ORIGINAL ARTICLE

CD4+CD25high regulatory cells in peripheral blood of cancer patients

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Abstract

AIM: Regulatory T cells (Treg) that prevent autoimmune diseases by suppression of self-reactive T cells may also suppress the immune response against cancer. Experimental tumor models in mice revealed that Tregs are potent inhibitors of an antitumor immune response. The purpose of the study was to identify a $CD4^+$ population of regulatory T cells expressing high levels of CD25($CD4^+CD25^{high}$) in the peripheral blood of cancer patients and provide the opportunity to determine whether cancer patients exhibit an expanded $CD4^+CD25^{high}$ pool.

METHODS: The frequency of $CD4^+CD25^{high}$ in the peripheral blood of 62 cancer patients and 15 healthy donors was determined by flow cytometry.

RESULTS: Compared with healthy donors, cancer patients have an increasing prevalence of $CD4^+CD25^{high}$ T cells in the peripheral blood with characteristics of Tregs, *i.e.* they are CD45-RA(-), CD69(-). Among patients, those with higher percentages of $CD4^+CD25^{high}$ T cells had a poor prognosis than did those with lower percentages.

CONCLUSION: We provide evidence of an increased pool of $CD4^+CD25^{high}$ in the peripheral blood of cancer patients, which may be related to immunosuppression and tumor progress in cancer patients. This finding suggests that the use of immunomodulatory therapy to treat cancer patients may be an effective strategy.

INTRODUCTION

It is indicated that malignant tumor cells may induce immune tolerance by multiple mechanisms. For example, some tumors do not express major histocompatibility complex (MHC) molecules so that these tumor cells do not elicit sufficient anti-tumor immunity [1, 2]. In other cases, tumor cells fail to generate new antigen or tumor-specific antigens; and as a result they are unable to evoke immune recognition and tumor destruction because of low intrinsic immunogenicity and tumor-associated immunosuppression [3, 4]. Moreover, even when tumor cells express tumor antigens which may act as an efficient target for MHC class I-restricted responses *in vivo,* the immune system still fails to produce an effective anti-tumor immune response [5,6]. The patholog-

ical interactions between cancer cells and host immune cells in the tumor microenvironment create an immunosuppressive network that promotes tumor growth, protects the tumor from immune attack and attenuates immunotherapeutic efficacy [7, 8]. All in all, malignant tumor cells may induce tolerance in the bodies bearing tumors [9].

Tolerance is the 'holy grail' in the field of immunology and is generally divided into central tolerance and peripheral tolerance. It has been suggested that Treg are responsible for inducing and maintaining peripheral tolerance and for negative regulation of tumor immunity so that they contribute to tumor growth in mice [10,12].

The recent description of a unique lineage of $CD4^+$ T cells that suppress T cell effector function has shed some light on the basic mechanisms of immune homeostasis [13]. These cells, called regulatory T cells (Treg), they are characterized by coexpression of CD4 and the IL-2R -chain (CD25).

Treg have been isolated from human peripheral blood mononuclear cells (PBMCs) and mouse spleen [14,15]. These cells are thought to be a functionally unique subset of T lymphocytes that play an important function in maintaining immune homeostasis and protecting the host against autoimmune diseases [16, 17]. In addition to CD4 and CD25 markers, Treg also constitutively express CD45RO and CD152 (CTLA-4). Ex vivo studies on these cells reveal a poorly proliferative cell population that secretes inhibitory cytokines such as TGF-beta and IL-10 [14]. They also inhibit the proliferation of $CD4^+25^-$ and CD8 lymphocytes [18]. Recently, Baecher-Allan et al [19] report the identification of a $CD4^+$ population of regulatory T cells in the circulation of healthy humans expressing high levels of CD25($CD4^+CD25^{high}$) that exhibit in vitro characteristics identical with those of the $CD4^+CD25^+$ regulatory cells isolated in mice. This $CD4^+CD25^{high}$ T cell subset in humans comprises 1–2% of circulating CD4 T cells, unlike that in rodents where 6–10% of $CD4^+$ T cells demonstrate regulatory function. Whereas the entire population of $CD4^+CD25^+$ T cells expressing both low and high CD25 levels exhibit regulatory function in the mouse, only the $CD4^+CD25^{high}$ population exhibits a similarly strong regulatory function in humans. In the present study, we demonstrate the possible involvement of $CD4^+CD25^{high}$ regulatory T cells in immune system impairment in patients with solid tumor and its clinical significancy.

