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ORIGINAL ARTICLE

Increased Frequency of $CD4^+CD25^{high}FoxP3^+$ Regulatory T Cells in Patients with Hepatocellular Carcinoma

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Abstract Accumulating evidence suggests regulatory T cells (Tregs) are associated with impaired antitumor responses. However, the relationship between the $CD4^+CD25^{high}FoxP3^+$ Treg and hepatocellular carcinoma (HCC) has not been well investigated. Levels of $CD4^+CD25^{high}FoxP3^+$ Tregs in peripheral blood mononuclear cells (PBMCs) from HCC patients and healthy donors, tumor infiltrating lymphocytes (TILs) extracted from HCC, and hepatic lymphocytes extracted from resected liver were measured by flow cytometry, and their effects on T-cell proliferation was determined by ^{3}H-thymidine incorporation. Serum levels of interleukin (IL)-10 and transforming growth factor (TGF)-β1 were measured by enzyme linked immunosorbent assay. The frequency of Tregs in PBMCs from HCC patients was higher than that from healthy donors. Similarly, the frequency of Tregs in TILs was higher than that of hepatic lymphocytes. On the other hand, the ^{3}H-thymidine uptake by TILs and PBMCs from HCC patients was decreased drastically when compared to the counterparts from normal controls. Furthermore, serum IL-10 and TGF-β1 levels increased significantly in HCC patients when compared to the healthy donors. This study identified an increased frequency of $CD4^+CD25^{high}FoxP3^+$ Tregs in patients with HCC. The elevated serum IL-10, TGF-β1 levels also correlated with impaired antitumor responses in these patients. Further effort is needed to establish new immunotherapeutic strategies designed to modulate Tregs to promote a competent antitumor response.

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Abbreviations

Treg	Regulatory T cell
HCC	Hepatocellular carcinoma
TIL	Tumor infiltrating lymphocyte
PBMC	Peripheral blood mononuclear cell
PHA	Phytohaemagglutinin
TGF	Transforming growth factor
IL	Interleukin

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of liver, which accounts for 85–90% of primary liver cancers (Llovet et al. 2003). Although remarkable advances in diagnostic and therapeutic techniques have been made in recent years, the prognosis for HCC patients remains extremely poor (Llovet et al. 2003). Despite the presence of tumor infiltrating lymphocytes (TILs) in HCC tissue, tumor progression is found in most of the patients, which suggests that there is an impaired local antitumor response.

Sakaguchi et al. (1995) for the first time described $CD4^+CD25^+$ T cells that had potent immunoregulatory functions and could control self-tolerance, now known as regulatory T cells (Tregs). It has been shown that Tregs play a critical role in controlling the immune equilibrium: on one hand, insufficient Tregs result in autoimmune disease and overwhelming tissue destruction during the infections; on the other hand, excessive Tregs undermine

the host antimicrobial and antitumor immune responses. Recently, a number of studies have demonstrated increased numbers of Treg cells in the peripheral blood and TILs in various cancers (Ichihara et al. 2003; Javia and Rosenberg 2003; Sasada et al. 2003; Schaefer et al. 2005; Woo et al. 2001), suggesting that Treg cells are closely associated with impaired antitumor immune responses enabling the tumor cells to escape from immune surveillance. It is well documented that interleukin (IL)-10 and transforming growth factor (TGF)-β1 exert a strong suppressive effect on Th immune reactions. Also, these cytokines interact closely with the immunoregulatory function of Tregs. Considering the above, the aim of this study was to investigate the role that $CD4^+CD25^{high}FoxP3^+$ Treg cells, IL-10 and TGF-β1 play in patients with HCC.

Materials and Methods

Patients and Healthy Donors

Forty-two HCC patients who underwent hepatectomy were included in this study and another 15 age-matched healthy volunteers donated their blood as normal control. In addition, resected liver from ten decompensated chronic hepatitis B patients who underwent living donor liver transplantation were used as a resource for hepatic lymphocytes. Informed consent was obtained from all subjects and this research protocol was approved by the Ethics Committee of West China Hospital in accordance with the Declaration of Helsinki.

Preparation of Peripheral Blood Mononuclear Cells

Fresh heparinized peripheral blood samples from the 42 patients were collected at the day of surgery. As previously described (Shabtai et al. 2003), peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation. The same technique was used for isolation of PBMCs from peripheral blood of the 15 normal donors.

