LAP⁺CD4⁺ T Cells Are Suppressors Accumulated in the Tumor Sites and Associated with the Progression of Colorectal Cancer

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Abstract

Purpose: Suppressor T cells are one of the determinants of colorectal cancer (CRC) clinical outcome. LAP⁺CD4⁺ T cells are a recently identified subset of suppressor T cells. This study was designed to investigate their clinical relevance in patients with CRC.

Experimental Design: Sixty patients with CRC and 24 healthy donors (HD) were enrolled in this study. The percentages of LAP⁺CD4⁺ T cells in peripheral blood and tumor tissue were measured. The phenotype and functional relevance of LAP⁺CD4⁺ T cells were analyzed subsequently.

Results: The percentages of LAP⁺CD4⁺ T cells in peripheral blood of patients with CRC were significantly higher than HD (HD vs. CRC: 3.1% ± 0.78% vs. 8.8% ± 5.8%, P < 0.0001) and in tumor tissue when compared with nontumor tissue (nontumor vs. tumor: 3.2% ± 1.1% vs. 9.5% ± 5.5%, P = 0.0002). In addition, LAP⁺CD4⁺ T cells with effector memory (EM) phenotype were more likely to accumulate in the tumor sites than in peripheral blood. These LAP⁺CD4⁺ T cells produced significantly higher levels of IFN-γ, IL-17 and comparatively lower IL-2 and very few IL-10. LAP⁺CD4⁺ T cells could suppress the proliferation of LAP⁻CD4⁺ T cells that were partially mediated by TGF-β. Furthermore, these LAP⁺CD4⁺ T cells accumulated in tumor site and increased further in the peripheral blood in patients with metastasis.

Conclusions: LAP⁺CD4⁺ T cells as a suppressor subset could accumulate in the tumor microenvironment and circulated more in the peripheral blood with tumor progression in patients with CRC. Clin Cancer Res; 18(19): 1–10. ©2012 AACR.

Introduction

The process of tumor progression involves complex interactions among different cells of the immune system and among different effector molecules. Effector T lymphocytes are key players that prevent tumor development and inhibit tumor progression (1). Previous studies had shown that T cells in the tumor microenvironment are relevant to the clinical outcome in patients with malignancies, such as colorectal cancer (CRC), breast cancer, and lung cancer (2–5). For example, in patients with CRC, a high density of tumor infiltrating memory T cells was associated with a low incidence of metastasis and/or relapse and a better rate of survival (2). On the other hand, the number of peripheral regulatory T cells (Treg cells) and myeloid-derived suppressor cells also increased and migrated to the tumor microenvironment to counteract the antitumor immunity and facilitate tumor growth (6–8). Furthermore, the cytokine networks related to tumor growth are composed of complicated and counteracting interactions and fluctuate during various stages of tumor development (9). Proinflammatory cytokines, such as IL-1-β, TNF-α, IL-6, and IFN-γ can enhance chemokine secretions to attract effector cells such as neutrophils, macrophages, natural killer cells, and cytotoxic T lymphocytes to the tumor microenvironment and augment the effector functions of these immune cells (10–13). On the other hand, a number of studies have provided evidence that several pro- and anti-inflammatory cytokines promote tumor growth by upregulating the adhesion molecules and metalloproteinases that favor tumor invasion (9, 14). More complicated are the greater productions of anti-inflammatory cytokines, such as TGF-β and IL-10, either secreted from tumor cells or from immune cells in the tumor microenvironment, for establishing the tolerogenic tumor microenvironment (15).

Latency-associated peptide (LAP) is a propeptide that noncovalently binds to the amino terminus of TGF-β and...
**Translational Relevance**

The main obstacle for successful immune control in cancer is the regulatory immune response. Foxp3+ regulatory T cells (Treg cells) are well-known key players in the regulatory immune response. Several clinical trials have focused on the depletion of these suppressor cells to evoke strong antitumor immune responses. In the present study, we provide evidence that LAP+CD4+ T cells are suppressors that are increased in patients with colorectal cancer (CRC). Furthermore, these cells exhibited suppressive functions in these patients. More importantly, they accumulated in the tumor microenvironment and also correlated with the serum levels of carcinoembryonic antigens and cancer progression in patients with CRC. Therefore, these suppressor cells play a pivotal role in the regulatory immune response in CRC and could possibly be targets for the development of therapeutic strategies.

produces a latent TGF-β complex (16). Recently, it was reported that LAP+CD4+ T cells, a new subset of non-Foxp3-positive suppressor cells in the human peripheral blood, suppressed the proliferation of other T cells in vitro (17). This suppression is partly mediated by TGF-β and IL-10 (16, 17). Furthermore, LAP+CD4+ T cells were found in mice, and they could suppress allergic inflammation in vivo (18). However, the role of LAP+CD4+ T cells in cancer has not been explored before.

