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Highly prevalent colorectal cancer-infiltrating LAP$^+$ Foxp3$^-$ T cells exhibit more potent immunosuppressive activity than Foxp3$^+$ regulatory T cells

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Although elevated CD4$^+$Foxp3$^+$ regulatory T cell (Treg) frequencies within tumors are well documented, the functional and phenotypic characteristics of CD4$^+$Foxp3$^+$ and CD4$^+$Foxp3$^-$ T cell subsets from matched blood, healthy colon, and colorectal cancer require in-depth investigation. Flow cytometry revealed that the majority of intratumoral CD4$^+$Foxp3$^+$ T cells (Tregs) were Helios$^+$ and expressed higher levels of cytotoxic T-lymphocyte antigen 4 (CTLA-4) and CD39 than Tregs from colon and blood. Moreover, ~30% of intratumoral CD4$^+$Foxp3$^-$ T cells expressed markers associated with regulatory functions, including latency-associated peptide (LAP), lymphocyte activation gene-3 (LAG-3), and CD25. This unique population of cells produced interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), and was ~50-fold more suppressive than Foxp3$^+$ Tregs. Thus, intratumoral Tregs are diverse, posing multiple obstacles to immunotherapeutic intervention in colorectal malignancies.

INTRODUCTION

Colorectal cancer (CRC) is the third and second most commonly diagnosed malignancy in males and females, respectively, with 1.2 million individual diagnoses and over 600,000 deaths worldwide each year.[1] Where possible, a colectomy to remove the primary tumor is performed; however, 40–50% of these patients will relapse or die from metastatic disease, despite the use of adjuvant chemotherapy.

The adaptive immune system can be directed against neoplastic, transformed cells.[2] Although there is evidence that an increased CD3$^+$ T cell infiltrate improves prognosis in CRC,[3] the fact that tumors still progress demonstrates a failure of antitumor immune responses to control the lesion effectively. Immunologically, there are several explanations that may work singularly or in conjunction to explain this observation. The tumor microenvironment seems to suppress immune responses as the tumor progresses, potentially reflecting a functional switch in tumor-infiltrating dendritic cells toward an immunosuppressive phenotype.[4] This switch is encouraged by suppressive cytokines and growth factors produced within the tumor, including interleukin-10 (IL-10),[5] transforming growth factor-β (TGF-β),[6] vascular endothelial growth factor,[7] and the activity of indoleamine 2,3-dioxygenase.[8] Hence, antitumor T cells become less responsive in advanced tumors.[9]

In addition to these factors, an antigen-specific suppression of effector T cell responses is mediated by a population of CD4$^+$Foxp3$^+$ regulatory T cells (Tregs),[10,11] either directly or through indirect effects on dendritic cells.[12,13] These Tregs have an important role in preventing autoimmunity, but may also control immune responses in a range of tumors.[14] We have previously found that the presence of CRC is associated with an expanded and distinct population of Tregs in blood, which specifically inhibits antitumor immune responses.[15] Resection of the primary tumor led to a reduction in the magnitude of this peripheral Treg population, and preoperative suppression of tumor-specific T cell function was associated with tumor

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recurrence 1 year later.[16] However, a number of reports have demonstrated decreased Foxp3$^+$ Treg infiltrates in more advanced tumors that correlate with disease outcome.[17,18] Differences in the proportions of peripheral and intratumoral Foxp3$^+$ Tregs could account for these seemingly disparate findings, but it is also possible that Treg populations other than Foxp3$^+$ Tregs mediate suppression of antitumor responses.

To understand the role of intratumoral Tregs, we undertook a detailed phenotypic and functional analysis of all CD4$^+$ T cell subsets that infiltrate colorectal tumors. We used multiparameter flow cytometry and functional assays of distinct populations isolated by fluorescence-activated cell sorting (FACS) to compare tumor-infiltrating lymphocytes (TILs) with the corresponding CD4$^+$ T cell subsets in healthy colon and peripheral blood. A marked difference in the phenotype of Foxp3$^+$ Tregs was observed, with intratumoral Tregs expressing far greater levels of markers associated with suppression, such as CD39 and cytotoxic T-lymphocyte antigen 4 (CTLA-4). Furthermore, we identified a major regulatory TIL population of CD4$^+$LAP$^+$ T cells that coexpressed lymphocyte activation gene-3 (LAG-3) and CD25, but not Foxp3 (forkhead box P3). These cells were ~50-fold more potent at suppressing effector T cells compared with conventional CD4$^+$Foxp3$^+$ T cells, and they did so through the secretion of immunosuppressive cytokines. These data reveal the presence of a novel suppressive CD4$^+$ T cell population within colorectal tumors that is phenotypically and functionally distinct from CD4$^+$Foxp3$^+$ T cells.

