightly stirred solution of $CaCl_2$. The next step was the formation of a semipermeable membrane on the surface of the gel beads induced by addition of polylysine (Sigma Chemical Co., St. Louis, MO). This was called polylysine-sodium alginate (PA) microencapsulation. By suspending the PA microencapsulation in a 1.2% low-viscosity sterile pharmaceutical-grade alginate, the APA was obtained. This process yielded microencapsulated cells containing viable tumor cells in liquid suspension. The microencapsulated cells were cultured in low sugar Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% FBS and 1.25 mL/l gentamycin (Gibco). Images of the cells were obtained with an inverted phase contrast microscope (Olympus Optical Co., Ltd., Japan).

2.6. Coculturing the Microencapsulated Cells and Macrophages. The microencapsulated SGC7901 cells were cultured for 14 days and the macrophages were cultured for 10 days individually. By that time, the macrophages were resuspended at a cell density of 3×10^4 cells/L. At last, the microencapsulated SGC7901 cells and the macrophages were cocultured together in a 6-well plate for 72 h.

2.7. MTT Assays. We plated microencapsulated HGC cells onto a 96-well plate (50 μl cells and 200 μl medium per well) and cultured cells for 14 days. We added 50 μl of MTT (Jun Chuang Chemical Co., Shanghai, China) solution (filtered 5 g/L MTT solution in serum-free medium) per well, covered the cells with aluminum foil, and incubated the samples at 37°C and 5% CO_2 overnight. The MTT solution was removed

and 150 μl of DMSO (Baofeng Chemical Co., Tianjin, China) was added to each well, followed by gentle shaking to dissolve the purple crystals in the well for 10 min at room temperature. The absorbency at 540 nm and 630 nm was measured in a lab-system microplate reader (Labsystems Co., Ltd., Finland). Three wells were examined every 2 days to obtain an average reading.

2.8. Glucose and Lactic Acid Readings. We transferred 150 μl supernatant from three wells every 2 days from the 96-well plate, which contained 50 μl microencapsulated HGC cells and 200 μl medium per well. Then, we froze the samples at −20°C. The glucose and lactic acid in the supernatant were evaluated by a biosensor (Shandong Academy of Sciences).

2.9. BrdU Proliferation Assays. The microencapsulated cells were cultured on a slide in a 35 mm dish. Cells were labeled with 5-bromo-2-deoxyuridine (BrdU) (ZSGB-BIO, Beijing, China) (0.3 g/L) and incubated at 37°C for 30 min. The labeling medium was removed and cells were washed with 1x PBS three times, fixed with 70% alcohol, and then air-dried. Next, the following reagents were added in turn: (a) 1 mol/L HCl, cultured at 37°C for 30 min; (b) 0.1 mol/L sodium borate, pH 8.5, at room temperature for 30 min; (c) 0.2% Triton X-100, pH 7.4, at room temperature for 10 min; (d) 5% normal goat serum (ZSGB-BIO, Beijing, China) block for 30 min. Cells were incubated with primary antibody (ZSGB-BIO, Beijing, China) for BrdU (1 : 50) at 4°C overnight. The slides were washed with PBS three times and stained with biotin-labeled secondary antibody (ZSGB-BIO, Beijing, China) for 30 min, followed by processing using the SP immune-histochemical kit (ZSGB-BIO, Beijing, China) staining reagent for 30 min at room temperature. The slides were washed with PBS three times and mounted in SlowFade Antifade (Jun Chuang Chemical Co., Shanghai, China). Positive staining was determined as brown or yellow staining in the nucleus.

2.10. Immunohistochemical Staining. The cocultured microencapsulated cells were fixed in 4% paraformaldehyde and then were embedded in paraffin. Paraffin-embedded sections were cut into standard 5 μm sections, deparaffinized in xylene, and rehydrated through graded alcohol solutions. Endogenous peroxidase was inactivated by immersing the sections in 0.3% hydrogen peroxide for 30 min at room temperature. The samples were washed three times with PBS-Tween (5 min each) and then incubated in 0.01% mol/L pH 6.0 sodium citrate buffer for antigen retrieval (microwave, 400 W, 15 min). Samples were cooled to room temperature and then sections were incubated with primary antibodies against PCNA (ZSGB-BIO, Beijing, China) and VEGF (ZSGB-BIO, Beijing, China) overnight at 4°C. PBS was used as the primary antibody for control. The sections were stained with secondary antibody and the SP immune-histochemical kit (ZSGB-BIO) as described above. Finally, the sections were counterstained with hematoxylin, mounted, and observed with a microscope (Olympus Optical Co., Ltd., Japan). PCNA and VEGF were detected in monolayer cultured cells and microencapsulated cells using the same method.

IHC results were independently evaluated by two specialized pathologists. The expression of PCNA protein was mainly observed in the nuclei of tumor cells and VEGF protein was observed in the cytoplasm. In the groups of microencapsulated cells and cocultured microencapsulated cells, the PCNA and VEGF expression levels were estimated by the percentage of positive stained cells and the staining intensity (0, negative; 1, weak; 2, intermediate; 3, strong).

2.11. Gelatin Gel Zymography. The supernatant of the cocultured microencapsulated cells was centrifuged at 2000 rpm for 10 min; next, it was frozen in −80°C. The supernatant of microencapsulated cells was used as a control. The supernatant samples (20 μl) and sample buffer were mixed together and then were vortexed and allowed to settle for 10 min before loading into wells in the gel. The voltage was first set to 70 V until the protein was stacked appropriately and then increased to 125 V for 90 min. The gels were then washed in 30 min washes using 1% Triton in distilled water to remove any remaining sodium dodecyl sulfate in the gels, followed by rinsing in distilled water to remove the remaining Triton. The gels were placed in developing buffer for 30 min and then incubated in a water bath at 37°C for 18 h. The gels were developed the next day using Coomassie Blue R-250 for 30 min and then destained using a destaining solution. The bands were scanned using a gel scanner. Intensities were analyzed by ImageJ 1.46 program (NIH) [15].

2.12. Statistical Analysis. The Mann–Whitney test was used to identify differences in nonparametric variables for two independent groups using GraphPad 7.0 software. (A value of $P < 0.05$ was considered statistically significant.)

3. Results

3.1. Phenotypic Characterization and Activity of the Microencapsulated SGC7901 Cells. Phase contrast imaging of the microencapsulated SGC7901 cells is shown in Figure 1. Microcapsules displayed a consistent appearance of a sphere with diameter of 500~600 μm. The surface of the capsule wall was clearly smooth. The number of cells contained in each capsule was approximately 600–800. Microencapsulated SGC7901 cells were evenly distributed in the beads on the day of encapsulation and started aggregating into spheres upon culture for 48 h to 72 h (data not shown). Over the first 14 days of culture, we detected an increase in the diameter of spheres and cell concentrations (data not shown). The maximum diameter of the microencapsulated cells was 300 μm.

To evaluate the viability of cells in the spheres, we performed MTT assay (Figures 2(a) and 2(b)). MTT assay was an established method for monitoring cell viability based on mitochondrial activity. In our study, the MTT assay was proposed for the in situ quantification of the living cell density of microencapsulated SGC7901 cells. After 24 h of incubation with MTT, the bluish-violet crystals appeared on the outer layer of the cell spheres, indicating that the cells proliferated and aggregated to be a sphere in the early

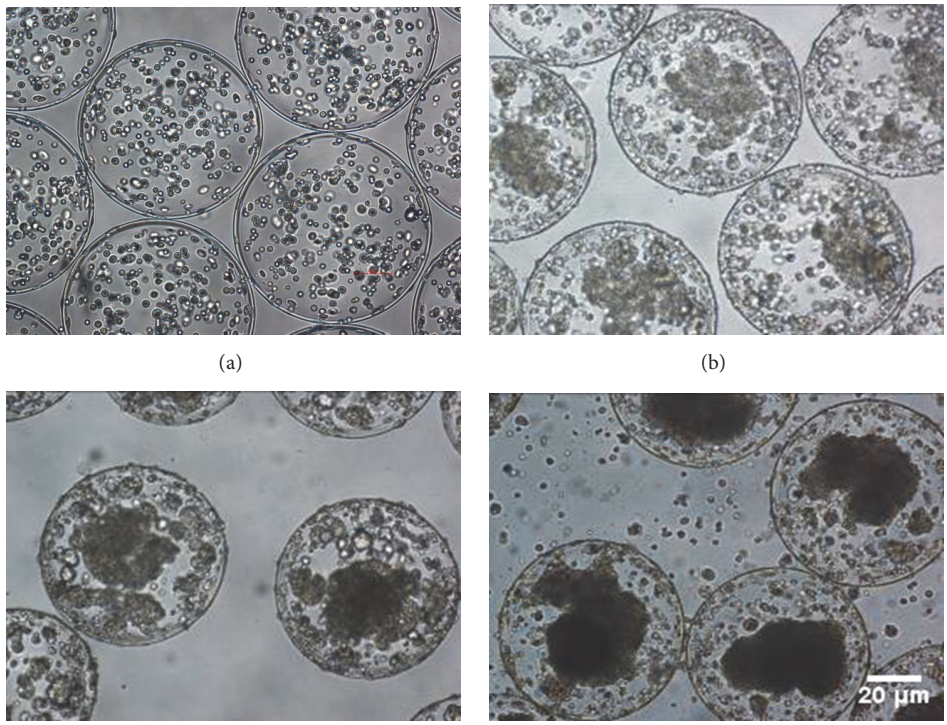


Figure 1: Phase contrast imaging of microencapsulated SGC9701 cells. (a) Microencapsulated cells on day 0 as control. (b) After culturing for 7 days, the cells tended to aggregate in spheres. (c) After culturing for 14 days, the spheres expanded rapidly and grew in three dimensions. (d) After culturing for 21 days, the sphere expansion slowed down and spheres showed necrosis in the center. Magnification: 200x.

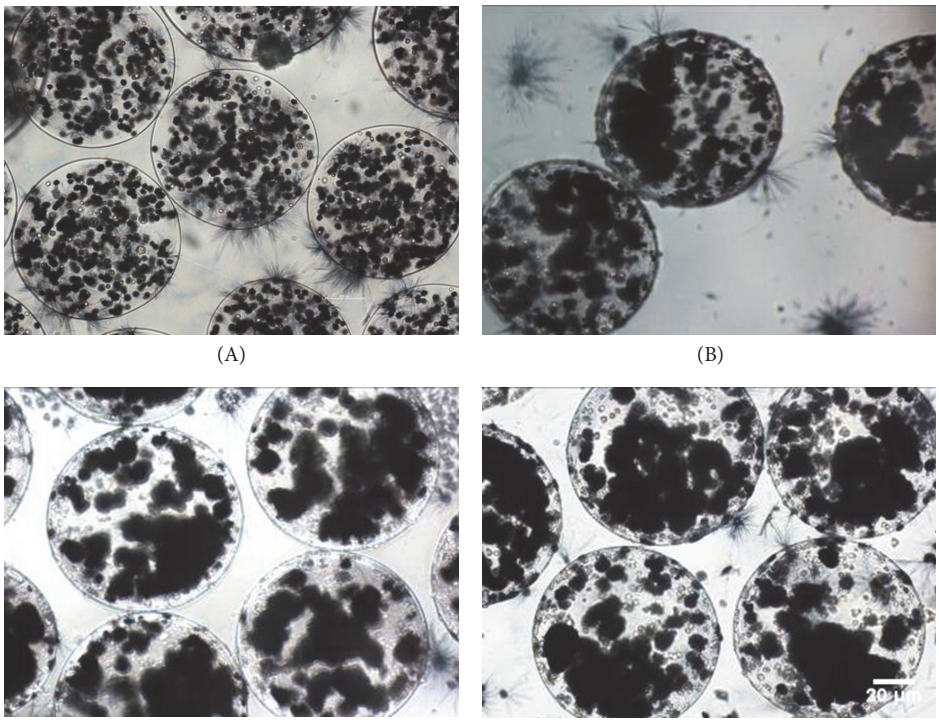
culturing time. We observed the maximum relative number of microencapsulated cells by day 14 of culture, and the cell number declined slightly over the following days (Figures 2(a) and 2(b)). These results show that the speed of cell death exceeded the rate of proliferation because of the limited nutrition and space in the late culturing time.

3.2. Metabolic Activity of the Microencapsulated Cells. To determine the metabolic activity of the cells in the spheres, we evaluated concentrations of glucose and lactic acid. We found that, over 21 days of culture of the cell spheres, these concentrations from the cultured microcapsules showed dynamic changes. By day 20, glucose levels decreased to 10 mmol/L compared with 110 mmol/L at day 1. In comparison, lactic acid increased from 40 mmol/L at day 1 to 100 mmol/L at day 20 (Figure 3). These data show that, with the increasing of the microencapsulated cells, the glucose was consumed while the acid was generated gradually. This suggested that the metabolic activity of the microencapsulated cells was the same as that of the tumor cells in the human body.

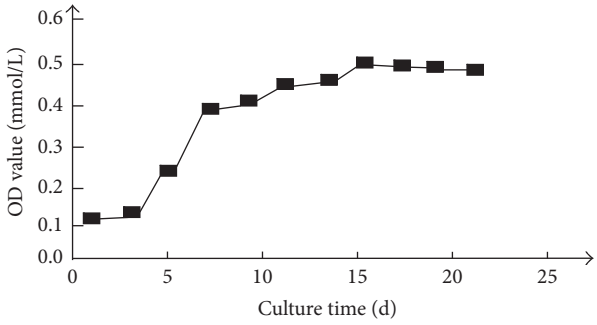
3.3. Proliferation of the Microencapsulated Cells. To evaluate proliferation rates of the microencapsulated cells, we performed BrdU staining analyses (Figure 4). The microencapsulated cells in the spheres showed positive BrdU staining at both day 7 and day 14. By day 21, the level of BrdU staining in some spheres was very low or even no staining in the center, while the cells outside still showed strong positive BrdU staining. Together, this indicates that the microencapsulated cells in the beans have normal proliferation cultured in the first 14 days. The proliferation rate of the cells decreased in the following days because of the nonaffluent nutrition and space in the capsule. Thus, cell death was more than cell proliferation especially in the center of the spheres.

3.4. Identification of Macrophages. CD68 was generally considered as a specific marker of macrophages, and therefore CD68 was used to identify TAMs in the macrophage culture system. Immunostaining for CD68 identified positive CD68-expressing cells as TAMs, which showed green fluorescence in the cytoplasm (Figure 5).

3.5. PCNA and VEGF Expression in Monolayer Cells, Microencapsulated Cells, and Microencapsulated Cells Cocultivated with Macrophages. As we all know, PCNA is considered as a specific marker for cell proliferation [16]. VEGF can induce the proliferation of vascular endothelial cells, promote the formation of new vessels, and increase vascular permeability. It contributes to metastasis and invasion of tumor cells. Previous clinical studies show that the higher the expression of VEGF in GC patients, the poorer the prognosis [17]. We next evaluated PCNA and VEGF expression in monolayer cells, microencapsulated cells, and microencapsulated cells cocultivated with macrophages. Both monolayer SGC9701 cells (Figures 6(a) and 7(a)) and microencapsulated SGC9701 cells showed expression of PCNA and VEGF. This result



(a) Crystal formation in MTT assays in microencapsulated SGC9701 cells. The bluish-violet crystals shown in the photos were the viable cells. (A) Microencapsulated cells on day 0 as control. (B) After culturing for 7 days, some viable cells were seen in the outer layer of the spheroids and increased obviously. (C) At 14 days of culture, there were the most viable cells throughout the culture. (D) At 21 days of culture, the viable cells slightly decreased compared to the culture on the 14th day. Magnification: 200x



(b) Growth curve of microencapsulated SGC9701 cells by MTT assays. The proliferation rate of viable cells increased rapidly over 7–14 days and increased slowly over the following days until reaching a plateau

Figure 2

suggests that the microencapsulated SGC9701 cells have the same potential on proliferation and protein expression as monolayer cells. In microencapsulated SGC9701 cells that were cultured for 7 or 14 days, the cell spheres were not large enough and still increased rapidly. Thus, the entire spheres showed expression of PCNA and VEGF (Figures 6(b), 6(c), 7(b), and 7(c)). On day 21, the cell spheres were large enough, and due to the limited nutrients of the microcapsules, with the proliferation of the microencapsulated SGC9701 cells, the center gradually developed hypoxia and necrosis. PCNA was only found at the outside of the microencapsulated cell spheres, but not in the center (Figure 6(d)), which was a similar trend to BrdU shown above. However, VEGF expression was detected throughout the spheres (Figure 7(d)). The number and density of the microencapsulated SGC7901 cells expressing PCNA or VEGF were increased when SGC7901 cells were cocultured with macrophages (Figures 6(e) and 7(e)). The average values of PCNA and VEGF expression in microencapsulated groups were 52%, 40%, 78%, and 80% in the cocultured group ($P < 0.05$). Meanwhile, the semiquantitative expressions of PCNA and VEGF were significantly different between microencapsulated culture and coculture with macrophages based on staining intensity ($P < 0.05$). Together, these results show that the expression of PCNA and

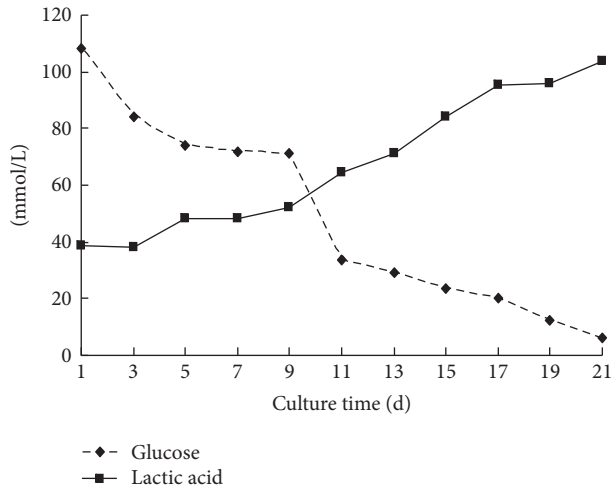
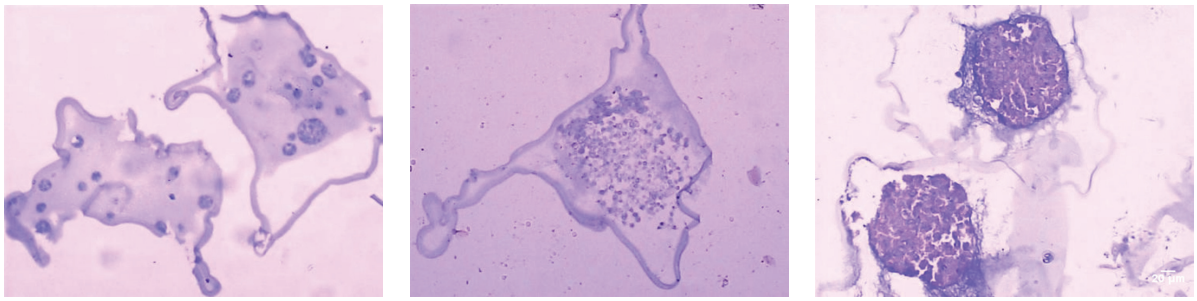


Figure 3: Concentration-time curve of glucose and lactic acid detected in the supernatant of microencapsulated SGC9701 cells.