In the current study, we found that this LAP+CD4+ T-cell population was increased in patients with CRC. We also characterized the phenotype, cytokine profile, and suppression function of LAP+CD4+ T cells in patients with CRC. In addition, we found that the population of these cells increased more in the advanced stage of CRC, and that the cells accumulated in the tumor microenvironment. These results implied that LAP+CD4+ T cells play an important role in tumor progression in patients with CRC.

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**Materials and Methods**

**Patients and blood/tissue samples**

From May 2010 to March 2012, 60 patients with different stages of CRC, who received surgical treatment in Chang Gung Memorial Hospital, Linkou Medical Center (Kweishan, Taoyuan, Taiwan), were enrolled in this study. We also enrolled 24 healthy donors (HD) during the same period (Table 1). Serum levels of carcinoembryonic antigens (CEA) were measured by ADVIA Centre CEA Assay using chemiluminescent technology (Siemens Healthcare Diagnostics). Peripheral blood mononuclear cells (PBMC) from these patients were isolated by the Ficoll/Paque PLUS density gradient centrifugation method. All patients underwent a radical colectomy for CRC. The whole specimens contained both tumor and nontumor tissues to ensure there was a safety margin for complete removal of all tumor cells. The nontumor tissue was obtained from the resection margin of the specimen, which was proved to be free of tumors as diagnosed in the routine pathologic examination. The fresh tumor samples and nontumor tissues from the same patients were collected during surgery and later chopped into small pieces using a razor blade in RPMI 1640 medium. These tissues were then filtered through a 70-μm nylon mesh. Single cell suspension was separated with Ficoll (Pharmacia), and leukocytes were recovered from the inter phase (19).

**Ethics statements**

All patients and HDs enrolled in this study provided written informed consent. This study protocol conformed to the ethical guidelines of the Declaration of Helsinki (59th World Medical Association General Assembly, Seoul, October 2008), and was approved by the ethical committees and Institutional Review Board of Chang Gung Memorial Hospital (Kweishan, Taoyuan, Taiwan).

**Antibodies and reagents**

PE (Phycoerythrin)-conjugated anti-LAP monoclonal antibody (mAb), allophycocyanin-conjugated anti-LAP mAb, and recombinant LAP (rLAP) were obtained from R&D.

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**Table 1.** Demographic features of patients with colorectal cancer and healthy donors

<table>
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<th>Tumor stage</th>
<th>Tumor without metastasis (N = 24)</th>
<th>Tumor with metastasis (N = 36)</th>
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<tr>
<td>CEA, ng/mL (mean ± SD)</td>
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*Including lymph node and/or distant metastasis.
Not applicable.
mAbs to CD4, CD8, CD62L, CCR7, CD45RA, CD25, CCR4, CCR5, IL-2, IL-10, Ki67, and IFN-γ were obtained from BD Bioscience, and mAbs to IL-17 and Foxp3 were from eBioscience. Intracellular staining of Foxp3 and Ki67 were conducted using eBioscience fixation and permeabilization buffers. Intracellular staining for IL-2, IL-10, IL-17, and IFN-γ was conducted as follows: lymphocytes were freshly isolated and activated with PMA/ionomycin for 5 hours and Golgi stop for the last 1 hour. Cells were stained for cell surface markers and then washed, fixed, and permeabilized with cytofix/cytoperm buffer (BD Bioscience) for intracellular cytokines staining. BD FACSCalibur was used to analyze the fluorescence intensity, and FlowJo cytometry analysis software (Tree Star) was used for analysis.

**Isolation of LAP⁺ CD⁴⁺ T cells**

LAP⁺ CD⁴⁺ T cells and LAP⁺ CD⁴⁺ T cells were sorted from PBMCs by FACSAria (BD Biosciences) with purity greater than 90%.

**Quantitative real-time PCR**

Total RNA was isolated by NucleoSpin RNA XS. The RNAs were reverse transcribed into cDNA using oligo(dT) primers and reverse transcriptase (Invitrogen). The primer sequences chosen for TGF-β, IFN-γ, IL-2, IL-17, and IL-10 were based on previous reports (20–22). For amplification, SYBR Green qPCR mix was used (Invitrogen). Each reaction was run in duplicate on the master cycle machine (Bio-Rad) and was normalized to transcripts of the housekeeping gene β-actin.

