

Hepatocellular carcinoma and macrophage interaction induced tumor immunosuppression *via* Treg requires TLR4 signaling

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Received: May 13, 2011 Revised: March 26, 2012

Accepted: April 2, 2012

Published online: June 21, 2012

Abstract

AIM: To investigate the interaction between toll-like receptor 4 (TLR4)-activated hepatoma cells and macrophages in the induction of tumor-immune suppression mediated by CD4+CD25^{high} family of transcription factor P3 (FOXP3) regulatory T cells (Tregs).

METHODS: The proportion of FOXP3+ Tregs was identified in peripheral blood and tumor tissues of 60 hepatocellular carcinoma (HCC) patients. TLR4 expression was examined in tumor tissues and cell lines. The correlation was examined between FOXP3+ Tregs in peripheral blood and TLR4 expression of HCC tissues. Following activation of TLR4 in H22 murine hepatoma cells pre-incubated with lipopolysaccharide (LPS) and co-cultured with macrophage cell line RAW246.7, the

synthesis of cytokines tumor necrosis factor- α , CCL22, and interleukin (IL)-10 by the two cell lines was detected and analyzed.

RESULTS: FOXP3+ Tregs were enriched in tumor sites, and circulating FOXP3+ Tregs were increased in HCC patients in correlation with multiple tumor foci and up-regulated TLR4 expression in HCC tissues. Semi-quantitative analysis indicated that TLR4 was over-expressed in HCC compared with the matched normal tissues. Cell cultivation experiments indicated that the mRNAs of IL-10 and CCL22 were significantly up-regulated in the RAW246.7 cell line when co-cultured with LPS pre-incubated H22 cells.

CONCLUSION: In hepatoma cell lines, TLR4 may indirectly facilitate the recruitment of Tregs to the tumor site and promote intrahepatic metastasis through its interaction with macrophages.

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Key words: CD4+CD25^{high}FOXP3+ regulatory T cell; Toll-like receptor; Tumor immunity; Hepatocellular carcinoma; Macrophage

Peer reviewer: Dr. Fabrizio Montecucco, Internal Medicine, University of Geneva, avenue de la Roseraie, 64, 1211 Geneva, Switzerland

Yang J, Zhang JX, Wang H, Wang GL, Hu QG, Zheng QC. Hepatocellular carcinoma and macrophage interaction induced tumor immunosuppression *via* Treg requires TLR4 signaling. *World J Gastroenterol* 2012; 18(23): 2938-2947 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i23/2938.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i23.2938>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most com-

mon cancer worldwide, and is the third most common cause of cancer related deaths^[1]. Previous studies have demonstrated that the majority of HCC patients develop tumor-specific immune responses; however, in most patients, tumors progress despite tumor-specific humoral and cellular immune responses. These findings imply that HCC escapes the anti-tumor immune response through various strategies.

Recently, many studies have suggested that the tumor microenvironment plays an important role in the establishment and progression of tumors. Lymphocytes contribute to the tumor microenvironment through immunity and inflammation. Through diverse strategies, tumor cells play an important role in the suppression of anti-tumor immunity in the surrounding microenvironment *via* interactions with infiltrated immune cells or macrophages, which in turn potentially facilitates growth of the tumor itself. Moreover, induction of the differentiation and/or recruitment of regulatory T cells (Tregs), a unique population of CD4+ T cells, potentially comprises one of the key mechanisms. Tregs are defined based on their expression of CD4, CD25 and forkhead, or winged helix family of transcription factor P3 (FOXP3), which is critical for the development and function of Tregs in mice and humans^[2]. Tregs play a critical role in immunologic self-tolerance and suppression in the tumor immune response^[3,4]. Early evidence indicates that Tregs (not FOXP3+ T cells but CD4+CD25+ T cells) are increased in patients with various types of cancer^[5-7]. It is hypothesized that their systemic and/or local accumulation promotes tumor growth through suppression of the host anti-tumor response^[8]. However, considerable uncertainty remains regarding the characteristics, functions, and regulation of Tregs. Therefore, we investigated the clinicopathologic significance of CD4+CD25^{high}FOXP3+ Tregs in HCC patients, and analyzed their ability to suppress the immune response. Elucidation of the mechanisms underlying Treg elevation is essential for the development of new approaches that aim to modulate the frequency and function of Tregs in order to enhance the efficacy of cancer immune-based therapies.

Toll-like receptors (TLRs) recognize specific structural regions of invading pathogens and initiate innate and adaptive immune responses; their expression has been detected in immune cells and also in many cancer cells^[9]. Lipopolysaccharide (LPS), a ligand for TLR4, stimulates immune cells and triggers the production of inflammatory cytokines and other mediators *via* TLR4, which in turn regulates the host immune defense system and eliminates pathogens^[10,11]. It has been reported that inflammatory cytokines induced by inflammatory stimuli counteract immune surveillance and facilitate tumor growth^[12,13]. However, various other reports have suggested that diverse TLRs potentially exhibit different effects on Tregs due to differences in pathogens and immune environment, resulting in either increased suppression or abrogation of suppression^[14-17]. The suppressive function of Tregs is tightly regulated to respond to the different requirements

of immunity. The mechanism underlying the selective control of Treg function remains obscure. Precise modulation of the suppressive function of Tregs is crucial for the development of effective cancer immunotherapy. Thus, in this study we investigated whether TLR4 is expressed in HCC, and whether tumor TLR4 is functionally active in inducing cytokines, and we also examined the clinicopathological correlation between tumor TLR4 signaling and CD4+CD25^{high}FOXP3+ Tregs in tumor immune escape.

Currently, the roles of CD4+CD25^{high}FOXP3+ Tregs and TLR4 in HCC and their regulatory activity in the tumor microenvironment remain unclear. In the present study, we described the clinicopathological significance of Tregs in 60 HCC patients, and the expression of TLR4 in hepatic cancer cells. Our findings indicated that TLR4 ligation promotes the secretion of inhibitory cytokine interleukin (IL)-10 and chemokine CCL22 from co-cultured macrophages, but not from the tumor cells themselves. Furthermore, the prevalence of Tregs significantly correlated with the presence of multifocal tumor. Our results suggest a mechanistic path for the indirect modulation of CD4+CD25^{high}FOXP3+ Tregs *via* tumor TLR4 signaling, and demonstrate that interactions between hepatoma cells and macrophages induce anti-tumor immune suppression *via* Tregs.