Preparation of TILs

The TILs from HCC tissue were prepared as previously described (Harada et al. 2004; Takagi et al. 1989). Briefly, freshly resected HCC specimens were minced into pieces smaller than 1 mm^3 and then treated with an enzyme cocktail containing 0.05% collagenase IV (Sigma-Aldrich) and 0.002% DNase I (Sigma-Aldrich) at room temperature overnight. Cell suspensions were filtered through 70 μm nylon mesh. The cells were washed twice and then centrifuged and separated using the discontinuous (45%/75%) Percoll (Sigma-Aldrich, St. Louis, MO, USA) gradient method. The TILs were harvested from the interface layer.

Preparation of Hepatic Lymphocytes

The hepatic lymphocytes from resected liver of decompensated chronic hepatitis B patients were prepared as previously described (Zhang et al. 2005). In brief, the resected livers were minced, digested and filtered as described above for the TILs. The cells were washed twice and then centrifuged and then separated using the discontinuous (40%/70%) Percoll (Sigma-Aldrich, St. Louis, MO, USA) gradient method. The hepatic lymphocytes were harvested from the interface layer.

Lectin Stimulation and Cell Proliferation

Proliferative lymphocyte responses to PHA were measured from cells cultured in RPMI-1640 medium supplemented with penicillin/streptomycin, L-glutamine, and human AB serum for 48 h in the presence of PHA (10 μg/10^6/ml), by the uptake of ^{3}H-thymidine as previously described (Shabtai et al. 2003). All lymphocyte proliferation assays were done in triplicate. During the last 16 h of culture, the cells were pulsed with 1 μCi per well of ^{3}H-thymidine. Cells were harvested and incorporated ^{3}H-thymidine was determined by scintillation counting.

Flow Cytometry

Flow cytometry was performed using a Beckman Coulter Epics Elite, 1×10^6 cells were acquired and data were analyzed using the software supplied by Beckman Coulter. Cells were surface stained with CD4 FITC and CD25 PE and subsequently intracellular stained with the PE-CY5 antihuman Foxp3 staining kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Cytokine Assays

Serum IL-10 (Abcam, Cambridge, UK) and TGF-β1 (Bender Med Systems, Burlingame, USA) levels were measured using commercially available human enzyme linked immunosorbent assay kits according to the manufacturer's instructions.

Statistical Analysis

Results are expressed as median and range. For continuous variables with normal distribution, the analysis of variance was employed. For continuous variables with non-normal distribution, analysis of variance was employed following

the rank transformation. Statistical significance was defined as $p < 0.05$.

Results

Increased Treg Frequency in PBMCs and TILs from HCC Patients

Because CD25 is not restricted to T cells and both Treg and activated effector T cell (Teff) populations are $CD25^+$ (Banham et al. 2006), the $CD4^+CD25^+$ phenotype does not solely represent the human Tregs. To exclude those $CD4^+$ T cells with intermediate to low levels of CD25 expression without immunoregulatory function (Baecher-Allan et al. 2001), the $CD4^+CD25^{high}FoxP3^+$ phenotype was recommended for identifying the human Tregs. The percentage of Tregs was determined as performed by Yokokawa et al. (2008). Lymphocytes were gated by plotting forward versus side scatter, followed by the gating of $CD4^+$ population. Then the $CD25^{high}$ and $FoxP3^+$ populations were gated. The $CD25^{high}$ population was separated from the $CD25^{low}$ population on the basis of the frequency of CD25 expression in $CD4^-$ T cells, as previously described (Cesana et al. 2006; Hoffmann et al. 2004) (Fig. 1).

TILs were successfully isolated from 37 out of the 42 HCC patients. The $CD4^+CD25^{high}FoxP3^+$ Treg frequency of TILs [2.6% (1.3–8.7%)] was significantly higher than that of hepatic lymphocytes [1.4% (0.7–1.8%)] ($p = 0.04$). Similarly, the $CD4^+CD25^{high}FoxP3^+$ Treg frequency of PBMCs from HCC patients [2.0% (1.1–6.2%)] was significantly higher than healthy donors [1.2% (0.3–1.6%)] ($p = 0.044$). In addition, the Treg frequency of TILs was higher than that of PBMCs from HCC patients ($p = 0.046$) while no statistical difference was found between the Treg frequency for hepatic lymphocytes and PBMCs from healthy donors ($p = 0.799$).

