Regulatory T cell depletion enhances tumor specific CD8 T-cell responses, elicited by tumor antigen NY-ESO-1b in hepatocellular carcinoma patients, *in vitro*

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Abstract. Immunotherapy in hepatocellular carcinoma based on one or a few tumor specific antigens have shown limited antitumor efficacy. As a major suppressive factor in tumor immune response, better understanding of the role of regulatory T cells (Tregs) in hepatocellular carcinoma might be important for design of future immunotherapy-based clinical protocols. Tregs from 49 HCC patients and 40 controls were identified by flow cytometric analysis for the phenotype. Functional studies were performed by analyzing their inhibition to immune responses. Finally investigating whether elimination of Tregs was capable of enhancing the immunostimulatory efficacy of NY-ESO-1b peptides. In HCC peripheral blood and tumor-infiltrating lymphocytes, we found increased numbers of Tregs, which expressed high levels of HLA-DR, GITR and CD103. The prevalence of Tregs increased with during progressive stages in HCC patients. Moreover, the elimination of Treg cells followed by stimulating with NY-ESO-1b peptide significantly improved the anti-tumor cytotoxic T lymphocytes responses in HCC patients compared with stimulating with NY-ESO-1b peptide alone. The immune response effi-ciency increased from 37.5 to 62.5%. In conclusion, the increase in frequency of Treg cells might play a role in suppression of the immune response against HCC and for the design of immunotherapy the incorporation of the Treg cell depletion strategy will achieve potent anti-tumor immunity with therapeutic impact.

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Abbreviations: HCC, hepatocellular carcinoma; TILs, tumor-infiltrating lymphocytes; HBV, hepatitis B virus; Treg, regulatory T cell; HCV, hepatitis C virus; PBMC, peripheral blood mononuclear cell; IFN-γ, interferon γ; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; HBcAg, hepatitis B core antigen; mRNA, messenger RNA; HBeAg, hepatitis B e antigen; DC, dendritic cell; BrdU, bromodeoxyuridine; PE, phycoerythrin; FITC, fluorescein isothiocyanate; FoxP3, forkhead box P3 transcriptional regulator; GITR, glucocorticoid-induced TNF receptor family gene; MLR, mixed-lymphocyte reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. CTLs, cytotoxic T lymphocytes. ELISPOT, enzyme-linked immunospot. SFDA, state food and drug administration; CT antigen, cancer-testis antigen; FCM, flow cytoMeter; CHB, chronic hepatitis B

Key words: T-lymphocytes, regulatory, carcinoma, hepatocellular carcinoma, immunotherapy, NY-ESO-1b, immune response

Introduction

As the fifth most common cancer worldwide and the third leading cause of cancer-related mortality, HCC causes a poor prognosis and limited survival (1). Despite improvements in HCC therapy including surgical resection, and liver transplantation in recent years, the outcome is still unsatisfactory. Thus, it remains a challenge to identify and establish alternative treatment options for this disease.

A promising new approach for the treatment of tumors is immunotherapy (2). The discovery that tumor antigens can elicit measurable immune responses has opened new possibilities for cancer treatment (3,4). Human tumor cells express diverse types of antigens during disease pathogenesis (5). Of these, tumor germ-line gene-encoded cancer-testis (CT) and melanocyte differentiation antigens have been shown to be widely immunogenic and are currently being tested for use in experimental therapeutic vaccines for cancer patients (6). One of the CT antigens, NY-ESO-1, induces specific cellular and humoral immune responses in melanoma, ovarian, and breast cancer patients without causing severe adverse effects (7,8). NY-ESO-1b peptide (p157-165, SLLMWITQC) has just been approved by the SFDA of China and clinical trials using NY-ESO-1b to treat HCC have been initiated in China. Previous reports by our laboratory have shown that NY-ESO-1b has the capacity to elicit tumor-specific cellular and humoral immune responses against this antigen in HCC patients (9). However, only 35.7% develop tumor-specific cellular immune responses and most patients (about 65% of all HCC patients) analyzed could not elicit measurable tumor-specific cellular immune response (9). As a result, it is critical to analyze possible mechanisms that impact the

potency of tumor-specific immune responses in patients with HCC.

$CD4^+CD25^+$ regulatory T cells (Treg) play a vital role in immunologic self-tolerance, anti-tumor immune responses, and transplantation (10,11,13). These T cells represent 5-10% of the human $CD4^+$ T cell population, are anergic, and thus do not proliferate in response to T cell receptor stimulation *in vitro* (12). Treg cells express CD4, CD25 (the interleukin 2 receptor chain), CD45RO, the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (12,13) and the forkhead/winged helix transcription factor gene (FoxP3) (14,15) and suppress the activation and proliferation of both $CD4^+$ and $CD8^+$ T cells (16,17). This cell type increases in patients with HCC (18-21), ovarian cancer (22), and gastric and esophageal carcinomas (23), and experimental tumor models show that removal of $CD25^+$ T cells alters the immune response to tumors both *in vitro* and *in vivo* (24). $CD25^+$ T cell depletion increases the tumor-specific immune response in patients with carcinoma (25-29). The presence of $CD4^+CD25^+$ Treg cells maybe can explain why some current immunotherapy protocols are ineffective. Thus, in order to design new immunotherapy-based clinical protocols, it will be important to gain a better understanding of the role of Treg cells in HCC development.