MATERIALS AND METHODS

Ethics

This study was conducted in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The Ethics Committee of Tongji Medical College (Wuhan, China) approved the study protocol. All patients provided informed written consent before blood and tumor sampling.

Patient and samples

Blood samples were collected from 60 HCC patients who underwent hepatic resection in the Center of Hepatobiliary Surgery, Union Hospital, Wuhan, China from March 2008 to October 2008. Control blood samples were obtained from 20 healthy volunteers. HCC patients were pathologically diagnosed following surgical resection. Among the 60 HCC patients, there were 51 males and nine females aged 17-77 years (mean, 51.3 years). According to the International Union against Cancer tumor-node-metastasis classification^[18], there were 19 (31.7%), 24 (40%) and 17 (28.3%) cases at stage I, II and III, respectively. No patient was treated with local ablative therapy, chemotherapy, or immunotherapy prior to surgery. Clinical and laboratory characteristics of the HCC patients are shown in Table 1. Blood samples (3 mL) were collected 2-3 d before operation from each patient in the early morning. Samples were placed in ethylenediaminetetraacetic acid anticoagulant tubes for flow cytometric detection in our hospital. Next, 1 cm × 1 cm × 1 cm tumor and normal tissues were obtained from each patient intraoperatively, avoiding

Table 1 Clinical and laboratory characteristics of the 60 hepatocellular carcinoma patients

Items	Results
Age (yr) median (range)	53.5 (17-77)
Gender (male/female)	51/9
Virus (HBV/HCV)	48/4
TNM stage (I / II / III/IV)	19/24/17/0
AFP ($\mu\text{g/L}$), median (range)	350 (1.8-127 278)
Blood neutrophil (%)	62.29 \pm 10.96
Blood monocyte (%)	7.21 \pm 1.69
Hemoglobin concentration (g/L)	124.47 \pm 17.04

TNM: Tumor-node-metastasis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: α -fetoprotein.

areas of necrosis, hemorrhage, and/or adipose tissues. One portion of each specimen was snap frozen in liquid nitrogen and the other part was fixed in 10% polyformaldehyde solution and embedded in paraffin.

Flow cytometric analysis

Peripheral blood mononuclear cells from each patient (100 μL) was separated into a heparinized container. Twenty microliters anti-human CD25-fluorescein isothiocyanate and anti-human CD4-PerCP was added to each tube, and incubated at room temperature in the dark for 15 min. After washing in 1 \times phosphate-buffered saline, fixed broken membrane buffer 1 mL (1% paraformaldehyde and 70% ice-alcohol; pH 7.4) was added. The remaining lymphocytes were incubated in 20 μL FOXP3-PE at 4 $^{\circ}\text{C}$ for 30 min. Subsequently, cells were analyzed by flow cytometry (FACS Calibur, BD) with Cellquest software (Version 3.3, BD Biosciences-Pharmingen, United States). All conjugated antibodies described above and their isotype-matched monoclonal antibodies were purchased from BD, United States.

Lymphocytes were gated on forward and side scatter profiles followed by gating on CD4+ T cells, and these cells were then analyzed for CD25 expression. For FOXP3 expression analysis, cells inside the CD4+CD25^{high} gate were analyzed.

Western blotting

Nuclear protein extracts were prepared in sodium dodecyl sulfate (SDS) lysis buffer containing protease inhibitors, pre-stained molecular weight markers were denatured in Laemmli buffer (10% glycerol, 2% SDS, 0.1 mol/L dithiothreitol, 50 mmol/L Tris, 0.01 mg/mL bromophenol blue; pH 6.8) at 90 $^{\circ}\text{C}$, and were separated by SDS-polyacrylamide gel electrophoresis. Resolved proteins were transferred onto polyvinylidene fluoride membrane in Trans-blot wet buffer (Bio-Rad Laboratories, United States). The membranes were blocked with 5% nonfat dry milk in 1 \times tris-buffered saline (TBS), then incubated with 2 $\mu\text{g/mL}$ mouse monoclonal anti-FoxP3 antibody (clone 22510; Abcam, United States) overnight at 4 $^{\circ}\text{C}$ followed by horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) for 1 h at room temperature, and washed with 1

\times TBS. Membranes were treated with enhanced chemiluminescence plus Western blotting detection kit (Transgen, Beijing, China), and bands were detected using STORM 840v2005 with ImageQuant software (GE, United States).

Cell lines and reagents

If not indicated otherwise, all substances were purchased from Gibco, United States. The H22 (murine) and HepG2 (human) hepatic cancer cell lines were a gift from Dr. Huang Bo (Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China). The immortalized murine macrophage line RAW246.7 was preserved in our laboratory. The cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. All experiments were performed under endotoxin-free conditions.

Co-culture assay

The various cellular components were grown in an artificial basement membrane in a modified Polyester-Transwell (Costar, United States) plate without direct cell-to-cell contact. 1×10^5 RAW246.7 cells/mL were seeded into the upper well of the Transwell plate (0.4 μm pore diameter), which consisted of a membrane permeable to liquids but not cells, whereas the lower well was filled to the top with RPMI + 10% fetal calf serum. H22 cells (1×10^6 cells/mL RPMI) were seeded into a 12-well plate. To activate TLR4 in H22 cells, LPS (Gibco, United States) was used at 1 $\mu\text{g/mL}$. The culture medium was removed after LPS-stimulating the H22 cells for 12 h. Next, the Transwells were inserted into the 12-well plate. Gene expression was compared between control and macrophages co-cultured with H22 cells after 24 h, and macrophages with or without conditioned tumor medium were analyzed using reverse transcriptase polymerase chain reaction (RT-PCR). All experiments were performed at least in triplicate.

