

Original Article

Distribution of LAP⁺CD4⁺ T cells in pancreatic cancer

Xi Ou¹, Jiecao Ying², Jingsen Chen¹, Jing Guan³, Peikai Tian¹, Xiaoping Liu¹, Jikui Liu¹, Yang Zhao⁴, Guangyin Yu³

Departments of ¹Hepatobiliary and Laparoscopic Surgery, ⁴Pathology, Shenzhen Hospital, Peking University, Shenzhen 518036, Guangdong Province, China; ²Department of General Surgery, Jinhua People's Hospital, Jinhua 321000, Zhejiang Province, China; ³Department of Obstetrics and Gynecology, Xiamen University Affiliated Zhongshan Hospital, Xiamen 361004, Fujian Province, China

Received February 1, 2016; Accepted April 7, 2016; Epub August 15, 2016; Published August 30, 2016

Abstract: Objective: The purpose of this study was to investigate the role of LAP⁺CD4⁺ T cell in pancreatic cancer immunity. Methods: LAP and CD4 double staining was performed for tumor tissues and peri-tumor tissues from 35 patients with pancreatic cancer, and normal pancreatic tissues from 35 patients with benign lesions. Average number of the LAP⁺CD4⁺ T cell in each visual field was quantified. Results: The number of LAP⁺CD4⁺ T cell/high-magnification microscope field (400×) in the pancreatic cancer tissues was 8.38±1.44, which was significantly higher than that (5.21±1.24) in the peri-pancreatic cancer tissues and that (1.49±0.64) in the normal pancreatic tissues with benign lesions. Furthermore, in the pancreatic cancer tissues, LAP⁺CD4⁺ T cells were present as clusters in the tumor stroma and closely associated with CD4⁺ T lymphocytes. In contrast, in the peri-pancreatic cancer tissues and normal pancreatic tissues, LAP⁺CD4⁺ T cells were distributed sparsely. Conclusions: LAP⁺CD4⁺ T cells was increased in pancreatic cancer tissues and may inhibit local anti-tumor immunity.

Keywords: LAP⁺CD4⁺ T cell, tumor microenvironment, pancreatic cancer

Introduction

Anti-tumor immunity includes cellular and humoral immune responses, of which cellular immune response plays a more important role. T lymphocytes, natural killer (NK) cells and macrophage are the main effector cells involved in the cellular immune response. Regulatory T cells (Treg) can produce inhibitory cytokines and suppress immune response, and thereby plays an important role in maintaining immune homeostasis. Treg is characterized by its anergy and immunosuppressive functions, which can reduce tumor antigen recognition and killing of tumor cells, induce immune tolerance and play a negative regulatory role in evasion of anti-tumor immune response. Treg accounts for 5%-10% of total CD4⁺ T cells. Treg-mediated negative immunomodulatory effect is one of the main mechanisms of tumor suppression of the immune system and immune evasion.

Previous studies of Treg cells focused on CD4⁺CD25⁺ Treg, T helper 3 cells (Th3), type 1 regulatory T cells (Tr1), immunomodulatory

CD8⁺ T cells, and natural killer T cells (NKT). LAP⁺CD4⁺ T cell is new subtype of Treg characterized by the cell surface marker LAP. LAP⁺CD4⁺ T cell lacks Foxp3 and expresses type II TGF-β receptor and its active marker CD69. In animal model, LAP⁺CD4⁺ T cells are associated with autoimmune disease and inflammatory disease progression. However, few studies regarding to the role of LAP⁺CD4⁺ T cells in tumor development have been conducted so far. In this study, we investigated the distribution of LAP⁺CD4⁺ T cells in pancreatic cancer tissues, peri-pancreatic cancer tissue and normal pancreatic tissues with an expectation to identify the role of LAP⁺CD4⁺ T cells in immune escape of pancreatic cancer.

