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Human Cancer Biology

Clinical Cancer Research

Accumulation of Foxp3$^+$ T Regulatory Cells in Draining Lymph Nodes Correlates with Disease Progression and Immune Suppression in Colorectal Cancer Patients

Liufu Deng[1,2], Haizeng Zhang[3], Yan Luan[1,2], Jianfeng Zhang[3], Qiao Xing[1], Shuxiao Dong[3], Xiaoran Wu[1,2], Mingyue Liu[1,2], and Shengdian Wang[1]

Abstract

Purpose: To assess the relation of Foxp3$^+$ regulatory T cells (Treg) in tumor draining lymph nodes (TDLNs) with tumor progression and immune suppression in colorectal cancer (CRC).

Experimental Design: Flow cytometry was used to analyze the densities of Tregs in lymphocytes of TDLNs, peripheral blood, and tumors from 34 patients with CRC. The frequency of Tregs was compared and evaluated for the association with disease stage. The effect of Tregs on the function of CD8$^+$ T cells was investigated by IFN-γ production.

Results: The density of Foxp3$^+$ Tregs in TDLNs was dramatically higher than that in peripheral blood lymphocytes, but significantly lower than that in tumor-infiltrating lymphocytes. Importantly, the frequency of Foxp3$^+$ Tregs in TDLNs, rather than that in tumors and peripheral blood, was positively correlated with disease stage. In addition, the functions of CD8$^+$ T cells were impaired in TDLNs compared with peripheral blood lymphocytes and were restored after Treg depletion.

Conclusions: Foxp3$^+$ Tregs in TDLNs are more correlated with disease progression and potentially influence CD8$^+$ T-cell functions. This study suggests that the frequency of Tregs in TDLNs may provide a valuable prognostic tool in the treatment of CRC. *Clin Cancer Res; 16(16); 4105–12. ©2010 AACR.*

A long-term dynamic cross-talk between tumor and immune system not only regulates tumor growth and metastasis but also changes the immune status of the host (1). It has been known that the presence of tumor-infiltrating lymphocytes (TILs) in primary colorectal cancer (CRC) presages an improved clinical outcome (2, 3). A high density of infiltrating memory and effector memory T cells within CRCs was associated with decreased invasiveness, lower stage, and improved survival (4). The type and density of T cells at the advancing tumor margin compared with the central core were recently proposed as having stronger prognostic significance than conventional TNM staging (5). These findings provide clear evidence that the host immune response plays an important role in determining the clinical outcome from CRC.

Regulatory T cells (Tregs) are a heterogeneous group of cells that are initially characterized by the CD4$^+$CD25$^+$ phenotype and more specifically identified by the nuclear transcription factor Foxp3 (6, 7). Tregs not only inhibit the development of autoimmune-mediated diseases but are also suspected of impeding antitumor immune responses (8, 9). It has been found that Tregs are increased in the peripheral blood and TILs of patients suffering from various malignant diseases, including CRC (9–11). Tregs from peripheral blood of CRC patients suppresses autologous T-cell responses (12, 13), which may be in an antigen-selective manner (14). However, there is no significant difference in tumor-infiltrating Treg density between advanced and early-stage CRC (15). Furthermore, the Treg density by immunohistochemistry assay was lower in tumor with higher American Joint Committee on Cancer (AJCC) or T stage. High density of Foxp3$^+$ cells in tumor tissue is associated with better survival and shows stronger prognostic significance than CD8$^+$ and CD45RO$^+$ lymphocytes densities, whereas high density of Foxp3$^+$ cell in normal tissue is associated with worse survival (16).

The tumor-draining lymph node (TDLN) is the site in which tumor antigens are typically first presented to the naïve immune system and antitumor immune responses are initiated (17). Meanwhile, this is also the preferential site of initial tumor metastases (18). Therefore, the local microenvironment in the TDLNs becomes a key

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Translational Relevance

The type, density, and location of lymphocytic infiltration surrounding the primary colorectal cancer (CRC) have been shown to have a strong prognostic significance. The tumor-draining lymph node (TDLN) is the site of the critical initial decision between immune activation and tolerance and has an outsized influence on host immune response. The current study analyzed in parallel regulatory T cells (Tregs) in TDLNs, tumor, and peripheral blood and showed that Tregs in TDLNs were more correlated with disease stage although the frequency of Tregs in TDLNs was markedly lower than that in primary tumor. Our studies indicate that Tregs in regional draining lymph nodes contribute to the immune-suppressive environment of CRC patients, and the frequency of Tregs in lymph node may provide a valuable prognostic tool in the treatment of CRC and possibly other malignancies.

determinant in setting the course of the subsequent immune response to the tumor. It was reported that a high density of $CD45RO^+$ T cells in lymph node metastasis of CRC was associated with improved prognosis (19). However, the distribution and function of Tregs in TDLNs have not been studied well. The aim of the present study was to analyze the Tregs in TDLNs of CRC patients and investigate their prognostic value and functions.