RNA extraction and RT-PCR

Total RNA was extracted from patient samples, H22, HepG2, and RAW246.7 cells, using Trizol reagent (Invitrogen, United States). Using a first strand cDNA synthesis kit (ToYoBo, Shanghai, China), cDNA was generated with Oligo dT primer. Primers for TLR4, tumor necrosis factor- α (TNF- α), IL-10, CCL22 and β -actin were designed using Premier 5.0 software (Table 2). Primers were synthesized by Sangon Inc. Shanghai, China.

Each reaction mixture contained 2.5 μL 10 \times buffer, 2.5 μL 2.5 mmol MgCl_2 , 0.5 μL 10 mmol of dNTP, 0.2 μL 5 U/ μL Taq DNA polymerase, 1 μL each of sense and antisense primer, and 1 μL cDNA in a final volume of 25 μL (Fermentas, United States). Reaction mixtures were incubated at 94 $^{\circ}\text{C}$ for 5 min to activate the Taq DNA polymerase, and then amplified using 40 cycles of 30 s at 94 $^{\circ}\text{C}$ (denaturation) and 40 s at annealing temperature for TLR4, TNF- α , IL-10, CCL22, and β -actin, respectively. PCR was performed using the Agarose Gel Electrophoresis Imaging Analysis System (Beijing, China).

Table 2 Primer sequences and conditions used for reverse transcription-polymerase chain reaction

Gene	Sequence	Product size (bp)	Annealing temperature (°C)
<i>mTLR4</i>	5'-GCTTTCACCTCTGCCTTCAC-3' 3'-AGGCGATACAATTCCACCTG-5'	259	57
<i>HuTLR4</i>	5'-GAAATGGAGGACCCCTTC-3' 3'-GAATATTCCTTGCATAGGT-5'	506	52
<i>CCL22</i>	5'-AAGACAGTATCTGCTGCCAGG-3' 3'-GATCGGCACAGATATCTCGG-5'	141	57
<i>IL-10</i>	5'-GGTTGCCAAGCCTTATCGGA-3' 3'-ACCTGCTCCACTGCCTTGCT-5'	190	60
<i>TNF-α</i>	5'-CATCTTCTCAAAATTCGAGTG ACAA-3' 3'-TGGGAGTAGACAAGGTACAA CCC-5'	175	58
<i>β-actin</i>	5'-TCACCCACACTGIGCCCATCT ACGA-3' 3'-GATAACCGTTGCTCGCCAAG GCTAC-5'	300	50

TNF: Tumor necrosis factor; IL: Interleukin; TLR: Toll-like receptor.

Immunohistochemistry

To assess TLR4 protein expression in HCC and normal tissues, a polyclonal rabbit anti-human TLR4 (ab47093; Abcam, United States) was used. Paraffin-embedded sections (5-μm thick) were fixed in freshly prepared 10% paraformaldehyde for 5 min. After blocking the endogenous peroxidase activity with 0.3% hydrogen peroxide in TBS for 15 min, the sections were immersed in horse serum diluted 1:10 in TBS for 30 min to reduce nonspecific binding, and then incubated with the primary antibody overnight at 4 °C after washing in TBS. Next, the sections were incubated in biotinylated horse anti-mouse or goat anti-rabbit IgG for 30 min, and avidin-biotin-peroxidase complex for 30 min. After each step of the staining procedure, the sections were given three 5-min washes in TBS. Immunoreactivity (IR) was visualized using 1 mg/mL diaminobenzidine as chromogen and 0.01% hydrogen peroxide as substrate. The peroxidase reaction was stopped after 5 min with distilled water, and the sections were counter-stained with Toluidine blue, dehydrated, and then mounted with Entellan.

Slides were evaluated under a light microscope (× 400 magnification). For digital image analysis, the software Adobe Photoshop version 7.0 was used. Results were scored by two independent investigators as positive, heterogeneous, or negative. The two scores were averaged.

Statistical analysis

SPSS version 10.0 (SPSS Inc., United States) was used for statistical analysis. All data were expressed as mean ± SE. Statistical analyses were performed using the Student's *t* test. If there was evidence of non-normality, Kruskal-Wallis one-way analysis of variance was used to test the difference in median among the groups. To analyze the correlation between Tregs and TLR4, Spearman's rank correlation coefficients were performed. Difference was considered statistically significant at $P < 0.05$.

RESULTS

CD4+CD25^{high}FOXP3+ Tregs accumulation in HCC tumor and peripheral blood

The prevalence of CD4+CD25^{high} and CD4+CD25^{high}FOXP3+ Tregs was analyzed in the peripheral blood of 60 patients with HCC. The population of CD4+CD25^{high} and FOXP3+ Tregs as a percentage of total CD4+ T and CD4+CD25^{high} T cells, respectively, was identified by flow cytometry. Representative dot plots of HCC patients and controls are shown in Figure 1A. The frequency of CD4+CD25^{high} and FOXP3+ Tregs in HCC patients was significantly higher than in the healthy controls (Figure 1B). The expression of FOXP3 in HCC patients was detected using Western blotting analysis. As shown in Figure 1C and D, both tumor and normal tissues exhibited FOXP3 protein expression, although the expression was weaker in normal tissues.

Clinicopathologic characteristics of HCC patients and prevalence of circulating CD4+CD25^{high}FOXP3+ Tregs

We analyzed the correlation between the proportion of CD4+CD25^{high}FOXP3+ Tregs in the peripheral blood and the clinicopathological characteristics of the subjects (Table 3). The proportion of CD4+CD25^{high}FOXP3+ Tregs was significantly higher in patients with high serum AFP levels and multiple tumor foci ($P = 0.009$). The proportion of CD4+CD25^{high}FOXP3+ Tregs did not correlate with other clinicopathological characteristics of HCC patients ($P > 0.05$).

Considering the potential impact of multifocal tumor on tumor size, the correlation of the proportion of CD4+CD25^{high}FOXP3+ Tregs with different tumor diameters was analyzed in 52 patients with a single lesion. Our findings indicated that the proportion of Tregs was low in patients with a small tumor (< 5 cm in diameter) (Figure 2).