Materials and methods

Clinical data

Tumor and peri-tumor tissues were collected from 35 patients (Male: 23; Female: 12; Age: 28-80 with an average of 52.7±14.13) with pancreatic ductal adenocarcinoma between

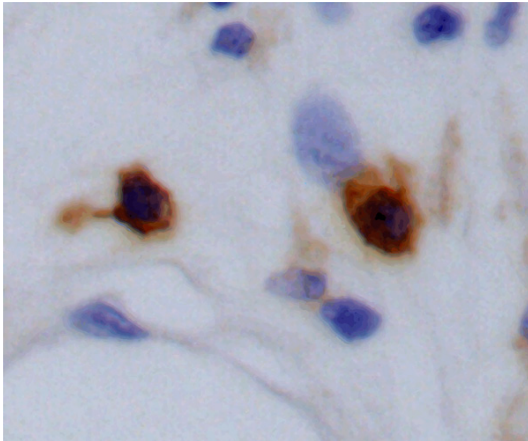


Figure 1. The LAP⁺CD4⁺ T cells positive staining.

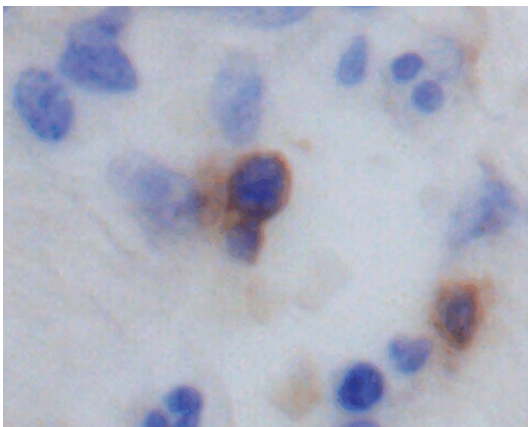


Figure 2. The CD4⁺ T cells positive staining.

August 2005 and May 2014. Patients did not receive chemotherapy and radiotherapy, and did not have distant organ metastasis before surgery. Peri-tumor tissues were collected at the area within 2 cm from the edge of the tumor. Necrotic tissues were not included in the sample. A total of 35 normal pancreatic tissues were also collected during pancreatic surgery for patients (male: 26; female: 9; Age: 25-76 with an average of 51.6 ± 13.39) with benign lesions. Paraffin sections were made for tumor tissues, peri-tumor tissues and normal pancreatic tissues. Age and gender were not significantly different between the pancreatic cancer group and the group with benign pancreatic lesions. All of the subjects did not have the history of immunotherapy and autoimmune diseases, e.g., systemic lupus erythematosus, rheumatoid arthritis, diabetes, and hyperthyroidism. The study was approved by the hospi-

Table 1. Distribution of LAP⁺CD4⁺ T cells in the tumor microenvironment of pancreatic cancer

Tissues	Case number	LAP ⁺ CD4 ⁺ T cells
pancreatic cancer tissue ①	35	8.38 ± 1.44
Peri-tumor tissue ②	35	5.21 ± 1.24
Normal pancreatic tissue ③	35	1.49 ± 0.64

Note: $P < 0.05$ for the comparisons between ① and ②, between ① and ③, and between ② and ③.

tal ethics committee. All subjects were informed of the study process and significance, the risk and impact on patient health before the specimen collection. An informed consent form was signed by all the subjects.

Immunohistochemistry analysis

Paraffin sections (4 μ m in thickness) were dewaxed and rinsed for three times with water. Antigen retrieval was conducted by incubating the sections with pepsin for 30 min at 37°C followed by two washes with PBS. The sections were immersed in a 3% hydrogen peroxide solution for 20 min in the dark condition to inactivate endogenous peroxidase activity and avoid non-specific staining. Non-specific binding was also blocked by incubating the sections with one drop of calf serum for 30 min at 37°C. Monoclonal rabbit anti-human CD4 antibody (R&D) was added to cover the entire sections and incubated overnight at 4°C. Following two washes with PBS, biotin-labeled secondary goat anti-rabbit antibody (R&D) was added and incubated for 30 min at 37°C. Following two washes with PBS, Streptavidin-biotin-peroxidase was added onto each section and incubated for 30 min at 37°C. DAB reagents were added to the sections and brown color was developed after 4 min. Similar procedures using goat anti-human LAP primary antibody and rabbit anti-goat secondary antibody (R&D) were performed to stain LAP. LAP was stained in red using AEC reagent.