(a) (b) (c)

Figure 4: Proliferation of microencapsulated SGC9701 cells by BrdU staining. Brown or yellow nuclear staining indicates live cells. After culturing for (a) 7 days or (b) 14 days, the cells tended to aggregate into spheres. The entire microencapsulated spheres showed positive BrdU staining. (c) After 21 days, the staining color faded or the center of the cells showed no staining while the cells outside still showed staining.

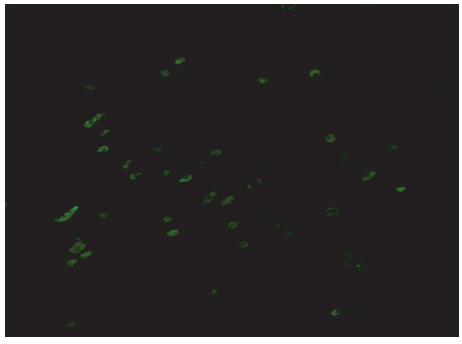


Figure 5: Identification of macrophages by CD68 immunofluorescence. Macrophages were differentiated from peripheral blood mononuclear cells and identified by CD68 immunofluorescence staining. Magnification: 100x.

VEGF in the microencapsulated cells is consistent with that in the monolayer cells. TAMs can promote PCNA and VEGF expression of the microencapsulated SGC9701 cells.

3.6. MMP-2 and MMP-9 in Microencapsulated Cells Cocultured with Macrophages. When the macrophages were induced into the tumor microenvironment, MMPs would be produced. MMPs play important roles in the responses of cells to their microenvironment, by effecting proteolytic degradation or activation of cell surface and extracellular matrix (ECM) proteins, which facilitate tumor cells proliferation, differentiation, migration, and survival [18].

Therefore, we next evaluated the levels of MMP-2 and MMP-9 in cells (Figure 8). Expression of MMP-2 and MMP-9 was not found within the supernatant of microencapsulated SGC7901 cells or macrophages cultured alone. However, MMP-2 and MMP-9 were detected in the supernatant of microencapsulated SGC7901 cells cocultured with macrophages. These data indicate that TAMs can promote the expression of MMP-2 and MMP-9 in microencapsulated SGC7901 cells because of the cross talk in the TME.

4. Discussion

Metastasis and drug resistance are the leading causes of death in GC patients. Currently, basic experimental studies are mostly dependent on GC cell two-dimensional culture *in vitro* and xenotransplantation *in vivo*. Both of these methods have advantages and disadvantages in research. For example,

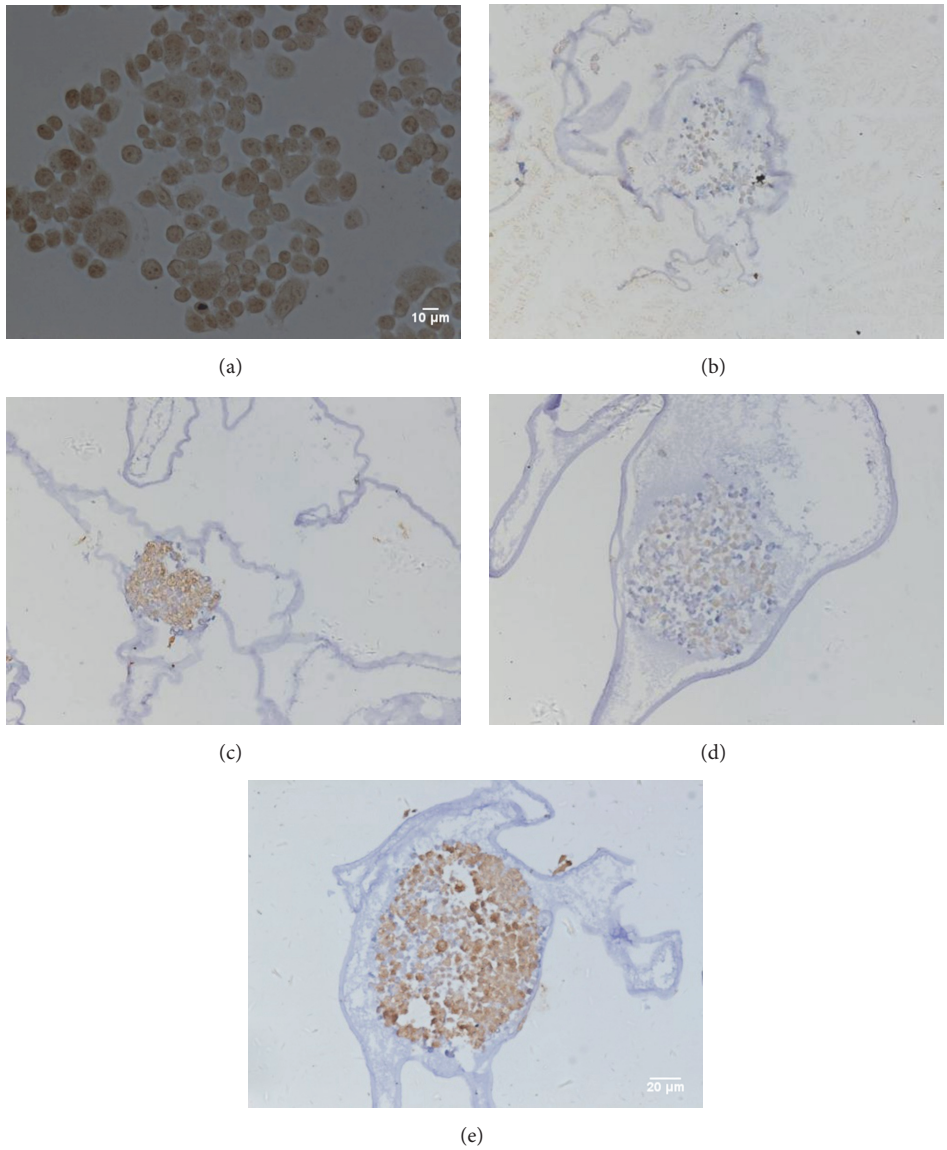
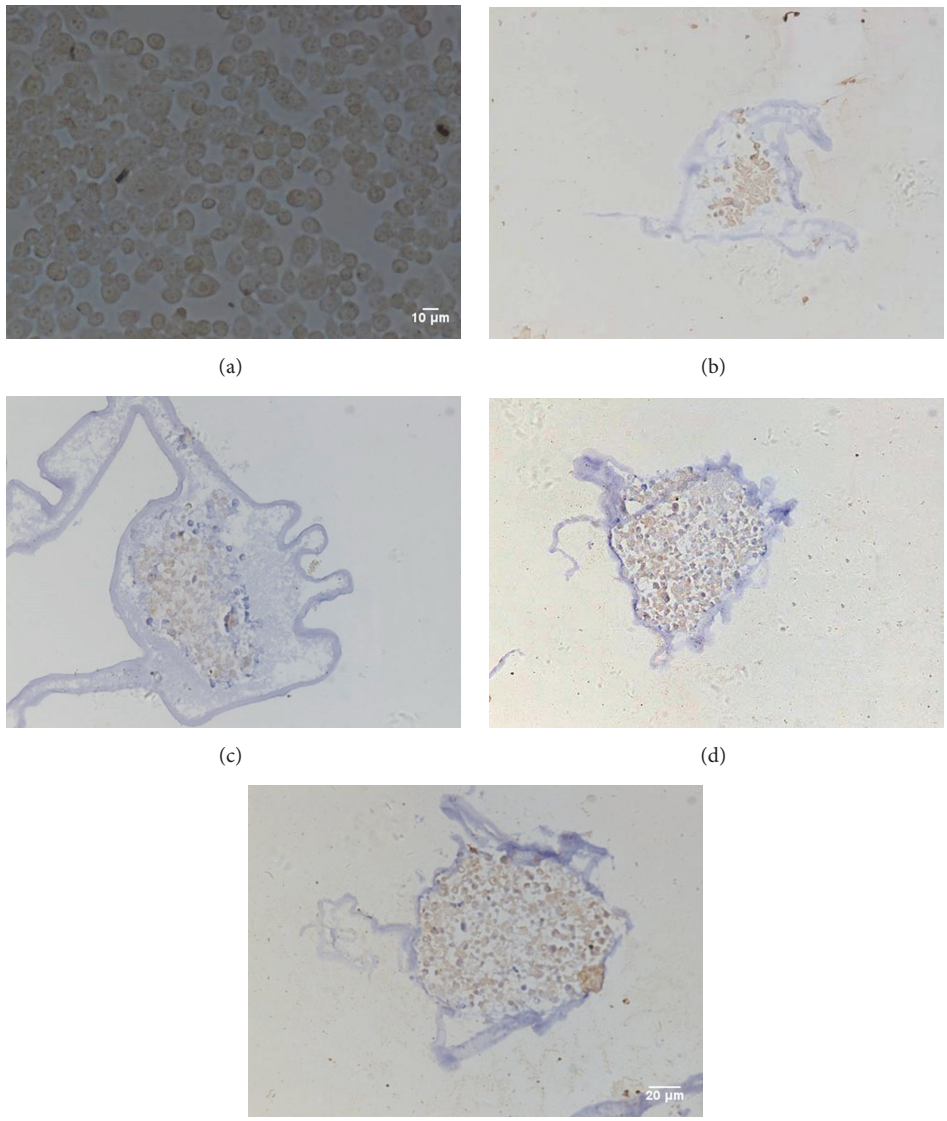


Figure 6: Expression of PCNA in the cells and spheres by H&E staining. Brown nuclei indicated positive PCNA staining. (a) Monolayer SGC9701 cells showed positive PCNA expression. (b, c) The microencapsulated cell spheres cultured for 7 days and 14 days: PCNA expression was observed throughout the entire spheres. (d) The microencapsulated cell spheres cultured for 21 days: PCNA expression was detected outside the spheres, but not in the center. (e) The microencapsulated cell spheres cocultured with macrophages for 3 days: the number and density of the spheres expressing PCNA were increased. Magnification: 200x.

tumor cells in animal xenograft models can grow in 3D conditions and also exist within a specific microenvironment that is similar to that observed in humans. However, animal experiments are relatively expensive, delicate, and highly susceptible to infection, and breeding and experimental conditions are strict. In contrast, although monolayer cells are more cost-effective, these cultured cells do not establish the special TME. Hence, there is an obvious gap between cell culture experiments and clinical application. Therefore, in our study, we aimed to establish a transitional culture model between the *in vivo* animal model and *in vitro* monolayer cell culture.

Here, we established a microencapsulated human GC cell model using the APA technique. The microencapsulated cells would be efficiently protected by the outer layer of the microcapsule and could undergo interaction with TMEs in the process of growth and activity. Moreover, prior studies have confirmed that microencapsulated tumor cells are more viable than monolayer cells, and the stability of tumor-associated genes is not affected [19]. In our study, the microencapsulated GC cells could grow in clusters within 48–72 h to form spheres. The maximal diameter of the spheres increased to 300 μm when culturing for 14 days and stopped increasing in the following culturing days. The cells in the microencapsulated spheres also showed a strong proliferation activity, and the metabolic curves of glucose and lactic acid were consistent with cell proliferation. Furthermore, in our study, the expression of PCNA and VEGF was detected both



(a) (b) (c) (d) (e)

Figure 7: Expression of VEGF in the cells and spheres by H&E staining. Brown nuclei indicated positive VEGF staining. (a) Monolayer SGC9701 cells showed positive VEGF expression. (b, c, d) The microencapsulated cell spheres cultured for 7, 14, and 21 days: VEGF expression was observed throughout the entire spheres. (e) The microencapsulated cell spheres cocultured with macrophages for 3 days: the number and density of the spheres expressing VEGF were increased. Magnification: 200x.

in the monolayer cell culture and in the microencapsulated cell culture. PCNA and VEGF are related to cell proliferation, metastasis, and invasion of the tumor cells [16, 17]. According to a previously published paper, the microencapsulated MCF-7 cell model was stably expressed with PCNA and VEGF in a single cell 3D culture. Both of these proteins were demonstrated as potential specific markers for the evaluation of basic biological functions in both the monolayer cell culture and the 3D microenvironment [20]. Therefore, we propose that the basic biological function of the tumor cells was still preserved after establishment of the microcapsules and the microencapsulated SGC9701 cell model. In further studies, we will detect more proteins to prove this.

The tumor is not composed only of the cancer cells. Instead, it is composed of heterogeneous tumor cells, endothelial cells, immune cells, smooth muscle cells, fibroblasts, and macrophages [21, 22]. There is also a dynamic and mutualist relationship between tumor cells and the surrounding stroma [23]. Moreover, tumor cells can secrete several factors that modify the surrounding stroma. The extracellular matrix is responsible not only for the structural support of the cells but also for storing important signaling molecules, such as chemokines [24]. This forms a special TME that can determine the malignant phenotype of tumor cells and promote tumor metastasis [25, 26].

Macrophages are differentiated from mononuclear cells which are the principal members of inflammatory cells in tumor stroma. These cells can be stimulated into TAMs under a low oxygen environment or by the chemokines and growth factors secreted by tumor tissues. They have an extremely

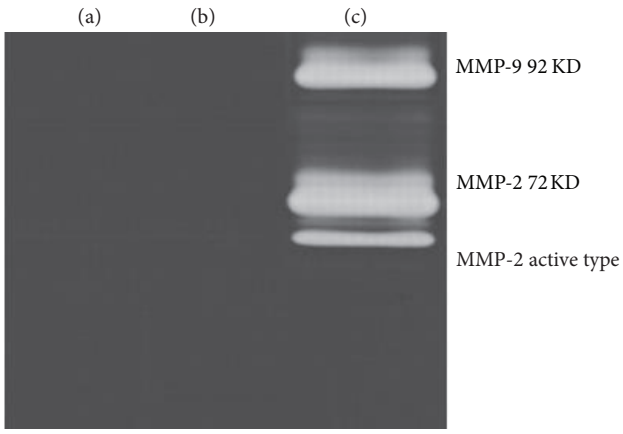


Figure 8: MMP-2 and MMP-9 expressions were detected by gelatin gel zymography. MMP-2 and MMP-9 expression in microencapsulated SGC7901 cells (a), macrophages (b), and microencapsulated SGC7901 cells cocultured with macrophages (c).

important action for tumor progression [27]. MMPs, a family of proteases secreted from myofibroblasts and tumor cells, are important for remodeling of the matrix and aid in the migration and invasion of tumor cells [26, 28].

In our study, the microencapsulated SGC7901 cells model can provide a TME between the tumor cells and the matrix due to the special microcapsule. When the microencapsulated SGC7901 cells were cocultivated with TAMs, we detected the expressions of MMP-2 and MMP-9 but not in microencapsulated SGC7901 cells cultured alone. In addition, an increased level of PCNA and VEGF was observed in the cocultured group compared with microencapsulated SGC7901 cells cultured alone. Thus, we presume that TAMs and microencapsulated cells that grow in three dimensions interact with each other to form a special microenvironment, which may be similar to the TME observed in the human body. We speculated that TAMs could facilitate the invasion and metastasis activity of GCs in the spheres by promoting the expression of PCNA and VEGF.

In conclusion, here we established a transitional *in vitro* 3D culture model, between *in vivo* animal models and *in vitro* single cell culture. The cells in this model showed normal growth and metabolism and stable protein expression. This microencapsulated SGC7901 cell model could form a TME which is similar to human while the monolayer cell culture could not. This model might provide a relative stable research platform for further study of the interaction between human gastric cancer cells and the host microenvironment.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Review Article

The Effect of Anesthesia on the Immune System in Colorectal Cancer Patients

Yangjie Dang,[1] Xingxing Shi,[1] William Xu,[2] and Mingzhang Zuo[3]

[1]*Department of Anesthesiology, Xi'an Children's Hospital, Xi'an, Shaanxi 710032, China*
[2]*Department of Anesthesiology, Carver College of Medicine, University of Iowa, Iowa City, IA 52246, USA*
[3]*Department of Anesthesiology, Beijing Hospital, National Center of Gerontology, No. 1 Dahua Road, Dongdan, Beijing 100730, China*

Correspondence should be addressed to William Xu; William-xu@uiowa.edu and Mingzhang Zuo; zuomz@163.com

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Colorectal cancer (CRC) is the key leading cause of high morbidity and mortality worldwide. Surgical excision is the most effective treatment for CRC. However, stress caused by surgery response can destroy the body's immunity and increase the likelihood of cancer dissemination and metastasis. Anesthesia is an effective way to control the stress response, and recent basic and clinical research has shown that anesthesia and related drugs can directly or indirectly affect the immune system of colorectal cancer patients during the perioperative period. Thus, these drugs may affect the prognosis of CRC surgery patients. This review is intended to summarize currently available data regarding the effects of anesthetics and related drugs on perioperative immune function and postoperative recurrence and metastasis in CRC patients. Determining the most suitable anesthesia for patients with CRC is of utmost importance.

1. Introduction

Colorectal cancer (CRC) is the third leading cause of death from cancer worldwide, accounting for approximately 135,430 new cases and 50,260 deaths in the United States in 2017 [1]. The interactions of the colon epithelium microbiome are considered to be important for the formation of colon cancer, and *Enterococcus faecalis* is thought to play an important role in the pathogenesis of CRC [2].

Anesthesia and related drugs can directly or indirectly affect the immune system of patients during the perioperative period and thus affect treatment and prognosis of CRC patients as surgery is currently the most effective treatment for CRC. The 2009 European Society of Anesthesiology (ESA) presented the new concept of "anticancer anesthesia technology" with the intention of identifying the most suitable anesthesia for patients with cancer. Exploring the effects of various anesthetic methods and their related drugs on the immune system of CDC patients continues to be of great significance.

2. Colorectal Cancer and Its Immunological Bases

Tumor generation requires the provision of nutrition in the surrounding microenvironment. Tumors grow and invade and infiltrate surrounding tissues and organs. The tumor microenvironment includes a large number of cells, including immune cells, endothelial cells, and interstitial cells, all of which are involved in the occurrence and development of tumors. Studies have shown that the immunological infiltration of CRC may be clinically related to those cells.

Immune response in the process of tumor development is not just a single factor, but it plays a multifaceted role affecting tumor initiation, growth, progression, and other processes. The immune system regulates and promotes cancer programs, a process known as "immunoediting." There are three phases to this process: elimination, balance, and escape [3].

Although experimental evidence shows that inflammation can also promote the occurrence and development of

tumors [2], the immune inflammatory response in colon carcinogenesis requires further study and is still under debate [4]. Some clinical data show that the immune response inhibits the tumor. However, other investigators have concluded that the opposite is true.

2.1. Immune Cells in Colorectal Cancer. Different immune cells play differing roles in the process of cancer: some cells affect its pathogenesis while others contribute to its recurrence.