Materials and Methods

Patient samples

Fresh blood, lymph nodes, and tumors samples were obtained from 34 sporadic CRC patients who underwent radical resection from December 13, 2007 to March 4, 2009 in a single group of the Cancer Hospital, Chinese Academy of Medical Science and Peiking Union Medical College. We excluded the cases that were treated with radiation, chemotherapy, or immunotherapy before surgery. Patients who had cancer history, autoimmune disease, inflammatory bowel disease, infectious diseases, and multiprimary cancer were also excluded. For tumor staging, the sixth edition of the AJCC Cancer Staging Manual was applied. The examination of regional lymph nodes was done by a single colorectal pathologist. The study was approved by the Ethics Committee of Cancer Hospital, Chinese Academy of Medical Science, and the Institutional Review Board of Institute of Biophysics, Chinese Academy of Sciences, Beijing, China. The written informed consent was obtained from each patient.

Cell isolation and Treg deletion

Peripheral blood mononuclear cells (PBMCs) were separated from fresh blood samples by Ficoll density gradient centrifugation. $HLA\text{-}A2^+$ PBMCs were sequentially incubated with anti-CD3-phycoerythrin (PE; BD Biosciences) and anti-PE MicroBeads (Miltenyi Biotec) for 15 minutes at 4°C and passed through a MACS LD column (Miltenyi Biotec) to get CD3-depleted PBMCs. Fresh tumor tissues were cut into small pieces, digested with RPMI 1640 containing 2% fetal bovine serum and 1 mg/mL type IV collagenase (Life Technologies, Inc.) for 2 hours at 37°C, and passed through a cell strainer to achieve a cell suspension. Fresh lymph node specimens were gently minced and passed through a cell strainer to achieve a cell suspension. After sequentially incubating with anti-CD25-PE (BD Biosciences) and anti-PE MicroBeads, the cell suspension was passed through the LD column to get CD25-deleted lymph node cells.

Lymphocyte stimulation

Fresh peripheral blood lymphocytes (PBLs) and lymph node cells were stimulated with 1 μg/mL soluble anti-CD3 (eBioscience, OKT3) and 1 μg/mL anti-CD28 (eBioscience, CD28.2) in the presence of anti-CD107a-FITC (10 μL/mL, eBioscience) for 1, 3, and 5 hours for

Table 1. Clinicopathologic characteristics

	All patients (n = 34), n (%)	Patients with LN available (n = 32), n (%)
Sex		
Male	23 (67.6)	21 (65.6)
Female	11 (32.4)	11 (34.4)
Age, y		
Median	57.6	57
Range	36-74	36-74
Tumor differentiation		
Good	3 (8.8)	3 (9.4)
Moderate	24 (70.6)	23 (71.9)
Poor	7 (20.6)	6 (18.7)
AJCC tumor stage		
I	5 (14.7)	5 (15.6)
II	16 (47.1)	16 (50.0)
III	10 (29.4)	10 (31.3)
IV	3 (8.8)	1 (3.1)
M stage		
0	31 (91.2)	31 (96.9)
1	3 (8.8)	1 (3.1)
ypT stage		
1	2 (5.9)	2 (6.2)
2	3 (8.8)	3 (9.4)
3	7 (20.6)	7 (21.9)
4	22 (64.7)	20 (62.5)
ypN stage		
0	22 (64.7)	22 (68.7)
1	3 (8.8)	3 (9.4)
2	9 (26.5)	7 (21.9)