**Immune suppression assay**

LAP⁺ CD⁴⁺ T cells were sorted by FACSAria (BD Biosciences) from the peripheral blood of patients with CRC. These cells were activated with anti-CD3 and anti-CD28 mAbs for 96 hours in the presence of LAP⁺ CD⁴⁺ T cells (responder) at a 1:1 ratio. The cells were pulsed with thymidine [³H] for 16 hours at the end of the incubation period.

**Statistical analysis**

The differences between groups were assessed using the Mann–Whitney U test, t test, paired t test, or one-way ANOVA. Multiple linear regression analysis was used to determine factors explaining variation in the percentage of LAP⁺ CD⁴⁺ T cells. P-values were graded as significant at P < 0.05 (*, P < 0.05; **, P < 0.01; and ***, P < 0.001). The statistical analyses were done by GraphPad Prism 5.0 software.

**Results**

The percentage of LAP⁺ CD⁴⁺ T cells increased in PBMCs and tumor tissues in patients with CRC

Previous studies have reported that LAP⁺ CD⁴⁺ T cells are a newly identified subset of suppressor T cells, few of which are found in healthy individuals (17). We proposed that this subset of T cells plays a suppressive role in patients with CRC because of their regulatory ability. As shown in Figures 1A & 1B, an increased percentage of LAP⁺ CD⁴⁺ T cells in PBMCs were found in patients with CRC compared with HDs (HDs vs. CRC: ref. 3. 1% ± 0.78% vs. 8.8% ± 5.8%, P < 0.0001). Furthermore, the percentage of LAP⁺ CD⁴⁺ T cells was also significantly higher in the tumor tissue than in nontumor tissue in the patients with CRC (nontumor vs. tumor: 3.2% ± 1.1% vs. 9.5% ± 5.5%, P = 0.0002, Fig. 1C). To further confirm the relationship between LAP expression and TGF-β expression, we sorted both LAP⁺ CD⁴⁺ T cells and LAP⁺ CD⁴⁺ T cells and examined their TGF-β expression by quantitative real-time PCR (qRT-PCR). The results showed higher levels of TGF-β expression in LAP⁺ CD⁴⁺ T cells but almost no expression in LAP⁺ CD⁴⁺ T cells in both HD and in patients with CRC (Fig. 1D). In addition, the relative levels of TGF-β gene expression in LAP⁺ CD⁴⁺ T cells were significantly higher in patients with CRC than in HDs (P = 0.0048, Fig. 1D).

LAP⁺ CD⁴⁺ T cells mainly display the effector memory phenotype in the tumor microenvironment

The developmental stage of LAP⁺ CD⁴⁺ T cells from patients with CRC was determined on the basis of the expression of CCR7 and CD45RA (23). As shown in Fig. 2A & 2B, LAP⁺ CD⁴⁺ T cells mainly displayed the central memory (CM) phenotype in the peripheral blood in both HDs and patients with CRC. However, a predominant effector memory (EM) phenotype was found in tumor tissue compared with nontumor tissue for LAP⁺ CD⁴⁺ T cells (Fig. 2B). We then comparatively analyzed the ratio of LAP⁺ CD⁴⁺ T cells to LAP⁺ CD⁴⁺ T cells (LAP⁺ /LAP⁻) in the peripheral blood and tumor tissue in patients with CRC. As shown in Fig. 2C, this ratio of LAP⁺ /LAP⁻ for the CM phenotype was significantly higher in peripheral blood (PBMCs vs. tumor tissue: 0.14 ± 0.06 vs. 0.07 ± 0.058, P < 0.05), and the ratio of LAP⁺ /LAP⁻ for the EM phenotype was significantly higher in tumor tissue (PBMCs vs. tumor tissue: 0.07 ± 0.02 vs. 0.14 ± 0.07, P < 0.05). These results indicate that the LAP⁺ CD⁴⁺ T cells with the EM phenotype were more likely to accumulate in the tumor tissue than in the peripheral blood compared with LAP⁺ CD⁴⁺ T cells.