Macrophages. Macrophages in CRC will appear as infiltration. Known as tumor-infiltrating macrophages, they are important contributors to tumors infiltrating the surrounding tissue. They are derived from peripheral blood mononuclear cells. According to the different functions and characteristics of tumor-infiltrating macrophages, they can be divided into two different subtypes: M1 and M2 [5]. Some cytokines, such as LPS and TNF-α, promote the conversion of classic activated macrophages to the M1 type. The remaining types are classified as M2. M1 is involved in the TH1-type immune response that can kill foreign pathogens and endogenous tumor cells. Glucocorticoids and IL-10 can induce the transformation of tumor-infiltrating macrophages to M2, which mainly causes TH2-type immune response and promotes the occurrence and development of tumors. Both types are involved in the immune response of colon cancer and can be transformed into each other under certain circumstances.

In short, both M1 and M2 participate in the occurrence and outcome of the tumor, and the conversion between the two determines the outcome and prognosis of tumors [6, 7]. Some studies have shown that macrophage infiltration in CRC seems to prefigure a better prognosis [8], since macrophage infiltration may be an important means of fighting against cancer via the regulation of endogenous mechanisms.

NK Cells. Natural killer (NK) cells are immune cells that maintain the defense function of the body. They are involved in antitumor and antiviral infection and immune regulation processes. Both murine [9, 10] and human [11, 12] models have shown that NK cells contribute to preventing tumor and controlling the effects of tumor growth and dissemination. In CRC, it has been reported that extensive infiltration of NK cells in a wide range of tumors can give rise to a better prognosis [13]. Although these studies suggest that NK cells play a beneficial role in tumor control, their specific mechanism of action remains unclear [14]. Therefore, as an important effector cell in innate immunity, NK cells have significant antitumor function and have the potential for positive applications and clinical significance in tumor immunotherapy.

T-Regulatory Cells (Tregs). Tregs, CD25 + regulatory T-cells, can not only prevent human autoimmune diseases, but also help protect against microbial infection and protect the fetus. There are indications that Tregs can indeed prevent human autoimmune diseases [15]. On the one hand, Tregs may control excessive immune response, but on the other hand, such control may also weaken the immune system's effectiveness in eliminating invaders. The most direct application for Tregs is in treating autoimmune diseases by increasing their activity. In addition, reducing the number of Tregs can also reduce tumor-associated immune responses. There is evidence that immune cells constantly monitor the molecular abnormalities that occur when cells become cancerous. To some extent, Tregs interfere with this monitoring and may help to root and grow malignant tumors [16]. On the basis of these studies, it was also found that tumor-associated T-cell-related immune responses are detected in tumor patients [17, 18]. Numerous studies have shown that T-cells infiltrate tumor tissue in patients who are less immunosuppressed. This is considered to be a good prognostic marker [19, 20]. Therefore, modulating the immune response of tumor patients can be an important way to improve the prognosis of patients, but further studies are needed.

2.2. Immune System and Colorectal Cancer. The innate immune response is a nonspecific immune defense already available at birth. This inherent immune response has a wide range of effects, including speed, efficiency, and stability. Innate immunity provides a nonselective rejection to a foreign body's entry into the body of antigens, a protective clearance function. The innate immune system is activated through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) which, in turn, produce a series of cytokines such as interleukins (ILs) and tumor necrosis factor (TNF). The innate immune response initiated by PRRs is important in the shaping of tumor immune microenvironment and tumorigenesis. PRRs are widely expressed in various innate immune cells of the host, such as dendritic cells and mononuclear macrophage NK cells, after which a series of immune responses are initiated. Among various PRRs, Toll-like receptors (TLRs) have attracted much attention and are involved in tumorigenesis through the recognition of the inflammation caused by PAMP. Among these receptors, the inflammasomes formed by the involvement of Nod-like receptors (NLRs) have also received much recent attention, as they have been shown to regulate the immune response and thus to inhibit tumorigenesis [21, 22].

Rakoff-Nahoum et al. [23] studied the dextran sulfate sodium- (DSS-) induced susceptibility to colitis and receptor-interacting protein 2 (RIP2) in TLR4, TLR2, and myeloid differentiation factor (MyD88) knockout mice in 2004. Compared with wild-type (WT) mice, they demonstrated that the MyD88-dependent axis is beneficial. MyD88 is a key linker molecule in the TLR-signaling pathway and plays an important role in the transmission of upstream information and disease development. It is therefore an important mediator of many molecular cascade reactions.

In addition, interleukin-17 (IL-17) is an early promoter of T-cell-induced inflammatory responses and can amplify the inflammatory response by promoting the release of proinflammatory cytokines. The main effector of T-helper interleukin-17-producing cells (Th17 cells) is IL-17. Th17 cells are capable of secreting IL-6 and tumor necrosis factor-α (TNF-α), among others. Subsequently, one study [24] has

demonstrated that IL-17 plays a role in the initiation of tumors in IL-17 knockout mice.

At present, the main treatment for CRC is surgery. Therefore, anesthetic management plays a vital role in the prognosis of CRC in the perioperative period.

3. The Effect of Anesthesia on the Immune System in Colorectal Cancer Patients

The immune system mainly includes two subsystems: nonspecific immunity and specific immunity. The effect of anesthesia on the immunization of colorectal cancer patients mainly includes the following. (1) Anesthesia affects the number and activity of immune cells: presently, the effects of anesthesia on immunity have been studied on NK cells, B-lymphocytes, T-lymphocytes, macrophages, leukocytes, and erythrocytes. These cells are involved in both nonspecific and specific immune functions. (2) Anesthesia affects the secretion of cytokines. Proinflammatory factors mainly include tumor necrosis factor-a (TNF-α), IL-1, IL-6, and IL-8. The anti-inflammatory factors mainly include IL-10. The levels of interferon-7 (IFN-7), interferon (IFN), TNF-α, and soluble interleukin-2 receptor (sIL-2R) are increased by general anesthesia. (3) Anesthesia affects the biological behavior of tumor cells. The proliferation, migration, and apoptosis are important biological characteristics of tumor cells, which are closely related to tumor growth and metastasis.

3.1. The Effect of Different Anesthesia Methods on Immunization in Colorectal Cancer Patients. A number of scholars have found that, compared to simple general anesthesia, general anesthesia combined with epidural anesthesia or simple spinal anesthesia can reduce the postoperative immunosuppression to varying degrees [25]. Studies on the effects of different anesthesia methods on the long-term survival rate of patients after colon cancer resection suggested that the combined application of epidurals can improve the survival rate of patients without metastasis to 1.46 years postoperatively, but there was no significant difference between the two groups for patients with metastasized tumors [26]. These studies have shown that, compared to general anesthesia, epidural anesthesia, paravertebral block, and other types of regional anesthesia can reduce the inhibition of immune function and improve the prognosis of patients with cancer.

Surgery can cause sympathetic adrenal medulla and hypothalamus-pituitary-adrenal cortical axis excitement based stress response by a variety of factors (pain, blood loss, low temperature, and psychological factors). Many factors produced in the stress response, such as glucocorticoids, catecholamines, and prostaglandin E2 (PGE2), can have an adverse effect on immune function, resulting in immune function inhibition in the perioperative period. Studies have shown that the inhibition of immune function by stress response is caused by inhibition of natural killer cell (NK cell) function. These cells are nonspecific components of cellular immunity. Their effect is not tumor-specific and the major histocompatibility antigen system (MHC) restriction plays an important role in early antitumor immunity. The effect of the stress response on the duration of NK cell activity inhibition is similar to the period of enhanced tumor metastasis [27]. The effect of the stress response on tumor metastasis is achieved by inhibiting the activity of NK cells. Epidural anesthesia can block the pain-conduction pathways, non-pain-conduction pathways, and sympathetic nerve fibers by blocking the spinal nerve root. However, opioid drugs commonly used in general anesthesia only block the pain-conduction pathway [28], indicating that epidural anesthesia can preserve NK cell function. Compared to general anesthesia, epidural anesthesia can better prevent nociceptive irritation transmission to the central nervous system and reduce the body's stress response, which may be beneficial for the prognosis of patients with cancer.

CD4+ T-cells first differentiate into T-helper 0 (TH0) cells after antigen stimulation and continue to differentiate into TH1 or TH2 cells. TH1 or TH2 subgroups secrete different cytokines: TH1 cells mainly secrete interferon-1 (IFN-1), which promotes cellular immunity, while TH2 cells secrete interleukin-4 (IL-4) and are associated with humoral immunity. These cytokines can also promote their own growth and inhibit the growth of other subpopulations. The proportion of IFN-γ IL-4 *in vivo* determines the ratio of TH1/TH2 which, in turn, determines the dominant *in vivo* immune response. Cellular immunity is important in controlling the immune response of the tumor, and thus a reduced TH1/TH2 ratio should be avoided in order to maintain the cell's immune response. Epidural anesthesia, compared to general anesthesia, is able to better preserve the TH1/TH2 ratio and thus can better preserve cellular immune function. Mrakovcic-Sutic et al. found that, in rectal cancer patients with postoperative analgesia, there was a lower inhibitory effect on NK cells than in patients in an intravenous analgesia group [29]. Animal experiments suggested that epidural anesthesia can increase the survival rate of colorectal cancer patients after surgery. The number of lymphocytes, NK cell activity, TH1/TH2 ratio, and tumor metastasis were significantly higher than those of general anesthesia group after spinal anesthesia, indicating that specific regional anesthesia could preserve the function of immune cells. Therefore, general anesthesia combined with epidural anesthesia or simple spinal anesthesia is better than simple general anesthesia in reducing surgery-related immune inhibition by reducing the stress response.

The protective mechanism of local anesthesia technology on immune function is not yet clear; however, there are three possible causes: (1) local anesthesia technology, by blocking the neurotransmitter incoming to the central nervous system, can significantly reduce the pain and surgical stress response; (2) the application of local anesthesia technology can reduce the dosage of opioids; and (3) general anesthesia combined with local anesthesia techniques can reduce the total use of general anesthetic drugs. Epidural anesthesia combined with general anesthesia can reduce the amount of general anesthetics and analgesics, and sustained epidural analgesia can also further reduce the use of opioids after surgery, which may be one of the reasons for reducing the immunosuppressive effects.

However, some scholars still believe that local anesthesia technology cannot protect the immune function and reduce

tumor recurrence and metastasis. Gottschalk et al. analyzed the data of 669 colorectal cancer patients undergoing radical surgery and found that the application of epidural analgesia during the perioperative period and a reduced recurrence rate of tumor after surgery are not necessarily linked [30]. The meta-analysis of Conrick-Martin et al. suggests that there was no significant difference between general anesthesia and spinal anesthesia on NK cell function after surgery [31]. The existing clinical research sample size is small, and retrospective studies have inherent bias and other deficiencies. In the future, more multidimensional, prospective, randomized clinical studies will be needed to further explore the linkages and mechanisms between anesthetics and the prognosis of malignant tumors in order to provide safer anesthesia for patients with cancer. For future study, retrospective studies will also require forward-looking large samples.

3.2. The Impact of Anesthesia-Related Drugs on Immunization in Colorectal Cancer Patients

3.2.1. Intravenous Anesthetics. Intravenous anesthesia can maintain a safe, constant concentration of drug treatment during surgery and can reduce the stimulation of surgical trauma. Intravenous anesthesia can also reduce the intraoperative inflammatory response albeit affecting the patient's immune system function. Immune system disorders or inhibition during the perioperative period can cause postoperative complications, especially in cancer patients. Immunosuppression after surgery can accelerate the spread of residual cancer cells and promote a new transfer.

Common opinion is that propofol does not inhibit the immune function. Inada et al. [32] found that propofol produces an antitumor effect by inhibiting cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2). Propofol is superior to inhaled anesthetics in postoperative immunoprotection. Propofol can increase the activity of NK cells, inhibit COX-2, prevent PGE2 generation, and protect the immune system function. It can also have a direct biological effect on the tumor. A series of cell culture experiments show that propofol at concentrations commonly used does not inhibit phytohemagglutinin- (PHA-) induced lymphocyte proliferation and does not significantly increase lymphocyte apoptosis. Propofol inhibits immune cells only at concentrations well above clinical concentrations [33–36].

Ketamine, thiophanate, and etomidate increase the chance of tumor retention and metastasis because of the reduced NK cell activity, reduced T-helper cells, and increased T-inhibiting cell viability. Ketamine inhibits NK cell activity due to the activation of alpha and beta adrenergic receptors [37].

Benzodiazepines are one of the most commonly used sedatives in intensive care patients [38]. Midazolam can inhibit the production of IL-2 and IL-8 to produce an immunosuppression effect [39]. Midazolam can inhibit the transcription activity of lipopolysaccharide-induced nuclear factor KB, the activity of TNF-α, the phosphorylation of p38 mitogen kinase, and the formation of peroxides, which can be blocked by peripheral benzodiazepine agonist PKII 195. Midazolam can inhibit neutrophil adhesion and tropism by inhibiting the level of IL-8, thereby reducing immune function [40]. Rapid administration of diazepam will produce a proinflammatory response and improve neutrophil function, but slow and sustained (60 days or longer) administration will inhibit the multinuclear leukocyte function and lead to immune response inhibition [41]. These results show that benzodiazepines have a significant inhibitory effect on innate immunity, but more research is needed on adaptive immunity.

The α2-agonists clonidine and dexmedetomidine can significantly increase tumor cell growth, but the α2-receptor antagonist yohimbine can reverse this effect [42].

3.2.2. Inhaled Anesthetics. Inhaled anesthetics include sevoflurane, halothane, isoflurane, and desflurane. The inhibitory effect of inhaled anesthetics on the immune function has been confirmed by many studies. Guptill et al. found that intravenous anesthesia with propofol and remifentanil is less immunosuppressive than inhaled anesthesia [43]. Inhaled anesthetics have greater immune cell inhibition than intravenous anesthetic drugs. Inhaled anesthetics can produce higher levels of plasma catecholamines and glucocorticoids to inhibit the release of IFN of NK cells in animal models and reduce the number of NK cells, the production of Th cytokines, and the expression of $Foxp_3$ mRNA in the human body [44].

Some studies have shown that halothane inhibits NK cell activity in a dose-dependent manner. *In vitro* experiments show that an increase in halothane concentration is accompanied with a significant reduction of NK cell activity. With an increase in halothane concentration, NK cell activity was significantly reduced in *in vitro* experiments [45].

Other studies have reported that sevoflurane can reduce the invasive ability of colorectal cancer [46].

Fleischmann et al. conducted a follow-up study with 204 colorectal cancer surgery patients and found that the use of nitrous oxide (N_2O) anesthesia does not increase the risk of recurrence of postoperative rectal cancer. However, the mechanism by which inhaled anesthetics affect the immune system remains unknown [47].

3.2.3. Opioid Analgesics. Acute pain is the main cause of the activation of the hypothalamic-pituitary-adrenal axis (HPA axis), the activation of which can cause immunosuppression, decreased NK cell activity, and Th cell imbalance. Opioid is the main analgesic in anesthesia, and now the study found that opioids and tumors have a complex association. The mechanism is unknown and even, to some extent, contradictory.

Opioid itself can directly affect the proliferation, apoptosis, and survival of normal cells and tumor cells. Opioid analgesics have the effect of inhibiting cell and humoral immunity. In isolated experiments, morphine, a commonly used opioid, can inhibit the formation of peroxides and the expression of cytokines. Morphine has also been found to inhibit the function of macrophages and NK cell activity [48].

Morphine regulates tumor cell invasion throughout adhesion, extracellular matrix degradation, and cell migration. An opioid represented by morphine can regulate angiogenesis,

promote endothelial cell proliferation and migration, promote angiogenesis, increase vascular permeability, and promote the proliferation of tumor cells in the blood vessels [49].

Another commonly used opioid, fentanyl, exhibited dose-dependent cytotoxicity against NK cells. Forget et al. found that fentanyl was able to inhibit the activity of NK cells in mice [48]. The effect of different doses of fentanyl on human immune function was observed. It was found that the activity of NK cells was reduced in patients with both low and high doses of fentanyl at 24 hours after operation. However, the activity of NK cells in the low-dose group returned to the preoperative level after 48 hours, while NK cell activity in the high-dose group remained low, indicating that the inhibitory effect of fentanyl on NK cells was time- and dose-dependent [49]. However, some studies have suggested that opioids can enhance the immune function by enhancing NK cell activity and the T-cell-mediated immune response. One possible mechanism involves morphine, which can activate the μ receptor and inhibit the release of NF-KB and NO [50, 51].

Sufentanil is a potent opioid analgesic, but it is unclear if it negatively affects the immune function.

Tramadol is a class of weak opioid receptor agonists that play an important role in the current clinical "multimodal analgesia" concept. They exhibit protective effects on cellular immunity in some studies.

3.2.4. Nonsteroidal Anti-Inflammatory Analgesics. Acute and chronic inflammation significantly increases the expression of cyclooxygenase (COX), causing increased prostaglandins, pain, and tumor metastasis rate. Nonsteroidal anti-inflammatory drugs (NSAIDs) have antipyretic, analgesic, anti-inflammatory, and antirheumatic effects. They work by reducing the biosynthesis of prostaglandin (PG) in the local tissue by inhibiting the activity of COX *in vivo*. Because of the strong analgesic effect of nonsteroidal anti-inflammatory drugs, researchers have been concerned in recent years about whether NSAIDs provide immune protection during the perioperative period and about whether or not they relieve the recurrence of tumor metastasis. NSAIDs can improve the immune function of patients and inhibit the recurrence of tumor metastasis. It has been suggested that NSAIDs can be used to reduce pain, reduce opioid dosage, and balance side effects of opioids in patients with radical neoplasm surgery. Animal models suggest that COX inhibitors can prevent tumor growth and metastasis by inhibiting apoptosis and reducing angiogenetic factors and tumor microvessel density. Epidemiological studies have shown that prolonged use of COX inhibitors can reduce the risk of cancer. The use of selective COX inhibitor celecoxib daily can reduce the risk of colorectal cancer by 69% [52]. While encouraging, this conclusion requires further study. In addition to inhibiting the synthesis of PG, NSAIDs also inhibit the release of bradykinin during inflammation, alter lymphocyte responses, and reduce the migration and phagocytosis of granulocytes and monocytes to regulate the immune response [53]. NSAIDs can inhibit the immunosuppression caused by trauma, pain, anesthesia, opioid use, surgery, and so forth to varying degrees [54–56].

We could use COX inhibitors to inhibit PG synthesis in order to slow down tumor progression. Anesthesia and analgesia are associated with the entire perioperative period, although they cannot resolve the issues of tumor spread and tumor residue. However, reducing NK cell activity inhibition and maintaining Th cell balance can improve the immunosuppression caused by surgical stress induced by some anesthesia intervention.

3.2.5. β-Receptor Blockers. The use of beta-blockers can reduce the risk of tumor metastasis by inhibiting the release of catecholamines and the activity of signal transducer and activator of transcription-3 (STAT-3), resulting in reduced NK cell activity inhibition and angiogenesis [57].

