

## RESEARCH ARTICLE

# Effective Response of the Peritoneum Microenvironment to Peritoneal and Systemic Metastasis from Colorectal Carcinoma

Min Yu, Zhi-Min Niu, Yu-Quan Wei\*

### Abstract

We here document discovery of a new and simple model of tumor seeding involving the mouse peritoneum. Irradiated tumor cells administered by i.p. injection provided effective vaccination against peritoneal carcinomatosis and distal metastasis with colorectal carcinomas. In flow cytometric analysis, CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, macrophages and myeloid-derived suppressor cells (MDSCs), which are easy to obtain in the peritoneal cavity, were revealed to have significant differences between immunized and non-immunized mice and these contributed to antitumor responses. We also observed that both serum and peritoneal lavage fluid harvested from immunized mice showed the presence of CT26-specific autoantibodies. In addition, increase in level of TGF- $\beta$ 1 and IL-10 in serum but a decrease of TGF- $\beta$ 1 in peritoneum was found. Taken together, these findings may provide a new vaccine strategy for the prevention of peritoneal and even systemic metastasis of carcinomas through induction of an autoimmune response in the peritoneum.

**Keywords:** Peritoneum - immune response - colorectal carcinoma - metastasis

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### Introduction

The leading cause of cancer related mortality is metastatic spread of tumor cells to vital organs. Peritoneal metastasis and carcinomatosis is the most common secondary cancerous disease to affect the peritoneal cavity for patients with cancers such as gastric, hepatic, ovarian and colorectal carcinoma, implying poor prognosis. Standard therapy used to be a cytoreductive surgery in combination with adjuvant chemotherapy. To improve the therapeutic outcome, developing a strong antitumor immunization before metastasis is therefore essential for metastatic prevention and therapeutic strategies assisting for local tumor.

Metastasis is a multistep process comprising intravasation, survival in the circulation, extravasation, and colonization of distant tissues (Chambers et al., 2002; Joyce et al., 2009), and the peritoneal dissemination and carcinomatosis processes consist of local inflammatory reaction, changes in the expression of adhesion molecules, proteolytic enzymes, and growth factors, destruction of peritoneum, attachment of carcinoma cells and colonization. The peritoneal cavity is proved to be home to a specific type of macrophage (Cailhier et al., 2005; Liu et al., 2006), rich effector memory T lymphocytes (Roberts et al., 2009), a resident population of self-renewing B lymphocytes (Hardy et al., 1994; Paciorkowski et al., 2000), a new subset of tissue-resident NK cells (Gonzaga

et al., 2011) and a newly described population found in fat-associated lymphoid clusters (Moro et al., 2010). These cell populations play a critical role in initiating acute peritoneal inflammation and host protection. Not much is known, however, about the alteration in quantity and function of the resident cell populations to produce autoimmune response for tumor in peritoneum. We speculate that these cells form part of the first line of defense against invading pathogens and make peritoneal to be a microenvironment, apart from subcutaneous, which could develop strong autoimmunization response against tumor metastasis in peritoneum and even systemic antitumor effect.

To test this concept, we describe the accumulation and quantification of T lymphocytes, B lymphocytes, macrophages, and (myeloid-derived suppressor cells) MDSCs by the primary tumor after peritoneal immunization in mouse cancer models, and observe the anti-tumor effect by i.p. immunization. In the study, the irradiated tumor cells as vaccine by i.p. injection is shown to be effective in both peritoneal and systemic antitumor immunity, in which autoreactive immunity may be directed against the tumor cells in both humoral and cellular immunity. These observations may provide a new vaccine strategy for the prevention of peritoneal and systemic metastasis of carcinomas through the induction of the autoimmune response in peritoneum, in addition to subcutaneous immunization.

