T cell immunity induced by a bivalent Salmonella-based CEACAM6 and 4-1BBL vaccines in a rat colorectal cancer model

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Received November 24, 2015; Accepted January 31, 2017

DOI: 10.3892/ol.2017.5938

Abstract. The present study investigated the anti-tumor mechanisms of recombinant non-specific cross-reacting antigen (CEACAM6) and 4-1BB ligand (4-1BBL) *Salmonella*-based vaccines, and the effect that these vaccinations have on memory T cells and T helper (Th) cell polarization. Colon tumors were induced in rats via 1,2-dimethylhydrazine (DMH) injections. Rats were then treated with injections of attenuated *Salmonella* typhimurium carrying pIRES-CEACAM6, pIRES-4-1BBL or pIRES-CEACAM6-4-1BBL. In total, 4 vaccine injections, one every other week, were administered during the 8 weeks subsequent to the DMH injection. Rats were sacrificed 18 weeks subsequent to the DMH injections, and the colons and spleens were collected for further analysis. Cluster of differentiation (CD) 45RO, interleukin (IL)-4 and IL-17 expression was analyzed in colon tumor tissues, and the expression of interferon (IFN)-γ, $CD3^+$, $CD4^+$, $CD8^+$, $CD56^+$, forkhead/winged-helix transcription factor box P3 ($FOXP3^+$), IL-4 and IL-17 were analyzed in splenic tissues. Compared with the pIRES/SL3261 group, the pIRES-CEACAM6-4-1BBL/SL3261 treatment group had a significantly higher number of $CD45RO^+$ expressing tumor infiltrating lymphocytes and lower expression levels of IL-4 and IL-17. Splenic tissues from the same treatment group exhibited significantly increased expression of IFN-γ, $CD3^+$ and $CD8^+$ and reduced expression levels of Foxp3, IL-4 and IL-7. $CD56^+$ T cell expression was increased in all groups except for the group that received no vaccine. The present study concluded that the combined CEACAM6 and 4-1BBL-attenuated recombinant *Salmonella* vaccine was able to inhibit the growth of DMH-induced colorectal tumors. This was mediated by generating an anti-tumor immune response, increasing the number of of $CD45RO^+$ memory T cells, decreasing the number of $FOXP3^+$ cells and promoting Th1 polarization.

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Abbreviations: CEACAM6, non-specific cross-reacting antigen; 4-1BBL, 4-1BB ligand; DMH, 1,2-dimethylhydrazine; FOXP3, forkhead/winged-helix transcription factor box P3; TIL, tumor-infiltrating lymphocyte; Th cell, T helper cell

Key words: recombinant attenuated *Salmonella,* colorectal cancer, 4-1BBL, CEACAM6, tumor-infiltrating lymphocytes, vaccine

Introduction

Colorectal cancer (CRC) is the fourth most common cause of cancer-associated mortality worldwide (1). In the advanced stages of the disease, the prognosis for patient with CRC remains poor due to the high frequency of recurrence, high rate of distant metastasis and resistance to chemotherapeutics. Therefore, novel treatments are urgently required and a number of different intervention strategies are being explored. The potential of immunotherapy is increasingly gaining attention. One reason for the growing interest in immunotherapy is that tumors that develop resistance to chemotherapy or radiation may continue to be suitable immunotherapy targets (2-4). CRCs are typically characterized by the infiltration of multiple types of stromal cells, including the tumor-infiltrating lymphocytes (TILs) that can serve as prognostic and predictive factors (5-8).

TILs include natural killer (NK) cells, cluster of differentiation (CD) 8^+ T cells and $CD4^+$ T cells, which are further subdivided into T helper (Th) 1, Th2, Th17 and regulatory T (Treg) cells. These various TILs exhibit different effects on tumorigenesis. Th1 cells produce the cytokines interleukin (IL)-2 and interferon (IFN)-γ, which possess anti-tumor activities. Additionally, IL-2 and IFN-γ perform a critical role in cellular immunity by activating $CD8^+$ T cells, which are the main effector cells involved in cytotoxic T lymphocyte (CTL) activity. Th2 cells produce IL-4, IL-5, IL-9 and IL-13 and protect against gastrointestinal parasites (9). Polarization of Th1 can overcome the ability of cancer cells to escape the immune response, thus killing more residual tumor cells. By contrast, Th2 polarization leads to an immunosuppressive

state that can result in tumor cells escaping immune detection. Th17 cells promote tumor growth by secreting IL-17, which enhances net angiogenic activity and promotes the growth of non-small cell lung cancer *in vivo* (10). IL-17 also supports the tumor promoting microenvironment at tumor sites, and its effects on myeloid derived suppressor cells represents an important tumor promoting mechanism (11). The critical role performed by TILs in cancer pathogenesis supports the hypothesis that immunotherapy has the potential to be an effective option for the treatment of CRC or for preventing relapse in patients with CRC.

Carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 6 is an important cell adhesion associated protein and a member of the CEACAM family. CEACAM family members are useful clinical biomarkers with promising therapeutic targets in melanoma and lung, colorectal and pancreatic cancer (12). CEACAM6 has been demonstrated to be an independent prognostic factor associated with a higher risk of CRC relapse (13). Therefore, CEACAM6 is a promising target for CRC malignancy and metastatic control.

The 4-1BB ligand (4-1BBL) is a type II surface glycoprotein member of the tumor necrosis factor (TNF) superfamily that is generally expressed by antigen presenting cells (APC), including dendritic cells, macrophages and activated B cells (13). 4-1BBL binds to 4-1BB [also termed cluster of differentiation (CD) 137], a member of the TNF receptor superfamily, and enhances T cell activation (14). The interaction of 4-1BB and 4-1BBL provides an important co-stimulatory T cell activation signal that is independent of CD28, and has attracted a lot of attention in previous immunology studies (15,16).

We previously demonstrated that recombinant CEACAM6 and 4-1BBL genes could be used to deliver CEACAM6 and 4-1BBL antigens effectively via attenuated *Salmonella*. This *Salmonella*-based vaccine efficiently inhibited the development of 1,2-dimethylhydrazine (DMH) induced colorectal tumors in rats (17). Tumor treatment using the *Salmonella*-based vaccine was accompanied by an increased number of $CD3^+$ and $CD8^+$ TILs and NK cells, and decreased forkhead/winged-helix transcription factor box P3 (FOXP3) positive T cells. The present study continues the investigation of this vaccine, and explores whether the vaccine is capable of inducing immune memory and also investigates the mechanisms by which the vaccine influences Th cell polarization. To investigate the mechanisms involved in the anti-tumor immunological activity of the recombinant DNA vaccine the present study analyzed $CD45RO^+$, IL-4 and IL-17 expression in induced rat tumors, and measured IFN-γ, $CD3^+$, $CD4^+$, $CD8^+$, $CD56^+$, $FOXP3^+$, IL-4 and IL-17 levels in splenic tissues.

Materials and methods

Animals. A total of 24 male 6-8 week old Sprague Dawley rats were obtained from the Experimental Animal Center of Soochow University (Soochow, China). The rats were housed with free access to water and food in a specific-pathogen-free facility under a 12 h light/dark cycle at 50±10% humidity and 21±2˚C. The body weight of each rat was measured weekly. All experimental protocols in this study were approved by the Ethics Committee of the First Affiliated Hospital of Soochow University (Suzhou, China).

Bacteria. *Salmonella enterica* serovars Typhimurium LB5000 and attenuated Typhimurium SL3261 were generously provided by Professor Stocker from Stanford University (Stanford, CA, USA). The plasmid pIRES2-EGFP was purchased from Clontech Laboratories, Inc. (Mountainview, CA, USA). The pIRES, pIRES-CEACAM6, pIRES-4-1BBL and pIRES-CEACAM6-4-1BBL plasmids were transformed into the SL3261 *Salmonella* strain, following a previously described method (17).

Rat colorectal tumor model establishment. A total of 20 mg/kg of subcutaneous DMH (Sigma-Aldrich; Merck Millipore, Billerica, MA, USA) was administered to all animals each week for 18 consecutive weeks. A total of 8 weeks subsequent to the first DMH administration, the rats were randomly divided into treatment groups and administered 2 ml PBS containing $2x10^9$ pIRES-transformed SL3261A, pIRES-4-1BBL-transformed SL3261B, pIRES-CEACAM6-transformed SL3261C or pIRES-CEACAM6-4-1BBL-transformed SL3261D via oral gavage every other week (4 doses over 8 weeks). DMH injections continued for 18 weeks. Subsequent to 18 weeks, the rats from the pIRES/SL3261, pIRES-4-1BBL/SL3261, pIRES-CEACAM6/SL3261 and pIRES-CEACAM6-4-1BBL/SL3261 groups were anesthetized by intravenous injections of pentobarbital (1.5 mg/100 g), blood samples were collected and the rats were subsequently sacrificed by using carbon dioxide. Following euthanasia, colons and spleens were dissected for immunohistochemical (IHC) staining.