RESULTS

The majority of CD4$^+$Foxp3$^+$ Tregs in colorectal tumors are thymus derived and express ICOS

The Treg transcription factor Foxp3 is readily detected by flow cytometry (**Figure 1a**). Compared with healthy age-matched controls, the overall proportion of CD4$^+$ T cells expressing Foxp3 in the peripheral blood of CRC patients ($n = 14$) was increased (patients $10.83 \pm 0.67\%$ vs. controls $7.77 \pm 0.68\%$, $P = 0.014$; **Figure 1b**). The relative proportions of Foxp3$^+$ T cells remained remarkably consistent in CRC patients between peripheral blood and unaffected background colonic tissue (blood $10.83 \pm 0.67\%$ vs. colon $10.60 \pm 0.83\%$); however, a significant increase was observed among colorectal tumor-infiltrating CD4$^+$ T cells (tumor $22.44 \pm 2.38\%$; $P = 0.0004$ vs. colon, $P = 0.0009$ vs. blood).

We reported previously that conversion of effector T cells into Tregs does not account for the increased proportion of Tregs found in a murine tumor model.[19] Helios, a member of the Ikaros family of transcription factors, has been identified as a marker of thymus-derived Tregs,[20] although several groups have recently disputed this.[21,22] Notably, we have found higher mean fluorescence intensities of Foxp3 on Helios$^+$ Tregs (**Supplementary Figure S1** online), indicative of greater suppressive potential.[23] Most Foxp3$^+$ Tregs in CRC patients were Helios$^+$, consistent with the notion that these populations predominantly comprise naturally occurring Tregs (**Figure 1c**). The proportion of intratumoral Foxp3$^+$ Tregs that expressed Helios was slightly lower compared with the corresponding peripheral blood populations, however, suggesting that the degree of conversion may be slightly increased in the tumor (tumor $57.13 \pm 2.00\%$ vs. blood $69.28 \pm 2.63\%$, $P = 0.012$). Nonetheless, this difference was small, indicating that conversion does not account for the substantial enrichment of Foxp3$^+$ Tregs observed in colorectal tumors. Based on the expression of Helios, approximately half of the Tregs in unaffected colon specimens appeared to be peripherally induced (colon $48.00 \pm 3.09\%$ vs. blood $69.28 \pm 2.63\%$, $P = 0.0004$). These findings are in line with previous reports demonstrating that the conversion of Foxp3$^-$ T cells into Foxp3$^+$ T cells physiologically expands the Treg repertoire in the gut.[24] Overall, these data are consistent with our previous findings in murine models indicating that tumor-infiltrating Tregs comprised mainly the naturally occurring Treg population.[19] In order to verify these findings, we attempted to stain samples with Neuropilin-1, another marker that was recently postulated to be selectively expressed on naturally occurring Tregs;[25] however, we failed to detect any Neuorpilin-1$^+$ CD4$^+$ T cells within the colon or colorectal tumors (**Supplementary Figure S2**).