3.2.6. Local Anesthetics. Most studies suggest that local anesthetics have antitumor effects and are suitable for anesthesia in patients with cancer. Martinsson found that the clinical concentration of ropivacaine can inhibit colon cancer cell proliferation in dose-dependent *in vitro* experiments [58]. Lucchinetti et al. found that bupivacaine can inhibit mesenchymal stem cell proliferation and negatively regulate tumor formation, metastasis, and cell differentiation [59].

3.3. The Effects of Other Anesthesia Interventions

3.3.1. Heat Preservation. As a stress response, hypothermia activates the sympathetic system and increases glucocorticoid release. A mild temperature (35.5°C) has some effect on cellular immunity, but a moderately low temperature (30°C) will directly inhibit NK cell activity and reduce resistance to tumor metastasis [60]. Therefore, the use of a warm blanket, hot air, infusion of liquid heating, and other measures ensures that patients maintain the appropriate body temperature perioperatively, which can offer protection for the immune function in cancer patients after surgery.

3.3.2. Blood Transfusion. While blood transfusion is an important tool for anesthesiologists during and after operation, perioperative blood use may influence cancer recurrence depending on the patient's nutritional health, anemic status, tumor type, stage and degree of resectability, blood loss, anesthesia type, stress level, and postoperative complications [61–67]. It has been reported that perioperative blood product transfusions, including packed red blood cells, platelets, and fresh frozen plasma, are linked to an increased risk of cancer recurrence in CRC patients [68–72]. Strict control of transfusions should be suggested to avoid unnecessary and excessive blood transfusion.

3.3.3. Mood. Anxiety in patients with cancer is associated with postoperative immunosuppressive levels, and the anesthesiologist can help to relieve the patient's anxiety about surgery and disease by preoperative conversation and medication [73].

Additional Points

Summary. Perioperative factors such as surgical trauma, volatile anesthetics, opioid use, physiological stress, hyperglycemia,

hypothermia, blood products transfusion, and mood can cause a significant TH1/TH2 imbalance between the antitumor and protumor environments in the human body and can have a profound effect on the initiation and progression of colon carcinogenesis, colon cancer metastasis, recurrence, response to standard antitumor therapy, and the final clinical outcome. In brief, regional anesthesia is significantly better than general anesthesia in the immune index and in later tumor recurrence and metastasis. General anesthesia combined with regional anesthesia is better than single-use general anesthesia. Most inhaled anesthetics, opioids, local anesthetics, and other intravenous anesthetics can reduce immunity to a certain extent, which sometimes leads to an increased recurrence of malignant tumors. However, tramadol, selective nonsteroidal anti-inflammatory analgesics, and propofol have protective effects on the immune function of the body and can reduce the recurrence and metastasis of the tumor. Therefore, it is important to make a careful anesthesia plan and to select appropriate narcotic drugs for patients with malignant tumors, since these decisions will have a crucial impact on the therapeutic effect and prognosis. In addition, red blood cells, platelets, and FFP transfusion during the perioperative period also directly cause immunosuppression and increase the risk of cancer recurrence in colorectal patients. Hypothermia, hyperglycemia, and even a patient's mood contribute to changes in immunity and clinical outcomes in colorectal cancer perioperatively. Existing clinical research sample sizes are small, and retrospective studies have inherent bias and other deficiencies. In the future, more basic research and large-scale, prospective, randomized clinical studies will be needed to further explore the linkage and mechanisms between anesthetic factors and the prognosis of malignant tumors to provide safer anesthesia for cancer patients.

Disclosure

Yangjie Dang and Xingxing Shi are first authors.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

All the authors have sufficiently contributed to this work to merit authorship.

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Research Article

Decreased Breg/Th17 Ratio Improved the Prognosis of Patients with Ulcerative Colitis

Xue Bing[1], **Liang Linlang**[1] **and Chen Keyan**[2]

[1]*Department of Endocrinology, General Hospital of Shenyang Military Area Command, No. 83 Wenhua Road, Shenyang, Liaoning Province 110016, China*
[2]*Department of Laboratory Animal Science, China Medical University, No. 77 Puhe Road, Shenyang North New Area, Shenyang, Liaoning Province 110122, China*

Correspondence should be addressed to Chen Keyan; kychen@cmu.edu.cn

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Objective. To investigate the effects of regulatory B (Breg) cells and T helper 17 (Th17) cells on pathogenesis of ulcerative colitis, explore the clinical significance of Breg/Th17 ratio on the prognosis of ulcerative colitis, and provide the theoretical basis for the targeted therapy, diagnosis, and prognosis of the disease. *Methods*. Peripheral blood and colonic mucosa were collected from patients with ulcerative colitis. Hematoxylin-eosin staining was used to observe the pathological changes of colonic mucosa. Flow cytometry was utilized to analyze the percentages of Breg cells and Th17 cells. Real-time fluorescent quantitative polymerase chain reaction and immunohistochemistry were applied to determine the expression of Breg cells-related cytokines IL-10 and Th17 cell transcription factor RORγT. Enzyme-linked immunosorbent assay was employed to detect serum IL-10 and IL-17 levels. *Results*. The colonic mucosa of ulcerative colitis patients presented massive inflammatory cell infiltration and hemorrhagic necrosis. The number of Breg cells and Th17 cells, the gene expressions of IL-10 and RORγT, and serum levels of IL-10 and IL-17 all increased in peripheral blood. Compared with nonremission group, the remission group showed that the percentage of Breg cells reduced, the percentage of Th17 cells increased, and thus the B10/Th17 ratio was significantly decreased in peripheral blood. In addition, serum IL-10 levels diminished, IL-17 levels increased, and thus IL-10/IL-17 ratio was remarkably reduced in remission group. B10/Th17 ratio and IL-10/IL-17 ratio were positively correlated with the severity of disease. *Conclusions*. Breg and Th17 cells participate in the occurrence and development of ulcerative colitis. B10/Th17 ratio and IL-10/IL-17 ratio can be used as prognostic markers for ulcerative colitis. This provides a theoretical basis for design of targeted treatment and prognosis assessment of the disease.

1. Introduction

Ulcerative colitis is a recurrent chronic immune disease. After the drug-induced remission, the symptoms and pathological examinations of the patients can be relieved to a great extent, but the condition still easily occurs again. Currently, in the treatment of ulcerative colitis, the induced remission can reduce the risk of ulcerative colitis-induced cancer [1]. In recent years, many serum [2, 3] and fecal [4, 5] markers have been applied to evaluate the prognosis of ulcerative colitis. However, regions of the intestinal mucosal inflammation, disease status, and drug administration can cause changes in these indicators [6, 7]. Thus, reliable biological markers are urgently needed for the diagnosis of ulcerative colitis and the evaluation of the disease prognosis.

B cells regulate immune responses by producing antigen-specific antibodies. However, specific B cell subsets also have the ability to modulate immune responses. Regulatory B cell (Breg), first proposed by Mizoguchi and Bhan [8], is a B cell subset that plays negative regulatory functions that inhibit immune response. Breg cells mainly affect the regulatory T cell activation and the production of immune response by producing interleukin-10 (IL-10), reducing the expression of MHC-II molecules on cell surface and downregulating tumor necrosis factor [9]. Breg exerts its immunomodulatory effects by secreting IL-10, known primarily as B10 cell [10]. T helper 17 (Th17) cell is a new type of effector T cell subset, and, like Th1 and Th2 subpopulation cells, it is differentiated from the progenitor T cells and strongly associated with autoimmune diseases and inflammatory responses [11]. Interleukin-17

(IL-17) is the major effector molecule secreted by Th17 cells. By secreting IL-17, Th17 mobilizes, recruits, and activates neutrophils and macrophages, mediates inflammatory cells to local invasion and tissue damage, and induces inflammatory responses [12].

This study further investigated the effects of Breg cells and Th17 cells on ulcerative colitis by determining peripheral blood Breg cells, Th17 cells, and related molecules in patients with ulcerative colitis and provided theoretical basis for targeted therapy and prognosis of ulcerative colitis.

2. Materials and Methods

2.1. Subjects and Group Assignment. Thirty-three patients with ulcerative colitis at the age between 23 and 59 years old were collected from the Department of Gastroenterology of the General Hospital of Shenyang Military of China from July 2016 to April 2017. These patients suffered from pus and blood in stool more than 3 times. The patients had not been treated with hormones, salicylic acid, other immunosuppressive agents, and antibacterial agents within 2 weeks. Thirty-four patients in remission orally took hormones, salicylic acid, or other immunosuppressive agents to achieve remission of clinical symptoms. Thirty-five patients in nonremission orally took hormones, salicylic acid, or other immunosuppressive agents to achieve remission of clinical symptoms. Thirty-five healthy controls in the control group were recruited from Hospital Physical Examination Center. Ulcerative colitis patients with one or more of the following conditions were excluded from this study: tumor and immune disease. The protocols were approved by the Ethics Committee of the General Hospital of Shenyang Military. Patients signed informed consent.

2.2. Sample Preparation. Venous blood (5 ml) was collected from healthy controls, ulcerative colitis patients, remission patients, and nonremission patients. 4 ml of the venous blood was used for isolation of mononuclear cells and 1 ml for serum isolation. All samples were stored at −80°C for enzyme-linked immunosorbent assay (ELISA). The colonic mucosa of ulcerative colitis patients, remission patients and nonremission patients was obtained by colonoscopy, fixed in formaldehyde, and stored at −80°C. Normal colons were harvested by colon surgery (except tumor patients).

2.3. Hematoxylin-Eosin Staining. The colons were fixed in 10% formaldehyde, dehydrated, embedded in wax, and sliced into sections. The sections were dewaxed, hydrated, stained with hematoxylin for 5 minutes, washed with running water for 5 minutes, differentiated with 1% hydrochloric acid in ethanol for 1–3 seconds, washed with running water for 30 seconds, stained with 0.5% eosin for 1–3 minutes, washed with distilled water for 5 seconds, dehydrated, permeabilized, and mounted with neutral resin. The changes in tissue structure were observed under the light microscope.

2.4. Isolation of Peripheral Blood Mononuclear Cells. Anticoagulated blood (4 ml) was collected from each group, diluted in an equal volume of PBS, lysed with an equal volume of erythrocyte lysate on the ice for 5 minutes, and centrifuged at 1000 rpm/min for 5 minutes. After removal of the supernatant, 5 ml PBS was added and centrifuged at 800 rpm/min for 5 minutes. The supernatant was discarded. RPMI-1640 cell culture medium was added for resuspension and cells were quantified.

2.5. Fluorescent Labeling of Peripheral Blood $CD24^{hi}CD27^{+}CD38^{hi}IL\text{-}10^{+}$ (B10) Cells and Th17 Cells. Cells (1×10^6) were placed in 12-well plates, and RPMI-1640 medium was added to a total of 1 ml. LPS (Sigma, L2880) 10 μg/ml was added and incubated in 5% CO_2 incubator at 37°C for 24 hours. 19 hours later, cells in each well were stimulated with PMA (Sigma, P1585), ionomycin (Sigma, I-0634), and Brefeldin A (BD, 555029) to reach the final concentrations as 10 ng/ml, 0.5 μg/ml, and 1 μl/ml, respectively. Afterwards, cells were centrifuged at 1500 rpm for 5 minutes in EP tubes. After removal of the supernatant, cells were washed twice with PBS 1 ml. The supernatant was discarded. PBS 100 ul was added, and then FITC-anti-CD24 (BD, 555427), APC-anti-CD27 (BD, 561400), PerCP-CY5.5-anti-CD38 (BD, 561106), and FITC-anti-CD4 (BD, 555427) were added and incubated at room temperature in the dark for 30 minutes. Samples were washed with cell staining buffer, incubated with 500 μl fixation/permeabilization solution (BD, 555028) at room temperature in the dark for 30 minutes, washed with 1 × BD perm/wash buffer, incubated with PE-anti-IL-10 (BD, 554498) and PE-anti-IL-17 (BD, 561400) at room temperature in the dark for 45 minutes, washed with 1 × BD perm/wash buffer, and resuspended with 300 μl flow washing liquid. Results were analyzed using flow cytometry.

2.6. Real-Time Fluorescent Quantitative Polymerase Chain Reaction (qRT-PCR). The colons after trituration and lymphocytes after 24 hours of stimulation were placed in EP tubes. Other procedures were conducted in accordance with the instruction of Trizol reagent (15596018; Invitrogen). After precipitation and drying, 50 μl of DEPC-treated water was added, and 1.2% MOPS-denaturing formaldehyde gel electrophoresis was conducted. In accordance with RevertAid™ First Strand cDNA Synthesis Kit (K1621, Thermo), RNA was reverse transcribed into first strand cDNA. Taking GAPDH as reference gene, IL-10 and RORγT were quantitatively analyzed with SYBR Green PCR kit (204054, Qiagen). The relative amount of each target gene/reference gene was calculated. According to the sequences of IL-10 and RORγT in Genbank, DNAman software was utilized for primer design. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd., China. Primer sequences are as follows:

IL-10-F: GACTCTATAGACTCTAGG
R: CATCAACTACATAGAAGC
RORγT-F: AACAACTTGGCCAAGGCA
R: GGGACAGGGCCCAGACAG
GAPDH-F: GCTCATTTGCAGGGGGGA
R: CACCACCAACTGCTTAGC

2.7. Immunohistochemistry. Paraffin sections were dewaxed, hydrated, and blocked with 3% H_2O_2 for 15 minutes to inactivate endogenous peroxidase. Antigens were retrieved with 10 mM sodium citrate buffer. After three washes with PBS, samples were blocked with goat serum for 30 minutes and incubated with anti-IL-10 (Abcam, ab134742) and anti-IL-17 (Abcam, ab92486) at 4°C overnight. After three washes with PBS, secondary antibody was added and incubated at 37°C for 2 hours. Nuclei were counterstained with hematoxylin. Samples were visualized with 3,3′-diaminobenzidine, mounted with neutral resin, and observed under the light microscope.

2.8. ELISA. Serum was collected from healthy controls, ulcerative colitis patients, remission patients, and nonremission patients. In accordance with the instructions of IL-10 (CCC, SEA056Hu) and IL-17 (CCC, SEA688Hu) reagents, optical density (OD) values were measured at 450 nm using a microplate reader. The standard curves used OD values as y-axis, and standard sample concentrations as x-axis. Curve equation and r value were calculated, and sample concentrations were measured.

2.9. Correlation Analysis between Percentages of B10 and Th17 Cells in Peripheral Blood and the Severity of the Disease. According to Mayo scoring system [13], the severity of the disease was assessed in all patients as follows: Mayo scoring ≤ 2 points, remission; 3–5 points, mild; 6–10 points, moderate; 11-12 points, severe (Table 1). Correlation analysis in peripheral blood B10 cells and Th17 cells as well as B10/Th17 ratio was carried out in patients in remission, with mild, moderate, and severe ulcerative colitis.

2.10. Statistical Analysis. All data were analyzed using SPSS 19.0 software. Data in each group were compared and analyzed using group t-test, one-way analysis of variance, and Spearman's correlation. The data were expressed as mean ± standard deviation. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Intestinal Morphological Changes after Ulcerative Colitis. In the control group, colonic mucosa was normal and cells were regularly distributed (Figure 1). In the ulcerative colitis group, colonic mucosa presented bleeding, edema, a large number of inflammatory cell infiltrations, intestinal epithelial cell degeneration, and necrosis. In patients in remission, a small number of inflammatory cell infiltrations and bleeding were visible in colonic mucosa; edema was remarkably lessened compared with ulcerative colitis patients. A large number of inflammatory cell infiltrations, intestinal epithelial cell degeneration, and necrosis were observed in the nonremission group.

3.2. B10 Cell and Th17 Cell Counts in Ulcerative Colitis Patients and Healthy Subjects. To study the B10 cell and Th17 cell counts in ulcerative colitis patients and healthy controls, cell counting was performed with flow cytometry. Our results revealed that $CD24^{hi}CD27^{+}CD38^{hi}IL\text{-}10^{+}$ (B10) percentage in peripheral blood was significantly higher in the ulcerative colitis patients than in controls ($P < 0.05$) (Figures 2(a)-2(b)). Moreover, the percentage of $CD4^{+}IL\text{-}17^{+}$ cells was significantly higher in the ulcerative colitis patients than in controls ($P < 0.05$) (Figures 2(c)-2(d)). These data suggested that the percentages of B10 cells and Th17 cells increased in peripheral blood of ulcerative colitis patients, which were possibly associated with the onset of disease.

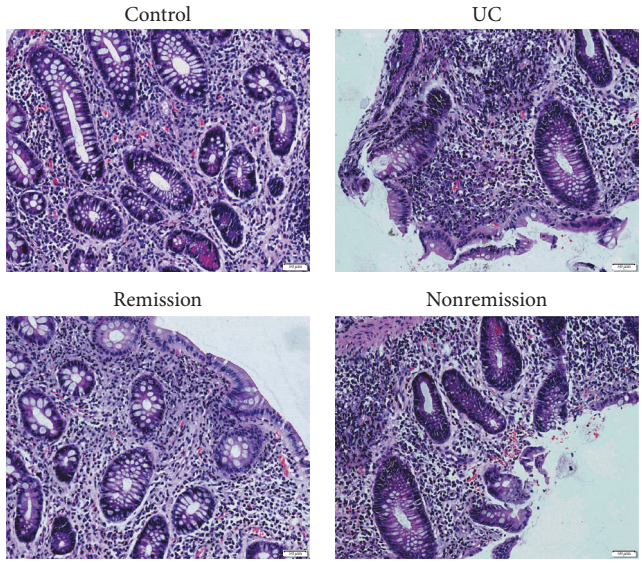

Figure 1: Intestinal morphological changes after ulcerative colitis by HE staining; UC group showed a lot of inflammatory cell infiltration. In remission, a small number of inflammatory cell infiltrations and bleeding were visible in colonic mucosa.

3.3. The Expressions of IL-10 and RORγT in Ulcerative Colitis Patients and Healthy Subjects. Based on the findings about the association between Breg and Th17 cells with pathogenesis of ulcerative colitis, the mRNA expression levels of IL-10 and RORγT were determined with qRT-PCR. Our results showed that, compared with the healthy controls, IL-10 mRNA expression was significantly increased in ulcerative colitis patients ($P < 0.05$) (Figure 3(a)). Compared with healthy controls, RORγT mRNA expression was also significantly increased in the UC patients ($P < 0.05$).

Immunohistochemistry results showed that IL-10 and IL-17 were also significantly increased in the UC patients (Figure 3(b)). In line with the above findings, these results suggested that the expressions of IL-10 and Th17 cell-specific transcription factors were upregulated in ulcerative colitis patients.