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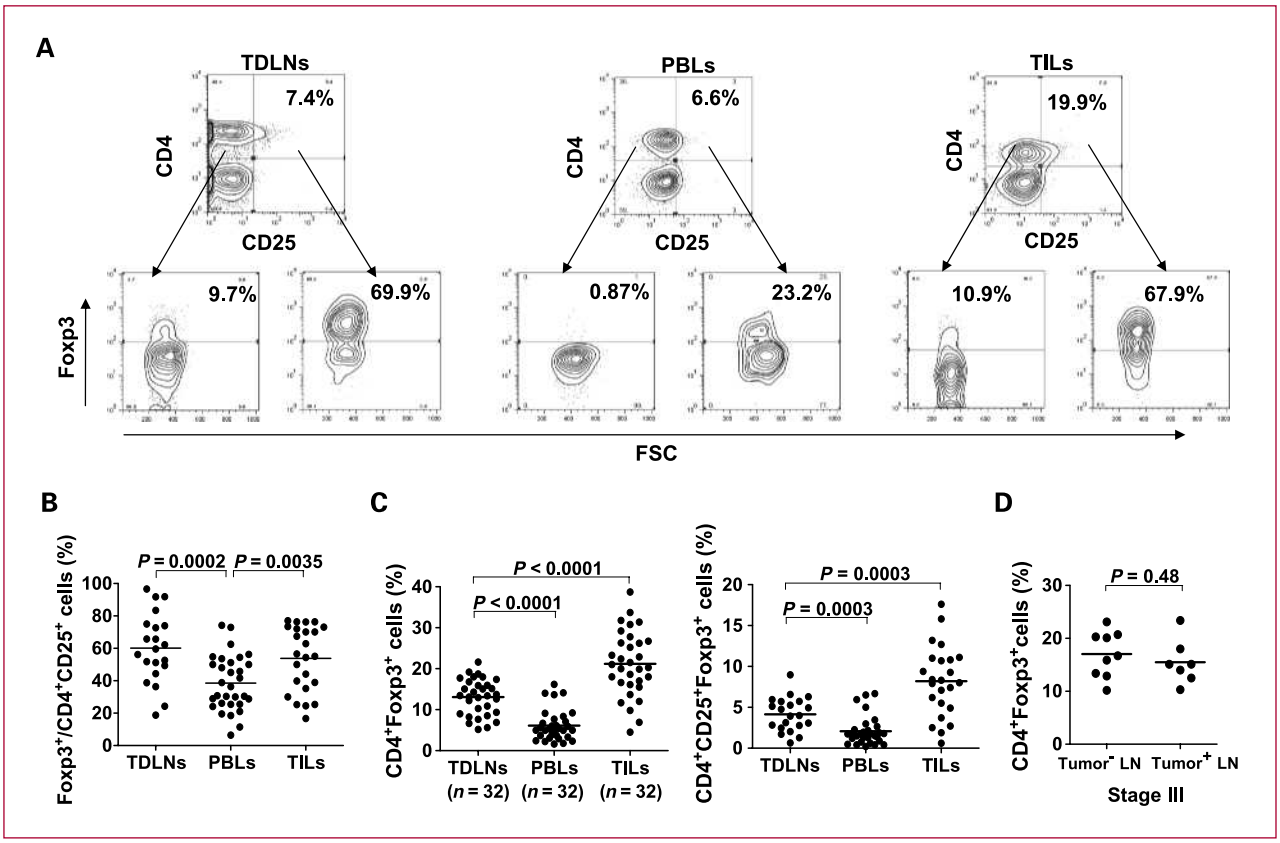

Fig. 1. Distribution of Tregs in TDLNs, PBLs, and tumor of CRC patients. A, representative flow cytometry analysis of lymphocytes of TDLNs, PBLs, and TILs stained with mAbs to CD4, CD25, and Foxp3. B and C, relative percentage of $Foxp3^+$ cells in $CD4^+CD25^+$ cells (B) and percentages of $CD4^+Foxp3^+$ and $CD4^+CD25^+Foxp3^+$ Tregs in TDLNs, PBLs, and TILs (C). D, percentage of $CD4^+Foxp3^+$ Tregs in individual lymph nodes with and without tumor metastasis in patients with stage III. Significance was indicated by *P* value. Each symbol represents a single individual.

CD107a staining and stimulated with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 μg/mL) for 4 hours for IFN-γ staining.

To study the effect of $CD25^+$ cells on the IFN-γ production of $CD8^+$ T cells, lymph node cells or CD25-deleted lymph node cells were stimulated with 5 μg/mL plate-bound anti-CD3 monoclonal antibody (mAb) and 2 μg/mL anti-CD28 mAb for 3 days or stimulated with CD3-depleted PBMCs pulsed with 10 μg/mL of influenza virus matrix peptide (Flu-$MA_{58\text{-}66}$, GILGFVFTL) or HIV pol peptide (HIV $pol_{476\text{-}484}$, ILKEPVHGV) or 200 μg/mL of tumor lysate in the presence of 10 units/mL interleukin-2 and 10 ng/mL interleukin-7 for 7 days. The tumor lysate was prepared and loaded onto PBMCs as described (20). In all stimulations, brefeldin A was added into the culture at the final 4 hours.