Table 1. Patients characteristics

	Healthy individuals	Malignancies
	(n= 15)	(n=62)
Age(yrs)	50.2+10.2	52.4 + 11.12
Male	9	40
Female	6	22
TNM stage		
I		3
II		12
III		21
IV		26

PATIENTS AND METHODS

Patients and normal donors. The current study involved 62 cancer patients (range age: 24–78 yrs) who were admitted to the cancer center, Union hospital afflcated to the Tong-ji medical college, Huazhong University of Science and Technology (Wuhan, China), between Oct. 2002 and Jan. 2006; Malignancies represented in the study population were diagnosed by pathology, included Non-small cell lung cancer (NSCLC, n=20), naso-pharyngeal carcinoma (n=18), lymphoma (n=10), gastric-intestinal carcinoma (n=9), breast cancer (n=3), others (n=2). Tumor stages were determined using TNM classification system. None of the patients received surgery, radiotherapy, chemotherapy, immunotherapy, or other medical interventions during this study. Characteristics of the study subjects are summarized in Table 1.

The 15 healthy donors also were included in the study, including 9 men and 6 women (age range, 25–70 yrs). Informed consent was obtained from all volunteers and all patients according to institutional review board approved protocol. Peripheral blood were obtained from all study participants.

Antibodies and Flow Cytometric Analysis. Blood mononuclear cells were prepared by Ficoll density gradient and stained with combinations of the following monoclonal antibodies (all purchased from BD Biosciences, San Jose, CA USA): Phycoerythrin (PE)-, fluorescein isothiocyanate (FITC)-and PE-Cychrome 5 (PC5) conjugated anti-CD4 antibodies; PE- and FITC-anti-CD25; Cy-chrome-conjugated anti-CD69; Cy-chrome-conjugated anti-CD45 RA; Flow cytometry was performed on a FACS-Calibur using CellQuest software (BD Biosciences, San Jose, CA USA).

Statistical analysis. Differences in cell surface molecule expression were determined by *t-test*, with $p < 0.05$ considered significant. NSCLC patient survival was the interval between diagnosis and death or the time of analysis. Data were censored at the last follow-up for patients who were disease-free or alive at the time of analysis. Median survival times were computed using Kaplan-Meier methods. 95% confidence intervals were computed where possible. Differences in survival functions were assessed using the log-rank test. All analyses were performed using SAS 8.2 software.

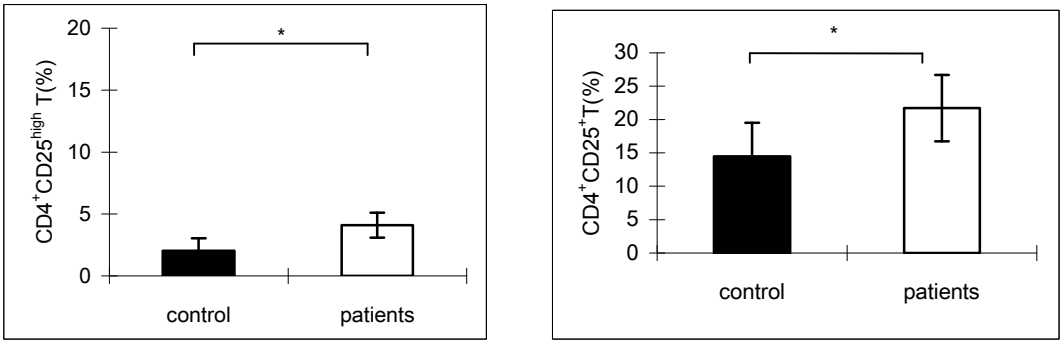

Figure 1. Treg in cancer patients. A. Cancer patients show a significant increase in $CD4^+CD25^{high}$ T cells in peripheral blood. B.Cancer patients show a significant increase in total $CD4^+CD25^+$ T cells in peripheral blood. *: $p< 0.001$.