3.4. Serum Levels of IL-10 and IL-17 in Ulcerative Colitis Patients and Healthy Subjects. Serums IL-10 and IL-17 in ulcerative colitis patients and healthy subjects were detected by ELISA. Our results demonstrated that serum IL-10 levels were significantly higher in ulcerative colitis patients than in controls ($P < 0.05$) (Figure 4). Similar results were obtained for the detection of serum IL-17 levels in the ulcerative colitis patients and controls ($P < 0.05$) (Figure 4). These findings suggested that, in line with the alterations in Breg and Th17

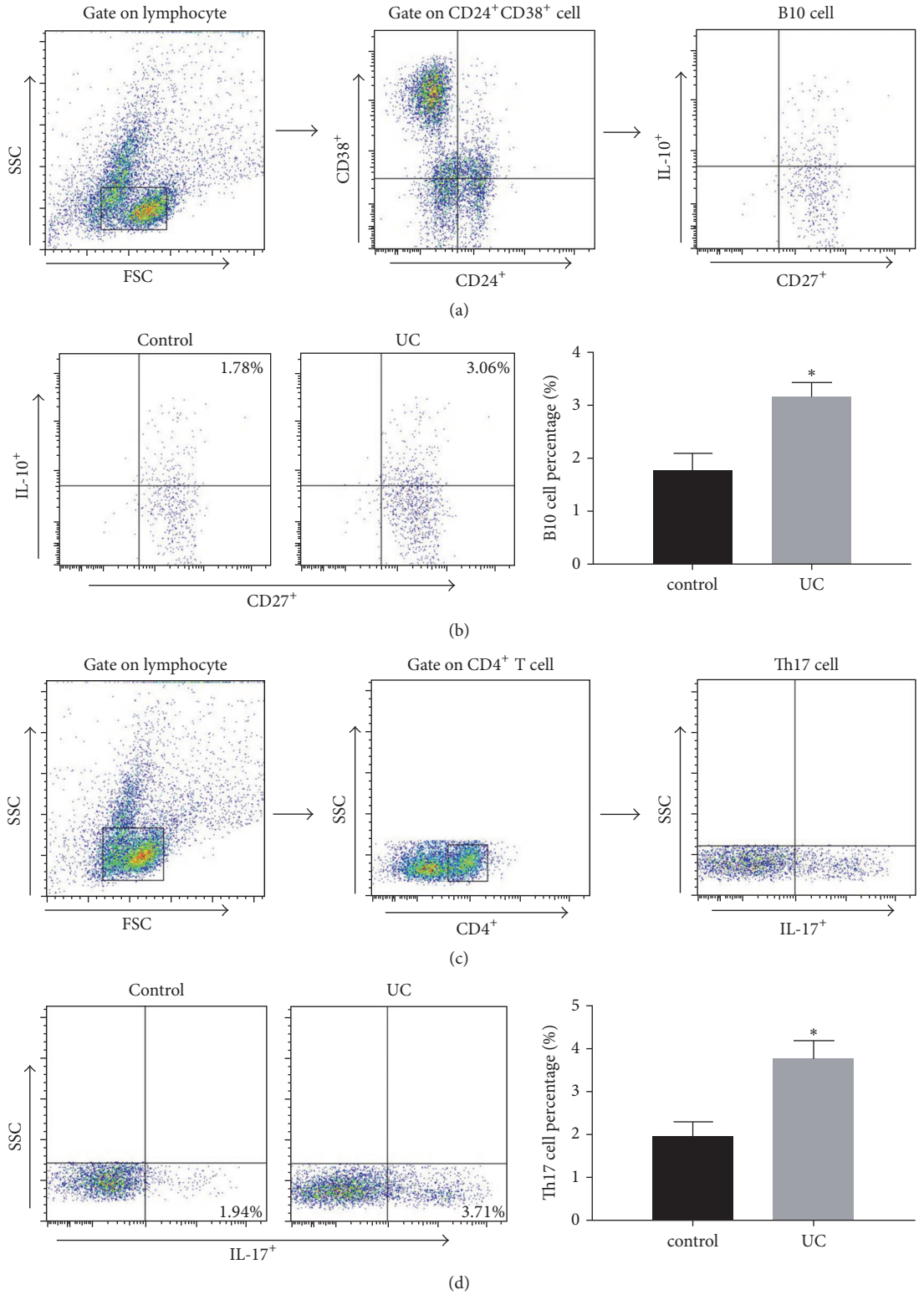

Figure 2: B10 cell and Th17 cell counts in ulcerative colitis patients and healthy subjects. (a-b) Gating criteria to define the $CD24^+CD27^+CD38^+IL\text{-}10^+$ Breg cell population. Different cell subsets were distinguished according to different cell labels. (c-d) The percentage of $CD4^+IL\text{-}17^+$ cells in peripheral blood. Compared with control group, $^*P < 0.05$.

Table 1: Mayo score.

Terms	≤2 points	3–5 points	6–10 points	11-12 points
Stool frequency	Normal	1-2 stools/day more than normal	3-4 stools/day more than normal	>4 stools/day more than normal
Rectal bleeding	None	Visible blood with stool less than half the time	Visible blood with stool half of the time or more	Passing blood alone
Mucosal appearance at endoscopy	Normal or inactive disease	Mild disease (erythema, decreased vascular pattern, mild friability)	Moderate disease (marked erythema, absent vascular pattern, friability, erosions)	Severe disease (spontaneous bleeding, ulceration)
Physician rating of disease activity	Normal	Mild	Moderate	Severe

Notes. Mayo scoring ≤ 2 points: remission; 3–5 points: mild; 6–10 points: moderate; 11-12 points: severe.

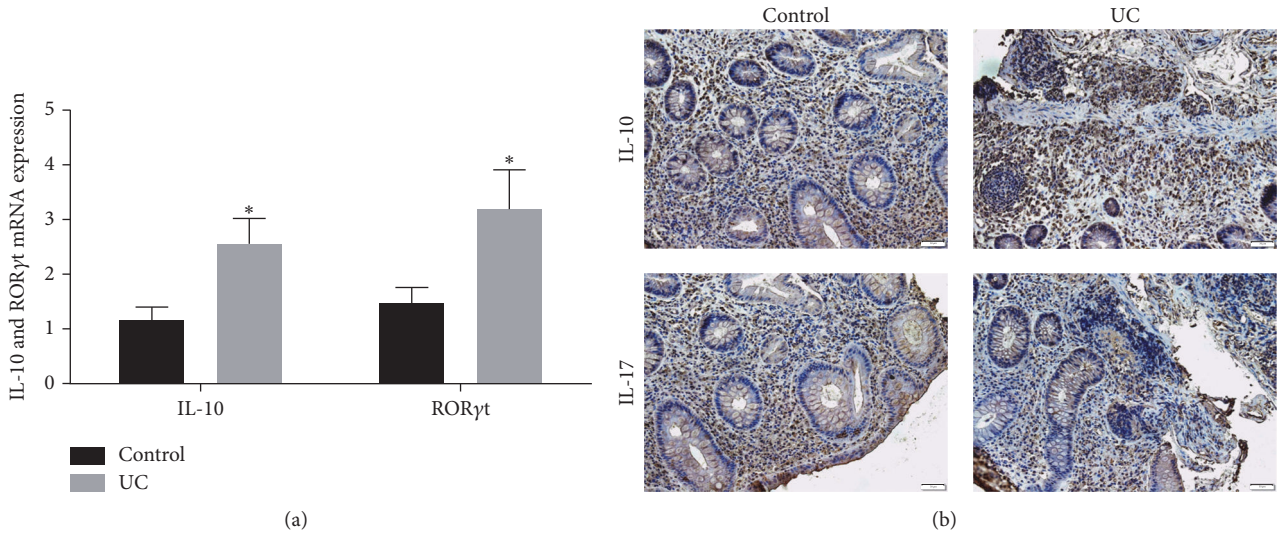

Figure 3: The expression of IL-10 and RORγT in ulcerative colitis patients and healthy subjects was upregulated. (a) The mRNA expression levels of IL-10 and RORγT were determined with qRT-PCR. (b) IL-10 and IL-17 expression by immunohistochemistry. Compared with control group, $^{*}P < 0.05$.

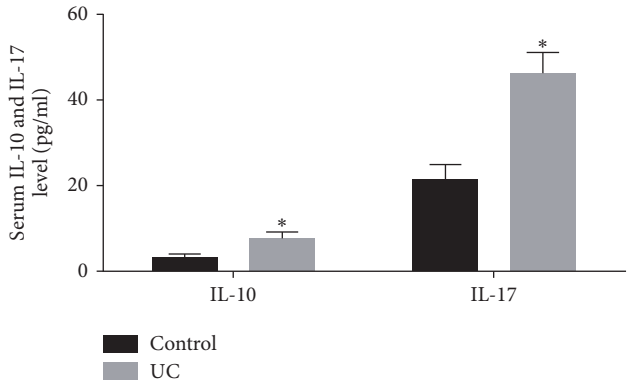

Figure 4: Serum levels of IL-10 and IL-17 in ulcerative colitis patients and healthy subjects were detected with ELISA. IL-10 and IL-17 levels were significantly higher, compared with control group, $^{*}P < 0.05$.

cells, the serum levels of IL-10 and IL-17 were increased in the ulcerative colitis patients.

3.5. Cell Counts and Cytokine Levels in Ulcerative Colitis Patients in Remission and Nonremission after Treatments. The differences in the cell counts and related cytokine levels between ulcerative colitis patients in remission and nonremission after treatment were next analyzed and compared. Flow cytometry analysis showed that the number of B10 cells significantly decreased in peripheral blood of ulcerative colitis patients in remission ($P < 0.05$, versus nonremission group) (Figure 5(a)); the proportion of Th17 cells significantly increased ($P < 0.05$, versus nonremission group) (Figure 5(b)). Accordingly, B10/Th17 ratio in the remission group was significantly lower than in the nonremission group (Figure 5(c)). The serum levels of IL-10 and IL-17 were also determined and compared between the remission and nonremission groups. Our results showed that serum IL-10 levels significantly diminished in the remission group ($P < 0.05$, versus nonremission group) (Figure 5(d)). However, IL-17 levels significantly increased ($P < 0.05$, versus nonremission group) (Figure 5(d)). The IL-10/IL-17 ratio was significantly reduced in the remission group compared with the

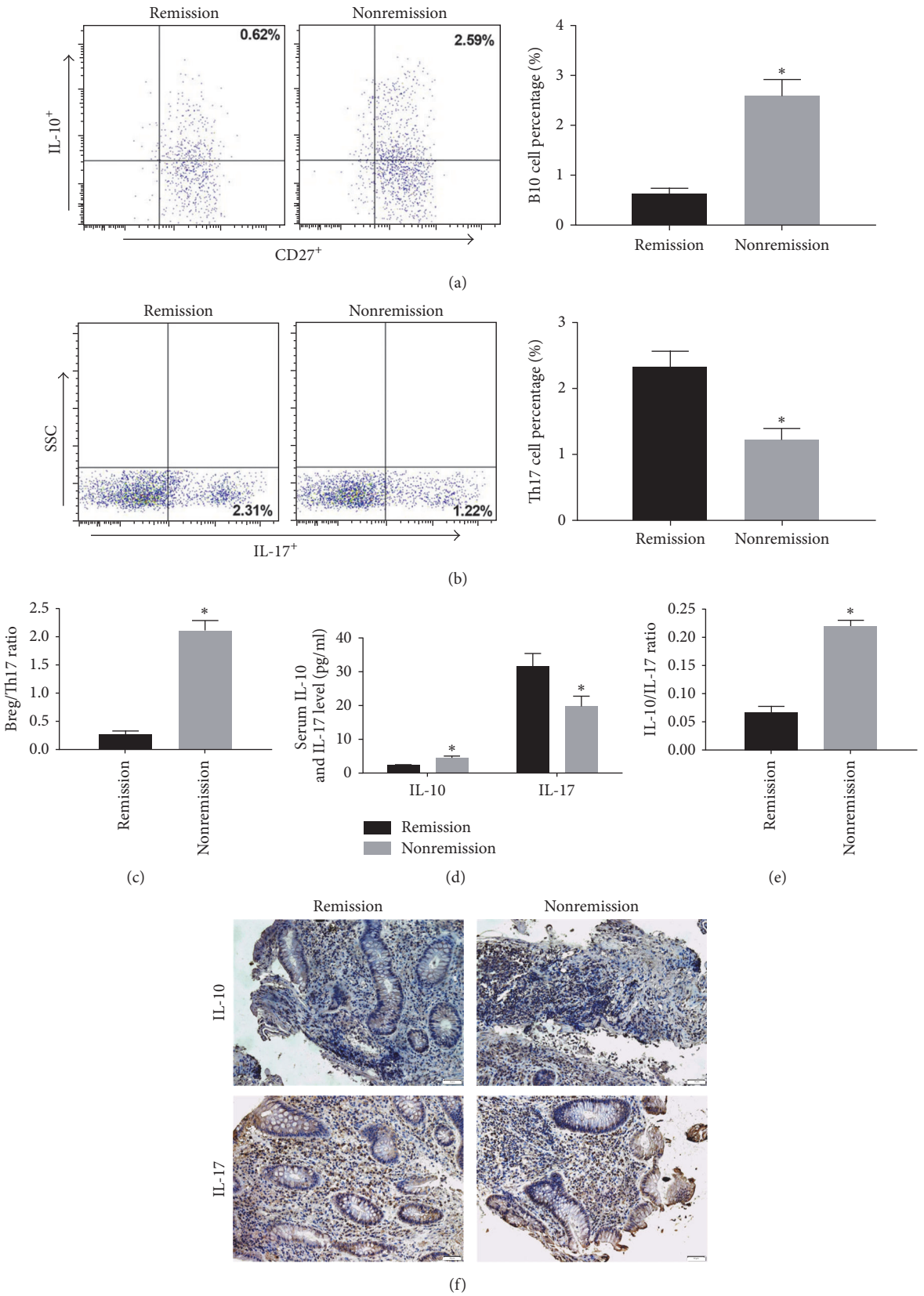

FIGURE 5: The differences in the cell counts and related cytokine levels between ulcerative colitis patients in remission and nonremission after treatment were analyzed with flow cytometry and ELISA. (a) Gating criteria to define the $CD24^+CD27^+CD38^+IL\text{-}10^+$ Breg cell population in the remission group and nonremission group. (b) The percentage of Th17 cells in the remission group and nonremission. (c) B10/Th17 ratio in the remission group and nonremission group. (d) Serum IL-10 levels and IL-17 levels were detected with ELISA. (e) The IL-10/IL-17 ratio in the remission group and nonremission group. (f) IL-10 and IL-17 expression by immunohistochemistry in in the remission group and nonremission group. Compared with remission group, $^*P < 0.05$.

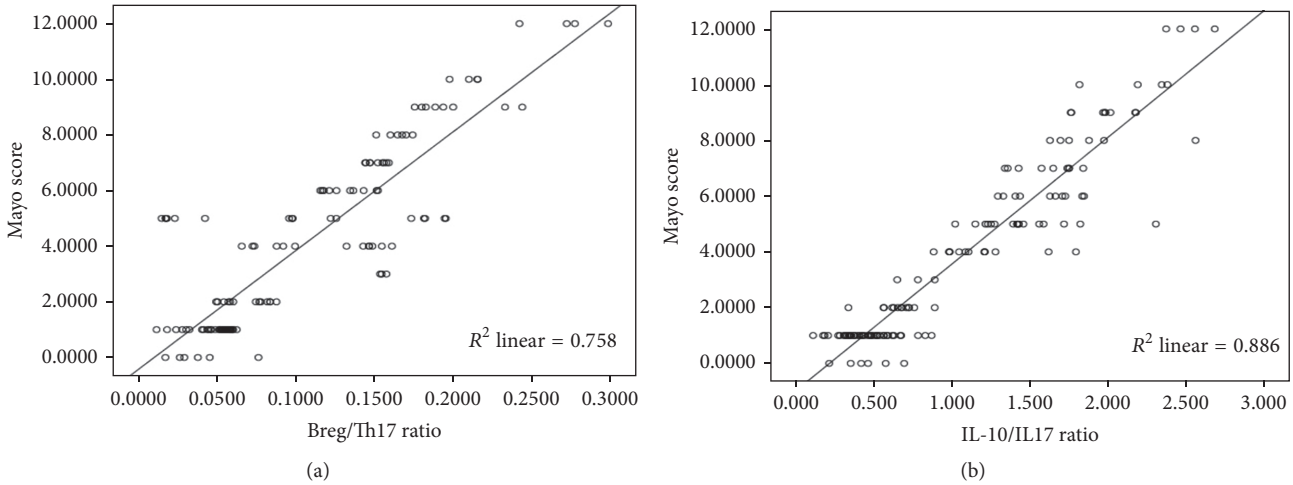

Figure 6: Correlation of cell counts and cytokine levels with Mayo scoring in ulcerative colitis patients, B10/Th17 ratio, and Mayo scoring were positively correlated; serum IL-10/IL-17 ratio and Mayo scoring were positively correlated. (a) Peripheral blood B10/Th17 ratio was positively correlated with Mayo in ulcerative colitis patients ($r = 0.758$, $P < 0.05$). (b) Serum IL-10/IL-17 ratio was positively correlated with Mayo in ulcerative colitis patients ($r = 0.886$, $P < 0.05$).

nonremission group ($P < 0.05$, versus nonremission group) (Figure 5(e)). The same results were also obtained by immunohistochemistry (Figure 5(f)). These results confirmed that the changes in the cell counts and related cytokine levels induced by drug treatment would contribute to the disease remission in ulcerative colitis patients.

3.6. Correlation of Cell Counts and Cytokine Levels with Disease Development in Ulcerative Colitis Patients. We analyzed the correlation of peripheral blood B10 cells, Th17 cells, and their related factors with Mayo scoring. Our results found that peripheral blood B10/Th17 ratio was positively correlated with Mayo in ulcerative colitis patients ($r = 0.758$, $P < 0.05$) (Figure 6(a)). Serum IL-10/IL-17 ratio was also positively correlated with Mayo ($r = 0.886$, $P < 0.05$) (Figure 6(b)). These data suggested that a high B10/Th17 or IL-10/IL-17 ratio indicated severe patient's condition, whereas a low B10/Th17 or IL-10/IL-17 ratio suggested good prognosis.

4. Discussion

The occurrence of ulcerative colitis is associated with heredity, immunity, infection, and environmental factors. The intestinal immune system is activated under the participation of environmental factors and intestinal flora [14, 15]. In the presence of persistent antigenic stimuli and (or) immune dysregulation, inflammatory cascade magnifies and local inflammatory mediators injure tissues, thereby resulting in the occurrence of ulcerative colitis. In this study, the number of peripheral blood Breg and Th17 cells increased in ulcerative colitis patients, which were possibly associated with the occurrence and development of disease. In remission, the percentage of B10 cells diminished, but the percentage of Th17 cells obviously increased in peripheral blood of ulcerative colitis patients. B10/Th17 ratio was significantly lower in the remission group than in the nonremission group. IL-10 levels remarkably reduced, but IL-17 levels noticeably increased in remission. IL-10/IL-17 ratio was significantly declined. These results indicated that, after drug treatment, the counts of B10 cells and Th17 cells and the expression of related cytokines contributed to the remission of ulcerative colitis.