Expression of TLR4 in patient samples and hepatoma cell lines

The expression of TLR4 in patient tumor samples and hepatoma cell lines was examined using RT-PCR. Tumor and normal tissues, as well as HepG2 and H22 cells, expressed TLR4 mRNA (Figure 3). We determined the relative expression of TLR4 mRNA (TLR4 mRNA level/β-actin mRNA level × 100%) and found that TLR4 mRNA expression level in HCC tissues was higher than in the normal tissues ($P = 0.01$) (Figure 3A). Additionally, immunohistochemical analysis confirmed the expression of TLR4 protein. TLR4 positive hepatocytes were present in paraffin-embedded sections. Moderate and strong IR for TLR4 was detected in 63.4% (38/60) of HCC specimens, and normal tissues displayed positive staining in 10% (6/60). TLR4 in cancer cells was stained more intensely than in the normal cells (Figure 3C). The distribution of TLR4 in a given HCC specimen was uneven, and the majority of positive hepatocytes exhibited expression on the membrane and in the cytoplasm. Scattered expression of TLR4 protein was also observed in a small number of liver sections derived from normal tissues.

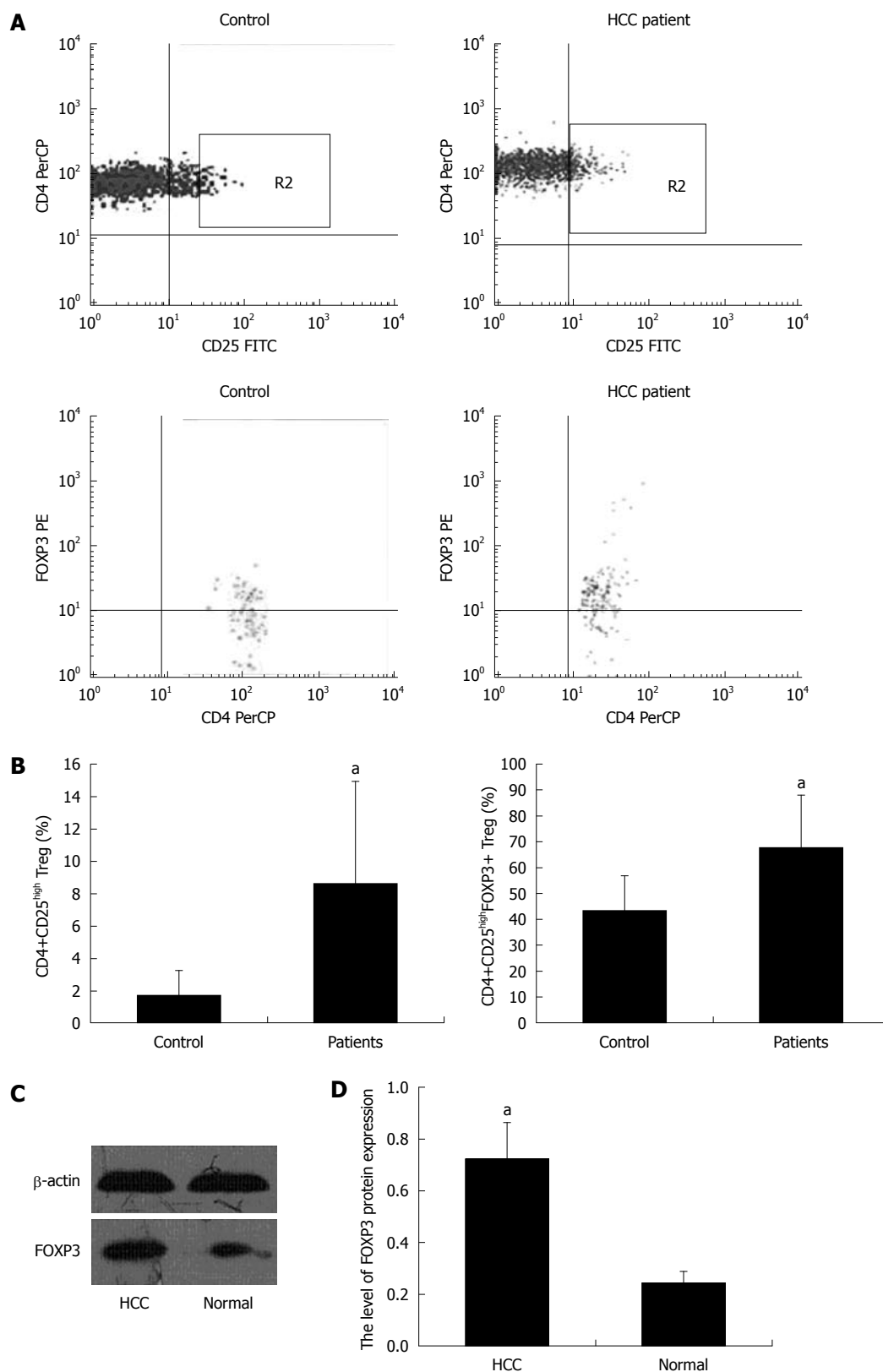


Figure 1 CD4+CD25^{high}FOXP3⁺ regulatory T cells accumulation in hepatocellular carcinoma and peripheral blood. A: Representative flow cytometry plots of CD4+CD25^{high} regulatory T cell (Treg) (region R2) and family of transcription factor P3 (FOXP3)+ Treg cells in peripheral blood from a healthy donor and hepatocellular carcinoma (HCC) patient; B: Percentage of CD4+CD25^{high} Treg and FOXP3+ Treg cells in peripheral blood from HCC patients and controls; C: The prevalence of CD4+CD25^{high} Treg (8.57% ± 6.31%, *P* = 0.002) and FOXP3+ Treg cells (67.51% ± 20.59%, *P* < 0.05) in peripheral blood from HCC patients was significantly higher than that of healthy donors (1.71% ± 1.59% and 43.35% ± 13.91%, respectively). Western blotting analysis of nuclear extracts prepared from HCC and normal tissues; D: The relative expression of FOXP3 protein (FOXP3 protein/β-actin protein × 100%). The FOXP3 protein expression in HCC tissues was stronger (0.72 ± 0.14) as compared with normal tissues (0.24 ± 0.05) (*P* < 0.05). FITC: Fluorescein isothiocyanate.