Data analysis

LAP⁺CD4⁺ T cell-positive staining was based on the criteria that cells were small and round with large nucleus and small cytoplasm (1); and both brown-stained and red-stained particles were located in the cytoplasm and cell membrane (**Figures 1, 2**). CD4⁺ T cell-positive stain-

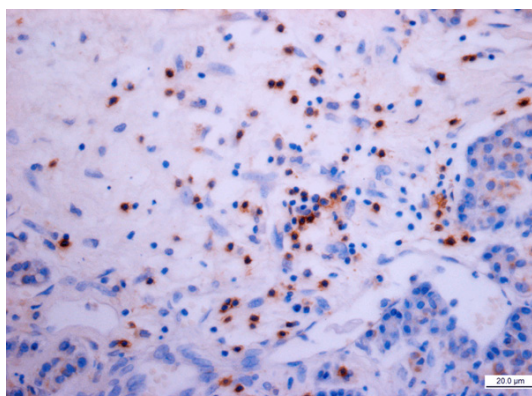


Figure 3. Distribution of LAP⁺CD4⁺ T cells in the pancreatic cancer tissue (high magnification field, 400×).

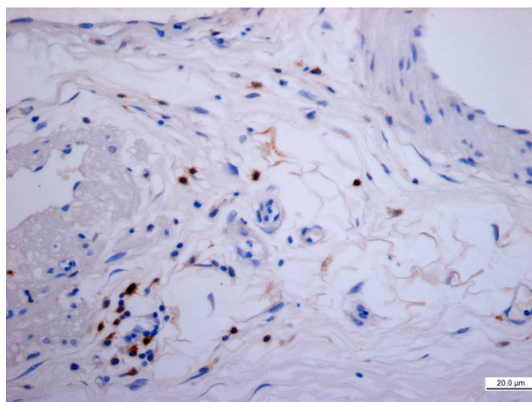


Figure 4. Distribution of LAP⁺CD4⁺ T cells in the pancreatic peri-carcinoma tissue (high magnification field, 400×).

ing was based on the criteria that cells were small and round with large nucleus and small cytoplasm (1); and only brown-stained particles were located in the cytoplasm and cell membrane (**Figure 2**) (2). Five high-magnification microscope fields were randomly selected and the number of LAP⁺CD4⁺ T cells and CD4⁺ T cells were counted and averaged.

Statistical analysis

The number of LAP⁺CD4⁺ T cells/one microscope field was represented as average \pm standard deviation ($\bar{x} \pm s$). Paired samples *t*-test was performed for the comparison between pancreatic cancer and peri-pancreatic cancer tissues. Independent samples *t*-test was performed for the comparison of normal pancreatic tissues with pancreatic cancer or peri-pancreatic can-

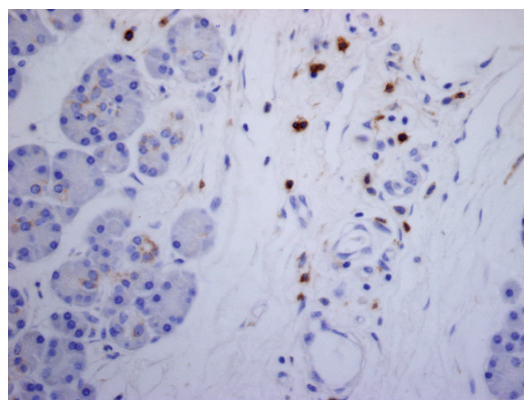


Figure 5. Distribution of LAP⁺CD4⁺ T cells in the normal pancreatic tissue (high magnification field, 400×).

cer tissues. $P < 0.05$ was considered as statistically significant. All statistical analyses were performed by SPSS17.0.

Results

LAP⁺CD4⁺ T cell distribution in tumor and peri-tumor tissues

The average number of LAP⁺CD4⁺ T cells/high magnification field (400×) in the pancreatic cancer and peri-pancreatic cancer tissues was 8.38 ± 1.44 and 5.21 ± 1.24 , respectively ($P < 0.05$). The average number of LAP⁺CD4⁺ T cells/high magnification field in the pancreatic tissues with benign lesions was 1.49 ± 0.64 , which was significantly lower than that in the pancreatic cancer and peri-pancreatic cancer tissues ($P < 0.05$) (**Table 1**).

Characteristic localization of LAP on the cells

LAP was localized on the cytoplasm and cell membrane of CD4⁺ T cells (**Figure 1**). In the pancreatic cancer tissues, LAP⁺CD4⁺ T cells present as clusters in the tumor stroma and were closely associated with lymphocytes (**Figure 3**). In contrast, in the peri-pancreatic cancer tissues, LAP⁺CD4⁺ T cells were mostly present individually (**Figure 4**). In the normal pancreatic tissues with benign lesions, LAP⁺CD4⁺ T cells were present even more sparsely (**Figure 5**).