At present, the negative immune regulatory function of Breg cells plays an important role in autoimmune response [16]. Harris [17] suggested that, in inflammatory conditions, B cells are similar to $CD4^+$ T cells and produce cytokines to fight against inflammation. Wolf et al. [18] found that the induction of experimental autoimmune encephalomyelitis in B cell-deficient mice aggravated the severity of the disease, but the same induction in wild-type mice could mitigate the symptoms. Xiao et al. [19] found that, in models of mutation of Fas gene, Breg cells (B10) that secreted IL-10 had abnormal function; mice presented severe lupus, and the pathogenic antibodies of lupus were also significantly elevated. It is thus clear that Breg cells from B cells have a negative immune regulation mechanism, and B cells play an immune regulatory role in autoimmune diseases.

Th17 cell is a new type of CD4 effect T cell that is different from the traditional Th1 and Th2 cells. It plays an important role in the pathogenesis of inflammatory diseases and autoimmune diseases [20]. IL-17 is the major effector molecule secreted by Th17 cells. By secreting IL-17, Th17 mobilizes, recruits, and activates neutrophils and macrophages, mediates inflammatory cells to local invasion and tissue damage, and induces inflammatory responses [21]. Recent data has demonstrated that biologics neutralizing IL-17 (ixekizumab and secukinumab) or its receptor (brodalumab) are highly effective with a positive safety profile in treating moderate to severe psoriasis [22]. In this study, we found that abnormal expression of Breg and Th17 cells in peripheral blood of UC patients may lead to immune imbalance, so that the effector type $CD4^+$ T cells migrate from the circulatory system to the local inflammatory site of the intestinal

tract, enrichment in the inflammatory site, leading to digestion mucosa in a highly active state, induced by the intestinal response, resulting in self-antigen intolerance and increased release of damage to cytokines, leading to mucosal injury.

In summary, Breg cells and Th17 cells participate in the occurrence and development of ulcerative colitis. B10/Th17 ratio and IL-10/IL-17 ratio can be used as prognostic markers of ulcerative colitis. This provides a theoretical basis for targeted therapy of clinical drugs and disease prognosis.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication ofthis article.

Authors' Contributions

Liang Linlang and Chen Keyan contributed equally to the manuscript.

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Review Article

Combination Immunotherapy Approaches for Pancreatic Cancer Treatment

Xianliang Cheng,[1] Gang Zhao,[2] and Yunqi Zhao [1]

[1]*School of Pharmaceutical Sciences & Yunnan Provincial Key Laboratory of Pharmacology for Natural Products, Kunming Medical University, Kunming, Yunnan, China*
[2]*The First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning, China*

Correspondence should be addressed to Yunqi Zhao; chloe_zyq@163.com

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Pancreatic ductal adenocarcinoma is a lethal malignant disease with a very low medium survival. Currently, metastatic pancreatic cancer poorly responds to conventional treatments and exhibits an acute resistance to most chemotherapy. Few approaches have been shown to be effective for metastatic pancreatic cancer treatment. Novel therapeutic approaches to treat patients with pancreatic adenocarcinoma are in great demand. Last decades, immunotherapies have been evaluated in clinical trials and received great success in many types of cancers. However, it has very limited success in treating pancreatic cancer. As pancreatic cancer poorly responds to many single immunotherapeutic agents, combination immunotherapy was introduced to improve efficacy. The combination therapies hold great promise for enhancing immune responses to achieve better therapeutic effects. This review summarizes the existing and potential combination immunotherapies for the treatment of pancreatic cancer.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer (approximately 90%), and it is the third leading cause of cancer death with an overall 5-year survival rate of 5–10% [1, 2]. Since PDAC is normally diagnosed at a late stage, the majority of patients with PDAC do not survive a year after diagnosis. The standard chemotherapy for metastatic PDAC is FOLFIRINOX, a combination of oxaliplatin, irinotecan, fluorouracil, and leucovorin [3]. However, concerns for toxicity and adverse side effects quickly restricted patients to the treatment. Due to rising incidence of PDAC, there is a major unmet need to develop novel promising therapeutic strategies.

Immunotherapy opens a new era in cancer treatment. People achieve great success in cancer vaccines and immunomodulators, such as checkpoint blockade to induce endogenous host immune response. Nevertheless, PDAC has non-immunogenic and immune-suppressive microenvironment, and immune checkpoint inhibitor monotherapy alone lacks efficacy in this disease. Tremendous efforts have been made to seek a new strategy to improve immunotherapy efficacy.

Innate immune cells express pattern-recognition receptors, such as dectin-1, on their surfaces. Dectin-1 was found highly expressed in human PDAC tumor and peritumoral inflammatory compartments. The dectin-1 signal transduction pathway opens a new area in the anticancer therapeutic application. It could be an attractive target for PDAC immunotherapy.

Adoptive immunotherapy utilizing chimeric antigen receptor-engineered T-cells is being exploited as a promising strategy to redirect patient's T-cells against tumors and reduce tumor load. Several antigens, such as carcinoembryonic antigen and mesothelin, have been chosen as the target of the engineered T-cells. Researches indicate that this strategy showed encouraging results. However, serious adverse events were associated with the treatment [4, 5] such as cytokine release syndrome and neurological toxicity [6]. Other therapeutic approaches need to be done to solve the safety issue.

In this review, we summarized recent findings in the development of novel combination immunotherapies to improve treatment efficacy in PDAC.

2. Immune Checkpoint

Immune checkpoints are involved in regulation of antigen recognition of T-cell receptor by costimulatory or inhibitory signaling transduction in the immune system. Immune checkpoint blockade therapy achieves great success in treating many types of cancers [7]. It targets T-cell regulatory pathways to enhance anticancer immune response. Since the immune response has dynamic nature, research indicates that combination therapies may provide a better survival benefit for cancer patients [8].

2.1. Cytotoxic T-Lymphocyte-Associated Antigen-4 (CTLA-4). T-cell exclusion is obviously evident in PDAC, in which effector T-cells are often scarce within tumor tissue and confined to peritumoral lymph nodes and lymphoid aggregates [9]. CTLA-4 is an immune checkpoint receptor expressed on regulatory T (Treg) cells and recently activated conventional T-cells [10]. It is a negative regulator of T-cell activation, and it is also known as CD152. CTLA-4 is homologous to CD28 and they share the same ligands. Both B7-1 (CD80) and B7-2 (CD86) ligands are expressed on antigen-presenting cells (APCs) and can render costimulatory signals to T-cells. Upon activation, T-cells express CTLA-4 on the cell surface. CTLA-4 engagement with B7 inhibits T-cell activation. CTLA-4 has higher affinity to B7 ligands compared to CD28. CTLA-4 ligation delivers an inhibitory signal to T-cells, whereas CD28 delivers a stimulatory signal [11, 12]. The anti-CTLA-4 antibody can blockade CTLA-4 interaction with B7 and prevents the inhibitory signal [13]. Targeting CTLA-4 with a human anti-CTLA-4 antibody has demonstrated therapeutic success in the treatment of melanoma [14]. Then blockade of CTLA-4 may be a promising new approach to cancer therapy and constitutes a novel approach to induce host responses against tumors. It could downregulate the immune system and produce durable anticancer responses [15]. However, there is no sufficient evidence showing that CTLA-4 is a potential therapeutic target for PDAC immunotherapy [16]. Little benefit has been achieved so far by applying CTLA-4 antibodies alone in PDAC treatment. This might be due to high tumor burden and the intrinsic nonimmunogenic nature of pancreatic cancer that cause immune quiescent, and the blockage of only one checkpoint is not enough for immunosuppressive reduction.

Ipilimumab (MDX-010) is a fully humanized IgG1 monoclonal antibody that works by blocking the ligand-receptor interaction of B7-1/B7-2 and CTLA-4. Thereby, ipilimumab has the potential to increase antigen-specific immune responses. In 2011, it is approved by US Food and Drug Administration (FDA) to treat metastatic melanoma [17], and the thread name is Yervoy. Ipilimumab, as a single agent, has been tested in PDAC patients. Despite the fact that ipilimumab at a dose of 3.0 mg/kg was minimally effective for the treatment of advanced pancreatic cancer, the delayed response case suggests that it deserves further investigation and complete assessment of immunotherapeutic approaches to pancreatic cancer [18]. Therefore, the concept of synergy between immune checkpoint blockade and cancer vaccines was brought up. It has shown encouraging results in PDAC in treatment combinations with granulocyte macrophage-colony stimulating factor (GM-CSF) cell-based vaccines (GVAX). In a phase I b trial study, ipilimumab 10 mg/kg + GVAX treatment group showed prolonged median overall survival and 1-year overall survival compared to ipilimumab 10 mg/kg treatment group. It indicates that checkpoint blockade in combination with GVAX has clinical benefit potential for PDAC patients [19].

Tremelimumab (CP 675206; CP-675; CP-675,206; CP-675206; Ticilimumab) is fully humanized IgG2 monoclonal antibody that antagonizes CTLA-4. It has been used for the treatment of various cancers, such as melanoma, colorectal cancer, prostate cancer, and pancreatic cancer [20]. A phase I study of tremelimumab combined with gemcitabine to treat pancreatic cancer was performed [21]. The study demonstrated a safe and tolerable profile and suggested that anti-CTLA-4 antibody in combination with standard chemotherapy might provide synergistic anticancer activity without increasing side effects.

2.2. Programmed Death 1 (PD-1). PD-1 protein, known as another immune checkpoint, is expressed on the surface of activated T-cells and is associated with programmed cell death [22]. PD-1, together with one of its ligands, programmed death-ligand 1 (PD-L1; also called B7-H1 or CD274), a B-7 family ligand, can suppress the overstimulation of immune responses and commit to the maintenance of immune tolerance to self-antigens [23]. It is an immunosuppressive pathway that is upregulated in tumor cells. PD-L1 is expressed by immune cells and various cancer cells, including breast, cervical, colorectal, gastric, glioblastoma, melanoma, non-small-cell lung, ovarian, pancreatic, and urothelial cancer [24]. Binding of PD-1 to its ligands inhibits T-cell activity and restricts tumor cell killing [25–28], leading to detrimental immune responses and preventing autoimmunity [29]. Blocking the ligation between PD-1 and PD-L1 should, therefore, augment immune response *in vitro* and initiate antitumor activity in preclinical models [30–32]. Because of this, targeting PD-1/PD-L1, as immune checkpoint blockade, has been developing in oncologic therapy for various cancers as of late. For instance, pembrolizumab (trade name Keytruda, 2014), nivolumab (trade name Opdivo, 2014), atezolizumab (trade name Tecentriq, 2016), and Durvalumab (trade name Imfinzi, 2017) were approved by US FDA for the treatment of metastatic non-small cell lung cancer, squamous cell carcinoma of the head and neck, metastatic melanoma, and bladder cancer, respectively. However, in the treatment of pancreatic cancer, there tends to be no apparent therapeutic effects of a single antibody [33, 34].

Therefore, to overcome the resistance of anti-PD-1/PD-L1 monotherapy, combination therapy strategies have been suggested for PDAC treatment. The current study shows that 92% clinical efficacy rate can be achieved when pembrolizumab is combined with gemcitabine plus nab-paclitaxel [35]. Meanwhile, combining radiotherapy with PD-1/PD-L1 blockade therapy could increase radiosensitization and enhance

the tumor cell immunogenicity [36]. Anti-PD-1/PD-L1 also can be combined with targeted therapies. Research indicates that the combination with poly (ADP-ribose) polymerase (PARP) inhibitors may be effective against pancreatic cancer with BRCA1/2 mutations [37]. Since many mechanisms are involved in immunosuppression of PDAC, two different immunotherapies also can be combined. When anti-PD-1 combined with GVAX, the murine survival rate was significantly improved compared to anti-PD-1 or GVAX monotherapy [38].

Therefore, as discussed above, the combination therapy of anti-PD-1/PD-L1 may overcome the immune resistance properties of PDAC and could improve the therapeutic efficacy of anti-PD-1/PD-L1 immunotherapy.

3. Dectin-1 and Innate Immune System

Dectin-1, also known as C-type lectin domain family 7 member A, is encoded by *CLEC7A* gene and is a pattern-recognition receptor expressed by myeloid-monocytic lineage cells [39]. Dectin-1 recognizes β-glucans polysaccharides in fungal cell walls and is directly associated with the innate immune system [40]. Dectin-1 was found highly expressed in both mouse and human PDAC tumors and macrophages. Ligation of dectin-1 with galectin-9, a member of the β-galactoside-binding family of lectins and a functional ligand for dectin-1, can accelerate the progression of PDAC in mice. The treatment by dectin-1 agonist could induce accelerated PDAC progression.

Galectin-9 is also overexpressed in both murine and human PDAC and upregulated in diverse PDAC-infiltrating myeloid cells and cancer cells. The blockade of galectin-9 could extend animal survival. Similarly, elevated galectin-9 expression associated with reduced survival in human PDAC [41]. The ligation of dectin-1 with galectin-9 in pancreatic cancer can cause mouse and human tolerogenic macrophages programming and adaptive immune suppression. The upregulated expression of either dectin-1 or galectin-9 plays a pivotal role in the ability of pancreatic tumor cells to evade the host's immune system, causing immunotherapy failure. Due to animal survival experiments and the limitation of targeting dectin-1 or galectin-9, additional treatments are required for the immunotherapy [41]. Therefore, immunotherapy regimen targeting PD-1 has been suggested to combine with therapies targeting either dectin-1 or galectin-9. This strategy might offer synergistic efficacy for cancer treatment.

4. Chimeric Antigen Receptors (CARs) and Adoptive Immune System

The better understanding of T-cell biology and genetic engineering allows us to modify T-cells by associating a synthetic molecule and infusing them into tumor tissue to enhance the immune response against malignant lesion [42]. Genetically engineered T-cells can specifically target cancer cells to eradicate tumor burden through a T-cell receptor or chimeric antigen receptors (CARs). CARs, also known as chimeric immunoreceptors, are engineered recombinant receptors with an intracellular signaling domain consisting of T-cell receptor-CD3-ζ domain and an extracellular single-chain variable antibody fragment [43]. CARs can directly bind to tumor-associated antigens, carbohydrates or glycolipids.

In August 2017, US FDA has approved a CARs therapy (tisagenlecleucel, Kymriah, Novartis) that used adoptive cell transfer technique to treat acute lymphoblastic leukemia. Actually, the CARs therapy technology was first introduced in 1989. The first generation of CARs, the targeting moiety, is coupled to a CD3-ζ module, which initiates T-cell activation and enables T-cell to mediate cytotoxicity [44]. This generation was shown clinically ineffective in patients with diverse solid tumors [45]. The second generation of CARs incorporate an additional costimulatory domains (CD28 or CD137), which have enhanced T-cell proliferation as well as cytotoxic activity [46]. The costimulatory effect may be imparted by receptors, for example, 4-1BB [47], CD28 [48], or ICOS [49]. A complete response of 90% was achieved in lymphodepleted patients treated with the second-generation CAR T-cells [50]. The third generation of CAR comprises CD3-ζ and two additional costimulatory signaling domains, CD28 and 4-1BB, or CD28 and OX40 [51]. T-cell targeting by a TCR faces a challenge because it is restricted by human leukocyte antigen (HLA) while CARs help T-cells to target tumor cells directly and are not restricted by HLA [52]. Both second- and third-generation CARs have shown preclinical efficacy in mesothelioma and ovarian xenograft models [53]. However, there is still a lot to learn about which method will be the safest and best suited to treat solid tumors [54].

The antigens overexpressed on solid tumor cells but with limited or no expression on normal cells can be promising targets for CAR T-cell therapy. Pancreatic cancer exhibits a number of tumor-specific antigens, such as carcinoembryonic antigen, mesothelin, HER-2, and MUC1, which are promising applicants for testing CARs T-cell therapy [55, 56].

4.1. Carcinoembryonic Antigen (CEA). CEA is a set of glycoproteins involved in cell adhesion, and the expression of CEA is low in healthy adults. The serum level of CEA can be elevated in some types of a cancer patient; for example, the antigen expressed in pancreatic adenocarcinomas is nearly 75% [57, 58]. CEA can be recognized by CARs T-cells, which makes it a valuable candidate target in CARs T-cell therapy for pancreatic cancer. In addition, the CEA level in serum can be a specific marker used routinely to monitor disease progression and tumor load. It was found that when patients received the highest dose of anti-CEA CARs T-cell, the levels of CEA declined [59]. In a clinically relevant model in murine, adoptive transfer of anti-CEA CAR-engineered T-cells was able to specifically and efficiently reduce the size of pancreatic tumors below the limit of detection in all mice and give continuing tumor eradication in 67% of mice [60]. This suggests the notion that CAR T-cells targeting CEA have the potential to treat pancreatic cancer.

4.2. Mesothelin (MSLN). MSLN is a glycosyl-phosphatidylinositol- (GPI-) linked membrane glycoprotein, which is highly expressed in mesothelioma, pancreatic, lung, ovarian,

and other cancers, but lowly expressed in normal tissue [61]. The aberrant expression of MSLN involves the aggressiveness and transformation of tumors through promoting cancer cell proliferation [62]. In a preclinical model, T-cells were engineered to express an affinity-enhanced TCR and were utilized to target MSLN antigen in a genetically engineered model of autochthonous PDAC. Engineered T-cells are known to accumulate in PDAC, inducing tumor death and stromal remodeling. Engineered human T-cells lyse PDAC cells in vitro, which further supports TCR-based strategy for the treatment of PDAC [63]. Other similar studies of CAR T-cells targeting MUC1 [64], CD24 [65], and HER2 [65] were tested, leading to tumor regression in mice. MORAb-009 is a chimeric antimesothelin monoclonal antibody that was utilized to target tumor-associated mesothelin overexpressed on pancreatic, ovarian, lung, and colorectal carcinoma. It was found that MORAb-009 reduced tumor growth in mesothelin-positive cancers and enhanced effectiveness of chemotherapy [66]. MORAb-009 was found to be safe in phase I clinical study of 24 mesothelin-positive patients, which included 7 pancreatic cancer patients [67].

4.3. Treatment Concerns. The treatment of pancreatic cancer through CARs T-cell therapy still remains challenging because of on-target and off-tumor effects. Identification of choosing an ideal tumor-restricted antigen is rare and rigorous. The CAR T-cell has toxicities to healthy tissue, causing trials cease, especially when the target tissue is expressed in central tissues, such as lungs, heart, or liver [6]. This has once again illustrated the importance of careful target antigen selection.

Cytokine release syndrome (CRS) and neurological toxicity are the most common severe side effects of CARs T-cell immunotherapy, and tocilizumab (IL-6 blocker) is used to treat patients with CRS. CRS correlates with disease burden [68] and is potentially due to the release of inflammatory cytokines produced by amounts of activated CARs T-cells [69].

Serious adverse events of CARs T-cells immunotherapy can be related to several factors. The current approaches of toxicity management have incorporated dual targeting strategies [70]. Suicide gene combined with cellular therapeutic products can eliminate the majority of CARs modified T-cells and prevent contiguous cells and/or tissues from collateral damage. This strategy has been advised to be combined together to reduce the side effects.