Flow cytometry

The cells were incubated with the anti-CD3, anti-CD4, anti-CD8, anti-CD25, and isotype control (eBioscience) antibodies for 30 minutes on ice. Foxp3 was stained with anti-Foxp3-PE (eBioscience) according to the manufacturer's protocol. For IFN-γ and Ki-67 intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences) for 20 minutes on ice, washed with Perm Wash Buffer (BD Biosciences), and then stained with labeled mAb. After two additional washes, cells were fixed, acquired on a flow cytometer, and analyzed with FlowJo software.

Statistical analysis

Statistical analysis was done with GraphPad Prism 5 software. The results are expressed as means with the SEM when appropriate. Two-tailed unpaired Student's *t* test was used to determine significance. A paired *t* test was used to compare the frequency of IFN-γ^+CD8^+ T cells in lymph nodes and PBLs for each patient. $P < 0.05$ (*) was considered significant, and $P < 0.01$ (**) was considered highly significant.

Results

Patient characteristics

Thirty-four patients (male 23, female 11) with CRC were enrolled in this study. The characteristics of

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Table 2. Frequency of $CD4^+Foxp3^+$ Tregs in lymph nodes for each patient

No. of patients	No. of LNs examined by H&E	No. of LNs for Treg analysis	LN1	LN2	LN3	LN4
Stage I						
1	25 (0)	4	15.8	18.2	14.8	15.1
2	20 (0)	2	13.6	11.7		
3	9 (0)	3*	6.7			
4	8 (0)	1	12.2			
5	19 (0)	1	6.9			
Stage II						
6	9 (0)	4	11	13.3	13.7	14.3
7	27 (0)	3	16.2	18.5	17.5	
8	5 (0)	2	13.9	13.6		
9	19 (0)	2	14.2	14.3		
10	30 (0)	2	13.5	13.1		
11	17 (0)	2	8.2	7.1		
12	27 (0)	2	9.9	8.6		
13	11 (0)	2	18.9	19.5		
14	5 (0)	4*	15			
15	9 (0)	3*	8.2			
16	7 (0)	2*	9			
17	21 (0)	2*	18			
18	18 (0)	1	5.6			
19	19 (0)	1	10.9			
20	18 (0)	1	8.5			
21	16 (0)	1	5.2			
Stage III						
22	17 (5)	2	23.4†	10.2		
23	9 (2)	2	23.1	20.1		
24	19 (8)	2	12.5†	13.4		
25	16 (4)	2	20.7	16.7		
26	22 (1)	2	14†	12.9		
27	17 (3)	2	20.3	15.1†		
28	62 (38)	1	10.3†			
29	16 (8)	1	15.9			
30	18 (10)	1	15.1†			
31	23 (17)	1	18.0†			
Stage IV						
32	13 (0)	2	15.1	15.2		
Total	571	63				
Mean	17.8	2.0				

NOTE: Numbers in parentheses represent the numbers of tumor-positive lymph nodes.
Abbreviation: LN, lymph node.
*Lymph nodes that were mixed together.
†Tumor-positive lymph nodes.

patients are shown in Table 1. A total of 63 lymph nodes from 32 patients were investigated. Ten patients had one lymph node available. Thirty-nine lymph nodes were individually analyzed in 17 patients who had two or more lymph nodes, whereas lymph nodes from each patient were pooled for analysis in five patients. Another two patients only have tumor tissue and blood available.

The frequency of Tregs is higher in TDLNs than in peripheral blood, but remarkably lower in tumor tissues

Tregs were identified by flow cytometry with CD4, CD25, and Foxp3 markers. Representative dot plots of Tregs in TDLNs, PBLs, and TILs of CRC patients are shown in Fig. 1A. $CD4^+CD25^+$ T cells seemed to markedly accumulate more in tumor than in TDLNs and PBLs (Fig. 1A).

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However, the frequency of Foxp3$^+$ cells in CD4$^+$CD25$^+$ population was dramatically higher in TDLNs and TILs than in PBLs (Fig. 1A and B). The frequency of CD4$^+$Foxp3$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ Tregs in TDLNs was significant higher than that in PBLs, but lower than that in TILs (Fig. 1C).