Discussion

From the perspective of immunology, tumor development is essentially the process of interactions between the different immune cells

and molecules. Tumor microenvironment in which tumor growth and metastasis occur is composed of tumor cells, infiltrating immune cells, stromal cells and their secreted active media. Tumor microenvironment determines the biological features, prognosis and outcome of tumors. Therefore, compared with peripheral blood, tumor microenvironment can more accurately reflect the host anti-tumor immune status. Better understanding of the characteristics of tumor microenvironment is important for studying immunogenicity of cancer and designing effective immunotherapy against cancers.

Regulatory T cells (Treg) play an important suppressive role in tumor immune response. The level of Treg infiltration in the tumor microenvironment determines the immune activation or immune tolerance against tumors. Enhanced infiltration of Treg and myeloid derived suppressor cells into tumor microenvironment can suppress the anti-tumor immune response and facilitate tumor growth [1, 2]. Previous studies have shown that Treg cells are significantly increased in the peripheral blood of patients with breast cancer, lung cancer, ovarian cancer, gallbladder cancer, pancreatic cancer, colon cancer and other malignant tumors. Furthermore, the level of Treg is closely correlated with the tumor stage and course [3-6]. Recent studies have also shown that in the peripheral blood and the tumor microenvironment, Treg cells are significantly increased, suggesting that Treg is closely correlated to tumor immune escape [7, 8]. Thus, Treg cells play an important negative immunomodulatory role in the tumor microenvironment.

LAP is a pro-peptide capable of non-covalent binding with the amino-terminus of TGF- β [9]. TGF- β is a type of multifunctional polypeptide growth factor, and is usually secreted as inactive or latent precursors outside of the cells. Upon activation, TGF- β can bind TGF- β receptor and exert biological activity. Before activation, TGF- β complex is composed of TGF- β homodimer, LAP and latent TGF- β binding protein (LTBP). Recently, Candhi et al. discovered a new subtype of cells that are Foxp3-negative and express TGF- β and LAP on the cell membrane. This new type of cells was designated as LAP⁺CD4⁺ T cells. LAP⁺CD4⁺ T cells are different from CD4⁺Foxp3⁺CD25^{high} Treg, but they can express TGF- β II and activated CD69. LAP⁺CD4⁺ T cells secrete TGF- β , IL-8, IL-9, IL-10 and interferon- γ (IFN- γ). Different from LAP⁺CD4⁺ T

cells, LAP⁺CD4⁺ T cells are low proliferative cells. However, it was demonstrated in vitro that proliferation of LAP⁺CD4⁺ T cells had suppressive effect and such effect was mediated by TGF- β and IL-10. In CD4⁺CD25⁺ Treg, expression of CD25 is closely correlated with their regulatory activity [10]. However, Nakamura et al. showed that regardless of CD25 expression, LAP⁺ T cells that express TGF- β 1 can suppress immune response. Therefore, LAP is superior to CD25 as a marker of Treg [11]. Chen et al. showed that LAP⁺CD4⁺ T cells co-express TGF- β and TGF- β receptor and thus exert stronger regulatory activity than conventional Treg via direct cell-cell interaction or TGF- β -dependent mechanisms [12].

Currently, most of the studies on LAP⁺CD4⁺ T cells focused on their role in auto-immune diseases and infectious diseases in animal models and the results have demonstrated that similar to Treg, LAP⁺CD4⁺ T cells play a negative immune regulatory role in these diseases. Wu et al. [13] showed that in the mouse arthritis model, LAP⁺CD4⁺ T cells can be induced to suppress arthritis-specific reactive T cell proliferation and specific antibody production. Ishikawa et al. [14] showed in a mouse model of diabetes that LAP⁺CD4⁺ T cells are induced and can inhibit the growth of T helper cells and negatively regulate immune responses. Mahalingam et al. showed that LAP⁺CD4⁺ T cell cluster together in the tumor microenvironment of patients with colorectal cancer, which are closely associated with clinical progression and metastasis. Furthermore, LAP⁺CD4⁺ T cells can inhibit LAP⁺CD4⁺ T cell proliferation in a TGF- β -dependent manner [15]. However, the mechanisms by which LAP⁺CD4⁺ T cells are involved in immune inhibition in the tumor microenvironment have not been elucidated. Furthermore, studies on the role of LAP⁺CD4⁺ T cells in other tumors, e.g., pancreatic cancer, have not been reported.