Another approach is to aim at coexpressing "inhibitory" CARs (iCARs) to avoid normal tissue targeting. iCARs can incorporate with CTLA-4 and PD-1 domain to transmit an inhibition signal instead of an activation signal [71]. Targeting multiple cancer-specific markers simultaneously could result in increased specificity and better therapeutic efficacy. "TanCAR" was introduced to avoid the off-target effect. TanCar mediates bispecific activation and targets to T-cells. This strategy could potentially offer a safer approach to minimize the severe adverse effects [72].

5. Summary

Pancreatic cancer remains a devastating lethal disease with poor prognosis. Immunotherapy uses the self-immune system to fight cancer and is emerging as the fourth pillar of cancer treatment, after surgery, chemotherapy, and radiation therapy. In the immune system, immune checkpoints are molecules involved in cell signaling transduction. By inhibiting T-cell signaling, many cancers can evade from the immune system elimination. Immune checkpoint therapy can target T-cells' regulatory pathways and enhance antitumor immune responses. Immune checkpoint inhibitors, such as anti-CTLA-4, anti-PD-1, and anti-PD-L1, can enhance antitumor immunity and mediate cancer regressions in many types of cancers. These findings have established immune checkpoint blockade immunotherapy as a viable treatment option for patients with advanced cancers. However, due to pancreatic cancer's unique characteristics, the treatment of single immune checkpoint inhibitor, anti-CTLA-4, anti-PD-1, or anti-PD-L1, has shown minimal clinical benefits in the treatment of advanced PDAC. Although some early success has been achieved with monotherapies blocking PD-1 pathways, the efficacy of an anti-PD-1/PD-L1 monotherapy may be ineffective when treating pancreatic cancer in an immune system suppressed by high tumor burden and intrinsic non-immunogenic nature [73]. However, preclinical models have indicated that combinatorial approaches will provide some favorable clinical outcomes. Therefore, combination immune therapy that targets PDAC immune checkpoints is currently the subject of intense study.

Besides, antibody blockers of novel immune checkpoints, which may be effective if employed in treatment combinations, are under development. These include lymphocyte activation gene 3 (LAG-3), killer inhibitory receptors (KIRs), B7-H3 (CD276), T-cell immunoglobulin and mucin-3 (TIM-3), V-domain Ig-containing suppressor of T-cell activation (VISTA), T-cell immunoglobulin and immunotyrosine inhibitory motif (ITIM) domain (TIGIT), and indoleamine 2,3-dioxygenase (IDO) [7]. The blockade of these checkpoints can be combined with anti-PD-1 or anti-PD-L1 to enhance antitumor immunity. Both innate and adaptive immunity are cooperating to promote tumor progression in PDAC. Dectin-1 plays a role in the innate immune response. The ligation of dectin-1 with galectin-9 in PDAC results in tolerogenic macrophage programming and adaptive immune suppression. The development of the therapeutics that target dectin-1/galectin-9 axis in combination with other immunotherapies will potentially be an attractive strategy for immunotherapeutic for human PDAC.

Adoptive immunotherapy using CARs T-cells is emerging as a novel approach to pancreatic cancer immunotherapy. Despite the improvement of CAR T-cells therapy within the last decade, its application as a treatment still remains in its infancy for PDAC. There are many obstacles in the clinical development of CARs-based immunotherapy for PDAC, such as significant toxicity profile and high cost. To ensure safety, a combination of two targets has been applied and will be investigated in clinical trials. Therefore, the selection of suitable targets to increase the precision of tumor targeting is crucial in future CARs development.

Immunotherapy has been successfully applied in treating various types of cancers. It also has the potential to treat pancreatic cancer. Even though PDAC does not have a good response to many single immune therapeutic agents, such as

immune checkpoint inhibitors, combination therapy opens new possibilities. Therefore, more preclinical and clinical studies are needed to further identify better combination immunotherapy for PDAC.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

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Research Article

EGCG Maintains Th1/Th2 Balance and Mitigates Ulcerative Colitis Induced by Dextran Sulfate Sodium through TLR4/MyD88/NF-κB Signaling Pathway in Rats

Xue Bing,[1] Liu Xuelei,[2] Dong Wanwei,[3] Liang Linlang,[1] and Chen Keyan[3]

[1]*Department of Endocrinology, General Hospital of Shenyang Military Area Command, No. 83 Wenhua Road, Shenyang, Liaoning 110016, China*
[2]*Department of Laboratory, General Hospital of Shenyang Military Area Command, No. 83 Wenhua Road, Shenyang, Liaoning 110016, China*
[3]*Department of Laboratory Animal Science, China Medical University, No. 77 Puhe Road, Shenyang North New Area, Shenyang, Liaoning 110122, China*

Correspondence should be addressed to Liang Linlang; liangllllj@sina.com and Chen Keyan; kychen@cmu.edu.cn

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Objective. To observe the protective effect of epigallocatechin gallate (EGCG) on dextran sulfate sodium- (DSS-) induced ulcerative colitis in rats and to explore the roles of TLR4/MyD88/NF-κB signaling pathway. *Methods.* Rat models of ulcerative colitis were established by giving DSS. EGCG (50 mg/kg/d) was given to assess disease activity index. HE staining was applied to observe histological changes. ELISA and qPCR detected the expression of inflammatory factors. Flow cytometry was used to measure the percentage of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}4^+$ in the spleen and colon. TLR4 antagonist E5564 was given in each group. Flow cytometry was utilized to detect $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}4^+$ cells. Immunohistochemistry, qPCR, and western blot assay were applied to measure the expression of TLR4, MyD88, and NF-κB. *Results.* EGCG improved the intestinal mucosal injury in rats, inhibited production of inflammatory factors, maintained the balance of Th1/Th2, and reduced the expression of TLR4, MyD88, and NF-κB. After TLR4 antagonism, the protective effect of EGCG on intestinal mucosal injury was weakened in rats with ulcerative colitis, and the expressions of inflammatory factors were upregulated. *Conclusion.* EGCG can inhibit the intestinal inflammatory response by reducing the severity of ulcerative colitis and maintaining the Th1/Th2 balance through the TLR4/MyD88/NF-κB signaling pathway.

1. Introduction

Ulcerative colitis (UC) is a refractory inflammatory disease of the large intestine [1], but the pathogenesis of UC is not yet clear. Currently, the treatment of UC includes surgical treatment and drug therapy. In addition to the routinely used corticosteroids and aminosalicylic acid [2], many new drugs including anti-tumor necrosis factor antibody are also widely used in clinical practice [3, 4]. Nevertheless, UC often relapses in a certain period of time after treatment, which brings serious economic and mental distress to patients and their families [5]. Therefore, understanding the pathogenesis of UC and identifying medications to effectively treat UC are in urgent need.

T cells occupy an important place in the immune system [6, 7]. Experiments have shown that local $CD4^+$ T cells infiltrate and exhibit abnormal functional status in inflammatory bowel disease [8]. $CD4^+$ T cells are divided into Th1 and Th2 cells according to their secretion of cytokines [9]. Under normal conditions, Th1 and Th2 in the body are in dynamic balance and regulate each other, so as to maintain the stability of the environment in the body. If Th1 and Th2 are out of balance, inflammation occurs in the tissues and organs and causes disease. It was found that the $CD4^+$ T cells in UC mice produced large amounts of IL-4 and IL-5, and the single IFN-γ was normal or decreased [10], suggesting that the occurrence and development of UC were strongly associated with the imbalance of Th1 and Th2 cells.

Toll-like receptors (TLRs) are transmembrane protein family receptors that play a key role in nonspecific or innate immune defense [11]. TLR4 is a key element in the TLRs family. A previous study confirmed that, after external stimulation, the organism initiates the innate immune response, upregulates TLR4 expression, and activates NF-κB via MyD88 dependent signaling pathway, thereby resulting in severe abnormality in intestinal mucosal epithelium [12]. Abnormal activation of TLR4/MyD88/NF-κB signaling pathway in colonic mucosa of UC patients causes persistent aggravation of intestinal inflammation and becomes targets for drug therapy in UC patients [13].

Epigallocatechin gallate (EGCG) is the most abundant catechin in tea and is the main component of the biological activities of tea polyphenols [14]. Numerous studies have verified that EGCG has antioxidant and anti-inflammatory effects in cardiovascular diseases and lung, liver, and kidney diseases and has a protective effect in a variety of animal models of acute and chronic kidney disease [15, 16]. Therefore, this study sought to investigate the protective effect of EGCG on UC and to explore the possible molecular mechanism.

2. Materials and Methods

2.1. Experimental Animals and Group Assignment. Forty specific-pathogen-free male Sprague-Dawley rats weighing 260–289 g were purchased from Department of Laboratory Animals, China Medical University, China. This experiment was approved by the Institutional Animal Care and Use Committee of China Medical University (IACUC number 2015048). All rats were randomly assigned to sham surgery group (sham group; $n = 10$), DSS-induced UC group (UC group; $n = 10$), UC + EGCG group (EGCG group; $n = 10$), and UC + EGCG + TLR4 inhibitor E5564 group (TLR4 group; $n = 10$).

2.2. UC Models. Rat models of DSS-induced UC were established in accordance with a previous study [17]. The rats were allowed free access to solution containing 5% DSS (molecular weight 5000) for 7 days and then given distilled water for 14 days. After model establishment, the rats were intraperitoneally injected with EGCG 50 mg/kg/d, for 10 consecutive days.

2.3. Disease Activity Index (DAI). According to body weight, stool, and blood in the stool, the rats were scored. Score 0 indicates no weight loss, normal stool, and no blood in the stool; score 1 indicates 1%–5% weight loss, loose stools, and fecal occult blood; score 2 indicates 5%–10% weight loss, loose stools, and fecal occult blood; score 3 indicates 10%–15% weight loss, watery stool, and gross blood stool; score 4 indicates more than 15% weight loss, watery stool, and gross blood stool.

2.4. Severity of Colonic Mucosal Injury. In accordance with Luketal's standards, score 0 indicates normal and no injury; score 1 indicates hyperemia and no ulcer; score 2 indicates hyperemia, bowel wall thickening, and no ulcer; score 3 indicates one small ulcer focus in 0-1 cm diameter; score 4 indicates big ulcer focus in 1-2 cm diameter and no bowel canal adhesion to the surrounding organs; score 5 indicates ulcer focus in 1-2 cm diameter, bowel thickening, and severe adhesion to adjacent organs.

2.5. Hematoxylin-Eosin Staining. All rats were sacrificed after EGCG withdrawal; rat colonic mucosa was fixed in 10% formaldehyde, decalcified, dehydrated, permeabilized, embedded in wax, and sliced into 5 μm thick sections with a microtome. Sections were dewaxed with xylene, hydrated with absolute ethanol, and mounted with hematoxylin and eosin. Histological changes were observed under a microscope.

2.6. Enzyme Linked Immunosorbent Assay (ELISA). ELISA kit (Cloud-Clone Corp., USA) was used to measure the changes in IL-2, IFN-γ, IL-4, and IL-10 in rat serum and tissue in each group in strict accordance with the instruction. Standard preparation (50 μl) was added in the first and second wells, followed by multiple proportion dilution. Forty μl sample diluent and 10 μl sample (diluent : sample = 4 : 1) were added in each detected well and incubated at 37°C for 30 minutes. After washes with washing liquid for five times, secondary antibody (50 μl) was added in each well and incubated at 37°C for 30 minutes. Chromogenic agents A and B (50 μl for each) were added in each well and incubated at 37°C in the dark for 15 minutes. Termination solution (50 μl) was added in each well. Microplate reader (Bio-Rad, USA) was used for detection. Standard curve was drawn and sample concentration was calculated.

2.7. Flow Cytometry for Determining $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}4^+$ Lymphocytes in the Spleen. Mononuclear cells were isolated from the colon of rats. After counting, cells were incubated in a 5% CO_2 incubator at 37°C for 24 hours. Nineteen hours later, cells in each well were treated with PMA (Sigma, P1585), ionomycin (Sigma, I-0634), and Brefeldin A (BD, 555029) to reach a final concentration of 10 ng/ml, 0.5 μg/ml, and 1 μl/ml. After stimulation, cells were placed in EP tube, incubated with FITC-anti-CD4 at room temperature in the dark for 30 minutes, washed with cell staining buffer, mixed with 500 μl fixation/permeabilization solution (BD, 555028), and incubated at room temperature in the dark for 45 minutes. After being washed with 1x BD perm/wash buffer, cells were incubated with PE-anti-IL-4 and APC-anti-IFN-γ at room temperature in the dark for 45 minutes, washed with 1x BD perm/wash buffer, and resuspended with 300 μl flow washing liquid. Samples were measured with flow cytometry and analyzed using FlowJo software.

2.8. Immunohistochemical Staining. Approximately 1 cm^3 block of rat colon was fixed in 4% paraformaldehyde for 30–60 minutes and washed twice with PBS, each for 2 minutes. Tissue was dehydrated at 50°C, embedded in paraffin, and sliced into 4 um thick sections. The sections were placed on a slide and received treatment for adhesions. All samples were dewaxed, hydrated, treated with 3% H_2O_2 to deactivate endogenous enzyme, and immersed in 0.01 mol/L

Table 1: qRT-PCR using gene primers.

Gene	Primer ($5' \rightarrow 3'$)
TLR4	Forward: TGAATCCCTGCATAGAGGTA
	Reverse: GACCGTTCTGTCATGGAAGG
MyD88	Forward: TACAAAGCAATGAAGAAGGA
	Reverse: TTGCATGAGGTAGTGGCACG
NF-κB	Forward: CAGCCTGGTGGGCAAGCACT
	Reverse: GAAGGATTTGGGGACTTT
β-Actin	Forward: TCCTCACTGAGGCCCCGC
	Reverse: CTGCCCCATGCCATTCTC

citrate for antigen retrieval. These sections were blocked with 5% bovine serum albumin for 20 minutes, incubated with primary antibody TLR4 (1 : 500), MyD88 (1 : 1000), and NF-κB (1 : 1000) at 37°C for 1 hour, and washed two or three times with PBS. Subsequently, the sections were incubated with biotinylated goat anti-mouse IgG at 20–37°C for 20 minutes, washed four times with PBS, each for 5 minutes, visualized with 3,3′-diaminobenzidine, washed with distilled water, dehydrated, permeabilized, mounted with resin, and observed with a microscope.

2.9. Real-Time Polymerase Chain Reaction (PCR). The colon was triturated and treated with Trizol reagent. RNA was precipitated, dried, and dissolved with 50 μl DEPC-treated water. According to the manufacturer's instruction of RevertAid^{TM} First Strand cDNA Synthesis Kit (K1621, Thermo), the first strand of complementary DNA (cDNA) was produced by reverse transcription. SYBR Green (204054, Qiagen) was used for real-time fluorescence quantitative PCR. The relative gene expression data were analyzed with the 2^(−Delta Delta CT) method. The primers were designed and synthesized with Sangon Biotech (Shanghai) Co., Ltd., China. The primers used for real-time PCR were listed in Table 1.

2.10. Western Blot Assay. The colonic tissue was treated with precooled lysate and centrifuged at 12000 rpm for 30 minutes. Total protein (the supernatant) was extracted and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred onto the membrane by the semidry method. The membrane was blocked with confining liquid for 2 hours, incubated with primary antibodies TLR4 (1 : 500), MyD88 (1 : 1000), NF-κB (1 : 1000), and GAPDH (1 : 2000) (Abcam, USA) at 4°C overnight, washed three times with TBST, and then incubated with secondary antibody for 1 hour. After four washes with TBST, the membrane was visualized with enhanced chemiluminescence reagent. Images were obtained using a gel imaging system. Gray value was read with Quantity One software.

2.11. Statistical Analysis. Data were analyzed using SPSS 19.0 software and expressed as mean ± SD. The difference between samples was compared using group t-test. The difference among groups was compared using one-way analysis of variance. For heterogeneity of variance, Tamhane-t or Dunnett-$T3$ test was used. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. EGCG Lessens Colonic Mucosal Injury in UC Rats. Compared with the sham group, DAI score was significantly higher in the UC group ($p < 0.05$) (Figure 1(b)). Compared with the UC group, DAI score was significantly decreased in the EGCG group ($p < 0.05$). In the sham group, the colonic surface of rats was smooth with no bleeding or ulceration. Intestinal hemorrhage, ulcer, and intestinal adhesions were seen in UC rats. After EGCG intervention, small ulcer foci were visible in the colonic tissue (Figure 1(a)). It was found that the score of colonic mucosal injury was significantly increased in the UC group and diminished after EGCG intervention (Figure 1(c)). Hematoxylin-eosin staining results demonstrated that mucosal epithelial cell degeneration/necrosis and inflammatory cell infiltration were observed in the UC group (Figure 1(d)). Mild epithelial cell degeneration/necrosis and small amounts of inflammatory cells were distinct in the EGCG group. Histomorphological score of mucosa in rats was significantly lower in the UC group than in the sham group. After intervention with TLR4 inhibitor, scores and HE staining were not significantly different compared with the UC group ($p > 0.05$).

3.2. EGCG Suppresses Inflammatory Response in UC Rats. Serum IL-2, IFN-γ, IL-4, and IL-10 levels were measured in UC rats with ELISA. Compared with the sham group, serum IL-2 and IFN-γ levels were higher ($p < 0.05$), but IL-4 and IL-10 levels were lower in the UC group ($p < 0.05$). After EGCG intervention, serum IL-2 and IFN-γ levels were decreased ($p < 0.05$), but IL-4 and IL-10 levels were increased ($p < 0.05$) (Figure 2). All data suggested that EGCG could improve inflammatory response in UC rats.

3.3. EGCG Improves the Th1/Th2 Balance in UC Rats. To identify that EGCG can improve the Th1/Th2 balance, flow cytometry was utilized to analyze Th1 cells and Th2 cells in the rat colon. Data suggested that, compared with the sham group, IFN-γ expression in $CD4^+$ T cells was significantly increased ($p < 0.05$) (Figure 3(a)), but IL-4 expression in $CD4^+$ T cells was significantly reduced ($p < 0.05$) (Figure 3(b)). After EGCG intervention, Th1 cells and Th2 cells were approximately balanced. Compared with the EGCG group, the number of $CD4^+$IFN-γ^+ cells was higher in the rat colon ($p > 0.05$), but the number of $CD4^+$IL-4^+ cells was lower ($p > 0.05$) in the EGCG group after inhibiting TLR4 expression. These findings verified that EGCG could improve the Th1/Th2 balance in UC rats probably through TLR4/MyD88/NF-κB signaling pathway.

3.4. EGCG Diminishes the Expression Levels of TLR4/MyD88/NF-κB Pathway-Related Proteins in the Colon of UC Rats. Compared with the sham group, the expression levels of TLR4, MyD88, and NF-κB proteins were significantly higher in the UC group ($p < 0.05$). After EGCG intervention, TLR4, MyD88, and NF-κB protein expression was significantly decreased ($p < 0.05$) (Figure 4(a)). After adding TLR4 inhibitor E5564, the protective effect of EGCG was weakened.