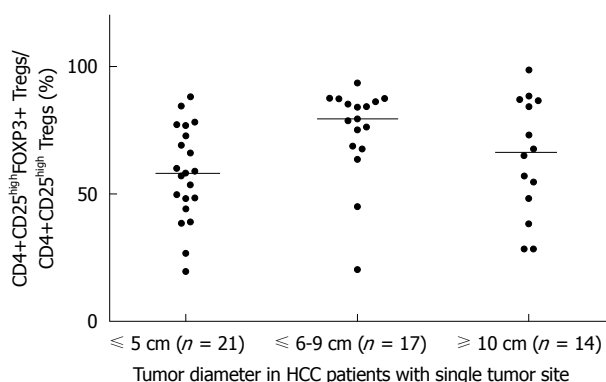


Figure 2 Correlation of regulatory T cells with tumor size of a single tumor. Circulating levels of CD4+CD25^{high}FOXP3+ regulatory T cells (Tregs) was low in patients with a small tumor (less than 5 cm in diameter). Comparisons of data of Tregs among 3 tumor size groups using a Kruskal-Wallis test. Statistically significant differences were found in all comparisons between the 3 groups (χ^2 : 7.365, $P = 0.0252$). HCC: Hepatocellular carcinoma.

Upregulation of IL-10 and CCL22 expression in RAW246.7 cells elicited by co-cultivation with LPS preincubated hepatoma cells

Hepatoma cell line H22 cells were pre-incubated with LPS. The RAW246.7 cells and the H22 cells were co-cultured in a transwell system for 24 h. Semiquantitative RT-PCR was performed to detect the expression of TNF- α , IL-10, and CCL22 in different cell lines. RAW246.7 cells co-cultured with LPS pre-treated H22 cells exhibited significant up-regulation of mRNA for genes IL-10 and CCL22 ($P < 0.001$), whereas expression of TNF- α remained unchanged (Figure 4). Tumor cells without LPS pre-incubation induced negligible gene expression changes in co-cultured RAW246.7 cells. H22 cells alone did not produce IL-10 and CCL22 under conditions of TLR4 activation following LPS stimuli or not (data not shown). These findings, taken together, suggested that activated TLR4 on H22 cells facilitated the induction of cytokine secretion of IL-10 and CCL22 by RAW246.7 cells.

Correlation between proportion of CD4+CD25^{high}FOXP3+ Tregs in peripheral blood of HCC patients and TLR4 in HCC tissues

We investigated the associations between tumor Union for International Cancer Control (UICC) stage, circulating CD4+CD25^{high}FOXP3+ Tregs, and TLR4 in the HCC tissues. The expression level of TLR4 protein in 60 HCC specimens was positively correlated with the frequency of CD4+CD25^{high}FOXP3+ Tregs in peripheral blood (Figure 5A). There was an increased frequency of Tregs in the peripheral blood of HCC patients, which exhibited a high level of expression of TLR4 protein. However, there was no significant correlation between the number of CD4+CD25^{high}FOXP3+ Tregs and tumor UICC stage (Figure 5B).

DISCUSSION

Increased FOXP3+ Tregs in HCC tissues and peripheral blood

T cells play an essential role in the immunosurveillance

Table 3 Correlation between the proportion of CD4+CD25^{high}FOXP3+ regulatory T cells in peripheral blood and clinicopathological characteristics of hepatocellular carcinoma

Items	n	Treg (%)	P values
Sex			0.201
Male	51	68.95 ± 20.71	
Female	9	59.37 ± 18.97	
UICC/TNM stage			0.781
I	19	66.41 ± 20.28	
II-III	41	68.02 ± 20.97	
AFP (μg/L)			<0.001
≤ 20	23	53.13 ± 17.96	
> 20	37	76.45 ± 16.84	
HBsAg			0.611
Positive	48	68.20 ± 19.69	
Negative	12	64.78 ± 24.66	
Margin			0.981
Clear	29	67.45 ± 19.74	
Unclear	31	67.57 ± 21.69	
Capsule			0.058
Complete	28	62.14 ± 21.92	
Incomplete	32	72.22 ± 18.43	
Tumor diameter (cm)			0.245
≤ 5	23	59.84 ± 19.06	
6-9	18	75.94 ± 18.43	
≥ 10	19	68.81 ± 21.84	
Tumor number			0.009
Single	52	63.83 ± 20.55	
≥ 2	8	86.41 ± 6.18	
Cirrhosis			0.881
Presence	23	68.02 ± 20.42	
Absence	37	67.19 ± 20.98	
Tumor differentiation			0.152
WD	11	56.61 ± 24.98	
MD	7	69.60 ± 20.45	
PD	42	70.02 ± 18.59	

MD: Moderately differentiated; WD: Well differentiated; PD: Poorly differentiated; AFP: α -fetoprotein; Tregs: Regulatory T cells; TNM: Tumor-node-metastasis; UICC: Union for International Cancer Control.

and destruction of cancer cells. Among CD4+ T lymphocytes, CD4+CD25+ Tregs are thought to comprise a functionally unique subpopulation of T cells that act to maintain immune homeostasis. The lack of CD4+CD25+ Tregs results in various autoimmune syndromes. Alternatively, Tregs-mediated suppression potentially hinders an effective immune response, which is crucial for elimination of tumors and infection^[19]. Humans with cancer exhibit increased numbers of peripherally circulating and tumor Tregs^[5,20,21]. Ormandy *et al*^[22] found that in HCC patients the number of Tregs in the peripheral blood was significantly increased. However, Yang *et al*^[23] found that the proportion of CD4+CD25+ Tregs in the peripheral blood of HCC patients was significantly decreased. Too few samples and different patient inclusion criteria has potentially led to conflicting results in previous studies. This study presents evidence of an accumulation of FOXP3+ Tregs in HCC tumor tissues and peripheral blood. A few previous studies have also investigated the clinicopathologic significance of Tregs, but conclusions regarding correlations with various clinicopathologic features were contradictory. Our results demonstrated