Characterization of LAP⁺CD4⁺ T cells in the tumor microenvironment of pancreatic cancer

LAP is an N-terminal pro-peptide of TGF- β and binds the N-terminus of TGF- β via non-covalent bond. Previous studies have confirmed that Treg can produce membrane-bound or secreted TGF- β , which play an important role in natural Treg cell differentiation, maintenance and the immune suppression. Our study showed that LAP was localized on the cell cytoplasm

and membrane of CD4⁺ T cells, suggesting that LAP⁺CD4⁺ T cells are similar to the traditional Treg cells, and can also produce membrane-bound or secreted TGF- β . These TGF- β play an important role in LAP⁺CD4⁺ T cell differentiation, maintenance and immune suppression. Our study also showed that LAP⁺CD4⁺ T cells were not expressed in normal pancreatic tissues, and were present individually in peri-tumor tissues and clustered together and associated with CD4⁺ T cells in the tumor stroma in pancreatic cancer tissues. These results suggest that these clustered LAP⁺CD4⁺ T cells in the local pancreatic cancer tissues negatively regulate effector T cells and thereby inhibit tumor-specific or non tumor-specific immune responses, which facilitates tumor cell proliferation and metastasis.

Distribution of LAP⁺CD4⁺ T in pancreatic cancer, peri-tumor and normal pancreatic tissues

Clinical and experimental studies have demonstrated that patients with pancreatic cancer usually have immune dysfunctions. Particularly, abnormal tumor immunological microenvironment results in the deficiency of immune defense, which is an important factor for pancreatic cancer immune escape, metastasis and recurrence. As a new subtype of Treg, LAP⁺CD4⁺ T cells have strong immune inhibitory effect on LAP⁺CD4⁺ T cells [16]. Thus, the expression of LAP⁺CD4⁺ T cells in tumor microenvironment has been extensively studied. Our results showed that LAP⁺CD4⁺ T cells in pancreatic cancer tissues were significantly increased compared to those in the peri-tumor or normal pancreatic tissues, suggesting that LAP⁺CD4⁺ T cells are more likely to be distributed to the tissues that are close to the tumors and may affect the tumor microenvironment. Increase of LAP⁺CD4⁺ T cells in the pancreatic cancer tissues may be correlated with IL-8. Gandhi et al., showed that upregulation of IL-8 could activate LAP⁺CD4⁺ T cells, while treatment with IL-8 monoclonal antibody dramatically decreases the LAP⁺CD4⁺ T cells, indicating that IL-8 is an effective stimulus for the activation of LAP⁺CD4⁺ T cells [10]. Previous studies have shown that IL-8 is increased in peripheral blood and tumor microenvironment in many cancers. Furthermore, IL-8 is closely correlated with immune cell chemotaxis and cell proliferation [17-19]. Haraguchi et al. showed that in the peripheral blood of patients with primary colorectal cancer, IL-8 level is significantly

increased compared with normal control group, and more importantly, IL-8 levels in patients with liver metastases were significantly higher than those without metastasis [20]. Chen et al. showed that higher level of IL-8 mRNA in non-small cell lung cancer (NSCLC) predicts a lower survival rate [21]. Therefore, we hypothesize that LAP⁺CD4⁺ T cells migrate to the pancreatic cancer tissues via IL-8 chemotactic effect, and within the pancreatic cancer tissues, LAP⁺CD4⁺ T cells alter the quantity and function of infiltrating lymphocytes, thereby inhibiting tumor-specific and non-specific immune response, and promoting tumor progression, invasion or metastasis.