Gross and histological examination. All colorectal tissue (from the cecum to the anus of each rat) was removed, washed with ice-cold saline, cut longitudinally and laid flat on a board. The total number of tumors was counted by two independent blinded investigators. Since it is difficult to distinguish lymph node metastasis in rats, Duke's staging system (18) was used with minor modifications to evaluate CRC disease stages as follows: Stage A, tumors are only present in the mucosa; stage B-C, tumors can be observed invading the muscularis propria, but no distant metastasis can be identified; and stage D, tumors with distant metastasis are present.

IHC analysis. All tumor specimens were analyzed with IHC for $CD45RO^+$ T cell infiltration, IL-4 and IL-17. Additionally, splenic tissues were analyzed for IFN-γ, $CD3^+$, $CD4^+$, $CD8^+$, $CD56^+$, $FOXP3^+$, IL-4 and IL-17. Tissue sections (4 μm) were prepared from 10% formalin-fixed (for 24 h at 25˚C) and paraffin-embedded tissues. Following deparaffinization and rehydration with ethanol (70-100%), the slides were heated to 100˚C in 10 mmol/l sodium citrate buffer (pH, 6) for 15 min to for antigen retrieval. Endogenous peroxidase activity was blocked by incubating at 25˚C with 0.6% H_2O_2 in methanol for 20 min. Sections were subsequently blocked with 10% normal horse serum (Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 5 min. Following blocking, sections were incubated with the following antibodies: Mouse monoclonal anti-rat CD3 (dilution, 1:100; catalog no. sc-20047;

Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-IL-4 (dilution, 1:100; catalog no. sc-71020; Santa Cruz Biotechnology, Inc.), anti-CD56 (dilution, 1:100; catalog no. ab80025; Abcam, Cambridge, MA, USA), anti-CD45RO (dilution, 1:10; catalog no. ab86080; Abcam), anti-FOXP3 (1:50; catalog no. ab450; Abcam, Cambridge), rabbit polyclonal antibodies anti-rat CD4 (dilution, 1:100; catalog no. sc-7219; Santa Cruz Biotechnology, Inc.), CD8 (dilution, 1:100; catalog no. sc-7188; Santa Cruz Biotechnology), IL-17 (dilution, 1:100; catalog no. ab134074; Abcam) and rabbit monoclonal antibody anti-rat INF-γ (dilution, 1:100; catalog no. ab134040; Abcam). Sections were incubated with primary antibodies at room temperature for 2 h.

The slides were incubated with streptavidin-horseradish peroxidase conjugated biotinylated secondary antibodies horse-anti-mouse immunoglobulin G (IgG) (dilution, 1:2,000; catalog no. K5006; Dako; Agilent Technologies, Inc.) and horse-anti-rabbit IgG (dilution, 1:2,000; catalog no. K5007; Dako; Agilent Technologies, Inc.) for 30 min at room temperature. Following incubation, an avidin/strepavidin complex (Dako; Agilent Technologies, Inc.) was added. A non-specific staining blocker (GeneTex Corporation, Shanghai, China) and enzyme-labeled sheep anti-rabbit IgG polymer reagent (GeneTex Corporation) were added according to the manufacturer's protocol. The antigen detection was conducted via a color reaction with 3,3'-diaminobenzidine (Dako, Agilent Technologies Denmark ApS, Glostrup, Denmark). Sections were counterstained using hematoxylin (AppliChem Inc., St Louis, MO, USA) and mounted with Aquatex (Merck Millipore). A total of 5 randomly selected fields (magnification, x400) were assessed using the Olympus BX53 microscope (Olympus Corporation, Tokyo, Japan), and areas of necrosis were avoided. The number of positive cells per field were estimated and assigned a number as follows: 0) None; i) <1/100 cells; ii) ≥1/100 to 1/10 cells; iii) >1/10 to 1/3 cells; iv) >1/3 to 2/3 cells; and v) >2/3 cells. Staining intensity was determined as follows: 0, none; 1,weak; 2, intermediate; and 3, strong. The sum of the first and second scores comprised the staining score, and the maximum possible score was 8 for any given tissue (19). The IHC analysis was performed by 2 independent blinded investigators (author 1 and 2) and the results were consistent between the two readings. Results were recorded as the mean ± standard deviation for each group.

