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ORIGINAL ARTICLE

Basic Study

Role of LAP$^+$CD4$^+$ T cells in the tumor microenvironment of colorectal cancer

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Abstract

AIM

To investigate the abundance and potential functions of LAP$^+$CD4$^+$ T cells in colorectal cancer (CRC).

METHODS

Proportions of LAP$^+$CD4$^+$ T cells were examined in peripheral blood and tumor/paratumor tissues of CRC patients and healthy controls using flow cytometry. Expression of phenotypic markers such as forkhead box (Fox)p3, cytotoxic T-lymphocyte-associated protein (CTLA)-4, chemokine CC receptor (CCR)4 and CCR5 was measured using flow cytometry. LAP$^-$CD4$^+$ and LAP$^+$CD4$^+$ T cells were isolated using a magnetic cell-sorting system and cell purity was analyzed by flow cytometry. Real-time quantitative polymerase chain reaction was used to measure expression of cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β.

RESULTS

The proportion of LAP$^+$CD4$^+$ T cells was significantly higher in peripheral blood from patients (9.44% ± 3.18%) than healthy controls (1.49% ± 1.00%, P < 0.001). Among patients, the proportion of LAP$^+$CD4$^+$ T cells was significantly higher in tumor tissues (11.76% ± 3.74%) compared with paratumor tissues (3.87% ± 1.64%, P < 0.001). We also observed positive correlations between the proportion of LAP$^+$CD4$^+$ T cells and TNM stage (P < 0.001), distant metastasis (P < 0.001) and serum level of carcinoembryonic antigen (P < 0.05). Magnetic-activated cell sorting gave an overall enrichment of LAP$^+$CD4$^+$ T cells (95.02% ± 2.87%), which was similar for LAP$^-$CD4$^+$ T cells (94.75% ± 2.76%). In contrast to LAP$^-$CD4$^+$ T cells, LAP$^+$CD4$^+$ T cells showed lower Foxp3 expression but significantly higher levels of CTLA-4, CCR4 and CCR5 (P < 0.01). LAP$^+$CD4$^+$ T cells expressed significantly larger amounts of IL-10 and TGF-β but lower levels of IL-2, IL-4, IL-17 and interferon-γ, compared with LAP$^-$CD4$^+$ T cells.

CONCLUSION

LAP$^+$CD4$^+$ T cells accumulated in the tumor microenvironment of CRC patients and were involved in immune evasion mediated by IL-10 and TGF-β.

Key words: LAP$^+$CD4$^+$ T cells; Colorectal cancer; Tumor microenvironment; Interleukin-10; Transforming growth factor-β

Core tip: Many carcinomas, including colorectal cancer, gastric and nasopharyngeal cancer, are associated with elevated numbers of T regulatory (Treg) cells. It is suggested that Treg cells promote tumor development and metastasis by inhibiting the proliferation of effector T lymphocytes. LAP$^+$CD4$^+$ T cells, a recently identified subset of CD4$^+$ Treg cells, have 50-fold more potent immunosuppressive ability than traditional CD4$^+$CD25$^+$ T cells. Here, we present several lines of evidence correlating LAP$^+$CD4$^+$ T cells with colorectal cancer progression.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common carcinoma in men and second most common in women, with > 1 million new cases and > 500000 deaths every year worldwide[1,2]. CRC progression is a complex process involving interactions between host cellular immunity factors and the tumor, which take place in the so-called tumor microenvironment[3,4]. This environment includes numerous factors that promote tumor growth, such as energy and nutrients in blood vessels, growth factors from immune cells and stromal cells, and proinflammatory mediators secreted by tumor cells[5]. The environment also contains numerous factors that can limit tumor growth, such as tumor-infiltrating immune cells and tertiary lymphoid structures[6]. This complex mixture of factors largely determines patient prognosis and serves as an attractive therapeutic target[6,7].

Several studies have suggested that during CRC progression, peripheral regulatory T (Treg) cells and myeloid suppressor cells increase in the tumor microenvironment, which is associated with worse prognosis[8-10]. Part of the reason appears to be that these cell populations counteract the host's antitumor immune response[11]. Downregulating Treg cells can render antitumor responses more effective, which may improve prognosis in patients with CRC and other malignant carcinomas[12].

LAP$^+$CD4$^+$ T cells are a newly identified subset of Treg cells that express latent-associated peptide (LAP), and function within the latent transforming growth factor (TGF)-β complex to block interaction between TGF-β and receptors on immune cells[13]. Among the various Treg cell populations, LAP$^+$CD4$^+$ T cells are endowed with more potent immunosuppressive function than traditional CD4$^+$CD25$^+$Foxp3$^+$ Treg cells[14], and they are associated with autoimmune disease progression[13,15-17]. However, we are unaware of studies examining whether LAP$^+$CD4$^+$ T cells contribute to CRC progression. Thus, we analyzed the abundance, phenotype and cytokine secretion of LAP$^+$CD4$^+$ T cells in the tumor microenvironment in patients with CRC.