Next, we individually analyzed the lymph nodes of each of the 17 CRC patients who had two or more lymph nodes. As shown in Table 2, there was no difference of frequency of CD4$^+$Foxp3$^+$ Tregs between lymph nodes in each patient in stage I and stage II. Although marked variation existed in the frequency of CD4$^+$Foxp3$^+$ Tregs between lymph nodes from each patient in half of patients with stage III, there was no significant difference in the frequency of CD4$^+$Foxp3$^+$ Tregs between lymph nodes with and without tumor metastasis in stage III patients (Fig. 1D). These data indicate that the presence of Tregs in TDLNs is not related with the distance of lymph node from the primary tumor and tumor invasion.

The frequency of Tregs in TDLNs is more correlated with disease stage

Next, we analyzed the relationship of Treg frequency in TDLNs with disease progression. Patients with stage IV were excluded, and the average percentage of CD4$^+$Foxp3$^+$ Tregs was calculated in the patients with two or more lymph nodes. As shown in Fig. 2A, the frequency of CD4$^+$Foxp3$^+$ Tregs in TDLNs in patients with lymph node metastasis was significantly higher than that in patients without lymph node metastasis. For the patients with advanced T stage of the primary tumor (T>2), the presence of CD4$^+$Foxp3$^+$ Tregs was also positively correlated with lymph node metastasis. The similar results were found with the percentage of both CD4$^+$CD25$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ Tregs (data not shown). However, there was no significant difference of the frequency of CD4$^+$Foxp3$^+$ Tregs in PBLs or TILs between the patients with and without lymph node metastasis in this cohort of CRC patients (Fig. 2B and C). These results suggest that the frequency of Tregs in TDLNs is more correlated with disease progression.

Tregs in TDLNs inhibit CD8+ T-cell functions

To determine the effects of Tregs on CD8$^+$ T-cell functions, we first compared the functional hallmarks of CD8$^+$ T cells in TDLNs with that in PBLs from the same patients. The IFN-γ production and expression of Ki-67 and CD107a of CD8$^+$ T cells were detected by flow cytometry. As shown in Fig. 3, the IFN-γ–producing CD8$^+$ T cells were markedly decreased in TDLNs versus in PBLs (Fig. 3A). Similarly, the Ki-67– and CD107a- expressing CD8$^+$ T cells were significant lower in TDLNs than in PBLs (Fig. 3B and C). These data suggest that CD8$^+$ T-cell functions are greatly decreased in TDLNs compared with PBLs.

The functional impairment of CD8$^+$ T cells in TDLNs may result from the suppression of Tregs. To test this hypothesis, we depleted Tregs from TDLNs and detected the IFN-γ production of CD8$^+$ T cells. Although both CD4$^+$ T and CD8$^+$ T cells express CD25, about 90% of CD25$^+$ cells are CD3$^+$CD4$^+$ T cells in TDLNs (data not shown). Thus, CD25 depletion was applied to remove Tregs from lymph node cells. As shown in Fig. 4A and B, depletion of CD25$^+$ cells dramatically increased IFN-γ–producing CD8$^+$ T cells of lymph node cells stimulated with anti-CD3 and anti-CD28 for 3 days. Moreover, we found that the majority of the increased IFN-γ–producing CD8$^+$ T cells were CD45RO$^+$CD8$^+$ T cells and depletion of Tregs increased IFN-γ production of both CD45RO$^-$CD8$^+$ and CD45RO$^+$CD8$^+$ T cells. Likewise, depletion of CD25$^+$ cells also restored IFN-γ production of CD8$^+$ lymph node cells stimulated with Flu-MA$_{58\text{-}66}$ peptide or tumor lysate. However, HIV pol$_{476\text{-}484}$ peptide, as a negative control, could not stimulate IFN-γ production of lymph node CD8$^+$ T cells (Fig. 4C and D). These studies suggest that CD8$^+$ T cells in TDLNs are functionally impaired and Treg depletion could reconstitute their functional activity.