Regulatory mechanisms LAP⁺CD4⁺ T cells in the microenvironment of pancreatic cancer

Regulatory T cells mediate immune escape of tumor cells via multiple molecular mechanisms. None of the single mechanism can fully explain all the regulatory functions. In vitro studies showed that CD4⁺CD25⁺ Treg mediate immune suppression via cell-to-cell contact and cytokine-independent mechanisms. However, in vivo studies showed that IL-10 (and potentially TGF- β) plays an important role in the Treg-mediated immune suppression [22]. Studies have shown that LAP⁺CD4⁺ T cells are mechanistically different from the traditional Treg. However, different studies showed different and sometimes even contradicted effector mechanisms of LAP⁺CD4⁺ T cells, indicating complex regulatory mechanisms of LAP⁺CD4⁺ T cells. Our studies showed a heterogeneous distribution of LAP⁺CD4⁺ T in the microenvironment of pancreatic cancer. LAP is localized in cytoplasm and membrane of CD4⁺ T cell. In tumor tissues, LAP⁺CD4⁺ T are distributed as clusters and are in close contact with the effector T lymphocyte. Therefore, we speculate that those LAP⁺CD4⁺ T cells inhibit the proliferation and function of effector T lymphocytes via a cell-to-cell contact mechanism. Future studies will be directed to verify if cell-to-cell contact is the main mechanism by which LAP⁺CD4⁺ T cells exert their negative regulatory function.

In conclusion, our studies showed that (1) LAP is localized on the cytoplasm and membrane of CD4⁺ T cell, suggesting that LAP⁺CD4⁺ T cells produced membrane-bound or secreted TGF- β , and TGF- β plays an important role in cell differentiation, maintenance and negative regulatory function of LAP⁺CD4⁺ T cells. (2) more LAP⁺CD4⁺

T cells are distributed in the tumor tissues than peri-tumor tissues and such tumor-oriented distribution may be due the IL-8 chemotactic function; (3) previous studies have suggested that the immunosuppressive role of LAP⁺CD4⁺ T cells is partly dependent on TGF- β [12, 17, 23, 24]. Our immunohistochemistry results demonstrated that LAP⁺CD4⁺ T cells exert negative regulatory function via cell-to-cell mechanism. Elucidation of the LAP⁺CD4⁺ T cell effector mechanisms and identification of approaches to inhibit LAP⁺CD4⁺ T cells may provide novel immunotherapy for pancreatic cancer.

Acknowledgements

A grant from the Shenzhen Science and Technology Bureau, No. JC200903180670A and No. 201103023.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaoping Liu, Department of Hepatobiliary and Laparoscopic Surgery, Shenzhen Hospital, Peking University, Shenzhen 518036, Guangdong Province, China. Tel: (86755)83923333-6000; E-mail: ninliu@163.com