RESULTS

The prevalence of $CD4^+CD\ 25^{high}$ Treg lymphocytes is higher in cancer patients than in normal individuals

The population of $CD4^+CD25^{high}$ cells as a percentage of total $CD4^+$ T cells was evaluated by flow cytometric analysis using the gating strategy (shown in Figure 1). Using FITC-conjugated CD25 antibody, the prevalence of $CD4^+CD25^{high}$ cells in cancer patients (4.2±1.9%: n=62) was significantly higher than that in normal individuals (2.04±1.03%: n=15, p<0.001). Representative dot plots of cancer patients and normal donors are shown in Fig. 1. Cumulative data for all the patients and normal donors are presented in a bar chart (Fig.lA). In addition, the increase in $CD4^+CD25^{high}$ T-cell proportion in patients with cancer was matched by an increase in the total proportion of $CD4^+CD25^+$ T-cells (incorporating both $CD25^{low}$ and $CD25^{high}$ populations, Fig. IB), expressed both as a percentage of $CD4^+$ T-cells (cancer patients: 21.71±8.28 %: normal: 14.49±4.69%; p<0.001).

The $CD4^+25^{high}$ T lymphocytes from cancer patients are phenotypically similar to T-regs from normal donors

Approximately one-half of the circulating normal human peripheral blood lymphocytes express CD4, and of these roughly 10% express the IL-2 growth factor receptor alfa-chain, CD25. Peripheral blood lymphocytes do not stain very strongly for CD25. Unlike what is seen in the mouse, the $CD25^+$ population in the human is not as clearly discernable. Rather, the $CD4^+$ T cells with the highest level of CD25 ($CD4^+CD25^{high}$) appear as a tail to the right from the major population containing both $CD4^+CD25^{low}$ and $CD4^+CD25^-$ cells. The $CD25^{high}$ cells represent 1–2% of the total $CD4^+$ T cell population, whereas the $CD25^{low}$ cells can represent up to 16% of $CD4^+$ T cells (Fig. 2A). To confirm that the more prevalent $CD4^+25^{high}$ lymphocytes in cancer patients are indeed similar to well-documented T-regs isolated from normal donors, we compared the surface marker expression by three-color staining and flow cytometry. As shown in Fig.2B, $CD4^+CD25^{high}$ T-cells derived from cancer patients had a broadly similar phenotype to equivalent populations in normal volunteers. In particular, we confirmed that $CD4^+$ $CD25^{high}$ T-cells of cancer patients had low forward scatter, low expression of CD45RA and lacked markers of recent activation (CD69).

The relationship of the $CD4^+25^{high}$ T lymphocytes to clinical stages

To determined whether the increase in the relative prevalence of the $CD4^+$ $CD25^{high}$ T cells was related to disease stage, we examined the prevalence by disease stag e of the $CD25^{high}$ subset among CD4 T cells in cancer patients. The proportion of $CD4^+CD25^{high}$ T cells in cancer patients with advanced (stage III/IV) stage (stage III: 4.0±1.51%, n=21; stage IV: 4.85±1.67%, n=26) was significantly higher than those in earlier stage (stage I and II: 2.41 ± 1.03%, n=15) (p<0.001)(Fig.3).