The inducible T-cell costimulator (ICOS) molecule is expressed on activated T cells, and interactions with the ICOS ligand enhance T cell proliferation, cytokine production, and survival.[26] ICOS is also expressed abundantly on CD4$^+$ CD25^{high}Foxp3$^+$ Tregs, particularly those found in tumors.[27] We identified a similar pattern in CRC patients, whereby ICOS was expressed mainly on CD4$^+$Foxp3$^+$ T cells compared with CD4$^+$Foxp3$^-$ T cells in blood, colon, and tumor (**Figure 1d**). The low levels of ICOS expression on CD4$^+$Foxp3$^-$ cells did not differ significantly by site (blood $13.91 \pm 2.56\%$ vs. colon $16.24 \pm 4.07\%$ vs. tumor $20.61 \pm 3.49\%$, $P = 0.37$). However, in line with previous reports, CD4$^+$Foxp3$^+$ TILs expressed significantly higher levels of ICOS than peripheral blood CD4$^+$Foxp3$^+$ T cells (blood $45.46 \pm 2.58\%$ vs. colon $39.10 \pm 6.81\%$ vs. tumor $60.78 \pm 4.42\%$, $P = 0.039$). Furthermore, there was a strong correlation between Foxp3 expression and ICOS expression on CD4$^+$ TILs ($P = 0.004$, $R^2 = 0.71$; **Figure 1e**). Purified CD4$^+$ICOS$^+$ T cells were more proliferative in response to stimulation with αCD3/28 beads compared with the corresponding CD4$^+$ICOS$^-$ T cells, a finding that was paralleled by *ex vivo* Ki67 staining (**Supplementary Figure S3**). Taken together, the expression patterns of Foxp3, Helios, and ICOS delineate a population of naturally occurring, highly proliferative Tregs that infiltrate colorectal tumors.

CD4$^+$Foxp3$^-$ and CD4$^+$Foxp3$^+$ T cells derived from blood, healthy colon, and colorectal tumors are phenotypically distinct

Next, we conducted a detailed phenotypic analysis of CD4$^+$ T cells using a panel of antibodies specific for the markers CD25, CTLA-4, CD39, LAG-3, CD103, ICOS, and Ki67, many of which are associated with natural Tregs.[28] Representative flow cytometry plots are shown in **Supplementary Figure S4**. Specifically, we compared CD4$^+$Foxp3$^+$ and CD4$^+$Foxp3$^-$ T cells from