MATERIALS AND METHODS

Ethics statement

All patients enrolled in this study provided written informed consent. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki (Fortaleza, Brazil; October 2013), and it was approved by the Research Ethics Committee of the First Affiliated Hospital of Guangxi Medical University, China.

Study participants and samples

This study involved 50 patients who underwent primary tumor resection for colorectal adenocarcinoma at the First Affiliated Hospital of Guangxi Medical University from January to August 2014. Samples of peripheral blood were obtained preoperatively, and colorectal tumor and paratumor tissues were obtained postoperatively from each patient. Paratumor tissue samples were taken from tissue near the

Table 1 Clinical characteristics of patients with colorectal cancer

Characteristics	Value[1]
Male	31
Female	19
Age, yr	57.4 (37-76)
Location of primary tumor	
Colon	22
Rectum	28
TNM stage	
Ⅰ/Ⅱ	23
Ⅲ/Ⅳ	27

[1]Values are *n* or mean (range).

resection margin (≥ 10 cm away from the tumor site) that was confirmed to be tumor-free based on routine pathology. The basic data regarding the study population are shown in Table 1.

Patients were excluded if they (1) had already undergone CRC surgery or had been diagnosed with locoregional recurrence; or (2) were receiving any anticancer therapy, corticosteroids or other nonsteroidal anti-inflammatory drugs at the time of peripheral venous blood collection.

During the study period, peripheral blood was also collected from 25 healthy donors serving as a control group. Healthy controls were free of chronic pain, cardiovascular complaints, or other chronic inflammatory diseases. They were matched with patients in age and sex and showed no significant differences from patients.

Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from patients using Ficoll density gradient centrifugation. Fresh tumor and paratumor samples were washed three times in RPMI 1640; after which, fatty, connective and necrotic tissues were removed. Samples were cut into 1-2-mm cubes, transferred to a 50-mL beaker, and incubated for 3 h at room temperature with a triple-enzyme digestion medium containing 1 mg/mL collagenase Ⅳ, 30 μg/mL DNase Ⅰ and 0.1 mg/mL hyaluronidase (Sigma, St. Louis, MO, United States). Dissociated cell suspensions were filtered through a 70-μm nylon mesh, then tumor-infiltrating lymphocytes (TILs) were isolated from cell suspensions using discontinuous density gradient centrifugation[18]. LAP$^-$CD4$^+$ T cells and LAP$^+$CD4$^+$ T cells were isolated using a Magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell purity was analyzed by flow cytometry as described below.

Flow cytometry

TILs and PBMCs were stimulated in culture for 4 h at 37 ℃ with 50 ng/mL phorbol-12-myristate-13-acetate, 1 μg/mL ionomycin, and 0.7 μL/mL GolgiStop reagent in a 5% CO_2 incubator. T cells were identified based on surface or intracellular expression of markers labeled using antibodies (eBioscience, San Diego, CA, United States) against the following human antigens: LAP, CD4, forkhead box (Fox)p3, cytotoxic T-lymphocyte-associated protein (CTLA)-4, chemokine CC receptor (CCR)4, and CCR5. Antibodies were conjugated with one of the following fluorophores: phycoerythrin (PE), fluorescein isothiocyanate, PEcy5.5, PEcy7, peridinin chlorophyll protein (PerCP)-cy5.5, or allophycocyanin. Labeled cell suspensions were analyzed using a FACS Calibur flow cytometer (BD Bioscience, Franklin Lakes, NJ, United States) and FlowJo software (Tree Star, Ashland, OR, United States).

Table 2 Primer sequences for polymerase chain reaction

Gene	Sequence (5′-3′)	Product (bp)	T (℃)
IL-2	F:5′ CAGCTACAACTGGAGCATTTAC	130	60
	R:5′ TCAGTTCTGTGGCCTTCTTG		
IL-4	F:5′ GACCGTAACAGACATCTTTGC	180	60
	R:5′ TCGAGCCGTTTCAGGAAT		
IL-10	F:5′ TTGCCAAGCCTTGTCTGA	160	60
	R:5′ ACAGGGAAGAAATCGATGAC		
IL-17	F:5′ CCTCAGAGATCAACAGACCAA	80	60
	R:5′ GGTGCCTTGATCAGACAGAA		
IFN-γ	F:5′ GGCAAGGCTATGTGATTACA	180	60
	R:5′ TAAAGCACTGGCTCAGATTG		
TGF-β1	F:5′ CACGTGGAGCTGTACCAGAA	219	60
	R:5′ GAACCCGTTGATGTCCACTT		
18srRNA	F:5′ CCTGGATACCGCAGCTAGGA	112	60
	R:5′ GCGGCGCAATACGAATGCCCC		