Materials and methods

Patients and healthy donors. Peripheral blood samples were collected from 49 HCC patients and 40 controls consisting of patients with chronic hepatitis B (who had no evidence of cirrhosis and no antiviral therapy during the six months prior to blood sampling), patients with liver cirrhosis, and healthy donors from the Hepatology Institute of Peking University People's Hospital (Peking, China) and the Guilin Medicial University (Guilin, China). All participants gave informed consent before blood donation and the Ethics Committee of Peking University People's Hospital approved the study protocol. Patient and control characteristics are shown in Table I.

HLA-A2 subtyping and tumor typing for NY-ESO-1 messenger RNA. The expression of HLA-A2 was assessed using PCR-sequence specific primers and sequence-based typing as described previously (30). The expression of NY-ESO-1 mRNA in tumor specimens from HCC patients was assessed by RT-PCR using the primers described previously (31).

NY-ESO-1b peptide. The HLA-A2-restricted NY-ESO-1b peptide, p157-165 (SLLMWITQC), was synthesized from Sangon Co. Ltd. (Shanghai, China). Purity after high-pressure liquid chromatography analysis was above 95%. An HCV peptide (NS31073-1081, CINGVCWTV) was used as a negative control to for tetrameric analysis.

Flow cytometric analysis. To determine the frequency and phenotype of Treg cells in PBMC and TIL, multicolor fluorescence-activated cell sorting analysis was performed using the following antibodies: anti-CD4, anti-CD25, anti-CD127, anti-CD62L, anti-HLA-DR, anti-CD45RA, anti-CD45RO, anti-CD152, anti-CD103, (BD PharMingen, Heidelberg, Germany), PE-conjugated anti-Foxp3 (Biolegend, USA) and anti-GITR (R&D Systems, Weisbaden, Germany). Three or four color flow cytometry was conducted using a Becton-Dickinson FACS Calibur and FlowJo 7.0 software. Isotype-matched antibodies were used as controls for all the samples.

Cell isolation and sorting. Peripheral blood mononuclear cells (PBMC) were isolated from freshly obtained blood using a Ficoll density gradient. To isolate $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells, PBMCs were further separated using the regulatory T cell isolation kit (StemCell, Canada), according to the manufacturer's instructions. Enriched cells were determined by flow cytometry and the purity after sorting was >90%.

Proliferation and suppression assays. $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were isolated and purified as described above. Cells from both fractions were cultured and stimulated with plate-bound anti-CD3 (Lab Vision, USA) and 1 μg/ml soluble anti-CD28 (R&D System, USA) in the presence of 20 μg/ml interleukin (IL)-2 (R&D System) in 96-well round-bottomed plates for 96 h. The suppressive effect of $CD4^+CD25^+$ T cells was assessed by co-culturing $CD4^+CD25^+$ and $CD4^+CD25^-$ cells with increasing concentrations of $CD4^+CD25^+$ cells in the presence of anti-CD3 and anti-CD28. Proliferation was measured by BrdU incorporation after 96 h (Roche, USA). BrdU was added to each culture and cell proliferation was measured by the incorporation of BrdU between 18 and 24 h later. The OD value was measured by calculating the stimulation index using an absorbance microplate reader (Bio-Rad 680, USA). The stimulation index was calculated according to the formula: SI = mean OD of stimulated culture/mean OD of non-stimulated control culture.

Isolation of TILs. Tumor specimens and non-tumor adjacent tissues were obtained from 12 HCC patients at the time of surgery. Samples were placed in RPMI supplemented with 10% fetal bovine serum and 0.02% EDTA, divided into small pieces, and incubated in complete medium for 2 h to obtain single cell suspensions. Hepatocytes were removed by centrifuging the samples at 50 x g. Supernatants were centrifuged at 2,000 x g and the pellets were washed twice in PBS and suspended in RPMI. Five to $10x10^5$ mononuclear cells were obtained.

$CD4^+CD25^+$ T cell depletion. $CD4^+CD25^+$ T cells were isolated from PBMC using the $CD4^+CD25^+$ Treg cell isolation kit (StemCell, Canada) according to the manufacturer's instructions. The efficiency of $CD4^+CD25^+$ T cell depletion was >90%. Untreated PBMC or $CD4^+CD25^+$ T cell depleted PBMCs were used to produce short-term T cell lines.

Expansion of CTLs. Total PBMCs or $CD4^+CD25^+$ depleted T cells were plated at $4x10^5$/well in U-bottomed 96 well plates, and stimulated in RPMI with 10% fetal calf serum containing the NY-ESO-1b peptides (20 μg/ml) and HCV peptides (20 μg/ml) used as a negative control. Recombinant IL-2 (30 U/ml, R&D Systems) was added after 24 h of culture, and the frequency and function of CTLs was analyzed 10 to 12 days later.

Table I. Patient and control characteristics.