Impaired Proliferative Response in Patients with HCC

The ^{3}H-thymidine uptake by TILs [972 (621–3,783)] was significantly lower than that of hepatic lymphocytes [5,227 (2,842–11,058)] ($p = 0.00016$) and the ^{3}H-thymidine uptake by PBMCs of HCC patients [1,071 (483–4,287)] was significantly decreased when compared to PBMCs of healthy donors [6,431 (3,162–10,326)] ($p = 0.001$) (Fig. 2). No statistical difference was found between the ^{3}H-thymidine uptake by hepatic lymphocytes and PBMCs from healthy donor ($p = 0.618$). Similarly, the ^{3}H-thymidine uptake by TILs and PBMCs from HCC patients were comparable ($p = 0.22$).

Elevated IL-10 and TGF-β1 Serum Concentrations in Patients with HCC

Serum IL-10 levels for HCC patients [42.09 pg/ml (26.47–78.52 pg/ml)] were significantly higher in healthy donors [28.61 pg/ml (14.95–47.23 pg/ml)] ($p = 0.042$). Similarly, serum concentrations of TGF-β1 for HCC patients [130.51 pg/ml (61.38–249.31 pg/ml)] was significantly higher in healthy donors [74.12 pg/ml, (57.39–136.04 pg/ml)] ($p = 0.01$) (Fig. 3).

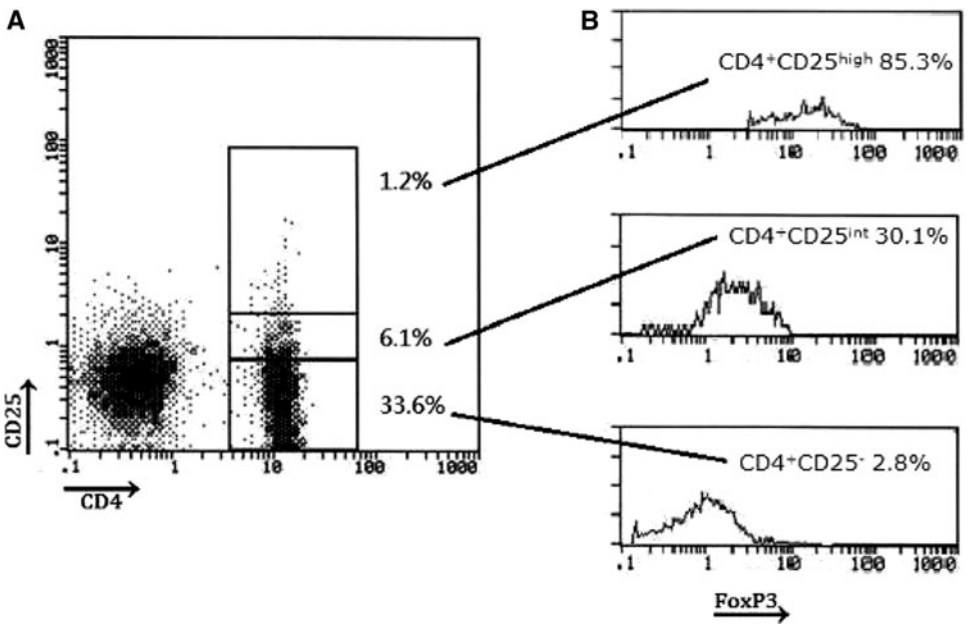

Fig. 1 Expression of FoxP3 in $CD4^+CD25^{high}$ Tregs from a healthy donor. **a** $CD4^+CD25^+$ T cell populations were gated as $CD25^{high}$, $CD25^{low}$ and $CD4^+CD25^-$ in regards to the level of CD25 expression in $CD4^-$ T cells. **b** FoxP3 expression within $CD4^+CD25^{high}$, $CD4^+CD25^{low}$ and $CD4^+CD25^-$ T cell populations

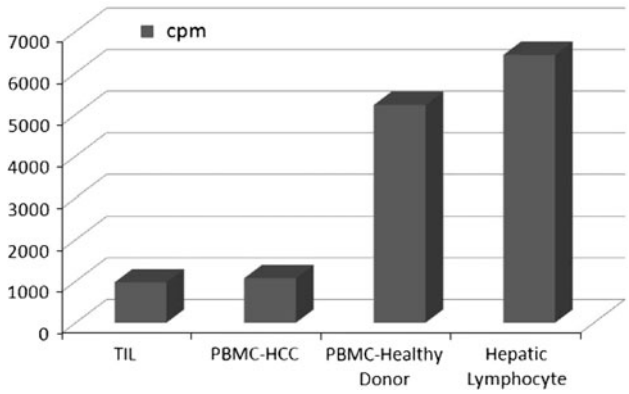

Fig. 2 The ^{3}H-thymidine uptake by TILs, PBMCs of HCC patients, PBMCs of healthy donors and hepatic lymphocytes