Statistical analysis. Data are represented as the mean ± standard deviation. Differences among different groups were assessed using one-way ANOVA, with the Tukey's honest significant difference post hoc test. Differences between two groups were assessed using the Student's t-test. Differences in the number of T-cells and cytokines were investigated using the non-parametric Wilcoxon Rank Sum test. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using Predictive Analytics Software (PASW; version 18.0; IBM SPSS, Armonk, NY, USA).

Results

SL3261D (pIRES-CEACAM6-4-1BBL/SL321) vaccination reduces the number of colorectal tumors and restricted the tumors to the mucosa. As demonstrated in a previous study (17), following colorectal tumor induction, 2 rats from the SL3261A (pIRES/SL3261) group exhibited bloody stools 18 weeks subsequent to the initial DMH treatment. Further analysis of the animals with bloody stools revealed severe bloody ascites and multiple tumor nodules of varying sizes in the mesentery and posterior peritoneum. However, none of the other groups exhibited an outcome this severe.

The number of tumors observed in the SL3261A (pIRES/SL3261; 11.7±2.1) group following DMH treatment (duration, 18 weeks) was increased compared with the average number in the SL3261B group (pIRES-4-1BBL/SL3261; 5.7±1.2) and the SL3261C group (pIRES-CEACAM6/SL3261; 5.0±1.4). The SL3261D (pIRES-CEACAM6-4-1BBL/SL3261) group had an average of 4.3±1.4 tumors, which was reduced compared to all other groups and significantly reduced compared with the SL3261A group ($P<0.05$). However, no significant differences were observed between the pIRES-4-1BBL/SL3261, pIRES-CEACAM6/SL3261 and pIRES-CEACAM6-4-1BBL/SL3261 vaccine groups ($P>0.05$). Additionally, a number of the tumors observed in the SL3261A group were advanced stage tumors (4 stage B-C tumors; 2 stage D tumors), while the majority of the tumors observed in the SL3261C groups were stage B-C (SL3261C, stage A, 1; stage B-C, 5). By contrast, the majority of the tumors observed in the SL3261D group were stage A tumors (stage A, 5; stage B-C, 1; $P<0.05$). This result suggested that the SL3261D tumors were mainly restricted to the mucosa with little or no invasion or metastasis.

Although no significant differences were identified for body weight of rats between the 4 groups, the SL3261D-vaccinated group had a marked but non-significant increase in body weight 16 weeks following DMH treatment (17).

Population analysis of immune cells and cytokines in the colon and spleen. In a previously published study, we observed that a significant reduction in the number of tumors was accompanied by higher densities of $CD3^+$, $CD8^+$ and $CD56^+$ and a lower number of $FOXP3^+$ TIL cells in SL3261D rats (17). Therefore, to investigate the mechanism underlying the tumor growth inhibition induced by this vaccine, the present study analyzed $CD45RO^+$ TIL infiltration and the expression of IL-4 and IL-17 by IHC analysis. The present study also analyzed splenic tissues for IFN-γ, $CD3^+$, $CD4^+$, $CD8^+$, $CD56^+$, $P3^+$, IL-4 and IL-17 expression. It was observed that colon tumor tissues from the vaccinated groups had significantly higher $CD45RO^+$ TIL antibody staining when compared with the SL3261A group ($P<0.05$). However, among the SL3261B, SL3261C and SL3261D groups, the SL3261D group exhibited the highest rate of $CD45RO^+$ cell infiltration (Fig. 1A). By contrast, the SL3261D group exhibited significantly lower staining for IL-4 and IL-17 when compared with the SL3261A group ($P<0.05$; Fig. 1B and C) (Fig. 2).

Splenic tissue analysis revealed that IFN-γ and $CD3^+$ (but not $CD4^+$) were expressed at significantly higher levels in the SL3261B and SL3261D groups when compared with the SL3261C and SL3261A groups. $CD56^+$ T cell expression was higher in the SL3261B and SL3261D groups when compared with the SL3261A group. Additionally, $CD8^+$ T cell expression was significantly higher in the SL3261D and SL3261B