Real-time quantitative polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, United States), and first-strand cDNA was generated using oligo (dT) primers and the SuperScript Ⅲ First-Strand Synthesis System (Invitrogen). Levels of mRNAs encoding cytokines secreted by LAP$^+$CD4$^+$ T cells and LAP$^-$CD4$^+$ T cells (TGF-β, INF-γ, IL-2, IL-4, IL-10 and IL-17) were determined using SYBR-based real-time polymerase chain reaction (7500 StepOnePlus system, Applied Biosystems, Carlsbad, CA, United States) and primers purchased from TaKaRa Biosystems (Table 2). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to levels of β-actin mRNA.

Statistical analysis

Data were expressed as mean ± SD. Differences between two groups were assessed for significance using the Mann-Whitney *U* test, *t*-test, or paired *t*-test, as appropriate. All statistical tests were performed using SPSS version 16.0 (SPSS, Chicago, IL, United States), and the threshold of significance was defined as $P < 0.05$.

Table 3 Proportions of LAP$^+$CD4$^+$ T cells in tumor tissues and in relation to clinicopathological characteristics

	n	LAP$^+$CD4$^+$ Treg (%)	*t*	*P* value	95%CI
Age, yr					
< 60	29	11.15 ± 2.03	0.747	0.458	-1.35-2.94
⩾ 60	21	11.96 ± 4.51			
Sex					
Male	31	11.37 ± 3.24	0.444	0.659	-1.60-2.50
Female	19	11.82 ± 3.89			
Location					
Colon	22	10.35 ± 3.45	0.652	0.517	-1.11-2.18
Rectum	28	11.89 ± 3.17			
TNM stage					
Ⅰ / Ⅱ	23	8.45 ± 2.98	4.973	0.000	3.85-9.07
Ⅲ / Ⅳ	27	14.90 ± 5.58			
Pathological pattern					
Tubular/ papillary	43	11.09 ± 3.54	1.335	0.188	-0.73-3.60
Myxoma/ ring cell	7	12.83 ± 3.26			
Differentiation					
High	42	10.50 ± 3.22	0.877	0.385	-1.45-3.70
Low	8	12.43 ± 3.87			
Metastasis					
Yes	9	12.51 ± 4.17	4.322	0.000	2.49-6.82
No	41	7.85 ± 2.61			
Ileus					
Yes	9	12.22 ± 3.49	0.904	0.470	-1.30-3.44
No	41	11.15 ± 3.14			
CEA (ng/mL)					
⩽ 5	34	9.94 ± 3.15	2.692	0.010	0.81-5.58
> 5	16	13.13 ± 4.06			
CA199 (U/mL)					
⩽ 37	32	11.34 ± 3.21	0.854	0.370	-1.51-3.85
> 37	18	12.42 ± 4.35			

RESULTS

LAP$^+$CD4$^+$ T cells are elevated in PBMCs and tumor tissue of CRC patients

PBMCs were isolated preoperatively and TILs were isolated postoperatively from patients who underwent radical resection for CRC. To understand further the roles of LAP$^+$CD4$^+$ T cells in the tumor microenvironment in patients with CRC, the proportion of LAP$^+$CD4$^+$ T cells in PBMCs and tissues was detected by flow cytometry (Figure 1). The proportion of LAP$^+$CD4$^+$ T cells was significantly higher in peripheral blood from patients (9.44% ± 3.18%) than healthy controls (1.49% ± 1.00%, $P < 0.001$; Figure 1B and C). Among CRC patients, the proportion of LAP$^+$CD4$^+$ T cells was significantly higher in tumor tissue (11.76% ± 3.74%) compared with paratumor tissue (3.87% ± 1.64%, $P < 0.001$; Figure 1B and D).

Relationship between proportion of LAP$^+$CD4$^+$ T cells in tumor tissues and clinicopathological characteristics of CRC

We also observed positive correlations between the proportion of LAP$^+$CD4$^+$ T cells and TNM stage ($P < 0.001$; Figure 2A), distant metastasis ($P < 0.001$; Figure 2B) and serum level of carcinoembryonic antigen (CEA) ($P < 0.05$; Figure 2C) (Table 3).