Cytokine profiles of LAP⁺ CD⁴⁺ T cells

We next analyzed the phenotype of LAP⁺ CD⁴⁺ T cells and LAP⁺ CD⁴⁺ T cells in patients with CRC. As shown in Fig. 3A, CCR4 and CCR5 expression was higher in LAP⁺ CD⁴⁺ T cells than in LAP⁺ CD⁴⁺ T cells (CCR4: LAP⁺ CD⁴⁺ T cells vs. LAP⁺ CD⁴⁺ T cells: 44.8% ± 23.3% vs. 27.3% ± 24.6%, P < 0.005; CCR5: LAP⁺ CD⁴⁺ T cells vs. LAP⁺ CD⁴⁺ T cells: 26.7% ± 6.7% vs. 10.1% ± 3.4%, P < 0.005). We also found a significantly increased percentage of Ki67⁺ cells in LAP⁺ CD⁴⁺ T cells compared with LAP⁺ CD⁴⁺ T cells (LAP⁺ CD⁴⁺ T cells vs. LAP⁺ CD⁴⁺ T cells: 7.8% ± 2.7% vs. 3.8% ± 1.1%, P = 0.08; Fig. 3A). We next analyzed the cytokine profiles of LAP⁺ CD⁴⁺ T cells and LAP⁺ CD⁴⁺ T cells from patients with CRC using intracytoplasmic staining (Fig. 3B and Supplementary Fig. S1). As shown in Fig. 3B, these LAP⁺ CD⁴⁺ T cells produced significantly
higher levels of IL-17 and lower levels of IL-2 compared with LAP⁺ CD4⁺ T cells (IL-17: LAP⁺ CD4⁺ T cells vs. LAP⁻ CD4⁺ T cells: 19.2% ± 11.5% vs. 36.6% ± 2.3%, P < 0.0001; IL-2: LAP⁺ CD4⁺ T cells vs. LAP⁻ CD4⁺ T cells: 11.0% ± 1.9% vs. 18.2% ± 4.99%, P < 0.05). On the other hand, both groups produced significant amounts of IFN-γ but no significant difference was observed between these 2 groups (LAP⁺ CD4⁺ T cells vs. LAP⁻ CD4⁺ T cells: 35.8% ± 16.9% vs. 28.6% ± 17.8%, P = 0.08). IL-10–producing cells were comparatively fewer in both groups (LAP⁺ CD4⁺ T cells vs. LAP⁻ CD4⁺ T cells: 2.1% ± 0.8% vs. 1.9% ± 1.2%, P = 0.3904). We also used qRT-PCR to measure the cytokine profiles in these cells (Fig. 3C). It was shown that IL-17 transcripts were significantly higher and IL-2 transcripts were significantly lower in LAP⁺ CD4⁺ T cells than in LAP⁻ CD4⁺ T cells. Similarly, IFN-γ transcripts were slightly higher in LAP⁺ CD4⁺ T cells than in LAP⁻ CD4⁺ T cells, but IL-10 transcripts were very low in both cell types. Therefore, the results suggested that LAP⁺ CD4⁺ T cells not only possess the ability to produce IFN-γ but can also produce IL-17. However, the ability of these cells to produce IL-2 is relatively less.

Figure 1. Increased frequency of LAP⁺ CD4⁺ T cells in PBMCs and tumor tissue in patients with colorectal cancer (CRC). A, identification of LAP⁺ CD4⁺ T cells. PBMCs and tissue-infiltrating lymphocytes were isolated and analyzed by fluorescence-activated cell sorting (FACS). The isotype control mAbs (IC) and recombinant LAP (rLAP) were used to confirm the reliability of staining. B, PBMCs from patients with CRC and HDs were isolated and the percentages of LAP⁺ CD4⁺ T cells in total CD4⁺ T cells were calculated by FACS analysis. C, the lymphocytes from either tumor or nontumor tissues were isolated from patients with CRC, and the percentage of LAP⁺ CD4⁺ T cells in total CD4⁺ T cells was calculated by FACS analysis. CRC-TIL: tumor-infiltrating lymphocytes from tumor in patients with CRC; CRC-NIL: nontumor tissue-infiltrating lymphocytes from nontumorous sections in patients with CRC. Each dot represents an individual sample. ***, P < 0.001 for statistical analysis by t test. D, LAP⁺ CD4⁺ T cells expressed higher levels of TGF-β transcripts in PBMCs of patients with CRC than in HDs. The level of TGF-β gene expression in sorted LAP⁺ CD4⁺ T cells and LAP⁻ CD4⁺ T cells was measured by qRT-PCR. Relative expression levels were adjusted by the level of β-actin mRNA for each sample. Results were expressed as the mean values of relative levels of TGF-β gene expression ± SD from 6 independent experiments. ***, P < 0.01 for statistical analysis by Mann–Whitney U test and paired t test.