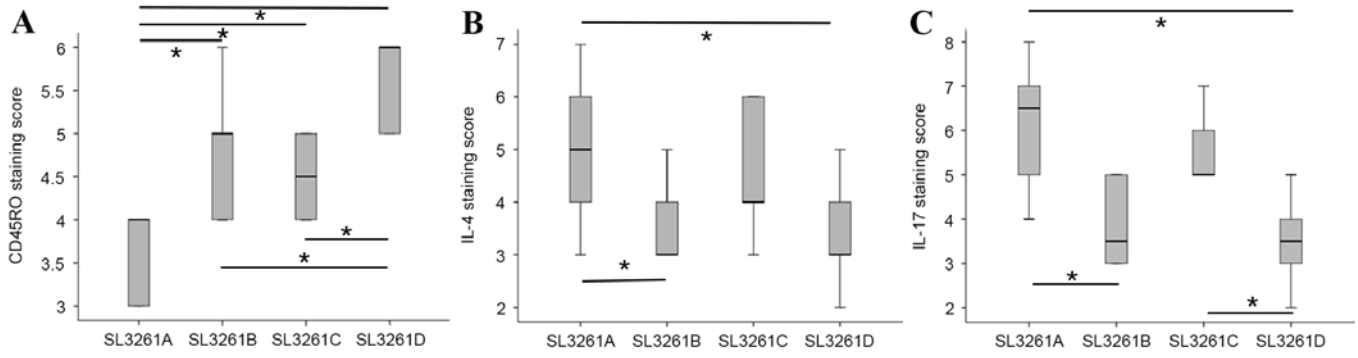

Figure 1. Immunohistochemical analysis of colon tissues. (A) Staining score for CD5RO in the 4 groups. (B) IL-4 staining score for the 4 groups. (C) IL-17 staining score for the 4 groups. Staining was performed using paraffin embedded tissue sections. Positive cells were quantified and staining scores were generated for all 4 groups. *P<0.05. CD45RO, cluster of differentiation 45RO; IL, interleukin.

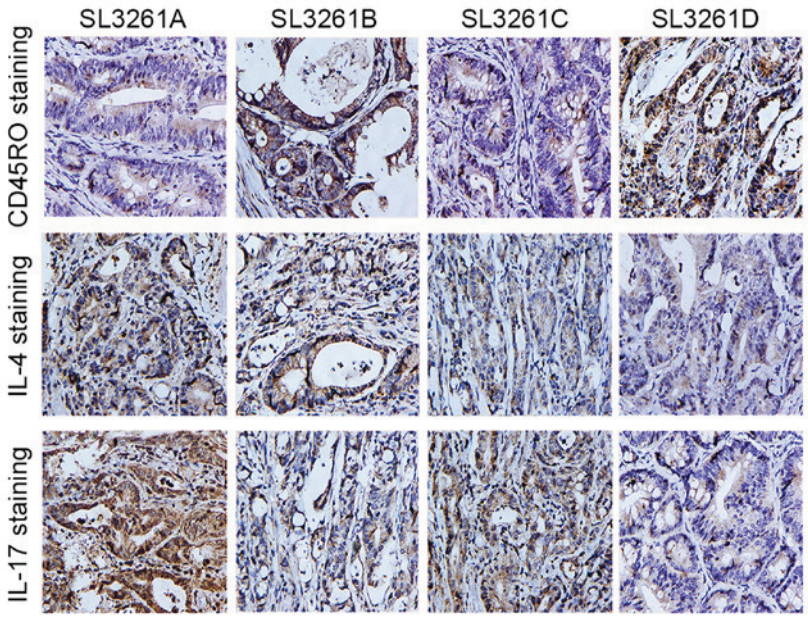

Figure 2. Representative images of immunohistochemical staining of colon tumor sections. CD45RO, IL-4 and IL-17 staining in colon tissue sections derived from the 4 treatment groups (SL3261A, SL3261B, SL3261C and SL3261D; magnification, x200). CD45RO, cluster of differentiation-45 RO; IL, interleukin.

groups when compared with the other groups. By contrast, the SL3261B and SL3261D groups exhibited significantly lower expression of FOXP3, IL-4 and IL-17 when compared with the SL3261A group (P<0.05). Finally, the SL3261D group exhibited the lowest IL-17 expression levels in the spleen among all the groups examined (Figs. 3 and 4).

Discussion

A previous study observed the appearance, subsequent to 12 weeks of DMH administration, of a cell mass exhibiting abnormal morphology and an accumulation of undifferentiated cells with pleiomorphic and conspicuous nucleoli in a small cluster of neighboring crypts of the colon (20). A total of 15 weeks subsequent to the initiation of DMH treatment, microscopic carcinomatous foci were observed (20). Therefore, in the present study, based on this background information, the vaccine was administered 8 weeks subsequent to the initiation of DMH treatment.

pIRES-4-1BBL/SL3261, pIRES-CEACAM6/SL3261 and pIRES-CEACAM6-4-1BBL/SL3261 vaccine treatment suppressed tumor growth when compared with pIRES/SL3261 treatment. The present study observed a small decrease in the number of colorectal tumors in the pIRES-CEACAM6-4-1BBL/SL3261 group.