LAP$^+$CD4$^+$ Treg cell phenotype in CRC microenvironment

Further studies of phenotypic marker expression revealed differences between LAP$^+$CD4$^+$ T cells and LAP$^-$CD4$^+$ T cells. In contrast to LAP$^-$CD4$^+$ T cells, LAP$^+$CD4$^+$ T cells showed lower Foxp3 expression but significantly higher levels of CTLA-4, CCR4 and CCR5 ($P < 0.01$; Figures 3 and 4).

Magnetic-activated cell sorting in vitro

Magnetic-activated cell sorting gave an overall enrichment of LAP$^+$CD4$^+$ T cells (95.02% ± 2.87%; Figure 3A) and enrichment was similar for LAP$^-$CD4$^+$ T cells (94.75% ± 2.76%; Figure 3B).

Cytokine expression

The expression levels of cytokine profiles were measured by real-time qPCR, LAP$^+$CD4$^+$ T cells expressed significantly larger amounts of IL-10 and TGF-β but lower levels of IL-2, IL-4, IL-17 and IFN-γ, compared with LAP$^-$CD4$^+$ T cells (Table 4).

DISCUSSION

Many carcinomas, including colorectal, gastric and nasopharyngeal cancer, are associated with elevated numbers of Treg cells[19-21], and it is suggested that Treg cells promote tumor development and metastasis by inhibiting the proliferation of effector T lymphocytes[22]. LAP$^+$CD4$^+$ T cells, a recently identified subset of CD4$^+$ Treg cells, have 50-fold more potent immunosuppressive activity than traditional CD4$^+$CD25$^+$ T cells[13,23]. Here we present several lines of evidence correlating LAP$^+$CD4$^+$ T cells with CRC progression. These cells were more abundant in peripheral blood and tumor tissue from patients with CRC compared with healthy controls. In CRC patients, the abundance of these cells correlated positively with TNM stage, metastasis, and serum level of CEA. CEA is the most widely used serum marker and is related to the prognosis of patients with CRC. The main use of CEA in CRC is in surveillance following curative resection for primary cancer[24,25]. These results suggest that LAP$^+$CD4$^+$ T cells, like traditional CD4$^+$CD25$^+$ Treg cells, accumulate in the tumor microenvironment and postoperative monitoring of the LAP$^+$CD4$^+$ T cells in CRC patients may be useful for assessing prognosis and predicting distant metastasis.

In our study, expression of CCR4 and CCR5 was higher in LAP$^+$CD4$^+$ T cells than in LAP$^-$CD4$^+$ T cells. CCR4 and CCR5 are highly expressed in tumor microenvironments and appear to act as proinflammatory cytokine receptors[26,27]. Some studies have reported that CCR4 and its ligands are associated with increased tumor recurrence and impaired overall survival in patients with gastric cancer[28,29]. Wang *et al*[30] have shown that the CCL5/CCR5 axis

Table 4 Cytokine expression by LAP$^+$CD4$^+$ T cells and LAP$^-$CD4$^+$ T cells in colorectal cancer microenvironment

	IL-2	IL-4	IL-10	IL-17	IFN-γ	TGF-β
LAP$^+$CD4$^+$	0.22 ± 0.01	0.32 ± 0.12	1.13 ± 0.23	0.38 ± 0.10	0.18 ± 0.08	1.40 ± 0.15
LAP$^-$CD4$^+$	1.49 ± 0.37	0.86 ± 0.23	0.86 ± 0.22	0.98 ± 0.23	0.69 ± 0.21	0.89 ± 0.11
t	8.811	5.505	-2.327	6.435	5.981	-7.316
P value	0.000	0.000	0.038	0.000	0.000	0.000