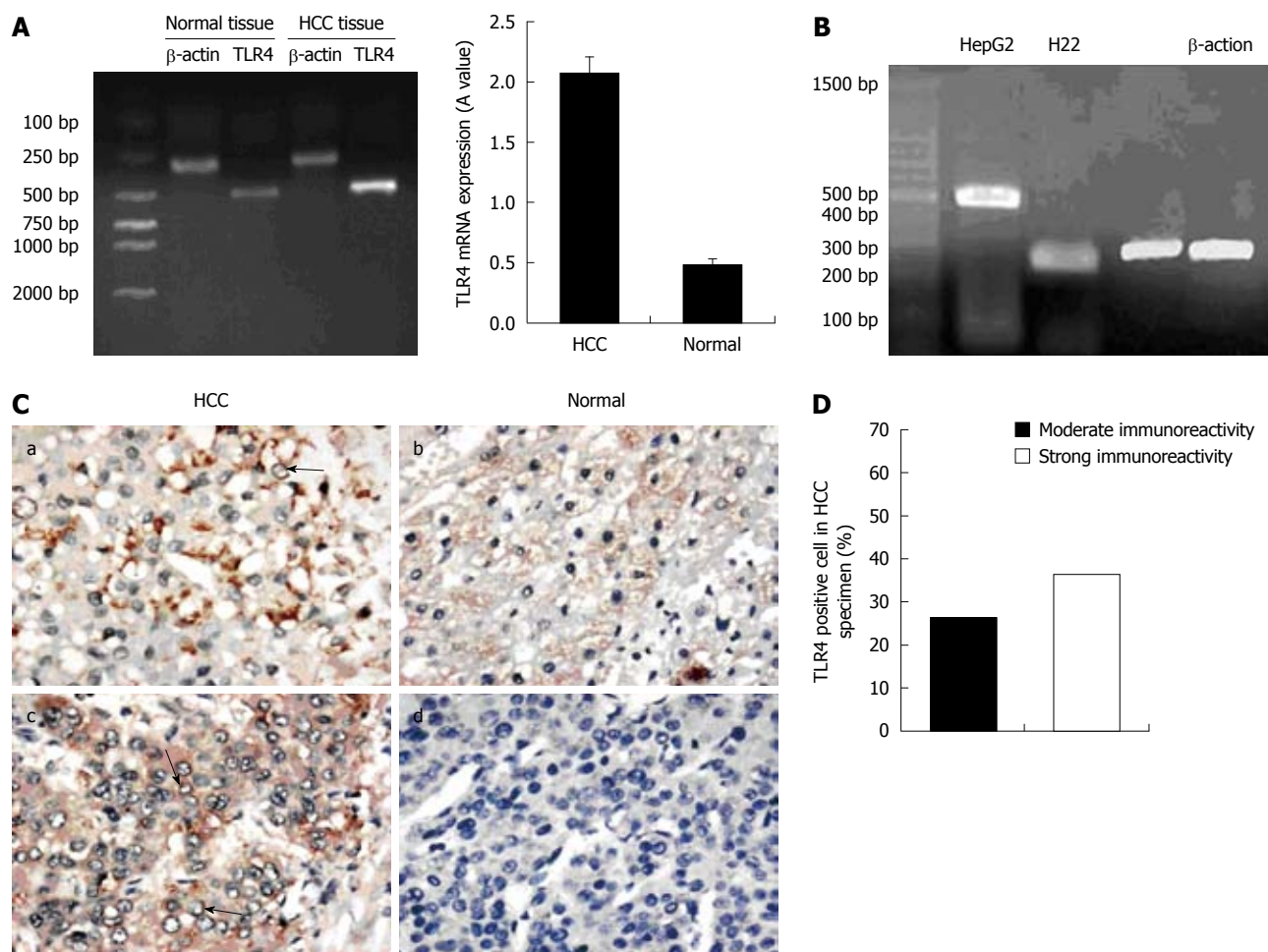


Figure 3 Expression of toll-like receptor 4 mRNA in hepatocellular carcinoma patients and hepatoma cell lines. A: Reverse transcriptase polymerase chain reaction analysis of total RNA from hepatocellular carcinoma (HCC) and normal tissues. The toll-like receptor 4 (TLR4) mRNA expression in HCC tissues was stronger (2.08 ± 0.14) compared to normal tissues (0.48 ± 0.05) ($P = 0.01$); B: TLR4 mRNA expressed in human HepG2 and murine H22 hepatoma cell lines; C: Expression of TLR4 protein in HCC and normal tissues by immunohistochemistry. a, c: Paraffin-embedded sections of HCC exhibiting positive expression on the membrane and in the cytoplasm in several positive hepatocytes (arrows) [diaminobenzidine (DAB) $\times 400$]; b, d: Paraffin-embedded sections of normal tissues showing weaker expression (DAB $\times 400$); D: TLR4 was detected by immunohistochemistry in tissue sections of patients with HCC (frequency of positive hepatocytes for TLR4 in paraffin-embedded sections).

that the proportion of Tregs was not correlated with hepatitis and cirrhosis, which was in concordance with other studies^[22,24]. Shen *et al*^[25] indicated that the increased prevalence and expanded function of Tregs in the tumor microenvironment of HCC correlated with cancer stage. However, no significant differences between number of Tregs and tumor UICC stage were observed in our HCC patients; other research groups also confirmed our findings^[26,27]. The discrepancy among different research groups potentially results from differences in patient profiles, e.g., Shen *et al*^[25] selected 31 patients who had undergone hepatectomy for their study. Moreover, we demonstrated a positive correlation between the number of Tregs and serum AFP levels and multifocal tumor. In order to exclude the influence of multifocal tumor, we separately analyzed 52 HCC patients with a single lesion, and found that the proportion of Tregs was higher in patients with a larger tumor size (Figure 3, $P = 0.0252$). Our results suggested that the number of Tregs in HCC potentially contributes to the inhibition of effective anti-

tumor immune responses and promotes intrahepatic tumor metastasis.