## Materials and Methods

### Mice and cell lines

Five- to six-week-old female BALB/c and C57BL/6 mice were purchased from Vital River and housed in microisolators until the age of 8 weeks before using them in experiments. CT26 colorectal carcinoma cell line, B16 melanoma cell line, and EG7 lymphoma cell line are used in this study. Colorectal cancer and lymphoma cells were maintained in RPMI medium 1640 with 10% FBS. Melanoma cells were grown in DMEM supplement with 10% FBS. CT26 colorectal cancer models were established in BALB/c mice. B16 melanoma and EG7 lymphoma models were established in C57BL/6.

### Immunization and peritoneal lavage fluid harvesting

Mice were immunized by peritoneal injection of irradiated tumor cells or saline alone (nonimmunized mice) for 3 times at day 0, 14, and 21, and challenged with tumor cells i.p. at day 28. According to the time point, the peritoneal cavity was lavaged with 5ml PBS for 2 times after sacrificing the mice immediately. The 10ml fluid was next centrifuged at 2000 rpm for 4 minutes and kept as single-cell suspensions in 1ml PBS for flow cytometric analysis and lavage supernatant for antibody and cytokine production evaluation, respectively.

### Flow cytometric analysis

Peritoneal lavage samples from groups of immunized and nonimmunized mice were pooled, spun, and resuspended in 1 ml of sterile PBS. Expression levels of Gr1, CD11b, F4/80, CD4, CD8, CD11c, MHC-II and CD69 on these lavage cells labeled with fluorescence-conjugated antibodies (BD PharMingen) and isotype-matched IgG controls were analyzed by flow cytometry (BD FACS Calibur). To assay the presence of CT26-specific autoantibodies, tumor cells were stained by an indirect method (Wei et al., 1996; Wei et al., 2000) using 1:50 to 3000 diluted serum or peritoneal lavage supernatant, and then FITC-goat anti-mouse IgG, IgM, and IgA (Sigma-Aldrich).

### Cytokine response

Mice were immunized for 3 times arranged at day 0, 14, and 21 and underwent peritoneal lavage before or following the i.p. injection of CT26 tumor cells. Lavage fluid was centrifuged, aliquoted, and stored at  $-80^{\circ}\text{C}$  until analyzed by ELISA for cytokine. Serum was also harvested at the same time from immunized and control group and evaluated for the production of TGF- $\beta$  and IL-10 by ELISA (RB).

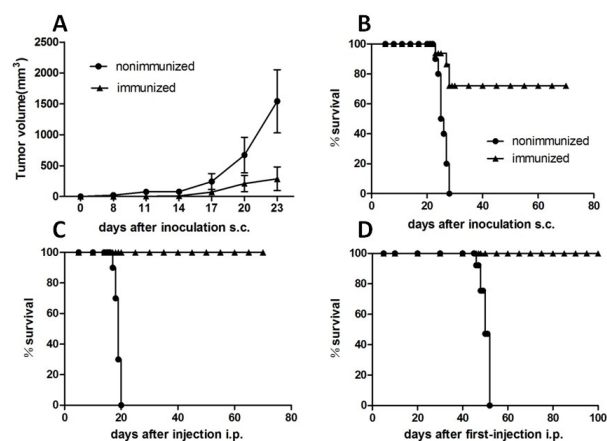
### Statistical analysis

For comparison of individual time points, ANOVA and an unpaired Student t test were used for our data analysis statistically (Sauter et al., 2000). Statistical significance was determined by the log-rank test (Peto et al., 1972). Error bars were from standard deviations, and p values less than 0.05 were considered significant. Survival curves were constructed according to the Kaplan-Meier method (Kaplan et al., 1958).