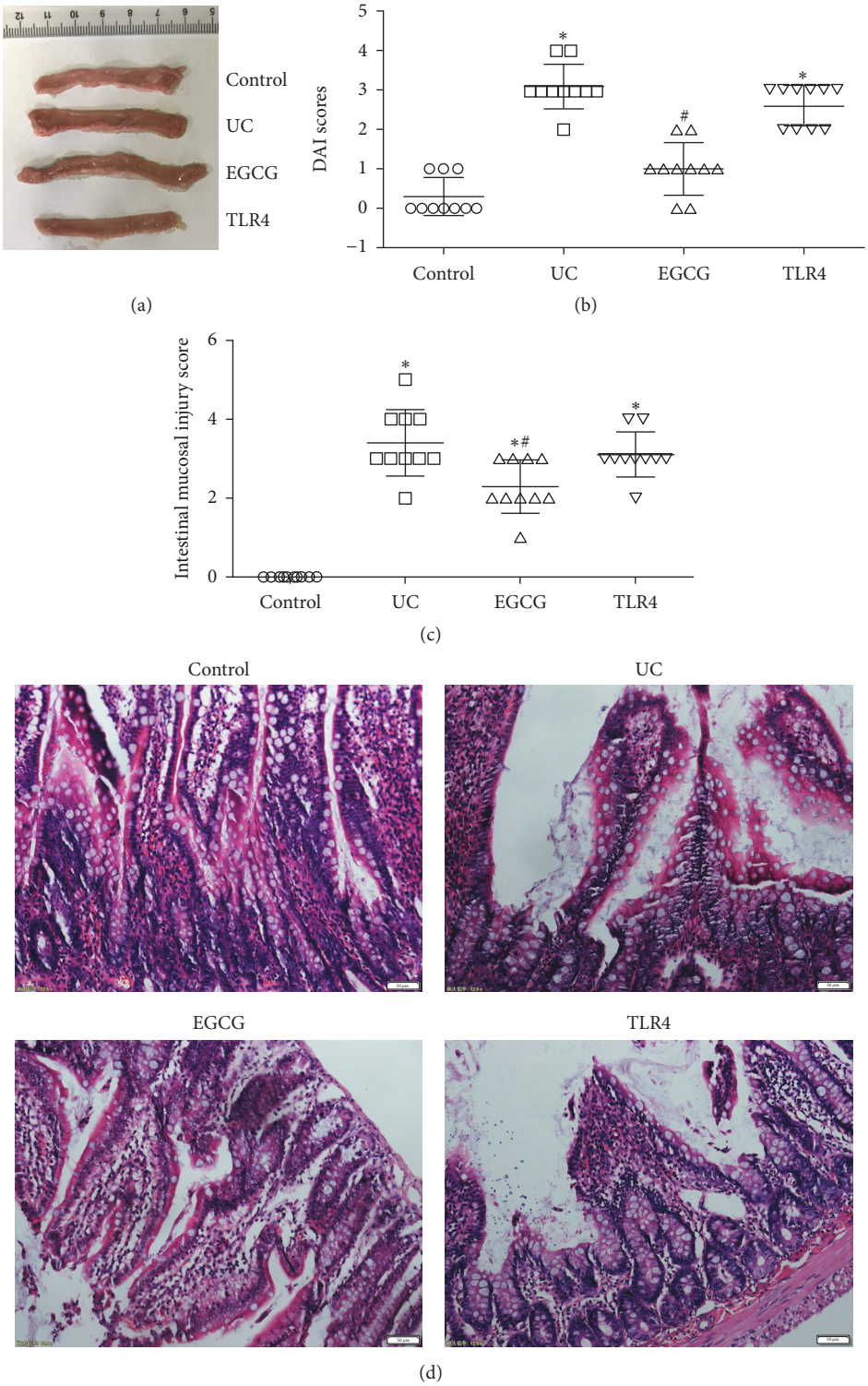

Figure 1: The UC model was established; the rats were intraperitoneally injected with EGCG 50 mg/kg/d for 10 consecutive days. The rat colonic tissue was collected, and colonic mucosal injury was observed (a). The rats were scored based on body weight, stool, and blood in the stool. (c) In accordance with Luketal's standards, colonic mucosal injury was scored. (d) The rat colonic tissue was collected, and the colonic pathological changes were observed after the HE staining (b). Data were collected from the control group, UC group, EGCG group, and TLR4 group ($n = 10$). Compared with control group, $^{*}p < 0.05$. Compared with UC group, $^{\#}p < 0.05$.

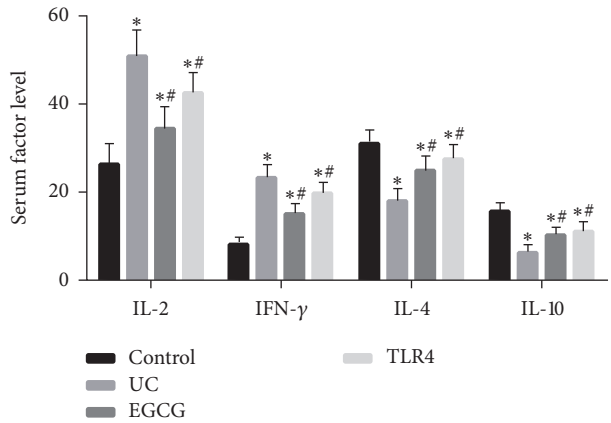

Figure 2: Peripheral blood was collected from the control group, UC group, EGCG group, and TLR4 group ($n = 10$), sera were isolated, and ELISA was used to detect the IL-2, IFN-γ, IL-4, and IL-10 levels. Compared with control group, $^{*}p < 0.05$. Compared with UC group, $^{\#}p < 0.05$.

TLR4, MyD88, and NF-κB mRNA expression (Figure 4(b)) and immunohistochemistry (Figure 4(c)) supported this conclusion. All data verified that EGCG improved Th1/Th2 balance and suppressed colonic mucosal injury in UC rats through TLR4/MyD88/NF-κB signaling pathway.

4. Discussion

UC is a chronic intestinal immune disease. UC can be alleviated by drug therapy, but it is easy to relapse and increase the risk of cancerization of colitis. This study established UC rat models and found that EGCG mitigated DSS-induced colonic mucosal injury, reduced epithelial cell degeneration/necrosis, and decreased inflammatory factor and apoptosis protein expression. Furthermore, EGCG could maintain Th1/Th2 balance and diminish DSS-induced MyD88 and NF-κB protein expression in the colonic mucosa TLR4 pathway. Our results indicated that EGCG had protective effect on UC by modulating TLR4/MyD88/NF-κB pathway.

EGCG is a major component in tea. EGCG has been shown to suppress inflammation, oxidation, tumor, and apoptosis. Katiyar and Mukhtar [18] found that EGCG significantly inhibited the activity of UVB-induced antioxidant enzymes and inhibited the oxidative stress induced by UVB. Brückner et al. [19] verified that EGCG suppressed the activity of oxygen free radicals in neutrophils [19] and exerted anti-inflammatory effects by scavenging free radicals and oxidants and inhibiting oxidative stress [20, 21]. We found that EGCG lessened the extent of DSS-induced colonic mucosal injury and epithelial cell degeneration/necrosis, and our results indicated that EGCG has protective effects on colon injury induced by DSS.

$CD4^{+}$ T cells induced by IFN-γ and IL-2 differentiate into Th1 cells and mediate cell immunity [22]. $CD4^{+}$ T cells induced by IL-4 differentiate into Th2 cells, secrete IL-4, IL-5, and IL-10 to activate B cells, and mediate humoral immunity and hypersensitivity [9]. The Th1/Th2 imbalance is associated with tumor immune escape, microbial infections such as bacteria and viruses, also involved in allergic diseases, autoimmune diseases, and transplant rejection, and plays an important role in mediating UC development [23–25]. To maintain intestinal balance, the normal intestinal mucosal immune system must maintain a balance between proinflammatory cytokines and anti-inflammatory cytokines or cytokine regulatory network. Once the balance is broken, it may cause excessive proliferation of the effector cells or regulate the decline of cellular function and aggravate the inflammatory response of the mucosa. Our study demonstrated that serum IL-2 and IFN-γ levels increased, but IL-4 and IL-10 levels decreased in UC rats, which suggested that it may be related to Th1/Th2 balance. Th1 cells and Th2 cells in the rat spleen were further analyzed by flow cytometry. Results demonstrated that the percentage of Th1 cells increased, but the percentage of Th2 cells diminished in the spleen of UC rats. After EGCG intervention, the percentage of Th1 cells decreased, but the percentage of Th2 cells increased, suggesting that EGCG improved the Th1/Th2 balance in rat intestinal mucosa, and promoted self-repair of intestinal mucosa.

TLR is an important component of innate immune recognition receptors and plays an important role in the identification of microorganisms in hospitals. TLR activates innate immune response, causes cytokine release, upregulates the expression of costimulatory molecules, and provides necessary activation signals for acquired immune response by identifying lipopolysaccharide, lipoproteins, and genetic material nucleic acids of microorganism in hospitals [26]. TLR4, an important component of TLR, can mediate the high reactivity of intestinal epithelial cells on cell wall components, activate NF-κB, cause effector cells to secrete cytokines such as tumor necrosis factor-α, and play an important role in inflammatory response. Our results showed that TLR4, MyD88, and NF-κB expression was high in UC rats. After EGCG intervention, TLR4, MyD88, and NF-κB protein expressions were remarkably decreased. EGCG may exert the

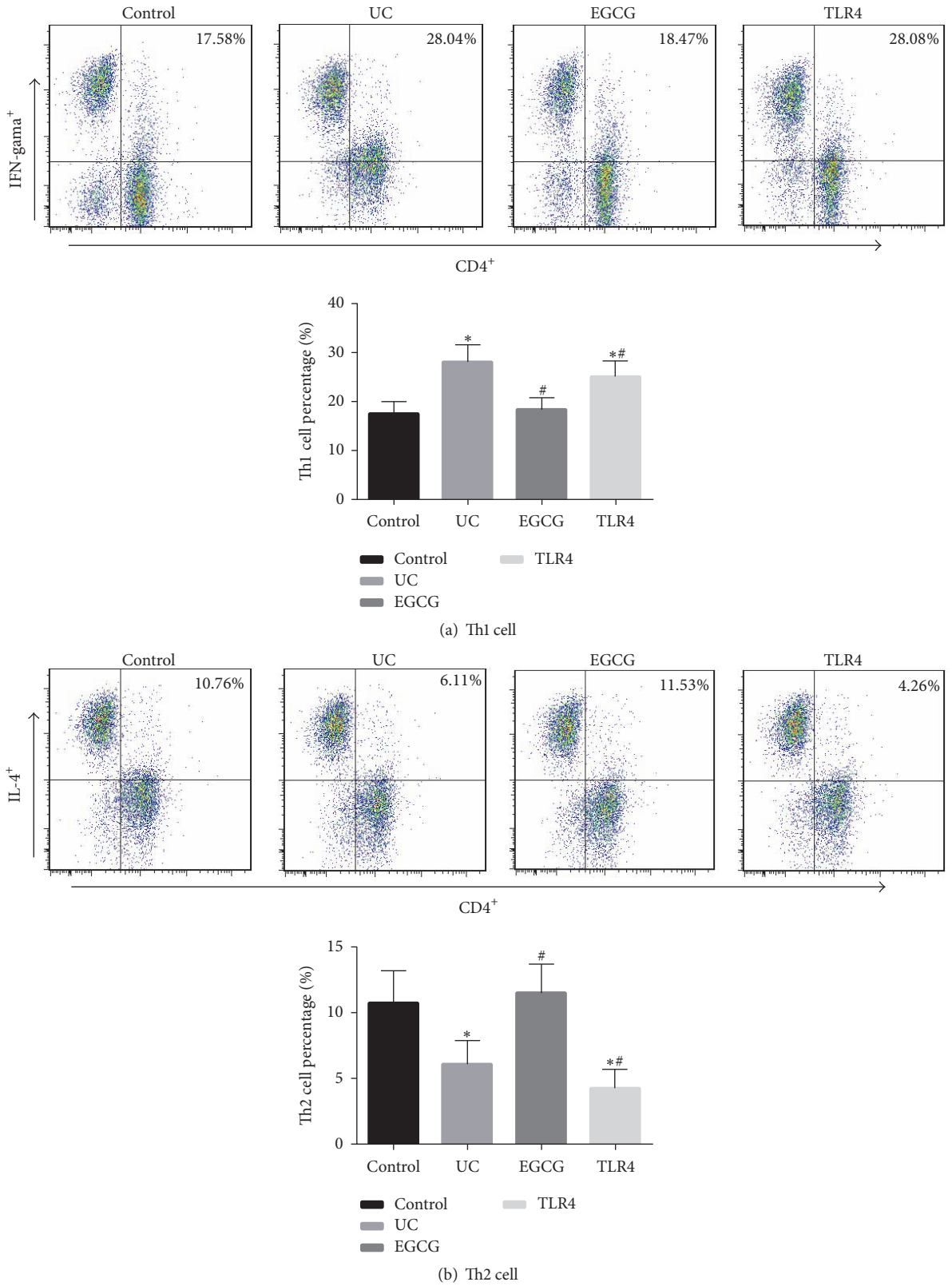

Figure 3: The colonic tissue was collected from UC group, EGCG group, and TLR4 group ($n = 10$), and PMBA was isolated from the colonic tissue. Flow cytometry was used to detect the $CD4^+IFN\text{-}\gamma^+$ (Th1) cell percentage (a). Different cell subsets were distinguished according to different cell labels. Flow cytometry was used to detect the $CD4^+IL\text{-}4^+$ (Th2) cell percentage (b). Different cell subsets were distinguished according to different cell labels. Compared with control group, $^*p < 0.05$. Compared with UC group, $^\#p < 0.05$.

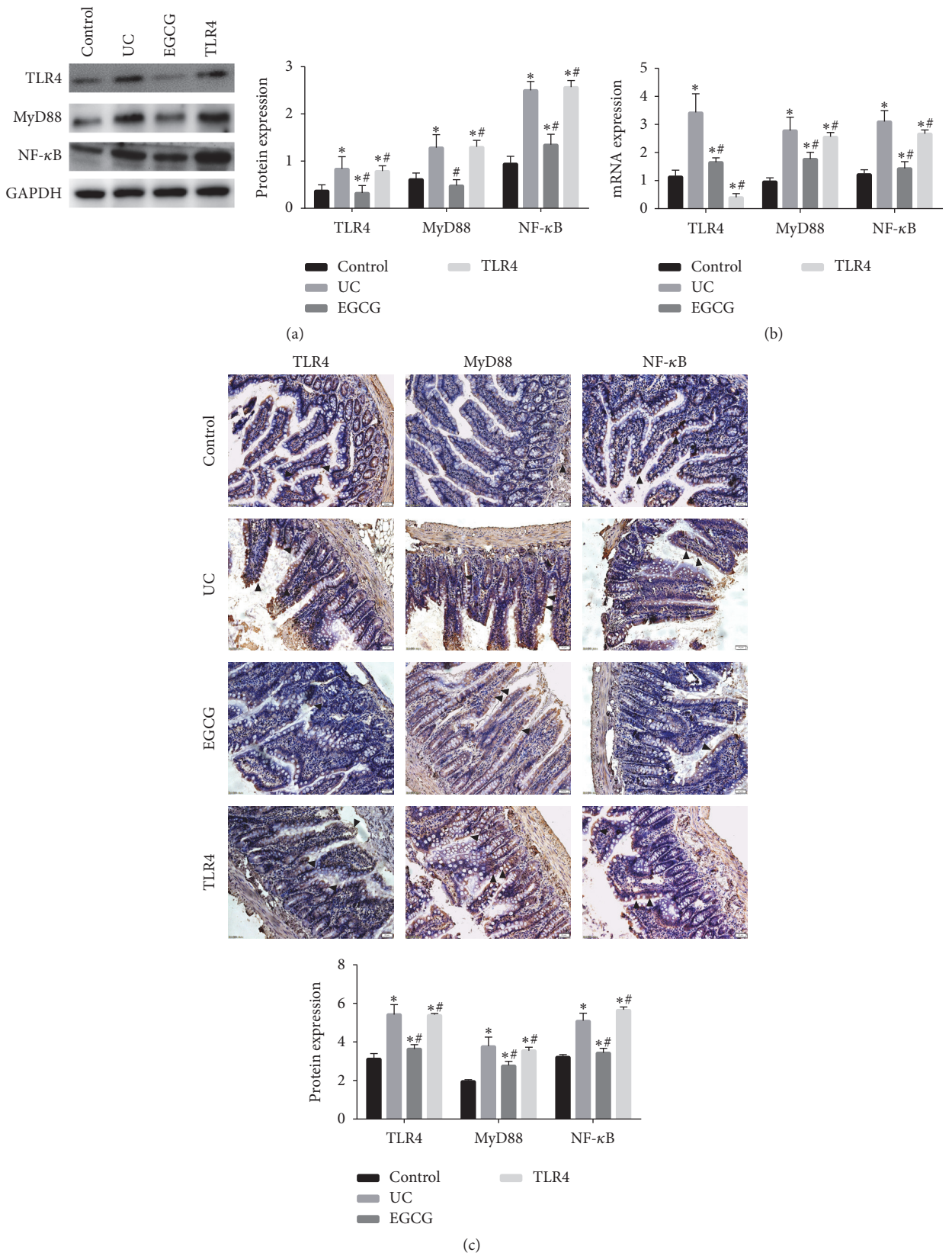

Figure 4: After establishing the UC model, EGCG and TLR4 inhibitor E5564 intervened. The colonic tissue was collected and the total protein was extracted. Western blot assay was used to detect the expressions of TLR4, MyD88, and NF-κB protein expressions (a). The 18 colonic tissues were collected, the total RNAs were extracted, and the first strand of DNA was synthesized by reverse transcription. QRT-PCR was used to detect the expressions of TLR4, MyD88, and NF-κB at mRNA level (b). The colon tissue was collected, fixed in 10% formaldehyde, embedded, and sliced into sections. Immunohistochemistry was used to detect the TLR4, MyD88, and NF-κB expressions (c). Compared with control group, $^{*}p < 0.05$. Compared with UC group, $^{\#}p < 0.05$.

anti-inflammatory effect and regulatory effect on Th1/Th2 balance through TLR4/MyD88/NF-κB signaling pathway. To further verify above results, we used TLR4 inhibitor E5564 to block the pathway and found that protective effect of EGCG disappeared; Th1/Th2 balance was broken; and the expression of TLR4, MyD88, and NF-κB significantly decreased. These findings further confirmed that EGCG maintained Th1/Th2 balance and suppressed immunoinflammatory response in colonic tissue through TLR4/MyD88/NF-κB signaling pathway.

In summary, EGCG can inhibit the intestinal immunoinflammatory response, reduce the severity of UC, and maintain the Th1/Th2 balance through the TLR4/MyD88/NF-κB signaling pathway. This lays theoretical foundation for developing target therapy for UC.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this article.

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