Suppressive properties of LAP⁺ CD4⁺ T cells in patients with CRC were partially mediated through TGF-β

Next, we examined the suppressive capacity of LAP⁺ CD4⁺ T cells in patients with CRC. We sorted LAP⁺ CD4⁺ T cells and evaluated their suppression abilities. As shown in Fig. 3D, LAP⁺ CD4⁺ T cells were anergic when stimulated with anti-CD3 and anti-CD28. However, when cocultured with LAP⁺ CD4⁺ T cells, the LAP⁺ CD4⁺ T cells partially suppressed the proliferation of LAP⁺ CD4⁺ T cells. In addition, this suppression could be partially reversed by anti–TGF-β mAbs (Fig. 3D).
LAP⁺CD4⁺ T cells are suppressors in colon cancer

To investigate the clinical relevance of LAP⁺CD4⁺ T cells in patients with CRC, we first analyzed the correlation between clinical parameters and the percentage of LAP⁺CD4⁺ T cells in the peripheral blood. As shown in Table 2, only the presence/absence of metastasis and the serum levels of CEA correlated with percentages of peripheral LAP⁺CD4⁺ T cells (presence/absence of metastasis: r², 0.147, P = 0.002; CEA levels: r², 0.151, P = 0.002). We then analyzed the impact of metastasis on the percentage of LAP⁺CD4⁺ T cells. As shown in Fig. 4A, the percentage of peripheral LAP⁺CD4⁺ T cells in patients with metastasis was significantly higher than in patients without metastasis or in the HDs. Interestingly, the percentage of LAP⁺CD4⁺ T cells in tumor tissues was similar in patients with or without metastasis, both of which were significantly higher than in the nontumor tissue samples (Fig. 4A). These results imply that LAP⁺CD4⁺ T cells accumulate in the tumor microenvironment and further increase in number in the peripheral blood of patients with metastasis.

Foxp3 has been shown to orchestrate the suppressor activities, especially in Treg cells (24). We therefore explored the expression of Foxp3 in LAP⁺CD4⁺ T cells. As shown in Fig. 4B and C, the expression of Foxp3 in LAP⁺CD4⁺ T cells was low but significantly higher in CRC-PBMCs than in HD-PBMCs (Foxp3⁺/LAP⁺CD4⁺ T cells in HD-PBMCs vs. CRC-PBMCs: 3.7% ± 0.7% vs. 4.3% ± 0.9%; P = 0.018). Although, LAP⁺CD4⁺ T cells could almost be deemed Foxp3⁺LAP⁺CD4⁺ T cells because of the relatively few Foxp3⁻LAP⁺CD4⁺ T cells (approximately 5%), we also analyzed the relationship between Foxp3 and LAP⁺CD4⁺ T cells and the patients’ disease status. As shown in Fig. 4D, similar to LAP⁺CD4⁺ T cells, the percentage of Foxp3⁺LAP⁺CD4⁺ T cells in the peripheral blood was significantly higher in patients with tumor metastasis than in those without metastasis or in the HDs. As for these cells in tumor tissue, the percentage of Foxp3⁻LAP⁺CD4⁺ T cells was similar in tumors with or without metastasis, both of which were significantly higher than in the nontumor tissue. Taken together, these results indicated that both LAP⁺CD4⁺ T cells and Foxp3⁻LAP⁺CD4⁺ T cells were similarly accumulated in tumor sites and correlated with the patients’ disease status.

Figure 2. Differentiation stages of LAP⁺CD4⁺ T cells and LAP⁺CD4⁺ T cells. A, dot blot figures represent the definition of naïve (N: CD45RA⁺CCR7⁻), central memory (CM: CD45RA⁻CCR7⁻), effector memory (EM: CD45RA⁻CCR7⁺), and terminal effector (TE: CD45RA⁻CCR7⁻) CD4⁺ T cells. B, differentiation stages of LAP⁺CD4⁺ T cells in PBMCs and patients with CRC, and in tumor and nontumor tissues from patients with CRC, were determined by flow cytometry. The dots represent the mean percentage ± SD. C, ratio of LAP⁺CD4⁺ T cells/LAP⁺CD4⁺ T cells (LAP⁺/LAP⁺) were calculated in each differentiation stage and compared between PBMCs and tumor-infiltrating lymphocytes of CRC patients. CRC-TIL: tumor-infiltrating lymphocytes from tumor tissue from patients with CRC; CRC-NIL: nontumor tissue-infiltrating lymphocytes from nontumorous tissue from patients with CRC. Each dot represents an individual sample. *, P < 0.05 for statistical analysis by paired t test.
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Clin Cancer Res; 18(19) October 1, 2012
Clinical Cancer Research