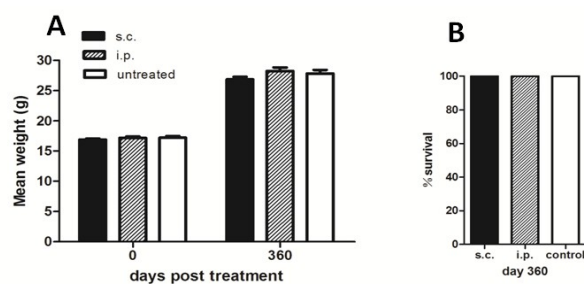
## Results

### Effective antitumor immunity of peritoneal cavity

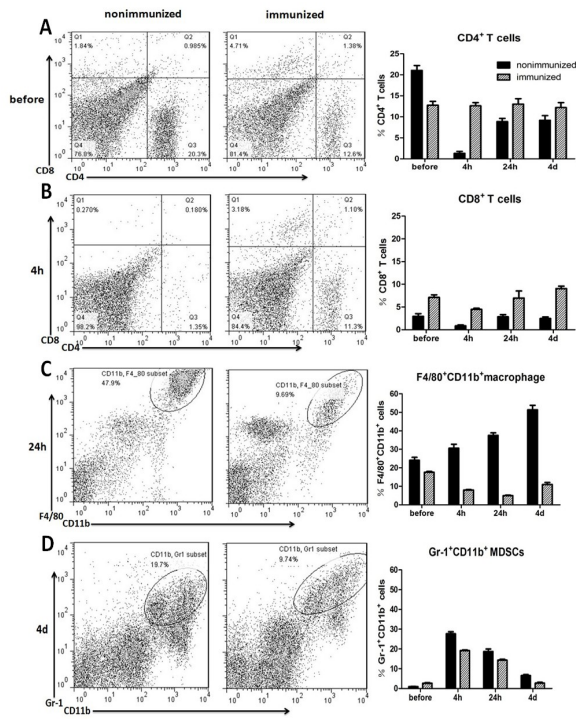
To investigate the protective antitumor immunity, we immunized mice (10 mice in each group) by the s.c. or i.p. injection of irradiated CT26 colorectal carcinoma or saline alone (nonimmunized mice) for 3 times arranged at day 0, 14, and 21, and then challenged mice with CT26 cells at day 28 s.c. or i.p., respectively. As shown in Figure 1, tumors grew progressively in all nonimmunized mice by s.c. inoculation (saline alone) (Figure 1A) and the survival



**Figure 1. Preventive Antitumor Immunity of Irradiated CT26 Cells.** Mice (10 mice in each group) were immunized s.c. with  $1 \times 10^6$  irradiated CT26 colorectal carcinoma or nonimmunized (saline alone) for 3 times arranged at day 0, 14, and 21. Mice were then challenged with  $5 \times 10^5$  live CT26 cells (A and B) s.c. 1 wk after the third immunization. A and B, Tumor volume (mm<sup>3</sup>) and percentage of survival in mice immunized and controls was shown. C and D, Percentage survival of mice treated. The mice were injected peritoneal the same way as immunization s.c. (C) and rechallenged with CT26 cells 30 days after the first injection. The immunized mice have been followed for >5 months. The survival rate of the mice was 40% at day 150 for immunized s.c. and 100% immunized i.p., respectively



**Figure 2. The Effects of the Irradiated CT26 Cell Vaccination on Body Weight and Life Span.** Normal BALB/c mice (8 wk old, 10 mice/group) were immunized i.p. with  $1 \times 10^6$  irradiated CT26 cells s.c. or i.p., or nonimmunized (saline alone) for 3 times at day 0, 14, and 21 as described in Fig. 1, but mice were not challenged with tumor cells. Mice in each group have been investigated for the potential adverse toxicity for at least 360 days. There was no weight loss in the mice immunized comparing with the nonimmunized group ( $p < 0.05$ ) (A). The results are expressed as mean weight and error bars represent  $\pm$  SEM. In addition, there was no decrease on life span in the mice immunized compared with the control group (B). Data represent the percentage of survival at day 360 after the vaccination