Characteristics	HCC patients	CHB patients	Healthy donors
Mean age ±SD/y	49.9±12.9	36.2±17.5	46.9±14.5
Sex, male/female	36/13	10/5	20/5
HBsAg +/-	29/20	15/0	Neg
HBeAg +/-	15/33	12/3	Neg
HBV DNA, ±SD	13/29/7	ND	ND
TNM stage II/III/IV	8/19/22	ND	ND
Serum AFP level >400/400-200/<200	22/15/12	ND	ND

HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; Neg, negative; ND, no detected.

Enzyme-linked immunospot assays. IFN-γ ELISpot assays were performed using $2x10^5$ PBMC in the presence or absence of $CD4^+CD25^+$ T cells (n=16). Ninety-six well Immobilon-P membrane microtiter plates (Millipore, Bedford, MA) were coated with 100 µl of 5 µg/ml anti-IFN-γ monoclonal antibody, and human IFN-γ enzyme-linked immunospots were performed according the manufacturer's instructions (ELISpot kit; Diaclone, France). The pre-incubated cell cultures (included four groups, medium group, PBMC without NY-ESO-1b peptides stimulated group, PBMC with NY-ESO-1b peptides stimulated group, PBMC with NY-ESO-1b peptides stimulated and Treg depleted group) were transferred to Immobilon-P plates as the responder T cells. NY-ESO-1b peptides were added together with responder T cells to each well. After incubation for 18-20 h at 37˚C, cells were removed, and the plates were developed with a second (biotinylated) antibody to human IFN-γ and streptavidin-alkaline phosphatase. Plates were developed for 10 min at room temperature in the dark, and the reaction was stopped by rinsing plates with distilled water. The membranes were air dried. The spots were counted using an ELISpot reader system (CTL Immunospot 3B, USA). The number of specific spot-forming cells was determined as the mean number of spots in the presence of an antigen minus the mean number of spots in the wells containing medium only, and expressed per $2x10^6$ PBMCs.

Staining with HLA-tetrameric complexes. HLA class tetramers were purchased (Proimmune, Oxford, UK). HLA-A2-restricted NY-ESO-1b peptide, p157-165 (SLLMWITQC), is an HLA-A2 tetramer specific for HLA-A2-restricted NY-ESO-1b peptide 157-165-specific CD8 cells. The tetramers were used to stain short-term lines produced from the patients' PBMCs. T cell lines were incubated for 10 min at room temperature with 10 µl of a phycoerythrin (PE)-labeled tetrameric complex. Cells were washed in PBS and incubated at 4˚C for 20 min with saturating concentrations of directly conjugated anti-CD8 FITC monoclonal antibody (Becton-Dickinson). After further washing, the cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry (FACS Calibur; Becton-Dickinson) using Flow Jo7.0 software.

Analysis of Foxp3 expression by real-time PCR. $CD4^+CD25^+$ and $CD4^+CD25^+$ cells were sorted as described above. Cell RNA was extracted using the RNeasy mini kit (Qiagen, USA) according to the manufacturer's instructions. Foxp3 mRNA was determined by RT-PCR, using GAPDH as an internal control. The primers used for Foxp3 and GAPDH were as follows: Foxp3 forward 5'-GAAACAGCACATTCCCAGA GTTC-3' and reverse 5'-CAACCTGAGTCTGCACAAG TGC-3', and GAPDH forward 5'-CCACATCGCTCAGACA CAT-3' and reverse 5'-GGCAACAATATCCACTTTACCAG AGT-3'. Real-time PCR products were generated in 35 cycles from cDNA in a Rotor-Gene 3000 real-time PCR apparatus (Corbett Research) in a mixture containing SYBR-Green (Molecular Probes) and 1 µl of 100 µM specific primers for differentially expressed genes.

SYBR-Green fluorescent signals were used to generate cycle threshold values from which mRNA ratios were calculated when normalized against the GAPDH housekeeping gene. Thermal cycling included 35 cycles of 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 1 min, in addition to one cycle of 72˚C for 8 min. PCR products were confirmed by ethidium bromide-stained 1.5% agarose gel electrophoresis. A semi-quantitative value for the initial target concentration in each reaction was determined on the basis of the kinetic approach using the LightCycler system software, version 2.0.

Statistical analysis. Continuous variables were expressed as the mean ± SD. Differences of continuous parameters between the groups were analyzed, and one-way analysis of variance followed by the Scheffe's post-hoc test was employed. Statistical analysis was done using the Student's t-test to assess differences between the study groups. A value of $P<0.05$ was considered significant. The data were analyzed using SPSS software version 11.0 (SPSS Inc, Chicago, IL, USA).