Correlation between Tregs levels and expression of TLR4 in HCC tissues

Despite the important role of Tregs in controlling immune responses to self-antigen and non-self-antigens and their natural ligands, the molecular mechanisms underlying the regulation and recruitment of Tregs in the tumor microenvironment remain poorly understood. One family of receptors involved in immune regulation is TLRs, a class of receptors that recognizes pathogen-associated molecular patterns or endogenous inflammation-associated molecules^[28]. Recently, TLRs were identified on cancer cells and T cells, including Tregs^[9,11]. Thus, TLR-signaling directly or indirectly regulates the immunosuppressive function of Tregs in the immune response^[29-32]. A few studies concerning indirect regulatory function have been conducted to clarify the complex cross-talk between TLRs and Tregs in tumors. Our data provide

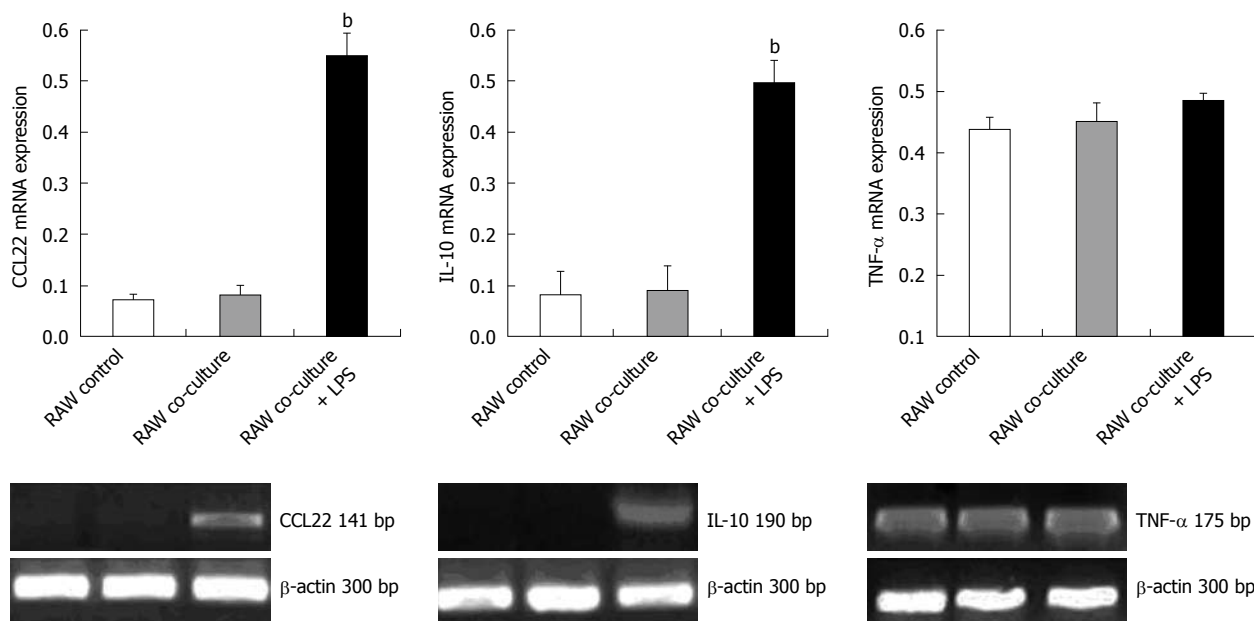


Figure 4 Tumor necrosis factor- α , interleukin-10, and CCL22 mRNA expression in RAW246.7 cells co-cultured with H22 cells \pm lipopolysaccharide. The bars indicate the relative amount of tumor necrosis factor (TNF)- α , interleukin (IL)-10 and CCL22 mRNA expression (reverse transcriptase polymerase chain reaction). LPS: Lipopolysaccharide. ^b $P < 0.001$ vs RAW co-culture.

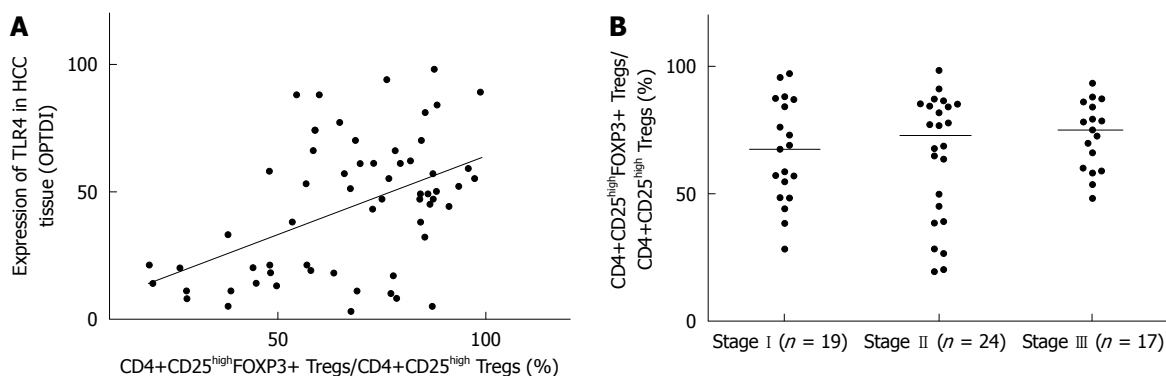


Figure 5 Correlation between the expression of toll-like receptor 4 in hepatocellular carcinoma specimens, tumor Union for International Cancer Control stage, and CD4+CD25^{high} FOXP3+ regulatory T cell. A: Toll-like receptor 4 expression was positively correlated with the number of CD4+CD25^{high}FOXP3+ regulatory T cells (Tregs) in peripheral blood. Analysis of data was performed using Spearman's rank correlation coefficients ($n = 60, r = 0.411, P < 0.001$); B: According to Union for International Cancer Control/tumor-node-metastasis classification, there were 19 cases, 24 cases, and 17 cases at stage I, II and III hepatocellular carcinoma, respectively. Statistically negative correlations were found in all comparisons between the 3 groups ($\chi^2 = 0.921, P = 0.631$) using the Kruskal-Wallis test.

some important clues regarding the interaction of TLR4 expression in tumor tissues and the number of Tregs in hepatocellular cancer. We demonstrated that TLR4 was expressed in diverse hepatoma cells and enriched in HCC tumor tissues, and that the expression of TLR4 in HCC positively correlated with the frequency of FOXP3+ Tregs in both circulation and tumor tissues ($P < 0.001$). These results suggest that the recruitment and proliferation of Tregs are indirectly regulated *via* TLR4 signaling.