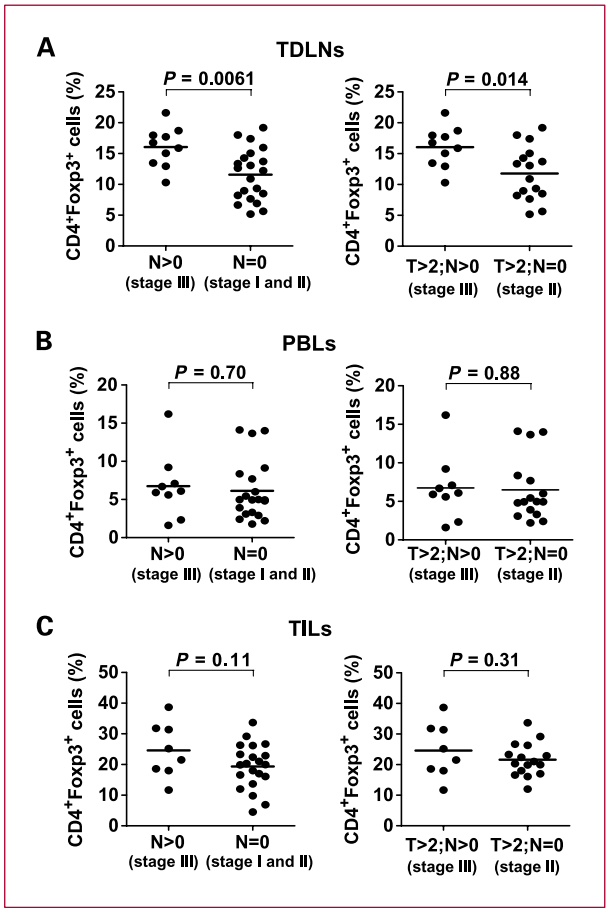

Fig. 2. Association of percentage of CD4$^+$Foxp3$^+$ Tregs with disease stage. Percentage of CD4$^+$Foxp3$^+$ Tregs in TDLNs (A), PBLs (B), and TILs (C) of patients with lymph node metastasis (N > 0, stage III) and without lymph node metastasis (N = 0, stage I and II; left) or patients with the advanced T category of the primary tumor (T > 2; right). Each symbol represents the percentage of CD4$^+$Foxp3$^+$ Tregs in a single individual. The average percentage of CD4$^+$Foxp3$^+$ Tregs was calculated in patients with two or more lymph nodes. Significance of correlation is indicated by *P* value.

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Discussion

The high density of $CD8^+$ and $CD45RO^+$ T cells within the tumor was associated with improved prognosis (4, 21). The frequency of Tregs was significantly higher in TILs than in lymphocytes from nonmalignant colon tissue. However, the correlation of Tregs with disease progression has not been well established in CRC (15, 16, 22). In agreement with an early report (15), the current study confirmed the high frequency of Tregs in tumor compared with that in PBLs. The novelty of this study was that Tregs was analyzed in TDLNs in parallel with TILs and PBLs. Although the frequency of Tregs in TDLNs was markedly lower than that in tumor, Tregs in TDLNs were more correlated with disease stage than those in primary tumor.

The density of $Foxp3^+$ Tregs was higher in tumor tissue compared with normal portion of the colon (23). Our study showed that the density of $Foxp3^+$ Tregs in tumor was also higher than that in TDLNs and PBLs. However, the presence of $Foxp3^+$ Tregs in tumor was not associated with disease stage (16, 22). Consistently, there was no significant difference in percentage of $CD4^+Foxp3^+$ Tregs in TILs between patients with and without lymph node metastasis in our cohort (Fig. 2C). Although the frequency of $CD4^+Foxp3^+$ Tregs was markedly lower in TDLNs than in TILs, the frequency of $CD4^+Foxp3^+$ Tregs in TDLNs was significantly higher in patients with lymph node metastasis than in patients without lymph node metastasis (Figs. 1C and 2A). It is possible that the difference in the density of $CD4^+Foxp3^+$ Tregs in tumor will become significant between patients with and patients without lymph node metastasis with the increase of cases in the cohort. However, our results at least suggest that the frequency of Tregs in TDLNs is more correlated with disease progression. In addition, the analysis of individual lymph node in each patient showed that the presence of Tregs in regional draining lymph nodes was not influenced by the distance of lymph nodes from the primary tumor and tumor invasion (Fig. 1D; Table 2). Thus, the presence of Tregs in regional draining lymph nodes is related with the disease stage of CRC patients.

It was shown that human CRCs with a high density of infiltrating memory and effector memory ($CD45RO^+$) T cells were less likely to disseminate to lymphovascular and perineural structures and to regional lymph nodes (4). The frequency of $CD45RO^+$ T cells in lymph node metastasis of CRC was also associated with improved prognosis (19). Herein, we showed that more $CD45RO^+CD8^+$ T cells produced IFN-γ than $CD45RO^-CD8^+$ T cells in