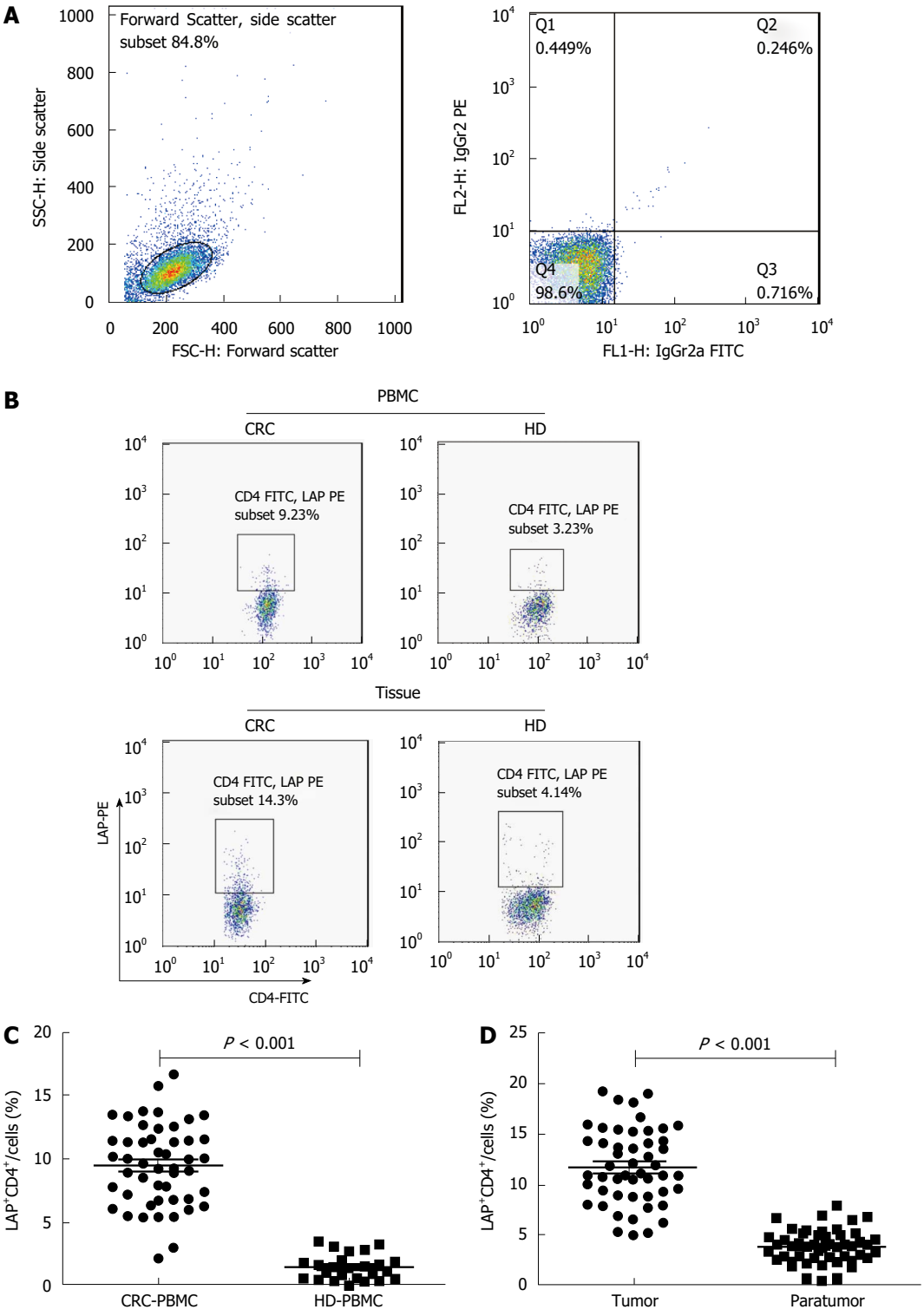


Figure 1 Abundance of LAP$^+$CD4$^+$ T cells in the colorectal cancer microenvironment based on flow cytometry. A: Gated on FSC/SSC, the proportion of LAP$^+$CD4$^+$ T cells in the CD4$^+$ subset is presented in quadrant Q2; B: Flow cytometry to measure the proportion of LAP$^+$CD4$^+$ T cells in PBMCs and tissues; C: Proportion of LAP$^+$CD4$^+$ T cells in PBMCs; D: Proportion of LAP$^+$CD4$^+$ T cells in tissues.