Activation of TLR4 on tumor cells and its interaction with macrophages indirectly suppress anti-tumor immune response via recruitment of Tregs

Although some evidence implicates Tregs in the immunopathogenesis of cancer, suppressive mechanisms in the tumor microenvironment and the underlying mecha-

nisms of regulation remain poorly understood^[33]. Early evidence suggests that CCL22 preferentially attracts activated antigen-specific T cells to dendritic cells^[34]. Curjel recently demonstrated that tumor cells and microenvironmental macrophages in ovarian carcinoma produce the chemokine CCL22, which mediates Treg trafficking to the tumor^[35]. Therefore, tumor cells potentially utilize this effect to attract Tregs to the microenvironment. Our data suggested that co-culture of macrophages with a hepatoma cell line leads to a significant increase in the expression of IL-10 and CCL22 ($P < 0.001$). The source of this CCL22 is co-cultured macrophages and hepatoma cells preincubated with LPS. IL-10 is a pleiotropic cytokine produced by myeloid cells and lymphocytes that displays immunoregulatory effects^[36]. IL-10 inhibits the production of other cytokines such as IL-2, IL-12 and TNF- α .

and plays an important role in suppression of T cell anti-tumor responses^[37-39]. In this study, we demonstrated that co-cultured macrophages and LPS pre-treated hepatoma cells significantly up-regulate IL-10 expression. Our findings indicated that the activation of TLR4 on hepatoma cells indirectly modulates the suppressive function of Tregs and enhances Tregs recruitment by inducing cytokines, resulting in the immune escape of HCC.

The accumulation of FOXP3+ Tregs in HCC suggests a trend toward intrahepatic metastasis. The modulation of TLR4 activation on tumor cells links between Tregs suppressive function and the immunopathogenesis of human cancer. The association between TLR4-signaling and functional control of CD4+CD25^{high}FOXP3+ Tregs indicates intriguing potential opportunities to suppress anti-HCC immunity and improve therapeutic effectiveness for the patients. Our study provides the evidence which is useful for the development of an improved immunotherapeutic approach to HCC, and further studies are warranted.

ACKNOWLEDGMENTS

We thank Ms. Yanli He for her technical assistance in flow cytometric detection.

COMMENTS

Background

The survival of hepatocellular carcinoma (HCC) is limited in the majority of such cases. The active suppression of immune responses against tumor is a major barrier to the likely success of cancer immunotherapy. There is now compelling evidence implicating CD4+CD25^{high} family of transcription factor P3 (FOXP3)+ regulatory T cells (Tregs) as being key players driving immune suppression. However, precise function regulation of CD4+CD25^{high}FOXP3+ Tregs remains obscure.

Research frontiers

Toll-like receptors (TLRs) have recently emerged as a critical pathogen-associated molecular pattern of the innate immune system for detecting microbial infection and activation of dendritic cell maturation programs to induce adaptive immune responses. TLR4, an important member of TLRs family, plays a central role in phagocytosis, signal transduction and cell apoptosis. New evidence suggests that TLR signaling may directly or indirectly regulate the suppressive function of Treg cells. TLR4 signaling pathway activation induces production of massive cytokines, and finally intrigues the downstream nuclear factor kappa B to shift the balance between CD4+ T-helper and Treg cells, in ways that may facilitate tumor evasion of immune surveillance.

Innovations and breakthroughs

Currently, the roles of CD4+CD25^{high}FOXP3+ Tregs and TLR4 in HCC and their regulatory activity in the tumor microenvironment remain unclear. In the present study, authors described the clinicopathological significance of Tregs in 60 HCC patients, and the expression of TLR4 in hepatic cancer cells. The findings indicated that TLR4 ligation promotes the secretion of inhibitory cytokine interleukin-10 and chemokine CCL22 from co-cultured macrophages, not from the tumor cells themselves. Furthermore, the prevalence of Tregs significantly correlated with the presence of multifocal tumor. The results suggest a mechanistic path for the indirect modulation of CD4+CD25^{high}FOXP3+ Tregs via tumor TLR4 signaling, and demonstrate that interactions between hepatoma carcinoma cells and macrophages induce anti-tumor immune suppression via Tregs.

Applications

The authors report that activation of TLR4 on hepatoma cells followed by its interaction with macrophages may indirectly facilitate the recruitment of Tregs into tumor site and promote the intrahepatic metastasis, which suggests innate immunity mediated cellular interference maybe an effective therapeutic target in the treatment of HCC patients.

Terminology

CD4+CD25^{high}FOXP3+ Treg: Tregs are defined based on their expression of CD4, CD25 and forkhead, or winged helix FOXP3, which is critical for the development and function of Tregs in mice and humans. Tregs play a critical role in immunologic self-tolerance and suppression in the tumor immune response; TLR: To recognize specific structural regions of invading pathogens and initiate innate and adaptive immune responses; their expression has been detected in immune cells and also in many cancer cells. TLR4, an important member of TLRs family, plays a central role in phagocytosis, signal transduction and cell apoptosis.

Peer review

The article shows that TLR on hepatoma cell lines may indirectly facilitate the recruitment of Treg cells within tumor site via the potential activation of macrophages. This is a good exploratory study in which authors analyze the relationship of tumor TLR4 signaling pathway and CD4+CD25^{high}FOXP3+ Treg cells in tumor immune escape.

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S- Editor Gou SX L- Editor Ma JY E- Editor Zheng XM