Results

$CD4^+CD25^+$ Treg cell numbers are higher in the peripheral blood of HCC patients. The $CD4^+CD25^{high}$ subset of $CD4^+$ T cells is often included in calculations of the $CD4^+CD25^+$ Treg frequency in humans (29-31). However, recent research has shown that Treg are defined as the population of $CD4^+$ $CD25^+FoxP3^+$ T cells as a percentage of total $CD4^+$ T cells. As a result, we determined the frequency of $CD4^+CD25^+$ Treg cells by measuring the number of $CD4^+CD25^+FoxP3^+$ T cells (Fig. 1). Our study revealed that the frequency of circulating

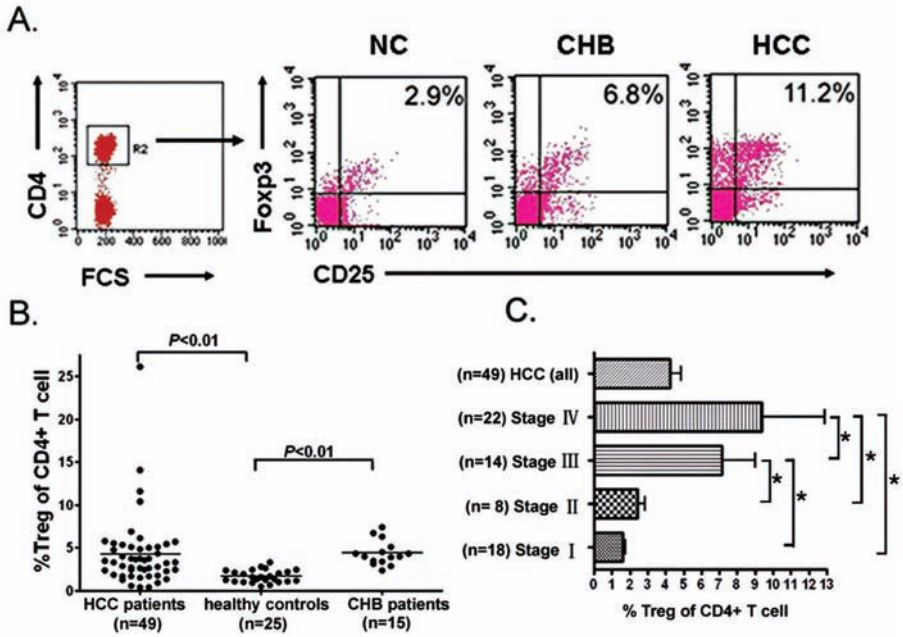

Figure 1. $CD4^+CD25^+$ Treg frequency is increased in the peripheral blood of HCC patients. (A) Representative prevalence of $CD4^+CD25^+FoxP3^+$ Treg of PBMC from an HCC patient, CHB patients and normal control (NC). (B) Percentage of $CD4^+CD25^+$ Treg cells in the peripheral blood of HCC patients (n=49), healthy donors (n=25), CHB patients (n=10). Individual cell frequencies in each patient are shown $CD4^+CD25^+FoxP3^+$ T cells are presented as a percentage of the total $CD4^+$ cell population. The prevalence of $CD4^+CD25^+FoxP3^+$ Treg cells in HCC and CHB patients was higher than the prevalence in healthy controls ($p<0.01$). (C) The prevalence of $CD4^+CD25^+$ Treg cells during the different progressive stages in HCC patients. Asterisks (*) represent the significant statistical difference compared to the HD ($p<0.05$).

Treg cells was significantly higher in HCC patients than healthy controls (4.26±4.18% and 1.67±0.73% in HCC patients and healthy controls, respectively; $p<0.01$). As shown in Fig. 1C, there was a significant difference in the prevalence of $CD4^+CD25^+$ Treg cells during the early and advanced disease stages in HCC patients. We found that HCC patients in stage III (7.16±4.52%) and stage IV (9.39±8.58%) had a higher percentage of circulating $CD4^+CD25^+$ Treg cells than patients in stage II (2.43±1.01%) (stage II vs. stage III and IV, $p<0.01$). Thus, a tumor-bearing host in an advanced stage of disease has a higher number of $CD4^+CD25^+$ Treg cells than individuals who are healthy or in the early stages of disease.

Phenotypic analysis of $CD4^+CD25^+$ Treg cells from HCC patients. Isolated PBMCs from all patient populations were further characterized by labeling them with CD4, CD25, CD127 and a series of cell surface markers. Surface marker expression of GITR, CD103, and HLA-DR on $CD4^+CD25^+CD127^{low}$ T cells were analyzed by flow cytometry. Cell surface expression of GITR, CD103, and HLA-DR on Treg cells from HCC increased compared to Treg cells from healthy donors (Fig. 2A and B). Thus, the $CD4^+CD25^+$ T cells in HCC patients and healthy donors were phenotypically similar to Treg cells described in previous reports. Furthermore, the expression of Foxp3 in magnetically purified $CD4^+CD25^-$ and $CD4^+CD25^+$ cells from HCC patients and healthy donors were used in real-time PCR. Fig. 2D shows sorted $CD4^+CD25^+$ cells from both HCC and healthy donors expressing Foxp3, whereas $CD4^+CD25^-$ cells expressed no or very little Foxp3 (Fig. 2C).

Increased prevalence of $CD4^+CD25^+$ T cells in TILs from HCC patients. Tumor specimens and non-tumor adjacent tissues were collected at the time of surgery to obtain single cell suspensions. The cells were washed twice and lymphocytes were isolated for FCM analysis. TIL from HCC patients (n=12) had more infiltrating $CD4^+CD25^+FoxP3^+$ Treg cells (15.77±1.45%) than non-tumor adjacent tissues (3.54±1.30%) (Fig. 3A and B).