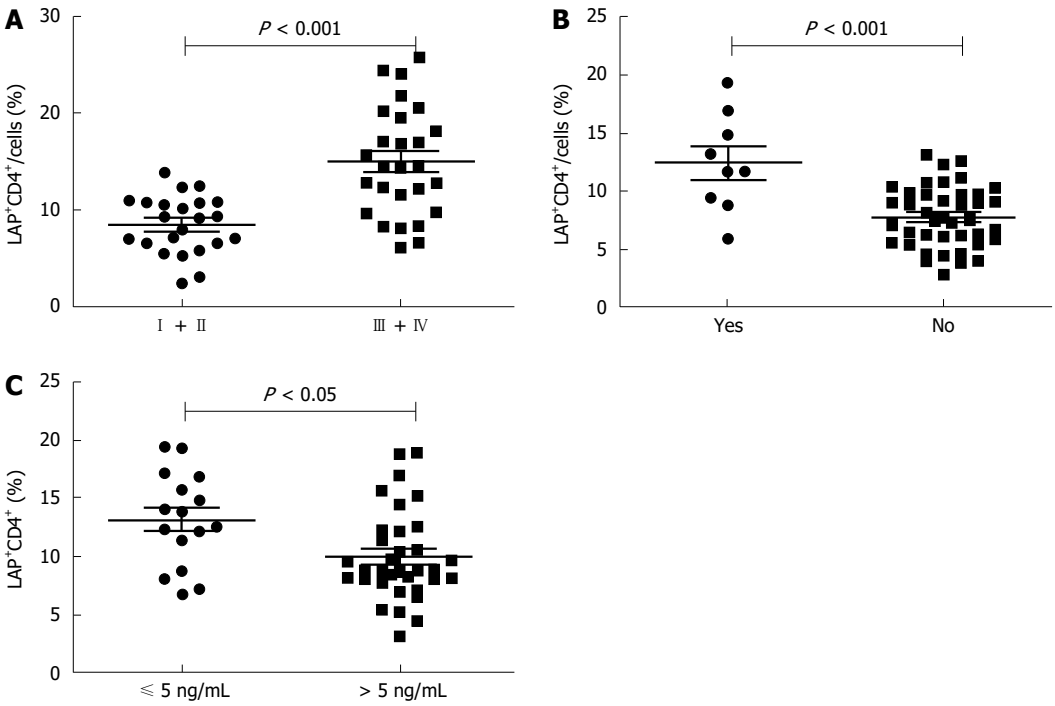
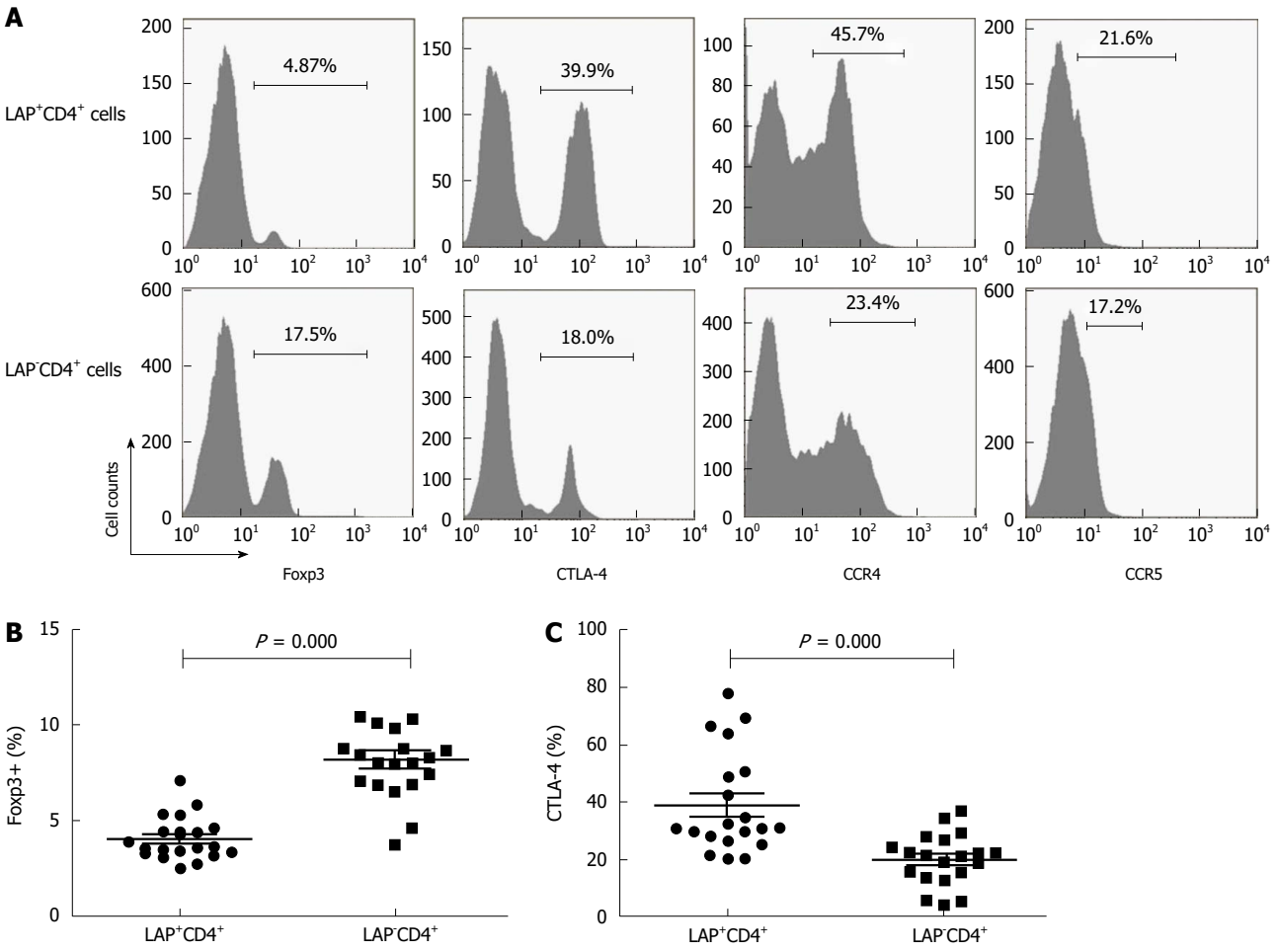