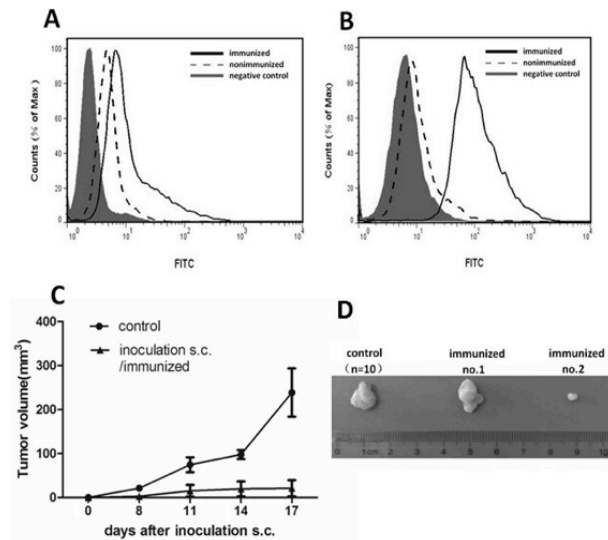


**Figure 3. Changes of Cell Population in Peritoneal Following Engraftment in Two Groups.** Mice were immunized i.p. as described before and challenged with  $5 \times 10^5$  live CT26 cells i.p.. The diversification of the immune cell composition in peritoneal was analysed at different time point (before the injection, 4h, 24h, and 4d later) for immunized and nonimmunized group respectively. A, B, C and D, Flow cytometry analysis for CD4<sup>+</sup> CD8<sup>+</sup> T cells, F4/80<sup>+</sup>CD11b<sup>+</sup> macrophages and Gr-1<sup>+</sup>CD11b<sup>+</sup> MDSCs in peritoneal upon tumor cell challenge of BALB/c mice over time (left panel). Quantification of cells is presented in the right panel (n = 5)

was significantly greater than that of untreated mice (Figure 1B,  $p=0.0182$ , by log-rank test). When injected i.p., the apparent protection from tumor growth was also shown in the group immunized and the mice even survived totally (Figure 1C).

The long-term immune efficacy of the irradiated tumor cell vaccine injected by i.p. was next tested. All the immunized mice survived after the first injection were rechallenged with CT26 cells 30 days later. The survival of the rechallenged mice was also significantly greater than that of the controls (Figure 1D,  $p=0.0004$ , by log-rank test). The protective effect was dose independent. Injection i.p. with a dose of  $1 \times 10^5$ ,  $2 \times 10^5$ ,  $1 \times 10^6$ ,  $5 \times 10^6$  cells did not show worse effect than that with  $5 \times 10^5$  CT26 cells used in Figure 1C. In addition, the protective effect with the peritoneal immunization of irradiated tumor cells such as B16 melanoma and EG7 lymphoma was also found, while the efficacy and survival revealed differently (60% and 100%, respectively).

The mice immunized with irradiated tumor cell vaccines s.c. or i.p. have been in particular investigated for the potential long-term toxicity. No adverse consequences in gross measures such as ruffling of fur, ascites, decreased appetite, weight loss (Figure 2A), and life-span (Figure 2B) were indicated and no pathologic changes of heart, liver, spleens, lung, kidney, etc. were found by the microscopic examination.