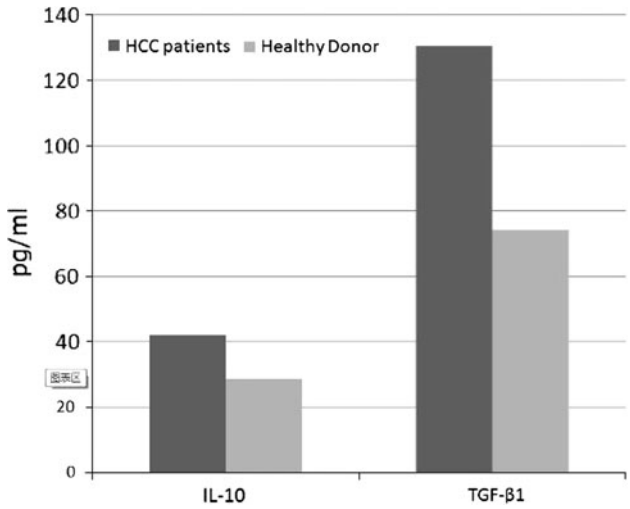

Fig. 3 Serum cytokine levels in HCC patients and healthy donors

Discussion

To achieve immunological tolerance, a network of Tregs exists to maintain a fine balance between the peripheral tolerance to autoantigens while preserving the potential to initiate protective immune responses in various contexts and inflammatory settings (McHugh and Shevach 2002; Powrie et al. 2003). Generally, there are two subsets of Treg: naturally occurring Tregs (nTreg) and induced Tregs (iTreg).The nTreg cells develop in the thymus and migrate to the periphery, playing an important role in keeping immune homeostasis and regulating the immune responses to tumors, grafts, and pathogens (Hori et al. 2003; Piccirillo and Thornton 2004; Shevach 2002). On the other hand, the iTreg cells are induced in the periphery and acquire their suppressive activity in response to stimulatory conditions, including the counter-regulatory IFN-γ-producing Th1 cells, IL-4-producing Th2 cells, IL-10-producing Tr1 cells and TGF-β-secreting Th3 cells (Toda and Piccirillo 2006). It has been shown that the $CD4^{+}CD25^{+}$ Treg frequency in HCC patients is increased (Ormandy et al. 2005; Yang et al. 2006), showing a connection between the elevated Treg levels and malignancy. However, it should be noted that the significance of those studies are considerably dampened due to their inability to exclude the possible Teff contamination (Baecher-Allan et al. 2001). This study employed the $CD4^{+}CD25^{high}FoxP3^{+}$ phenotype to identify the human Tregs and demonstrated the frequency of Tregs in peripheral blood and the tumor microenvironment were increased in HCC patients. The increase of Tregs in peripheral blood might be caused by the Tregs spreading from the tumor microenvironment. On the other hand, the observation that the Treg frequency in the tumor microenvironment were significantly higher than in PBMCs from HCC patients, suggests that the tumor microenvironment might lead to Treg expansion or Treg migration from the periphery into the tumor tissues. However, the exact mechanism why Tregs are enriched in the tumor tissue is uncertain, but the role that they play is clear: blocking the generation of immunity to tumor antigens in the periphery and neutralizing the TILs (Knutson et al. 2007). The present study revealed a negative correlation between the Treg frequency and lymphocyte proliferation: as the Treg frequency increased in lymphocytes, the uptake of ^{3}H-thymidine dramatically decreased. Obviously, this finding showed that the $CD4^{+}CD25^{high}FoxP3^{+}$ T cells from HCC patients had a potent suppressive ability on lymphocytes proliferation. There are several explanations for the mechanism mediating the immunosuppressive effects. Early studies (Dieckmann et al. 2001; Levings et al. 2001) demonstrated the suppressive effect of Treg cells that requires cell-to-cell contact. Also, it was reported that the immune response mediated by myeloid dendritic cells (Houot et al. 2006) and natural killer cells (Ghiringhelli et al. 2005) can be inhibited by $CD4^{+}CD25^{high}$ Tregs. In addition, Tregs may lead to apoptotic or necrotic T cell death through the perforin/granzyme pathway (Grossman et al. 2004). Although there are multiple pathways by which Tregs exert their immunosuppressive function, further efforts are needed to unveil the exact mechanism by which Treg suppresses the immune responses.