Figure 2 Correlation of LAP$^+$CD4$^+$ Treg cell abundance with clinicopathological characteristics of colorectal cancer, based on flow cytometry. A: TNM stage; B: Distant metastasis; C: Level of CEA.



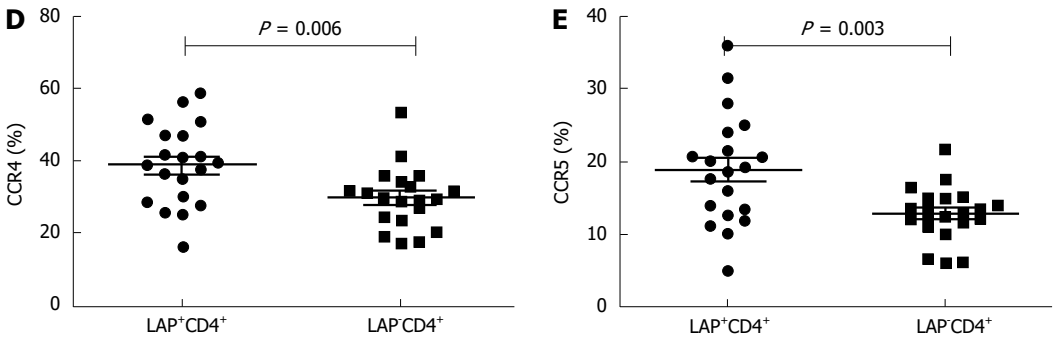


Figure 3 Expression of phenotypic markers by LAP$^+$CD4$^+$ T cells and LAP$^-$CD4$^+$ T cells. A: Typical histograms of the expression of Foxp3, CTLA, CCR4 and CCR5 in LAP$^+$CD4$^+$ T cells or LAP$^-$CD4$^+$ T cells are depicted. The expression levels of Foxp3, CTLA, CCR4 and CCR5 were measured and compared between LAP$^+$CD4$^+$ T cells and LAP$^-$CD4$^+$ T cells; B: Foxp3; C: CTLA-4; D: CCR4; E: CCR5.

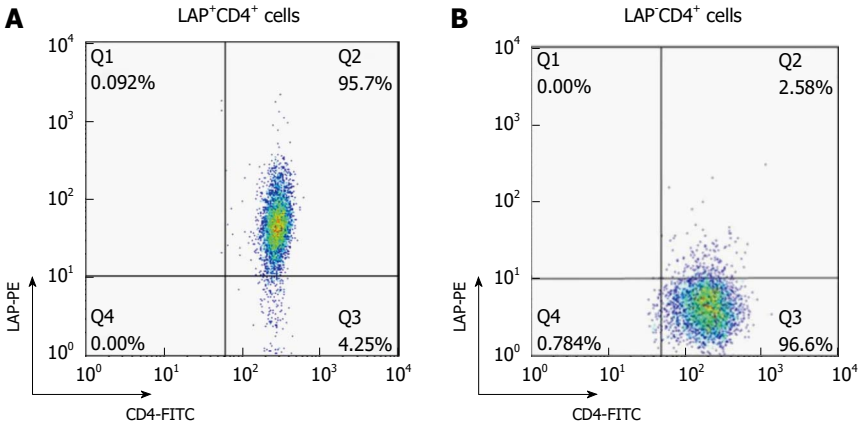


Figure 4 Purity of cells. A: LAP+CD4+ T cells; B: LAP−CD4+ T.