Staging of the observed tumors revealed that the majority of the tumors in the pIRES/SL3261 groups were in a late stage, while the majority of tumors in the pIRES-4-1BBL/SL3261 and pIRES-CEACAM6/SL3261 groups were in an early or middle stage. By contrast, the pIRES-CEACAM6-4-1BBL/SL3261 group primarily exhibited early stage tumors. These data indicate that the individual recombinant bacteria-based 4-1BBL and CEACAM6 vaccines effectively inhibit CRC development, but the recombinant bacteria-based combined CEACAM6+4-1BBL vaccine synergistically inhibits CRC development and tumor growth.

To investigate the mechanism behind the tumor suppressive activity of these vaccines, the present study studied the

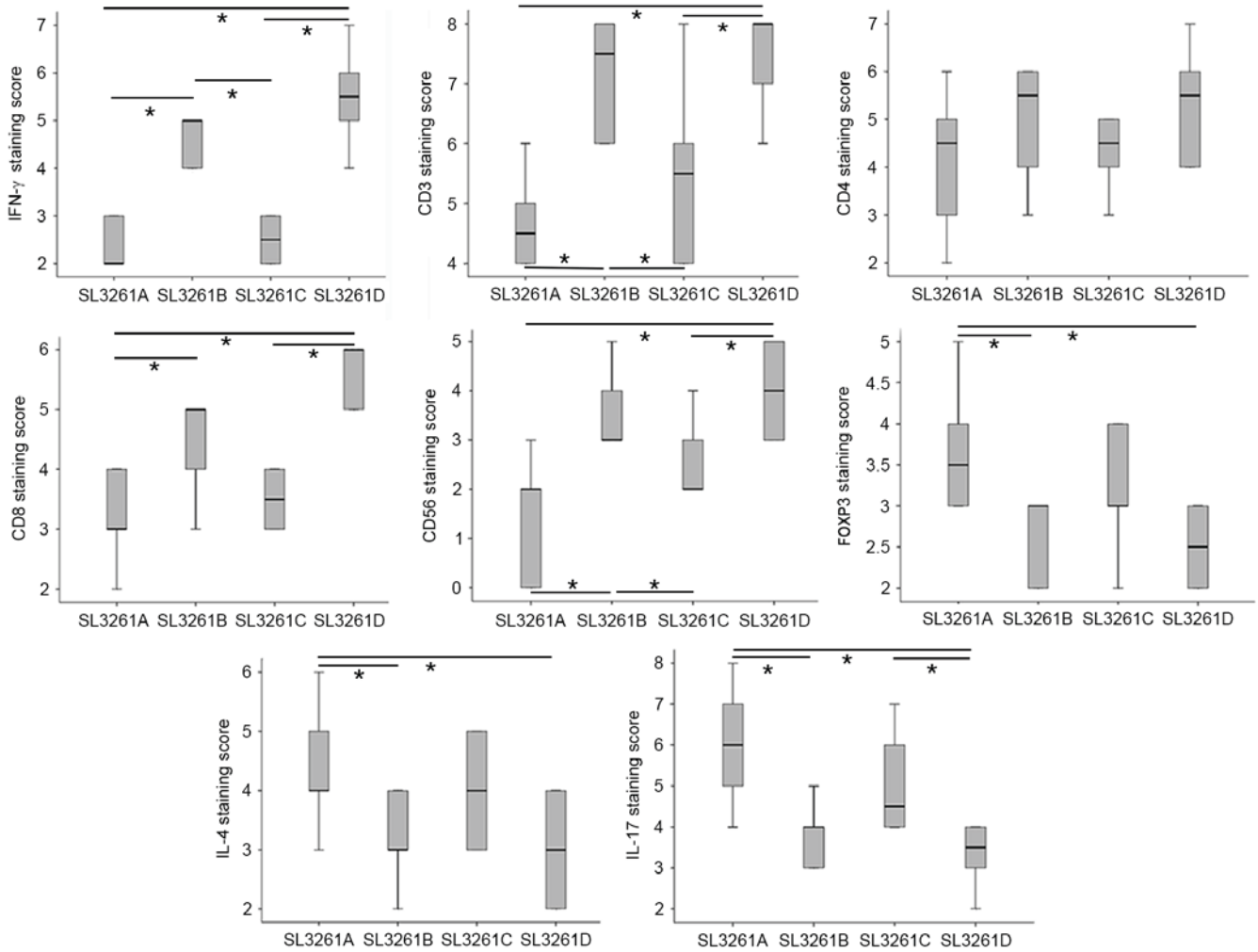

Figure 3. Immunohistochemical staining of splenic tissue. Immunostaining scores for IFN-γ, $CD3^+$, $CD4^+$, $CD8^+$, $CD56^+$, $FOXP3^+$, IL-4 and IL-17 in splenic tissues from the 4 treatment groups. *P<0.05 IFN, interferon; CD, cluster of differentiation; FOXP3, forkhead/winged-helix transcription factor box P3; IL, interleukin.

subsets of immune cells that infiltrated colon tumor tissues and the spleen in the DMH treated rats. It was observed that the pIRES-CEACAM6-4-1BBL/SL3261 group exhibited significantly higher $CD3^+$, $CD8^+$ and $CD56^+$ and reduced $FOXP3^+$ TIL. It is important to note that this group had the lowest number of tumors. Furthermore, analyzing cytokine expression revealed that the pIRES-CEACAM6-4-1BBL/SL3261 and pIRES-4-1BBL/SL3261 groups had reduced IL-4 and IL-17 staining when compared with the pIRES/SL3261 and pIRES-CEACAM6/SL3261 groups. A similar distribution of these molecules was observed in the splenic tissue analysis. Among all groups, splenic IFN-γ expression was highest in the pIRES-CEACAM6-4-1BBL/SL3261 group. These results suggest that the recombinant *Salmonella*-based CEACAM6 and 4-1BBL vaccine inhibited the development of rat CRC by inducing specific tumor suppressive immune responses and enhancing T cell immunity.