The current study demonstrated that serum IL-10 and TGF-β1 levels in HCC patients were significantly increased when compared to those with healthy donors. It is reported that IL-10 and TGF-β1 are necessary for Treg suppressive function (Dieckmann et al. 2002; Jonuleit et al. 2002) and could be produced by Treg cells (Barrat et al. 2002; Nakamura et al. 2001; Sundstedt et al. 2003; Zhang et al. 2001). These two cytokines play an important role in regulating the antitumor immunity. Knutson et al. (2007) suggest that the tumor tissue establishes a concentration

gradient of antigens and other factors (TGF-β, chemokines, etc.) that leads to the elevated Treg levels in peripheral blood due to the convergence of lymphatics and blood vessels. Strauss et al. (2007) showed that $CD4^+CD25^{high}$ T cells could produce IL-10 and TGF-β1 and the addition of neutralizing antibodies to these two cytokines completely abrogated the suppression, regardless of the presence of a transwell. However, when the transwell was present, but in the absence of the blocking antibodies, suppression was only reduced by 50% (Strauss et al. 2007). Thus, they suggested that these cytokines might be required for the activation of surface molecules on Tregs that are involved in suppression mediated by cell-to-cell contact. Taken together, their findings suggest that IL-10 and TGF-β1 are largely responsible for the Treg immunosuppressive function. Therefore, we hypothesize that down-regulation of serum IL-10 and TGF-β1 levels may inhibit the immunosuppressive function of Tregs and boost the host antitumor responses.

This study demonstrated an increased Treg frequency in peripheral blood and the tumor microenvironment, which correlated with the impaired lymphocyte proliferation in patients with HCC. Furthermore, serum IL-10 and TGF-β1 levels were significantly increased in patients with HCC. Significant progress in understanding the role of Tregs in immune responses to the tumors has been made in the past few years. However, further effort is needed to establish new immunotherapy strategies designed to modulate the Tregs to promote competent antitumor responses.

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Conflict of interest All the authors who have taken part in this study declared they have nothing to disclose regarding competing interests or funding from industry with respect to this manuscript.

References

Baecher-Allan C, Brown JA, Freeman GJ et al (2001) CD4+CD25high regulatory cells in human peripheral blood. J Immunol 167:1245–1253

Banham AH, Powrie FM, Suri-Payer E (2006) FOXP3+regulatory T cells: current controversies and future perspectives. Eur J Immunol 36:2832–2836

Barrat FJ, Cua DJ, Boonstra A et al (2002) In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. J Exp Med 195:603–616

Cesana GC, DeRaffele G, Cohen S et al (2006) Characterization of CD4+ CD25+ regulatory T cells in patients treated with high-dose interleukin-2 for metastatic melanoma or renal cell carcinoma. J Clin Oncol 24:1169–1177

Dieckmann D, Plottner H, Berchold S et al (2001) Ex vivo isolation and characterization of $CD4^+CD25^+$ T cells with regulatory properties from human blood. J Exp Med 193:1303–1310

Dieckmann D, Bruett C, Ploettner H et al (2002) Human $CD4^+CD25^+$ regulatory, contact-dependent T cells induce interleukin 10-producing, contact-independent type 1-like regulatory T cells. J Exp Med 196:247–253

Ghiringhelli F, Menard C, Terme M et al (2005) $CD4^+CD25^+$ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. J Exp Med 202:1075–1085

Grossman W, Verbsky J, Berchet W et al (2004) Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity 21:589–601

Harada N, Shimada M, Okano S et al (2004) IL-12 gene therapy is an effective therapeutic strategy for hepatocellular carcinoma in immunosuppressed mice. J Immunol 173:6635–6644

Hoffmann P, Eder R, Kunz-Schughart LA et al (2004) Large-scale in vitro expansion of polyclonal human $CD4^+$CD25 high regulatory T cells. Blood 104:895–903

Hori S, Takahashi T, Sakaguchi S (2003) Control of autoimmunity by naturally arising regulatory $CD4^+$ T cells. Adv Immunol 81:331–371