Discussion
It is already known that tumor-infiltrating immune cells are a mixture of heterogeneous cells with effector and regulatory phenotypes (25, 26). LAP⁺CD4⁺ T cells are a newly identified population of T cells expressing the membrane-bound TGF-β and exhibit suppressive activity in healthy human peripheral blood (17). In the present study, we observed increased numbers of LAP⁺CD4⁺ T cells in the peripheral blood in patients with CRC. In addition, LAP⁺CD4⁺ T cells still exhibit a suppressive activity in patients with CRC, and they also synthesize significant amounts of IFN-γ and IL-17. Furthermore, this subset of T cells accumulated in tumor tissue, and its percentage in the peripheral blood had further increased in patients with metastasis. Therefore, our results imply that this recently identified suppressor subset of CD4⁺ T cells play an important role in tumor progression in patients with CRC.

LAP noncovalently binds the TGF-β peptide dimer to form a small, latent TGF-β that facilitates the release and targeting of TGF-β1 to the extracellular matrix (27). In

Figure 3. Phenotype, cytokine profile, suppression ability of LAP⁺ CD4⁺ T cells, and suppressive properties were partially mediated by TGF-β. Cell suspensions were made from the blood obtained from patients with CRC, and the phenotype markers of CD62L, CCR4, CCR5, and Ki67 were examined as described in the Materials and Methods. Results represent the percentage of each population were made from the blood obtained from patients with CRC, and the phenotype markers of CD62L, CCR4, CCR5, and Ki67 were examined as described in the Materials and Methods. Results represent the percentage of each population were made from the blood obtained from patients with CRC, and the phenotype markers of CD62L, CCR4, CCR5, and Ki67 were examined as described in the Materials and Methods. The expression of TGF-β was adjusted by the level of anti-CD28 mAb. The percentage of suppression in the absence/presence of anti-TGF-β mAb. The expression levels of cytokines were compared between LAP⁻/CD4⁺ T cells and LAP⁺/CD4⁺ T cells. The statistical results were analyzed by a paired t test. These cells were also stimulated in vitro as described in the Materials and Methods, and the cytokine profiles (including IFN-γ, IL-17, IL-2, and IL-10) were analyzed. A, the dot blot represents the expression of the cytokine profile. The expression levels of cytokines were compared between LAP⁻/CD4⁺ T cells and LAP⁺/CD4⁺ T cells by a paired t test. C, gene expressions of IFN-γ, IL-17, IL-2, and IL-10 of sorted LAP⁻/CD4⁺ T cells and LAP⁺/CD4⁺ T cells were measured by qRT-PCR. Relative expression was adjusted by the level of β-actin mRNA for each sample. Results were expressed as the mean values of the relative levels of gene expression of IFN-γ, IL-17, IL-2, and IL-10 ± SD from 6 independent experiments. D, LAP⁺/CD4⁺ T cells and LAP⁻/CD4⁺ T cells in different combinations were stimulated with anti-CD3 mAb and anti-CD28 mAb for 96 hours as described in the Materials and Methods. Proliferation of T cells measured by a thymidine [³H] incorporation assay expressed as the mean ± SD, pooled from 3 independent experiments. Anti–TGF-β mAbs with a final concentration of 10 µg/mL was added to LAP⁺ CD4⁺ T cells cocultured with LAP⁺ CD4⁺ T cells in a 1:1 ratio and activated by anti-CD3 mAb and anti-CD28 mAb. The percentage of suppression in the absence/presence of anti–TGF-β mAbs was expressed as the mean ± SD, pooled from 3 independent experiments. ****, P < 0.0001; ***, P < 0.001; **, P < 0.01; and *, P < 0.05 for statistical analysis by paired t test.