The present study proposes that CEACAM6 presented on the surface of APCs is the first T cell activation signal, and this signal is aided by the co-stimulatory molecule 4-1BBL. Thus, the combination of CEACAM6 and 4-1BBL leads to a strong induction of specific anti-CEACAM6 CTLs. Additionally, the exogenous addition of 4-1BBL increases the functional activity of $CD3^+CD56^+$ NK cells. Furthermore, these vaccines may also promote Th1 polarization and inhibit Th2 polarization. This, in turn, promoted the formation of IFN-γ and inhibited the formation of IL-4. The elevated IFN-γ then inhibited the development of Th17 cells, which explains the reduction of IL-17. Finally, this chain of molecular events led to the inhibition of inflammation and tumor blood vessel formation, which resulted in the observed reduction in tumor incidence.

Once memory T cells encounter an antigen, due to prior infection, cancer or a vaccine, they can mount a fast and strong immune response when the same antigen is encountered a second time. CD45RA and CD45RO are specific markers that distinguish T cells from memory T cells (21). $CD45RO^+$ T cells are considered memory T cells since they proliferate in response to recalled antigens and following PHA activation by cord blood mononuclear cells (21). $CD45RO^+$ T cells have also been identified as the main anti-tumor effector cells in early CRCs and high $CD45RO^+$ T cell infiltration levels are correlated with the absence of early metastatic invasion, less advanced pathologic stages and increased survival rate (22,23). The present study observed higher $CD45RO^+$ TIL cell staining in the pIRES-CEACAM6-4-1BBL/SL3261 group, which also exhibited the lowest number of tumors among the 4 groups.