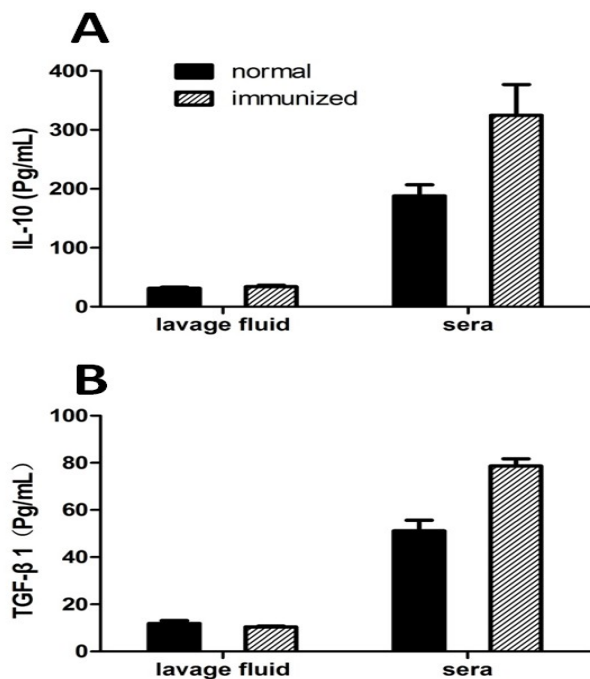


**Figure 4. Identification of Autoantibodies in Blood and Peritoneal and Their Systemic Antitumor Efficacy.** CT26 cells were stained by an indirect method using 1.5:100 (1.5  $\mu$ l/100  $\mu$ l PBS) diluted serum or 100  $\mu$ l peritoneal washing supernatant, and then FITC-goat anti-mouse IgG in flow cytometric analysis. A and B, CT26 cells both showed positive staining for serum and peritoneal lavage fluid supernatant isolated from immunized mice and negative staining for the serum isolated from the nonimmunized mice and other control groups. In addition, these cells also showed positive staining for serum isolated from the immunized mice with or without subcutaneous tumor-bearing. Mice were immunized in peritoneal and challenged with CT26 cells on day 28 s.c.. C and D, Tumor volume ( $\text{mm}^3$ ) and macroscopy of tumor nodules derived from immunized and control group on day 45 were shown. A 100% incidence in control group (n=10) was shown and tumor nodules were only turned out in two mice (no.1 and no.2, n=10)

#### Cellular immune response in peritoneum induced by the irradiated tumor cells

In an attempt to explore the possible mechanism by which the antitumor activity was induced with irradiated tumor cells, we investigated the differences of the immune cell composition in peritoneal that could influence dissemination and metastasis. We stained the cell populations at different time point (before the injection, 4h, 24h, and 4d later) for two groups, and found that a small amount of CD4<sup>+</sup> (Figure 3A) and CD8<sup>+</sup> T (Figure 3B) lymphocytes exist in the peritoneal of mice in two groups, decrease after the i.p. inoculation in nonimmunized group, but maintain nearly the same percentage in immunized group, suggesting that T cells may be required for the antitumor response. Besides, the amount of CD4<sup>+</sup>T lymphocytes (1.35%-20.3%) is always higher compared to CD8<sup>+</sup>T lymphocytes (0.45%-8.08%) in both immunized or nonimmunized mice. No differences in numbers of CD19<sup>+</sup> B lymphocytes (34.4%-39.9%) could be identified between two groups by flow cytometry. However, immunized mice displayed reduced numbers of F4/80<sup>+</sup>CD11b<sup>+</sup> macrophages (4.94%-16.6%) but a obvious increase in nonimmunized group was shown (24.9%-47.9%) (Figure 3C), suggesting that the macrophages may be no longer needed or less but more active for antitumor activity. In addition, we investigated whether myeloid cell populations are recruited to CT26





**Figure 5. Production of Cytokines in Sera and Peritoneal Lavage.** Serum and peritoneal lavage supernatant were harvested from normal (nonimmunized) and immunized mice, and tested for IL-10 (A) and TGF- $\beta$ 1 (B) by ELISA. The serum isolated from immunized mice showed the increase both in the level of IL-10 and TGF- $\beta$ 1. A weak increase in IL-10 and a decrease of TGF- $\beta$ 1 was found in the lavage supernatant from immunized group. The results are expressed as mean with error bars representing  $\pm$  SEM (n=5)

cells in peritoneal. Significant association of Gr-1<sup>+</sup>CD11b<sup>+</sup> cells (MDSCs) with the injection of CT26 colorectal tumor cells into peritoneal was observed shortly after the treatment. There is only a small amount of MDSCs in the peritoneal of immunized or nonimmunized mice (0.89%-2.05%). However, distinguished increase showed up 4h later after the inoculation (18.7%-28.4%) but decreased over time (Figure 3D). Quantification of the MDSCs revealed that the nonimmunized mice could recruit larger amount cells than immunized ones, indicating that the recruit may be inhibited and MDSCs are needed for the first inflammatory response.

#### *Autoantibodies both in sera and peritoneal and their systemic antitumor efficacy*

Mice were immunized in peritoneal three times as before. Serum and peritoneal lavage supernatant harvested from immunized mice at day 28 were assayed for the presence of CT26-specific autoantibodies in flow cytometric analysis. Tumor cells were stained with sera or peritoneal lavage supernatant isolated from immunized and nonimmunized mice. As a result, tumor cells showed the positive staining both with sera or supernatant from immunized mice, but negative staining for unimmunized mice (Figure 4A-B).