modulates angiogenesis and metastasis that dictate cancer development in the tumor microenvironment. We identified similarities and differences between LAP$^+$CD4$^+$ T cells and traditional CD4$^+$CD25$^+$ Treg cells. Foxp3, previously identified as important in the differentiation and development of Treg cells[31,32], was expressed at detectable levels in only 4% of LAP$^+$CD4$^+$ T cells. This means that LAP$^+$CD4$^+$ T cells differ from traditional CD4$^+$CD25$^+$ Treg cells in marker expression and their immunosuppressive activity is independent of Foxp3. In contrast, LAP$^+$CD4$^+$ T cells expressed abundant levels of CTLA-4, which is used by CD4$^+$CD25$^+$ Treg cells to modulate immune responses[33]. CTLA-4 on CD4$^+$CD25$^+$ Treg cells has been shown to suppress immune function through several mechanisms[34,35]: increasing numbers of CD4$^+$CD25$^+$ CTLA-4 T cells; inhibiting production of proinflammatory factors such as IFN-γ; increasing production of IL-2, IL-4, IL-10 and TGF-β1; and blocking tryptophan synthesis by antigen-presenting cells[36]. Under normal circumstances, these mechanisms can promote self-tolerance and prevent autoimmune disease and transplant rejection. Our results suggest that the CTLA-4 on LAP$^+$CD4$^+$ T cells help CRC tumors evade the host immune system, and one mechanism may be by inhibiting proliferation of effector T lymphocytes.

Our results reproduce most of those of Mahalingam *et al*[37], using different procedures. We isolated LAP$^+$CD4$^+$ T cells and LAP$^-$CD4$^+$ T cells using a magnetic cell sorting system and analyzed cell purity by flow cytometry. Our results revealed that, after sorting, the purity of these two cells was > 90%. This is the first time that LAP$^+$CD4$^+$ T cells were isolated using a magnetic cell sorting system. In contrast to Mahalingam *et al*[37], we found that LAP$^+$CD4$^+$ T cells expressed high levels of IL-10 and TGF-β. These cytokines play key roles in suppressing immune responses in mouse models of cerebral meningitis and allergic inflammation[13,38,39]. The immunoregulatory activity of Treg cells has been linked to several molecules, such as CTLA-4, TGF-β, and IL-10[40,41]. TGF-β has been shown to play an important role in the differentiation, maintenance and function of natural Treg cells[42-45]. However, several studies have revealed the role of IL-10 in Treg cell suppression. It has been demonstrated that IL-10 is required for the homeostatic maintenance of the T cell number by Treg cells[46] and is involved in Treg-cell-mediated suppression in murine models of transplantation, graft-versus-host disease, chronic parasite infection, colitis, and a rat model of type 1 diabetes[47]. Like classical CD4$^+$CD25$^+$ Treg cells, our experiments suggest that the immunosuppressive activity of LAP$^+$CD4$^+$ T cells

could be mediated by IL-10 and TGF-β.

In conclusion, we provide evidence that patients with CRC have elevated proportions of LAP$^+$CD4$^+$ T cells in the peripheral blood and tumor microenvironment, and their accumulation at tumor sites correlates with CEA level, TNM stage and distant metastasis. LAP$^+$CD4$^+$ T cells express high levels of IL-10 and TGF-β, which may be involved in tumor immune evasion. Our findings suggest that investigating the functions and regulation of LAP$^+$CD4$^+$ T cells in CRC may improve our understanding of disease progression and treatment.

COMMENTS

Background

LAP$^+$CD4$^+$ T cells are a newly identified subset of regulatory T (Treg) cells that express latent-associated peptide (LAP). They function within the latent transforming growth factor (TGF)-β complex to block interaction between TGF-β and receptors on immune cells, of the various Treg cell populations, LAP$^+$CD4$^+$ T cells are endowed with more potent immunosuppressive function than traditional CD4$^+$CD25$^+$Foxp3$^+$ Treg cells, and they have been associated with autoimmune disease progression. However, they are unaware of any studies examining whether LAP$^+$CD4$^+$ T cells contribute to colorectal cancer (CRC) progression. Thus, The authors analyzed the abundance, phenotype and cytokine secretion of LAP$^+$CD4$^+$T cells in the tumor microenvironment in patients with CRC.

Research frontiers

This is the first time that LAP$^+$CD4$^+$ T cells were isolated by using magnetic cell sorting system.

Innovations and breakthroughs

The authors found that LAP$^+$CD4$^+$ T cells accumulated in the tumor microenvironment of CRC patients and were involved in immune evasion mediated by interleukin-10 and TGF-β.

Applications

These findings suggest that investigating the functions and regulation of LAP$^+$CD4$^+$ T cells in CRC may improve our understanding of disease progression and treatment.

Terminology

LAP$^+$CD4$^+$ T cells are a newly identified subset of Treg cells that express LAP, and function within the latent TGF-β complex to block interaction between TGF-β and receptors on immune cells. CRC progression is a complex process involving interactions between host cellular immunity factors and the tumor, which take place in the so-called tumor microenvironment.

Peer-review

"The role of LAP$^+$CD4$^+$ T cells in the tumor microenvironment of colorectal cancer" from Zhong *et al* is a decent and interesting manuscript, and worthy to be published.

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