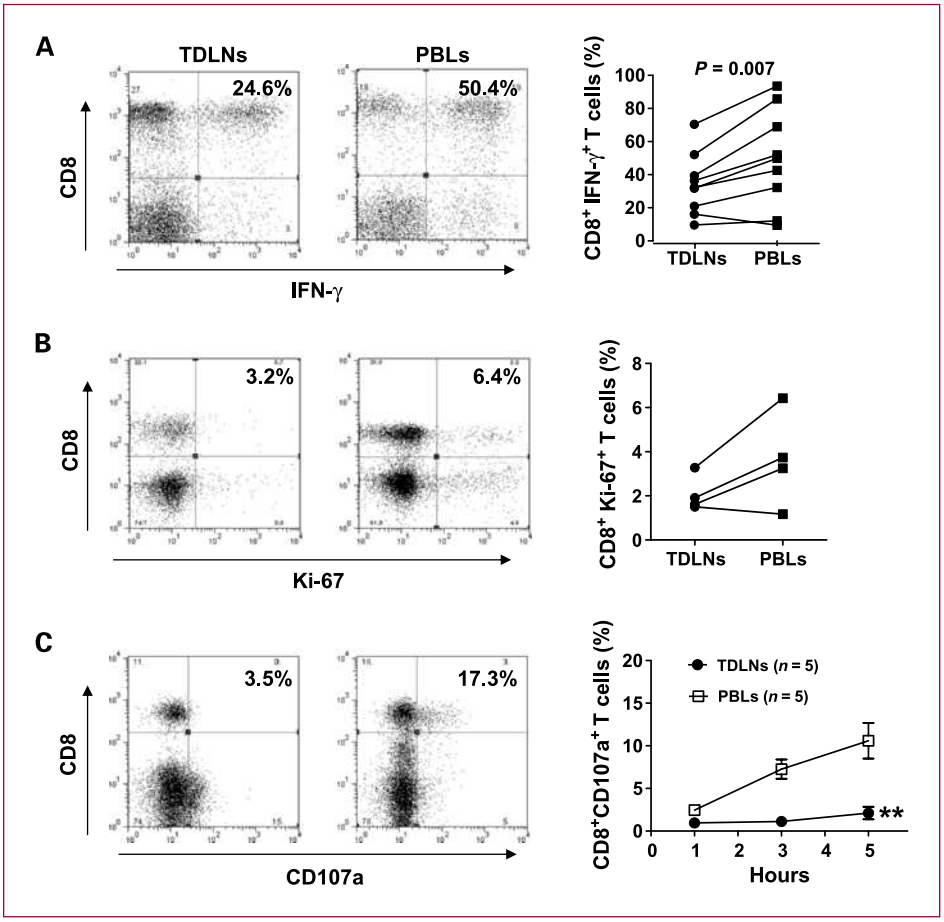

Fig. 3. The functions of $CD8^+$ T cells are decreased in TDLNs compared with PBLs. Flow cytometry analysis of PBLs and TDLNs stained with mAbs to CD8, Ki-67, IFN-γ, and CD107a. A, percentage of IFN-γ–producing $CD8^+$ T cells in TDLNs and PBLs stimulated with phorbol 12-myristate 13-acetate and ionomycin for 4 h. B, percentage of Ki-67^+ $CD8^+$ T cells in freshly prepared TDLNs and PBLs. C, percentage of CD107a-producing $CD8^+$ T cells in TDLNs and PBLs stimulated with anti-CD3 and anti-CD28 stimulated for 5 h (left) or the indicated time points (right). Significance of correlation is indicated by *P* value. Each symbol represents a single individual in A and B.