Published OnlineFirst August 9, 2012; DOI: 10.1158/1078-0432.CCR-12-0211

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LAP noncovalently binds the TGF-β peptide dimer to form a small, latent TGF-β that facilitates the release and targeting of TGF-β1 to the extracellular matrix (27). In
addition, this small, latent TGF-β can be expressed on the cell membrane of many immune cells, including platelets (28), dendritic cells (6), and Treg cells (29, 30), and it actively participates in immune regulation. Recently, Gandhi and colleagues identified a population of CD4⁺ T cells with membranous, latent TGF-β (LAP⁺CD4⁺ T cells) in human peripheral blood. These LAP⁺CD4⁺ T cells are hypoproliferative and inhibit the proliferation of LAP⁻/CD4⁺ T cells in vitro (17). Therefore, these cells were thought to be suppressor cells (17). In addition, a previous study showed that LAP⁺CD4⁺ T cells are suppressor cells in both in vitro and in vivo in a murine lung model of allergic inflammation (18). In the present study, we extended these observations and found that the number of LAP⁺CD4⁺ T cells had significantly increased in patients with CRC and that it correlated with the stages of CRC.

Table 2. Correlation determinant ($r^2$) between clinical parameters and percentage of LAP⁺CD4⁺ T cells in peripheral blood in patients with colorectal cancer

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<th>P-value</th>
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*Multiple linear regression analysis.
²Including lymph node and/or distant metastasis.
³NS: not significant.

A

Figure 4. Percentage of LAP⁺CD4⁺ T cells in patients with different stages of CRC. A, the patients with CRC were divided into groups according to the absence/presence of lymph node or distant metastasis. The percentage of LAP⁺CD4⁺ T cells in total CD4⁺ T cells in PBMCs was compared among these patients with different stages of CRC and HDs. The percentage of LAP⁺CD4⁺ T cells in tissue-infiltrating lymphocytes was compared between tumor tissue and nontumor tissue obtained from these patients with different stages of CRC. Statistical analysis was determined by one-way ANOVA; *, $P < 0.05$. B, the dot blot showed the expression of Foxp3 on LAP⁺CD4⁺ T cells from HD-PBMCs and CRC–PBMCs. C, PBMCs from patients with CRC and HDs were isolated and the percentage of Foxp3⁺/LAP⁺CD4⁺ T cells was calculated after FACS analysis. Each dot represents an individual sample. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ for statistical analysis by Mann–Whitney U test. D, the percentage of LAP⁺Foxp3⁺CD4⁺ T cells in total CD4⁺ T cells in PBMCs was compared among patients with CRC with or without lymph node metastasis and HDs. The percentage of LAP⁺Foxp3⁺CD4⁺ T cells in total CD4⁺ T cells in tissue-infiltrating lymphocytes was compared between tumor and nontumor tissues obtained from these patients with different stages. Statistical analysis was determined by one-way ANOVA; *, $P < 0.05$. 

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cells remained hypoproliferative with suppressive abilities. Therefore, it is reasonable to propose that LAP+ CD4+ T cells play a role in facilitating tumor progression through their suppression ability.

Treg cells were reported to confer growth and metastatic advantages to tumors by inhibiting antitumor immunity (31). On the other hand, TGF-β, an anti-inflammatory cytokine, is one of the important cytokines secreted by Treg cells that can suppress other immune cells (32–34). Here, we investigated another kind of TGF-β–secreting cell, LAP+ CD4+ T cells. Our results showed that LAP+ CD4+ T cells from patients with CRC suppressed other T cells partially through TGF-β; this finding was similar to that of a previous report (17). However, interestingly, LAP+ CD4+ T cells also expressed substantially high levels of IFN-γ and IL-17 but a relatively low level of IL-2 and very little IL-10. It is intriguing that these suppressive cells produce high levels of IFN-γ and IL-17. IFN-γ has long been known as one of the proinflammatory cytokines. However, a paradoxical “good/bad” role for IFN-γ was recently proposed (35, 36). It was reported that IFN-γ has dual functions by either promoting Th1-driven inflammatory responses or inducing the development of Treg cells for controlling immune responses (37). Furthermore, it has been shown that Treg cells can also be expanded ex vivo by IFN-γ (36). In addition, the IFN-γ–producing Treg cells behaved similar to the other Treg cells, either in vitro or in vivo (36). Therefore, although LAP+ CD4+ T cells from patients with CRC produced IFN-γ, these cells also behaved as suppressor cells, at least in vitro and possibly in vivo. The IL-17 produced by LAP+ CD4+ T cells was also an interesting observation. It was a controversial issue that IL-17 has a dual role in tumor progression with both pro- and antitumorigenic effects, partly through angiogenesis or antiangiogenesis, or through enhancement or suppression of the antitumor immune responses (38–40). However, an interesting observation has recently been published in which high intratumor IL-17 correlated with poor prognosis in patients with CRC (3). Similar to our study, they showed that LAP+ CD4+ T cells in patients with CRC secreted IL-17 and could preferentially migrate into tumor sites. Therefore, it was suggested that IL-17–secreting LAP+ CD4+ T cells facilitate tumor progression, especially when they migrate into the tumor microenvironment.