Proliferative and suppressive function of $CD4^+CD25^+$ T cells in HCC patients. To investigate the proliferative and suppressive function of $CD4^+CD25^+$ regulatory T cells in HCC patients, $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were isolated from HCC patients and healthy donors as described in Materials and methods. $CD4^+CD25^-$ cells were stimulated with plate-bound anti-CD3, soluble anti-CD28, and increasing concentrations of $CD4^+CD25^+$ cells for 96 h. Cell proliferation was determined by incorporation of BrdU. The $CD4^+CD25^-$ population proliferated robustly in HCC patients in response to anti-CD3 and anti-CD28 stimulation (Fig. 4) and was inhibited in the presence of $CD4^+CD25^+$ T cells. $CD4^+CD25^+$ T cell-induced suppression of $CD4^+CD25^-$ T cells occurred in a dose-dependent manner (n=7 for HCC patients and n=9 for healthy donors), and was similar in HCC patients and healthy donors (Fig. 4).

Depletion of $CD4^+CD25^+$ T cells enhanced the NY-ESO-1b-mediated anti-tumor immunity responses of HCC patients. Total PBMC or $CD4^+CD25^+$ T cell depleted PBMC from HCC patients were stimulated with selected peptides corresponding to HLA-A2-restricted NY-ESO-1b epitopes. NY-ESO-1b-specific $CD8^+$ T cell frequency was based on IFN-γ production and HLA-tetrameric complex analysis after 10-12 days of *in vitro* expansion (Fig. 5A). It should be noted that the

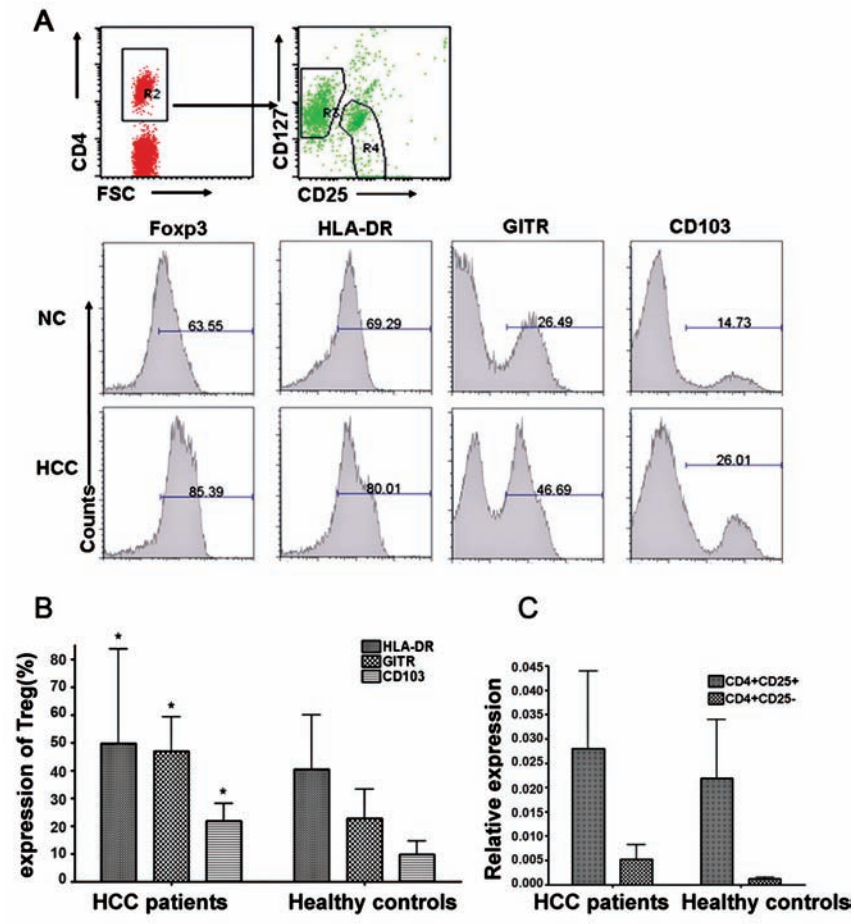

Figure 2. The expression of extracellular and intracellular markers of Treg cells. (A) Representative expression of FoxP3 in $CD4^+CD25^+CD127^{low}$ T cells of PBMC from an HCC patient and normal control (NC). (B) Freshly isolated PBMC from HCC patients or normal controls were labeled with anti-CD127, anti-CD4, anti-CD25, and anti-Foxp3, anti-GITR, anti-HLA-DR and anti-CD103. The expression of GITR, HLA-DR and CD103 on $CD4^+CD25^+CD127^{low}$ T cell populations (see Fig. 2A, gray histogram represented the expression of extracellular and intracellular markers of Treg cells gated from R4) were analyzed separately in normal control and HCC patients. Cell surface expression of GITR, CD103, and HLA-DR on Treg cells from healthy donors, HCC patients. (C) cDNA obtained from magnetically purified populations of $CD4^+CD25^-$ and $CD4^+CD25^+$ cells was analyzed by quantitative RT-PCR using primers specific for Foxp3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