Houot R, Perrot I, Garcia E et al (2006) Human $CD4^+$CD25 high regulatory T cells modulate myeloid but not plasmacytoid dendritic cells activation. J Immunol 176:5293–5298

Ichihara F, Kono K, Takahashi A et al (2003) Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. Clin Cancer Res 9:4404–4408

Javia LR, Rosenberg SA (2003) $CD4^+CD25^+$ suppressor lymphocytes in the circulation of patients immunized against melanoma antigens. J Immunother 26:85–93

Jonuleit H, Schmitt E, Kakirman H et al (2002) Infectious tolerance: human $CD25^+$ regulatory T cells convey suppressor activity to conventional $CD4^+$ T helper cells. J Exp Med 196:255–260

Knutson KL, Disis ML, Salazar LG (2007) CD4 regulatory T cells in human cancer pathogenesis. Cancer Immunol Immunother 56:271–285

Levings M, Sangregorio R, Roncarolo MG (2001) Human $CD25^+CD4^+$ T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. J Exp Med 193:1295–1302

Llovet JM, Burroughs A, Bruix J (2003) Hepatocellular carcinoma. Lancet 362:1907–1917

McHugh RS, Shevach EM (2002) The role of suppressor T cells in regulation of immune responses. J Allergy Clin Immunol 110:693–702

Nakamura K, Kitani A, Strober W (2001) Cell contact-dependent immunosuppression by $CD4^+CD25^+$ regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med 194:629–644

Ormandy LA, Hillemann T, Wedemeyer H et al (2005) Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. Cancer Res 65:2457–2464

Piccirillo CA, Thornton AM (2004) Cornerstone of peripheral tolerance: naturally occurring $CD4^+CD25^+$ regulatory T cells. Trends Immunol 25:374–380

Powrie F, Read S, Mottet C et al (2003) Control of immune pathology by regulatory T cells. Novartis Found Symp 252:92–98 [discussion 98–105, 106–114]

Sakaguchi S, Sakaguchi N, Asano M et al (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 155:1151–1164

Sasada T, Kimura M, Yoshida Y et al (2003) CD4 + CD25 + regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression. Cancer 98:1089–1099

Schaefer C, Kim GG, Albers A et al (2005) Characteristics of $CD4^+CD25^+$ regulatory T cells in the peripheral circulation of patients with head and neck cancer. Br J Cancer 92:913–920

Shabtai M, Ye H, Kono K et al (2003) Immune inhibitory effects of renal cell carcinoma extract on lectin and alloantigen-induced peripheral blood and tumor infiltrating lymphocyte blastogenesis. Urol Oncol 21:27–32

Shevach EM (2002) $CD4^+CD25^+$ suppressor T cells: more questions than answers. Nat Rev Immunol 2:389–400

Strauss L, Bergmann C, Szczepanski M et al (2007) A unique subset of CD4 + CD25highFoxp3 + T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment. Clin Cancer Res 13(15 Pt 1): 4345–4354

Sundstedt A, O'Neill EJ, Nicolson KS et al (2003) Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo. J Immunol 170:1240–1248

Takagi S, Chen K, Schwarz R et al (1989) Functional and phenotypic analysis of tumor-infiltrating. Lymphocytes isolated from human primary and metastatic: liver tumors and cultured in recombinant interleukin-2. Cancer 63:102–111

Toda A, Piccirillo CA (2006) Development and function of naturally occurring $CD4^+CD25^+$ regulatory T cells. J Leukoc Biol 80: 458–470

Woo EY, Chu CS, Goletz TJ et al (2001) Regulatory $CD4^+CD25^+$ T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res 61:4766–4772

Yang XH, Yamagiwa S, Ichida T et al (2006) Increase of $CD4^+CD25^+$ regulatory T-cells in the liver of patients with hepatocellular carcinoma. J Hepatol 45:254–262

Yokokawa J, Cereda V, Remondo C et al (2008) Enhanced functionality of $CD4^+CD25$(high)$FoxP3^+$ regulatory T cells in the peripheral blood of patients with prostate cancer. Clin Cancer Res 14:1032–1040

Zhang X, Izikson L, Liu L et al (2001) Activation of $CD25^+CD4^+$ regulatory T cells by oral antigen administration. J Immunol 167:4245–4253

Zhang J, Dong Z, Zhou R et al (2005) Isolation of lymphocytes and their innate immune characterizations from liver, intestine, lung and uterus. Cell Mol Immunol 2:271–280