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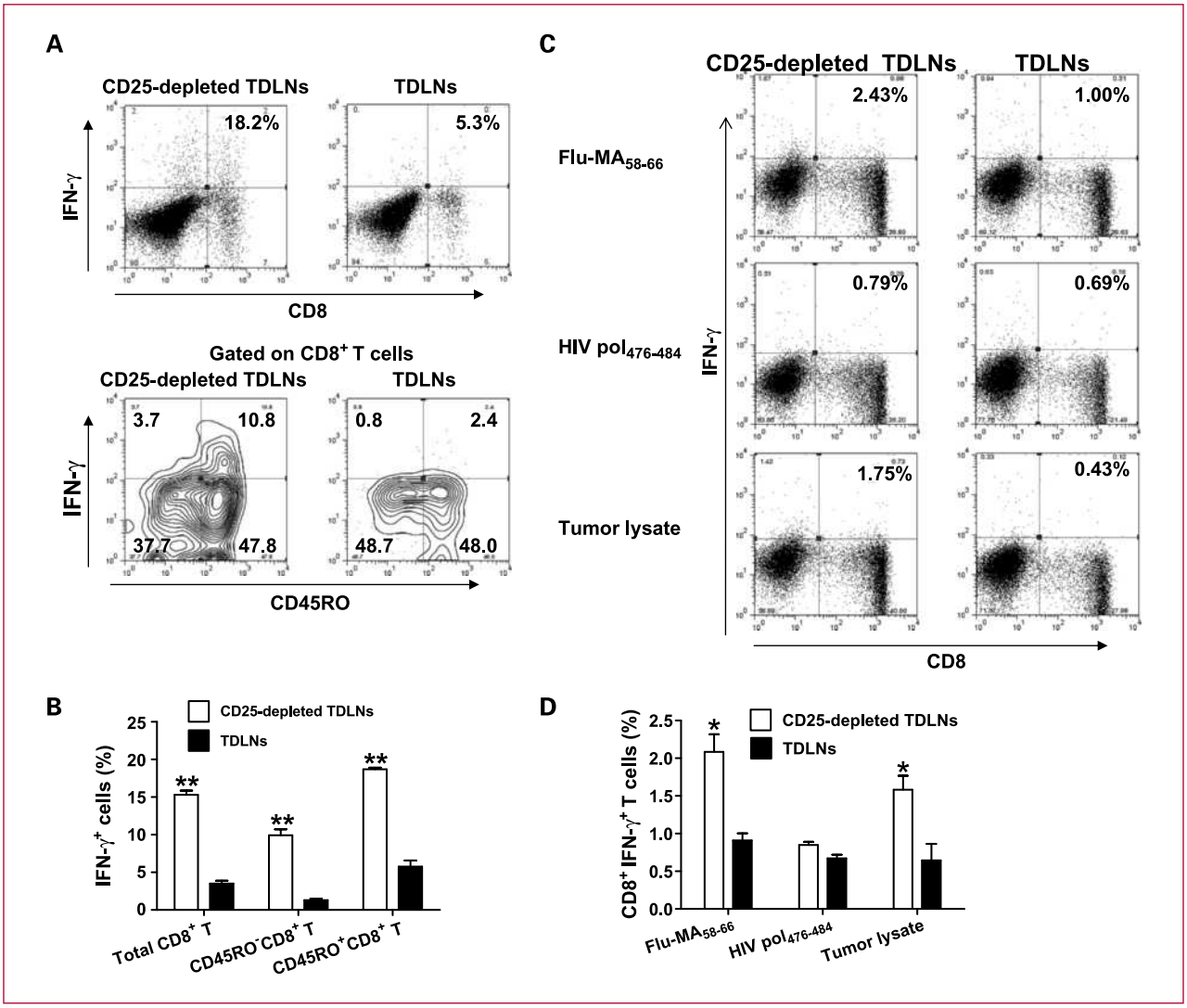

Fig. 4. Depletion of Tregs increases IFN-γ production of $CD8^+$ T cells in TDLNs. A and B, lymph node cells were stimulated with anti-CD3 and anti-CD28 for 3 d and stained with mAbs to IFN-γ, CD8, and CD45RO. Representative flow cytometry analysis of IFN-γ–producing $CD8^+$ T cells in lymph node cells (top) and IFN-γ–producing $CD45RO^+$ and $CD45RO^-$ cells in $CD8^+$ T cells (bottom; A). Percentage of IFN-γ production cells in total, $CD45RO^+$, and $CD45RO^-CD8^+$ T cells in lymph nodes and CD25-depleted lymph nodes stimulated in triplicate (B). Representative of three samples with similar results. C and D, the lymph node cells were stimulated with CD3-depleted PBMCs pulsed with Flu-MA_{58-66} or HIV $pol_{476-484}$ peptide or with tumor lysate for 7 d and then stained with mAbs to IFN-γ, CD8. Representative flow cytometry analysis of IFN-γ–producing $CD8^+$ T cells (C). Percentage of IFN-γ production cells in total $CD8^+$ T cells in lymph nodes and CD25-depleted lymph nodes stimulated with different CD3-depleted PBMCs in triplicate (D). Representative of two samples with similar results.

TDLNs stimulated *in vitro* (Fig. 4A and B), supporting that $CD45RO^+$ T cells are important effectors of antitumor responses. Tregs markedly suppressed IFN-γ production of $CD45RO^+CD8^+$ T cells in TDLNs. This may provide mechanistic support for the association of Tregs in lymph nodes with disease stage of CRC.

In conclusion, our studies indicate that Tregs in regional draining lymph nodes contribute to the immune-suppressive environment of CRC patients. The frequency of Tregs in lymph node is associated with disease stage and may therefore be a valuable prognostic tool in the treatment of CRC and possibly other malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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