Another interesting issue is the relationship between Foxp3 expression and LAP+ CD4+ T cells. In the present study, similar to previous reports (17, 18), almost all the LAP+ CD4+ T cells from healthy volunteers did not express Foxp3. In patients with CRC, the expression of Foxp3 was slightly increased, although these Foxp3-expressing cells were still a minor percentage of the LAP+ CD4+ T cells. At present, it is difficult to determine whether these Foxp3+ LAP+ CD4+ T cells were derived from Foxp3+ CD4+ T cells that acquired LAP expression or from LAP+ CD4+ T cells that acquired Foxp3 expression. Moreover, some complex evidence indicated that Foxp3 expression was observed in not only Treg cells but also in some effector CD4+ T cells, although the latter were transiently expressed (41). For the purpose of excluding the effect of Foxp3 in our study, we also analyzed the relationship between the percentage of Foxp3+ LAP+ CD4+ T cells and the patients’ disease status, and we obtained similar results. Therefore, the expression or absence of Foxp3 in LAP+ CD4+ T cells did not impair their impact on disease progression.

The LAP+ CD4+ T cell subset predominantly expresses both central memory (TCM) and effector memory (TEM) phenotypes in the peripheral blood of patients with CRC, whereas the TEM phenotype is primarily expressed tumors. TCM (CD45RA+ CCR7+ CD4+ T cells) are nonmemory T cells that mainly circulate in the lymphoid organs. TEM (CD45RA− CCR7− CD4+ T cells) are effector memory T cells that can migrate to inflamed tissues and exhibit an immediate effector function (42). Furthermore, it has already been shown that the proinflammatory chemokine receptors, CCR4 and CCR5, were highly expressed on T cells in the tumor microenvironment (42, 43). In the present study, we also found higher expression of CCR4 and CCR5 in LAP+ CD4+ T cells than in LAP+ CD4+ T cells. At the same time, we also found a significantly higher TEM ratio of LAP+ CD4+ T cells/LAP+ CD4+ T cells in the tumor site than in the peripheral blood. All these observations indicated that LAP+ CD4+ T cells had higher tumor tissue-homing tendency than LAP+ CD4+ T cells, and these cells therefore accumulated in the tumor microenvironment.

The clinical correlation between the percentage of LAP+ CD4+ T cells and the disease stage reinforces the role of LAP+ CD4+ T cells in cancer progression. A correlation of serum CEA levels with the percentage of LAP+ CD4+ T cells in the peripheral blood of patients with CRC is intriguing. CEA is a classic tumor marker that correlates with the disease prognosis (44). CEA is also the marker of choice for monitoring the response of metastatic disease to systemic therapy and tumor recurrence after surgical resection for CRC (45). Similarly, a significantly higher percentage of LAP+ CD4+ T cells were observed in our study in the peripheral blood of patients with metastasis than in patients without metastasis. Therefore, the increase in peripheral LAP+ CD4+ T cells paralleled tumor progression, as reflected in serum CEA levels and metastasis.

In conclusion, an expanded population of suppressor (LAP+ CD4+) T cells is found in patients with CRC. These cells could possibly accumulate in the tumor microenvironment and circulate in the peripheral blood as the tumor progresses. These suppressor cells synthesize not only TGF-β but also both IFN-γ and IL-17. Furthermore, the percentage of these cells correlated with serum levels of CEA and the presence of metastasis. These observations imply that this recently identified subset of suppressor T cells may play an important role in the progression of human CRC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Grant Support
This work was supported by NMRI, 97-2314-B-182A-027-MY3, and 100-3112-B-182-002 from the National Science Council, Taiwan (NSC Grant) and CMRPG, CMRPG392081, and CMRPG39096 from Chang Gung Memorial Hospital (CMRPG).

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Received January 22, 2012; revised July 11, 2012; accepted August 1, 2012; published OnlineFirst August 9, 2012.

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LAP⁺CD4⁺ T Cells Are Suppressors Accumulated in the Tumor Sites and Associated with the Progression of Colorectal Cancer

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Clin Cancer Res  Published OnlineFirst August 9, 2012.