Increases in tumor $CD4^+25^{high}$ Treg cells predict poor survaval of NSCLC

We predicted that the $CD4^+25^{high}$ Treg cells in peripheral blood of cancer patients would adversely affect survival. To test this prediction, we followed up NSCLC patients from Feb. 2003 to Jan. 2006. they were divided into three groups according to the prevalence of $CD4^+CD25^{high}$ regulatory Treg cells in peripheral blood, including five patients of $CD4^+CD25^{high}$ <3%; seven patients of 3% <$CD4^+CD25^{high}$ <4%; eight patients of $CD4^+CD25^{high}$> 4%. There was a significant correlation between the $CD4^+25^{high}$ Treg cells in peripheral blood and survival (p = 0 . 0 026) (Fig. 4).

DISCUSSION

In recent years, much attention has been given to factors that regulate immune responses to malignant cells contribute to the establishment and progressive growth of tumors. There are many proposed explanations for why tumor cells do not stimulate immune responses and/or are able to evade an immune attack in some cases, but two of these explanations are dominant: 1) the failure of the host immune system to recognize tumor antigens

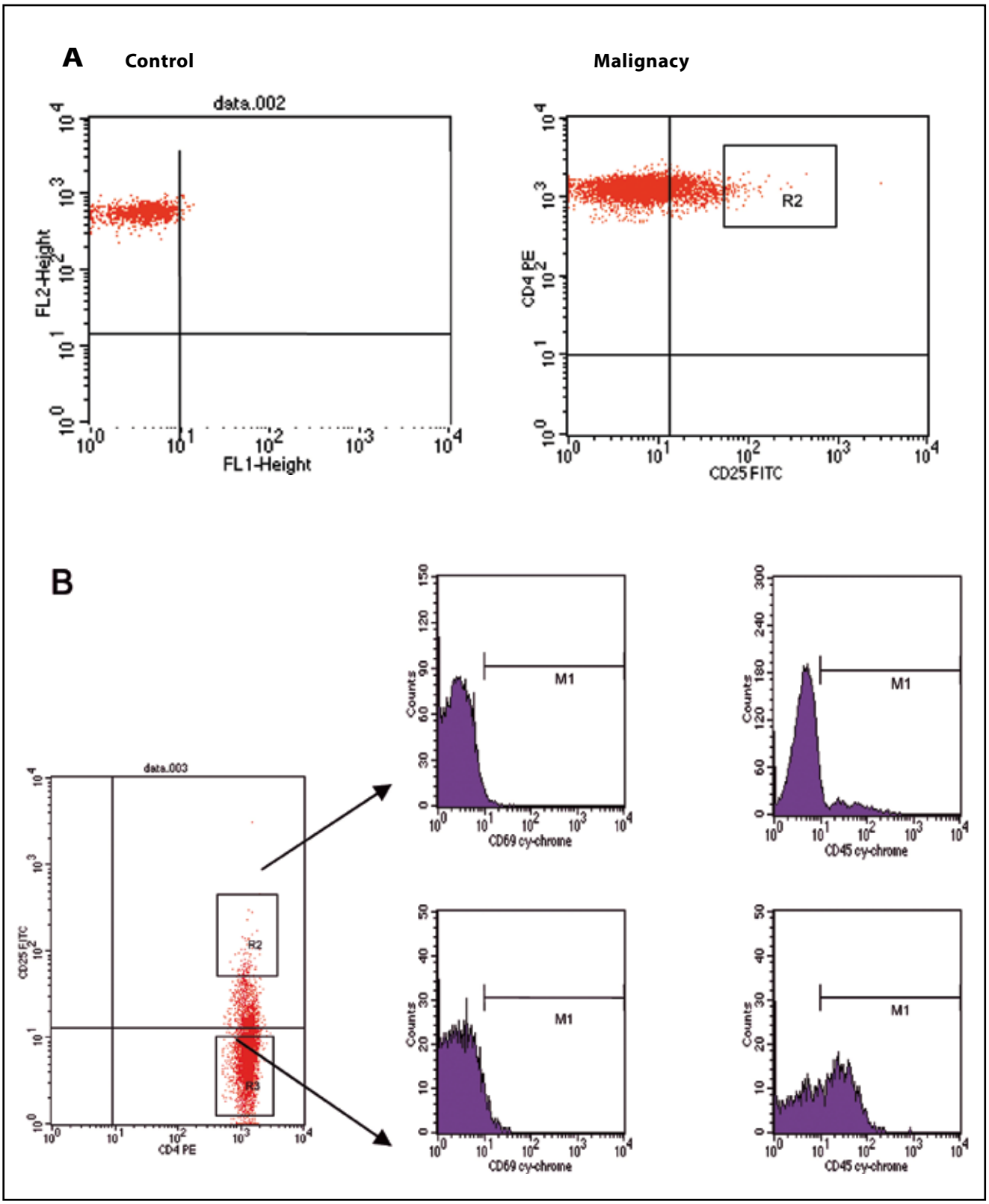

Figure 2. CD4+CD25high T cells from peripheral blood of cancer patients exhibit a Treg-phenotype.
A) a representative staining for CD4+CD25high T cells is shown for one cancer patient and one healthy control.
B) Gate CD4+CD25high T cells (upper panels, CD45RA−, CD69−) and CD4+CD25− T cells (low panels, CD45RA+, CD69−) were analyzed for expression of CD45RA, CD69.

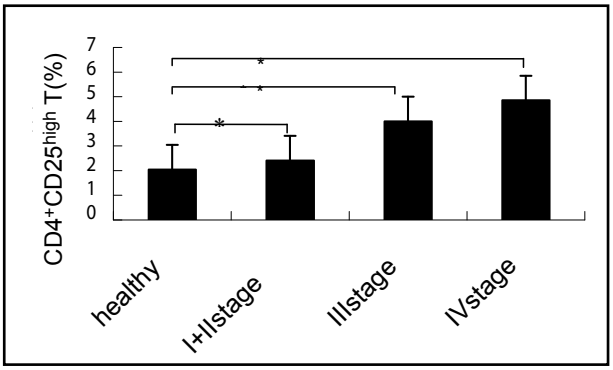

Figure 3. $CD4^+CD25^{high}$ T cells from peripheral blood of cancer patients with different Stages. *: > 0.05; **: < 0.001

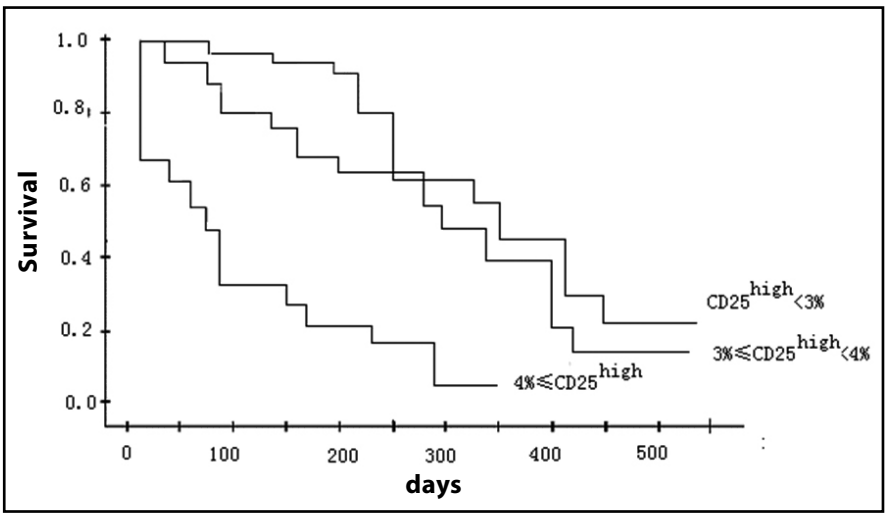

Figure 4. Accumulation of tumor $CD4^+CD25^{high}$ Treg cells predict poor survival individuals with NSCLC. A Kaplan-Meier curve for overall survival. Patients were divided into three groups on the basis of the prevalence of $CD4^+CD25^{high}$ Treg cells in peripheral blood. Survival significantly decreased as a increase of $CD4^+CD25^{high}$ Treg prevalence.