The systemic antitumor efficacy induced by irradiated tumor cell was next tested. Mice were immunized in peritoneal and then challenged with CT26 cells on day 28 s.c.. Tumor growth in all mice by s.c. inoculation was recorded (Figure 4C). Tumor nodules were only turned

out in two mice (20%) until day 17 in immunized group, while they got a 100% incidence in unimmunized mice (Figure 4D), suggesting that intraperitoneal immunization could induce an effective systemic antitumor efficacy.

#### *Cytokine response*

Serum and peritoneal lavage supernatant harvested were evaluated for the production of TGF- $\beta$ 1 and IL-10 by ELISA. As shown in Figure 5A-B, the increase in the level of TGF- $\beta$ 1 and IL-10 was found in the serum when compared with the controls. The lavage fluid supernatant from immunized mice only got a weak increase of IL-10 and a decrease of TGF- $\beta$ 1.

## Discussion

Peritoneal cavity is a microenvironment with rich resident and specific cells and surrounded by profuse arteriovenous network system. Yet its immune function has not been identified. In the present study several observations have been made concerning the immunization vaccine by i.p. injection, autoimmune response, and antitumor effect. The result, to our knowledge, first demonstrated that the microenvironment of peritoneal cavity could develop a strong peritoneal and systemic antitumor immunization effect by i.p. injection of normal irradiated tumor cell vaccines in mouse cancer models. Furthermore, our findings suggest that autoreactive immune response in peritoneum against the tumor cells may be provoked by the alteration in quantity and function of the resident cell populations such as T cells, macrophages and MDSCs, and that the antitumor activity following i.p. vaccination may be involved in both T cell effectors and autoantibodies. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells showed more stable and higher level in immunized mice. Macrophages and MDSCs found to be less in percentage. Autoantibodies in sera, peritoneum and on the tumor cells were identified. IgG subclasses were substantially increased in response to vaccine by i.p. injection. The inhibition of the *in vivo* tumor growth by s.c. inoculation was found when the vaccine was injected i.p.. These findings suggest that humoral immunity may be responsible for the antitumor activity by the vaccination.

We found in the present study that *in vivo* immunized mice have lower level of CD4<sup>+</sup> T lymphocytes and higher CD8<sup>+</sup> T lymphocytes comparing to normal mice. But these cell populations could keep the level after peritoneal tumor cells inoculation in immunized mice, while the decrease of T lymphocytes was found in nonimmunized mice. These findings suggest that T cells may be required for the antitumor response, and these suggestions were further supported by the important roles of CD4<sup>+</sup> T lymphocytes in the antitumor immunity (Bennett et al., 1997; Carbone et al., 1998; Hung et al., 1998; Ossendorp et al., 1998; Pardoll et al., 1998; Toes et al., 1999; Hu et al., 2000). Besides, it has been reported that CD4<sup>+</sup> T lymphocytes are required for the generation and maintenance of cytolytic CD8<sup>+</sup> T cells (Pardoll et al., 1998; Hu et al., 2000) and believed to be essential for the developing of both a cellular and humoral antitumor immune response (Bennett et al., 1997; Hung et al., 1998; Ossendorp et al.,