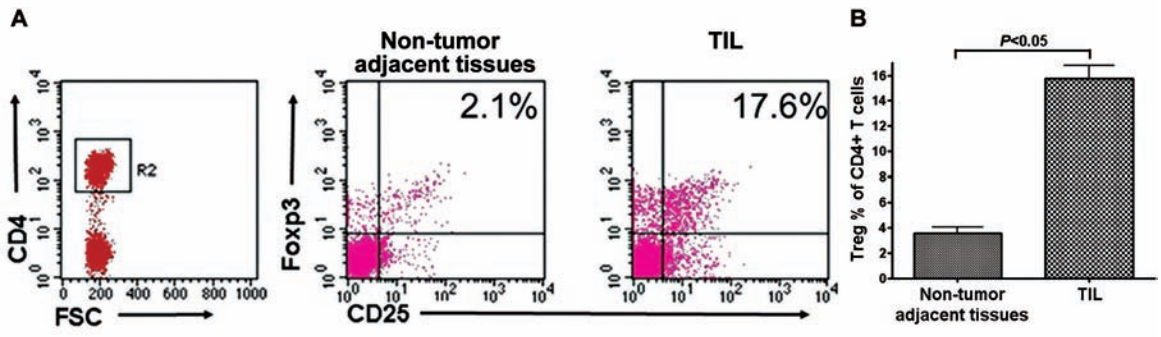

Figure 3. Increased numbers of $CD4^+CD25^+$ Treg cells in tumor specimens from HCC patients. (A and B) The number of $CD4^+CD25^+FoxP3^+$ T cells was evaluated as a percentage of total $CD4^+$ cells in tumor specimens and non-tumor adjacent tissues by flow cytometric analysis. A higher percentage of $CD4^+CD25^+FoxP3^+$ Treg cells was found in tumor specimens than non-tumor adjacent tissues ($p<0.01$).

patients analyzed were carefully selected from a larger group of HLA-A2 positive and NY-ESO-1 or LAGE-1 mRNA positive HCC patients. We selected 16 patients who had HLA-A2-restricted NY-ESO-1-specific $CD8^+$ T cells. The frequency of NY-ESO-1-specific $CD8^+$ T cells was higher in the $CD4^+CD25^+$ depleted PBMCs from HCC patients than the total PBMC population of all patients tested (Fig. 5A). Analysis of specific $CD8^+$ expansion using HLA tetramers (NY-ESO-1b p157-165) showed results similar to those obtained by ELISPOT (Fig. 5B and C).

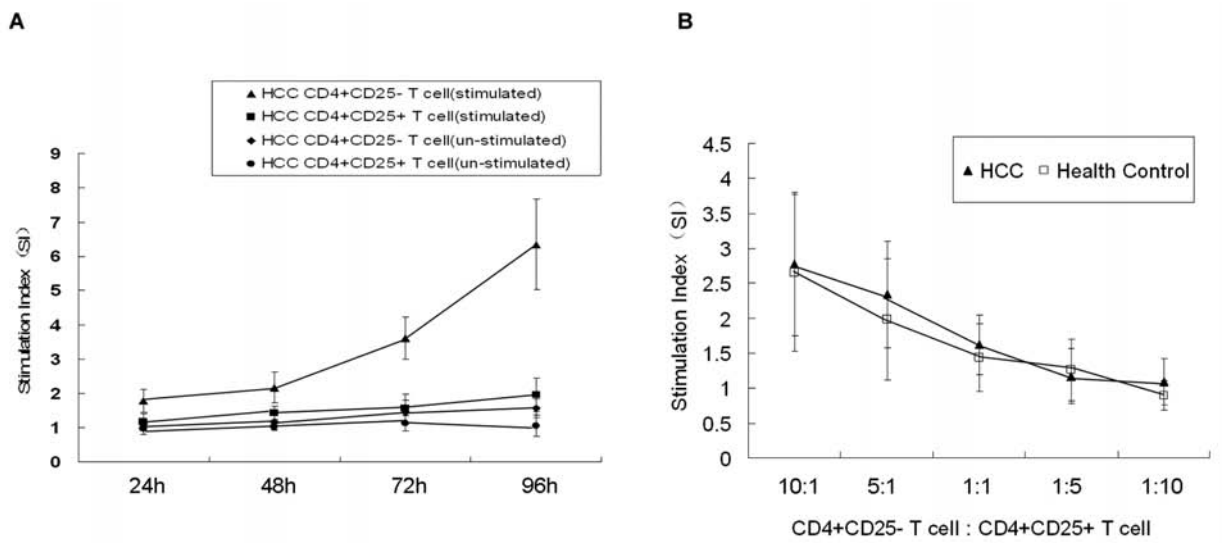

Figure 4. The suppressive function of $CD4^+CD25^+$ T cells from HCC patients and healthy donors. (A) The proliferation of HCC $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells with CD3/CD28 antibody sitmulated or without CD3/CD28 antibody sitmulated from 24 to 96 h. (B) $CD4^+CD25^-$ T cell proliferation was inhibited by co-culture with $CD4^+CD25^+$ T cells in a dose-dependent manner, in which the level of suppression correlated with the ratio of the two cell populations. The suppressive effect of $CD4^+CD25^+$ T cells from HCC patients and healthy donors were equivalent.