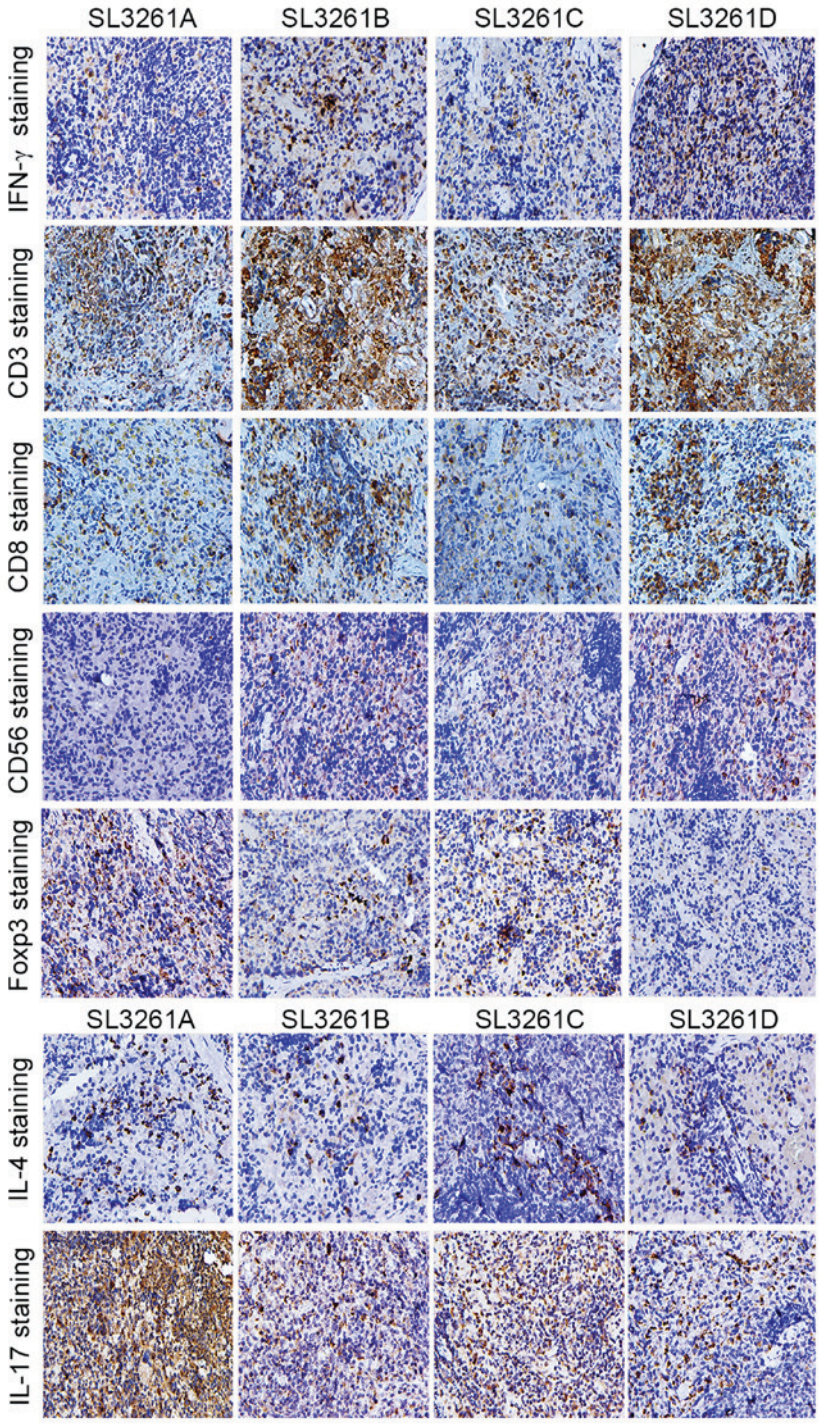

Figure 4. Representative images of immunohistochemical staining of spleen sections. IFN-γ, $CD3^+$, $CD8^+$, $CD56^+$, $FOXP3^+$, IL-4 and IL-17 staining in spleen sections derived from the 4 treatment groups (SL3261A, SL3261B, SL3261C and SL3261D; magnification, x200). IFN, interferon; CD, cluster of differentiation; FOXP3, forkhead/winged-helix transcription factor box P3; IL, interleukin.

This indicates that the vaccine activates tissue-resident memory T cells and induces an effective secondary immune response.

Tregs are a small subset of $CD4^+CD25^+$ T cells that are identified by FOXP3 expression. FOXP3 is a transcription factor that is critical for the differentiation and suppressive function of Tregs (24). $FOXP3^+$ expression has been associated with disease progression in several malignancies (25,26). The present study observed lower $FOXP3^+$ expression in the pIRES-CEACAM6-4-1BBL/SL3261 group. This finding suggests that the recombinant *Salmonella*-based CEACAM6 and 4-1BBL vaccine may contribute to CRC inhibition in part by decreasing FOXP3 expression. However, this mechanism of action needs to be confirmed by future studies.

In summary, the present results indicate that the *Salmonella* based vaccine efficiently inhibits DMH-induced colorectal tumor development in rats. This inhibition was accompanied by an increase in the number of $CD45RO^+$ TIL cells, a decrease in the number of $FOXP3^+$ cells, which promoted Th1 polarization and inhibited Th2 and Th17 polarization. These

findings have the potential to provide the basis for new immunotherapies designed to treat or suppress CRC.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (grant no. 81172166), the Natural Science Foundation of the Jiangsu Province of China (grant no. BK2008171) and the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (grant no. 11KJB320016).

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