(ignorance) and 2) the failure of tumor-specific T cells to proliferate and function at the levels required for eradicating tumors (immuno-suppression) [20). Curiel *et al.* [21] also presented evidence that human Treg have an important immunopathological role in human cancer by suppressing tumor-associated-antigen-specific T-cell immunity. Because a number of studies have demonstrated that Treg-mediated immunosuppression is one of the crucial mechanisms of tumor immune-evasion and the main obstacle of successful tumor immunotherapy [21–23].

There is increasing evidence that most tumor associated antigens are self antigens [24]; this evidence suggests that immunosuppression in tumor-bearing hosts may be associated with regulatory $CD4^+CD25^+$ T cells, which play a key role in the maintenance of immunologic self-tolerance via the inhibition of activated T cells. Tumor-induced tolerance mediated by T cells has been demonstrated in a variety of tumor types in mice. This has been well documented in a large body of literature which has been reviewed by R. J. North [25].

Human CD4 regulatory function was observed only when the cells expressed high levels of CD25 ($CD4^+CD25^{high}$) and were isolated apart from the $CD25^{low}$ T cells, because the features of the mouse CD4 CD25 and the human $CD4^+CD25^{high}$ regulatory cell populations are essentially identical, so they represent homologous populations. With TCR cross-linking, $CD4^+CD25^{high}$ cells did not proliferate but instead totally inhibited proliferation and cytokine secretion by activated $CD4^+CD25^-$ responder T cells in a contact-dependent manner. Thus, regulatory CD4 T cells expressing high levels of the IL-2 receptor and class II MHC are present in humans.

This study provides the evidence for increased prevalence of $CD4^+CD25^{high}$ Treg lymphocytes in the peripheral blood of patients with invasive cancer. In 62 cancer patients, the prevalence of $CD4^+CD25^{high}$ Treg in peripheral blood was significantly higher than that in 15 normal donors. These $CD4^+CD25^{high}$ T cells were $CD69^-$ but $CD45RA^-$. Now, the fact that the increased population of $CD4^+CD25^{high}$ is observed in peripheral blood of malignancies is established. Furthermore, we examined the prevalence by disease stage of the $CD25^{high}$ subset among CD4 T cells in cancer patients. The proportion of $CD4^+CD25^{high}$ T cells in cancer patients with advanced (stageIII/IV) stage was significantly higher than those in earlier (stage I and II) stage (p<0.001).These findings suggest that the relative prevalence of $CD4^+CD25^{high}$ regulatory T cells may be a determinant for predicting the prognosis of cancer patients.

It is well known that Treg are responsible for inducing and maintaining peripheral tolerance and the neg-

ative regulation of immunity [26,27]. Treg have been suggested to play an important immunopathological role in human cancer by 'lowering' the intrinsic T-cell immunity towards tumor-associated antigen, resulting in tumor immune evasion [21–23]. Some reports have indicated that Treg depletion can be useful in tumor biotherapy [28, 29]. Treg depletion would eliminate immune-suppression mediated by Treg leading to enhanced T-cell activity. When Treg are depleted using monoclonal antibody (mAb), transplanted tumors in mice are efficiently rejected by the host immune system [30, 31]. This finding suggests that Treg, which function as a protective mechanism against autoimmunity, may also mitigate the immune response against cancers. Now, the recognition the importance of $CD4^+CD25^{high}$ regulatory T cells in cancer patients will allow the rational design of more effective treatment. A strategy of treating cancer patients with Abs targeting $CD4^+CD25^{high}$ Treg offer promise. Furthermore, the efficacy of any vaccine based immunotherapy may be greatly enhanced by combining such vaccines with Abs that deplete $CD4^+CD25^{high}$ Treg.

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