References

- [1] Gandhi R, Anderson DE, Weiner HL. Cutting Edge: Immature human dendritic cells express latency-associated peptide and inhibit T cell activation in a TGF-beta-dependent manner. *J Immunol* 2007; 178: 4017-4021.
- [2] Kusmartsev S, Gabrilovich DI. Role of immature myeloid cells in mechanisms of immune evasion in cancer. *Cancer Immunol Immunother* 2006; 55: 237-899.
- [3] Sasada T, Kimura M, Yoshida Y, Kanai M, Takabayashi A. CD4+CD25+ regulatory T cells in patients with gastrointestinal malignancies: Possible involvement of regulatory T cells in disease progression. *Cancer* 2003; 98: 1089-1099.
- [4] Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, Linehan DC. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol* 2002; 169: 2756-2761.
- [5] Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, Rubin SC, Kaiser LR, June CH. Regulatory CD4+CD25+ T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 2001; 61: 4766-4769.
- [6] Woo EY, Yeh H, Chu CS, Schlienger K, Carroll RG, Riley JL, Kaiser LR, June CH. Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol* 2002; 168: 4272-4277.
- [7] Yamamoto T, Yanagimoto H, Satoi S, Toyokawa H, Hirooka S, Yamaki S, Yui R, Yamao J, Kim S, Kwon AH. Circulating CD4+CD25+ regulatory T cells in patients with pancreatic cancer. *Pancreas* 2012; 41: 409-415.
- [8] Yamamoto M, Kamigaki T, Yamashita K, Hori Y, Hasegawa H, Kuroda D, Moriyama H, Nagata M, Ku Y, Kuroda Y. Enhancement of anti-tumor immunity by high levels of Th1 and Th17 with a combination of dendritic cell fusion hybrids and regulatory T cell depletion in pancreatic cancer. *Oncol Rep* 2009; 22: 337-343.
- [9] Miyazono K, Ichijo H, Heldin CH. Transforming growth factor-beta: latent forms, binding proteins and receptors. *Growth Factors* 1993; 8: 11-22.
- [10] Gandhi R, Farez MF, Wang Y, Kozoriz D, Quintana FJ, Weiner HL. Cutting edge: human latency-associated peptide+ T cells: a novel regulatory T cell subset. *J Immunol* 2010; 184: 4620-4624.
- [11] Nakamura K, Kitani A, Fuss I, Pedersen A, Harada N, Nawata H, Strober W. TGF-beta1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. *J Immunol* 2004; 172: 834-842.
- [12] Chen ML, Yan BS, Bando Y, Kuchroo VK, Weiner HL. Latency-associated peptide identifies a novel CD4+CD25+ regulatory T cell subset with TGFbeta-mediated function and enhanced suppression of experimental autoimmune encephalomyelitis. *J Immunol* 2008; 180: 7327-7337.
- [13] Wu HY, Maron R, Tuckpah AM, Weiner HL. Mucosal anti-CD3 monoclonal antibody attenuates collagen-induced arthritis that is associated with induction of LAP+ regulatory T cells and is enhanced by administration of an emulsome-based Th2-skewing adjuvant. *J Immunol* 2010; 185: 3401-3407.
- [14] Ishikawa H, Ochi H, Chen ML, Frenkel D, Maron R, Weiner HL. Inhibition of autoimmune diabetes by oral administration of anti-CD3 monoclonal antibody. *Diabetes* 2007; 56: 2103-2109.
- [15] Mahalingam J, Lin YC, Chiang JM, Su PJ, Fang JH, Chu YY, Huang CT, Chiu CT, Lin CY. LAP+CD4+ T cells are suppressors accumulated in the tumor sites and associated with the progression of colorectal cancer. *Clin Cancer Res* 2012; 18: 5224-5233.

- [16] Ochi H, Abraham M, Ishikawa H, Frenkel D, Yang K, Basso AS, Wu H, Chen ML, Gandhi R, Miller A, Maron R, Weiner HL. Oral CD3-specific antibody suppresses autoimmune encephalomyelitis by inducing CD4⁺ CD25⁻ LAP⁺ T cells. *Nat Med* 2006; 12: 627-635.
- [17] Duan W, So T, Mehta AK, Choi H, Croft M. Inducible CD4⁺LAP⁺Foxp3⁻ regulatory T cells suppress allergic inflammation. *J Immunol* 2011; 187: 6499-6507.
- [18] Piconese S, Valzasina B, Colombo MP. OX40 triggering blocks suppression by regulatory T cells and facilitates tumor rejection. *J Exp Med* 2008; 205: 825-839.
- [19] Brode S, Raine T, Zacccone P, Cooke A. Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of CD4⁺CD25⁺Foxp3⁺ regulatory T cells. *J Immunol* 2006; 177: 6603-6612.
- [20] Haraguchi M, Komuta K, Akashi A, Matsuzaki S, Furui J, Kanematsu T. Elevated IL-8 levels in the drainage vein of resectable Dukes' C colorectal cancer indicate high risk for developing hepatic metastasis. *Oncol Rep* 2002; 9: 159-165.
- [21] Chen JJ, Yao PL, Yuan A, Hong TM, Shun CT, Kuo ML, Lee YC, Yang PC. Up-regulation of tumor interleukin-8 expression by infiltrating macrophages: its correlation with tumor angiogenesis and patient survival in non-small cell lung cancer. *Clin Cancer Res* 2003; 9: 729-737.
- [22] Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. *Trends Mol Med* 2007; 13: 108-116.
- [23] Wu HY, Center EM, Tsokos GC, Weiner HL. Suppression of murine SLE by oral anti-CD3: inducible CD4⁺CD25⁻LAP⁺ regulatory T cells control the expansion of IL-17⁺ follicular helper T cells. *Lupus* 2009; 18: 586-596.
- [24] Oida T, Zhang X, Goto M, Hachimura S, Totsuka M, Kaminogawa S, Weiner HL. CD4⁺CD25⁺T cells that express latency-associated peptide on the surface suppress CD4⁺CD45RB high-induced colitis by a TGF-beta-dependent mechanism. *J Immunol* 2003; 170: 2516-2522.