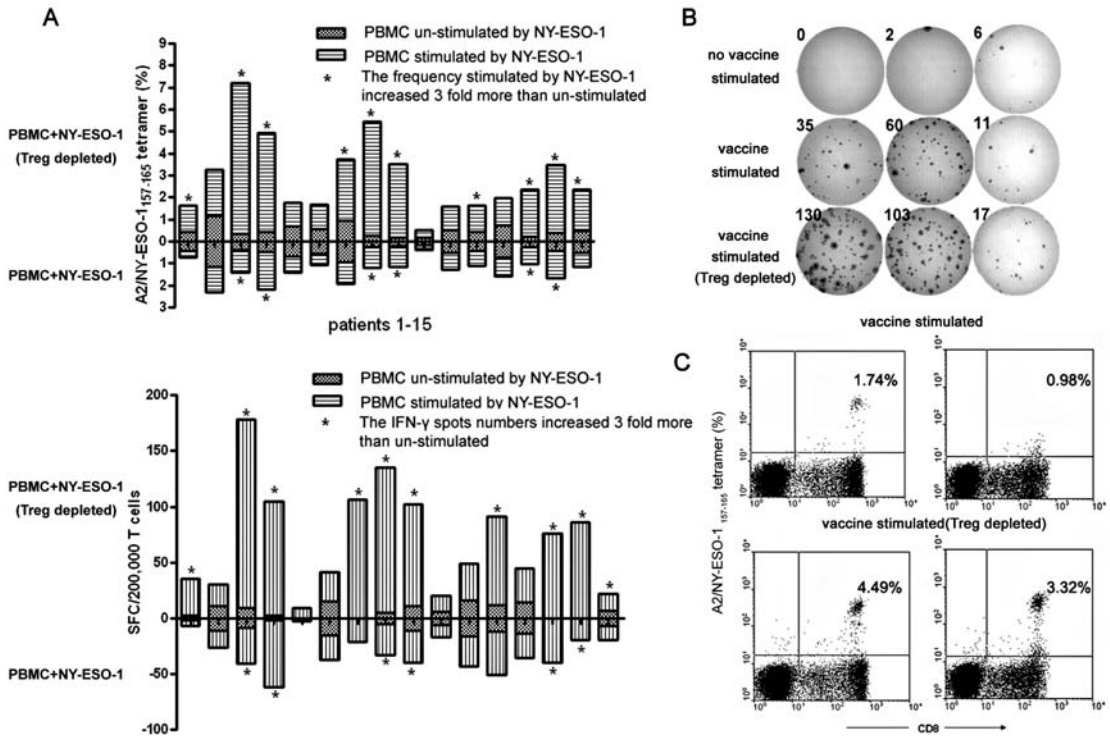

Figure 5. $CD8^+$ T cell responses to HLA-A2-restricted NY-ESO-1b peptide in HCC patients. (A) Representative data from all patients showing that the $CD8^+$ T cell population is specific for the NY-ESO-1b peptide. T cell frequency and the amount of NY-ESO-1-specific $CD8^+$ T cell-induced IFN-γ production was increased in $CD4^+CD25^+$ T cell depleted PBMC compared to total PBMC from all patients tested. *Represented NY-ESO-1-stimulated T cell and $CD8^+$ IFN-γ^+ T cell frequencies that were 3-fold more than the spontaneous NY-ESO-1-specific $CD8^+$ T cell frequency after no stimulation. (B) IFN-γ released by NY-ESO-1-specific $CD8^+$ T cells from total and $CD4^+CD25^+$ T cell depleted PBMC in HCC patients was detected by ELISPOT on day 12. (C) Tetramer analysis of NY-ESO-1b-specific $CD8^+$ T cells from total and $CD4^+CD25^+$ T cell depleted PBMC in HCC patients was performed on day 12.

An effective NY-ESO-1-specific $CD8^+$ T cells immune response was defined as the frequency of activated IFN-γ producing NY-ESO-1-specific $CD8^+$ T cells that was 3-fold higher than the frequency in unstimulated PBMCs from HCC patients. According to this criterion, after stimulation with NY-ESO-1b peptide, six out of sixteen patients (6/16, or 37.5%) acquired an effective NY-ESO-1-specific $CD8^+$ T cell response analyzed by ELISPOT and HLA tetramer staining. The efficiency of the immune response against CT antigen in HCC patients correlated with our published results (9).

Importantly, nine out of the sixteen patients (9/16) did not elicit an effective NY-ESO-1-specific $CD8^+$ T cell immune response after stimulation with NY-ESO-1b peptide. But, when we depleted $CD4^+CD25^+$ T cells from the PBMC of HCC patients, four of the remaining nine patients (4/9) acquired the ability to mount an effective NY-ESO-1-specific $CD8^+$ T cell immune response. The six cases that acquired effective immune response in the very beginning acquired more effective immune response after $CD4^+CD25^+$ T cell depletion than the total PBMC population. Thus, after $CD4^+CD25^+$ T cell depletion, ten out of sixteen HCC patients (10/16, or 62.5%) were able to elicit an effective NY-ESO-1-specific $CD8^+$ T cell immune response (Fig. 5A).