1998; Toes et al., 1999). Macrophages were revealed to have had a superior proangiogenic activity in vivo, and increased in numbers as tumors progressed (Movahedi et al., 2010). Our results were consistent with this completion in normal mice, while in immunized mice, a significant lower level of macrophages was shown as tumor growth. The decrease may be supported by the hypothesis that infiltrated macrophages which could provide an immunosuppressive microenvironment for tumor growth (Hao et al., 2012) would be inhibited in immunized environment to prevent tumor colonization and growth. The phenotype of MDSCs is GR1<sup>+</sup>CD11b<sup>+</sup> in mice, co-expression of the myeloid-cell lineage differentiation antigen cells (Kusmartsev et al., 2004), and they consist myeloid progenitor cells and immature myeloid cells (IMCs), that are immature macrophages, immature granulocytes and immature dendritic cells. It has been proved that MDSCs contribute to the negative regulation of immune responses and shown to be an important immune-evading strategy used by tumors (Gabrilovich et al., 2009; Ostrand-Rosenberg et al., 2009), with its remarkable ability to suppress T-cell responses (Gabrilovich et al., 2009). MDSCs were immediately recruited into peritoneum after tumor cells injection and decreased over time in the flow cytometric analysis. In the immunized mice, the percentage of MDSCs was always lower than nonimmunized mice, which demonstrated that the negative regulation of immune responses and T-cell suppression were reduced by immunization.

The mechanism by which antitumor immune response against colorectal tumors with irradiated tumor cell vaccine by i.p. injection in the present study may be involved in both humoral and cellular immunity was also supported by the characteristics of cytokine response. M2 macrophages express a wide array of anti-inflammatory molecules, such as IL-10 and TGF- $\beta$ 1. TAMs also release growth factors such as VEGF, transforming growth factor  $\beta$  (TGF- $\beta$ ), and a member of the FGF family, to promote angiogenesis in many tumors (Tanaka et al., 2002; Siveen et al., 2009; Solinas et al., 2009). Transforming growth factor- $\beta$ 1 was proved to facilitate cell adhesion to ECM (Dawes et al., 2007), induce peritoneal fibrosis and may provide a favorable environment for the dissemination of gastric cancer, by significantly stimulating the expression of collagen III and fibronectin in mesothelial cells through the Smad2 signal transduction pathway (Lv et al., 2010; 2012). Interleukin-10 (IL-10) is generally thought to support tumor growth as a broadly acting immune inhibitory cytokine, and shown to be produced by dendritic cells (DCs), and potently induce regulatory T cell responses (Almand et al., 2001). While recently, new findings also establish that endogenous IL-10 inhibits tumor development, growth, and metastasis by hampering the production of inflammatory cytokine production and the development of Treg cells and MDSCs (Tanikawa et al., 2012). Because of the dilution by 10mL PBS lavage, the diversity of IL-10 and TGF- $\beta$ 1 in lavage supernatant reduced. However, the increase of IL-10 both in lavage fluid and sera was found in immunized mice, which showed a supporting to its potentially antitumor effect. The increase of TGF- $\beta$ 1 in serum and decrease in lavage fluid

might revealed its different function in peritoneum and system. As in peritoneal cavity, the favorable environment for the dissemination of tumor cells induced by TGF- $\beta$ 1 was possibly inhibited.

Although the preventive protocol in the present study is effective in the inhibition of inoculated tumor growth and prolongation of the survival, its therapeutic effect was not shown in treating mice with established tumors and most of the studies indicated that tumor vaccine is only partially effective against preexisting tumors in mouse models (Staveley-O'Carroll et al., 1998). However, the vaccine might be acceptable before the preexisting tumor would get a surgical treatment. Besides, the exact mechanism of the discordant antitumor immune responses generated in tumor-free and tumor-bearing vaccine recipients might have been explained by such diverse factors as the generation of tumor-induced suppressor T lymphocytes (Awwad et al., 1998), alterations in T cell signal transduction (Mizoguchi et al., 1992; Nakagomi et al., 1993), T cell apoptosis or anergy (Mizoguchi et al., 1992; Nakagomi et al., 1993; Hahne et al., 1996; Awwad et al., 1998; Staveley-O'Carroll et al., 1998), and the development of peripheral tolerance to tumor antigens (Ye et al., 1994), or of immunological ignorance (Ochsenbein et al., 1992).

Taken together, these observations may provide a new vaccine strategy for the prevention of peritoneal and systemic metastasis of carcinomas through the induction of the autoimmune response in peritoneum, in addition to subcutaneous immunization, and may be of importance to the further exploration of the role of the peritoneal resident cells in other immune diseases.

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