Discussion

An increase in $CD4^+CD25^+$ Treg cell numbers is observed in patients with carcinoma, and experimental tumor models illustrate that removal of this cell population alters the anti-tumor immune response both *in vitro* and *in vivo* (24). Indeed, depletion of $CD25^+$ T cells in patients with carcinoma results in a potent tumor-specific immune response (25,26). Immune suppression by $CD4^+CD25^+$ regulatory T cells might be explain why some immunotherapy protocols are associated with a poor outcome.

In this study, we used several cell surface markers to distinguish $CD4^+CD25^+$ Treg cells from activated cells. Although CD25 is the typical cell surface marker used to identify Treg cells, it is also expressed on other types of activated T cells. Foxp3 is uniquely restricted to these cells and more precise (36). Moreover, we identified Treg cells by examining the expression of more specific surface markers, including CD127, CD45RO, CD152, GITR, HLA-DR, CD62L, and CD45RA (32,35,38,39). This allowed us to distinguish Treg cells from other activated $CD4^+$ T cells in peripheral blood from HCC patients and control individuals (38,39). As a result, our data indicate that Treg cell frequencies increased in both the peripheral blood and the tumor, suggesting that this cell type may suppress immune activation at the tumor site. Our data also showed a significant increase in Treg cell numbers in CHB patients compared to healthy controls. In this study, significantly fewer Treg cells were observed in the peripheral blood of early stage (II) than late stage (III and IV) patients.

The proliferative capacity of $CD4^+CD25^-$ T cells was inhibited by the presence of $CD4^+CD25^+$ T cells in a dose-dependent manner, in which the level of suppression correlated with the ratio of the two cell populations. The suppressive effects of $CD4^+CD25^+$ T cells from HCC patients and healthy donors were not statistically significant. The phenomena are similar with the result from another group (18), which were caused by the use of $CD4^+CD25^{high}$ isolated micro-bead kit and suppressive test conducted *in vitro*. These data suggest that tumor progression result in an increase in Treg cell numbers but do not affect $CD4^+CD25^+$ T cell function. It was not clear whether Tregs suppress T cells directly or through APC (16,37). In our research, Treg may directly suppress $CD4^+CD25^-$ Teff cells and the depletion of Treg in HCC patients *in vitro* could contribute to NY-ESO-1 specific CTL expansion induced by NY-ESO-1 peptides. The NY-ESO-1 specific CTL expansion ability induced by NY-ESO-1 peptides after depletion of Treg were higher than CTL expansion which come from purified CD8 T cells stimulated by the DC in our previous studies (9) (effective NY-ESO-1-specific $CD8^+$ T cell response, 35.7 vs. 62.5%). It suggested that the strategies of Treg depletion in HCC patients as the sensitization protocol could show better improvements in CTL expansion than the protocols via the DC induced purified CD8 T cells *in vitro*.

$CD25^+$ T cell depletion in patients with carcinoma is shown to induce a potent tumor-specific immune response (24,25). The main objective of our study was to enhance the immunostimulatory efficacy of the NY-ESO-1b peptide by selectively eliminating $CD4^+CD25^+$ Treg cells from HCC patients. We used the $CD4^+CD25^+$ regulatory T cell isolation kit to deplete this cell population, and showed that the number of NY-ESO-1-specific $CD8^+$ T cells increased after Treg depletion and demonstrated tumor specificity. Tumor-specific cellular immune responses could only be induced in 35.7% of HCC patients that expressed NY-ESO-1 and HLA-A2 (9). In our research, only six out of sixteen patients (6/16, or 37.5%) acquired an effective NY-ESO-1-specific $CD8^+$ T cell response analyzed by ELISPOT and HLA tetramer staining , and nine of sixteen HCC patients (9/16) could not elicit an effective NY-ESO-1-specific $CD8^+$ T cell immune response after stimulation with NY-ESO-1. However, after $CD4^+CD25^+$ T cell depletion, four of the nine remaining patients (4/9) acquired the ability to mount an effective NY-ESO-1-specific $CD8^+$ T cell immune response. In the six cases that acquired effective immune response in the very beginning, the frequency of NY-ESO-1-specific $CD8^+$ T cells was higher after the $CD4^+CD25^+$ T cells depleted than the total PBMC population of all patients tested. Thus, depletion of regulatory T cells enhanced the NY-ESO-1b-mediated anti-tumor immune response in patients with hepatocellular carcinoma. These results are may be helpful for overcoming the low efficiency of anti-tumor response in tumor immunotherapy clinical trials used by tumor specific antigens.

In conclusion, our findings suggest that Treg cells may help to modulate the immune response against HCC. Incorporation of Treg depletion strategies in the design of immune-based therapies could dramatically enhance anti-tumor immunity.

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