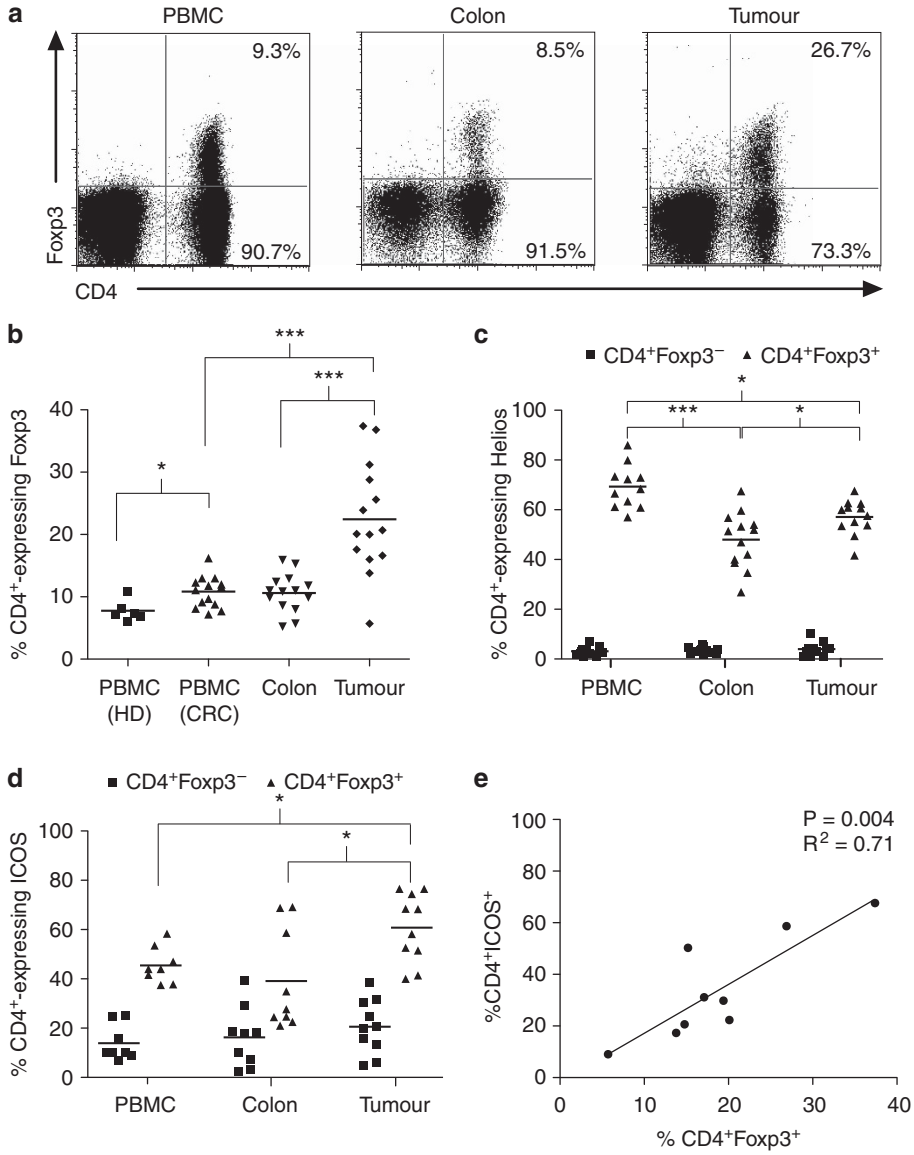

Figure 1 *Ex vivo* phenotypic analysis of regulatory $CD4^+Foxp3^+$ T cells in colorectal cancer (CRC) patients. (**a**) Representative bivariate flow cytometry plots showing Foxp3 (forkhead box P3) expression on live $CD4^+$ T cells obtained from matched peripheral blood, unaffected colon, and colorectal tumor samples. (**b**) Percentage of live $CD4^+$ T cells expressing Foxp3 in peripheral blood mononuclear cell (PBMC) samples ($n = 6$) from age-matched healthy donors (HD; mean age, 71 years), and PBMC, unaffected colon, and tumor samples from CRC patients ($n = 14$; mean age, 72 years). (**c**, **d**) Intracellular expression of the (**c**) Helios transcription factor and (**d**) cell surface expression of ICOS were assessed on $CD4^+Foxp3^+$ regulatory T cell (Tregs; ▲) and $CD4^+Foxp3^-$ T cells (■) isolated from blood (PBMC), unaffected colon, and tumor samples from CRC patients. Significant differences are indicated: $^*P < 0.05$, $^{***}P < 0.001$. (**e**) Linear regression comparing the percentage of intratumoral $CD4^+$ T cells expressing inducible T-cell costimulator (ICOS) with the percentage of intratumoral $CD4^+Foxp3^+$ Tregs.

blood, healthy colon, and tumor (**Figures 2a–f** and **Tables 1–3**). $CD4^+$ T cells derived from each compartment expressed distinct patterns of these phenotypic markers. Expression levels of several markers, such as CTLA-4 and CD39, increased steadily on both $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells when comparing blood with healthy colon to tumor (**Figures 2a–c**). Many markers associated with Tregs, such as the IL-2 receptor α-chain CD25 and CTLA-4, are also known to be present on activated T cells.[29] Accordingly, this upregulation could reflect an altered state of activation in these distinct colonic and tumoral niches. This is illustrated by CD25 expression (**Figure 2a**), which remained relatively stable on $Foxp3^+$ T cells but was significantly increased among $Foxp3^-$ T cells (blood $9.69 \pm 1.08\%$ vs. colon $28.93 \pm 3.51\%$, $P = 0.0009$; blood vs. tumor $41.23 \pm 3.05\%$, $P < 0.0001$). Similarly, a greater proportion of intratumoral $CD4^+Foxp3^-$ T cells proliferate in comparison with blood and colon, as denoted by Ki67 expression (blood $5.39 \pm 0.46\%$ vs. colon $7.37 \pm 1.43\%$, $P = 0.27$; blood vs. tumor $26.07 \pm 3.96\%$, $P = 0.0015$; colon vs. tumor, $P = 0.0039$; **Figure 2d**). Reduced levels of the IL-7 receptor CD127 and the anti-apoptotic protein BCL-2 were also observed among the $CD4^+Foxp3^-$ TILs (**Supplementary Figure S5**). CTLA-4 (**Figure 2b**), which is an important negative immunomodulator that decreases cellular cytokine production and proliferation,[30] followed a similar

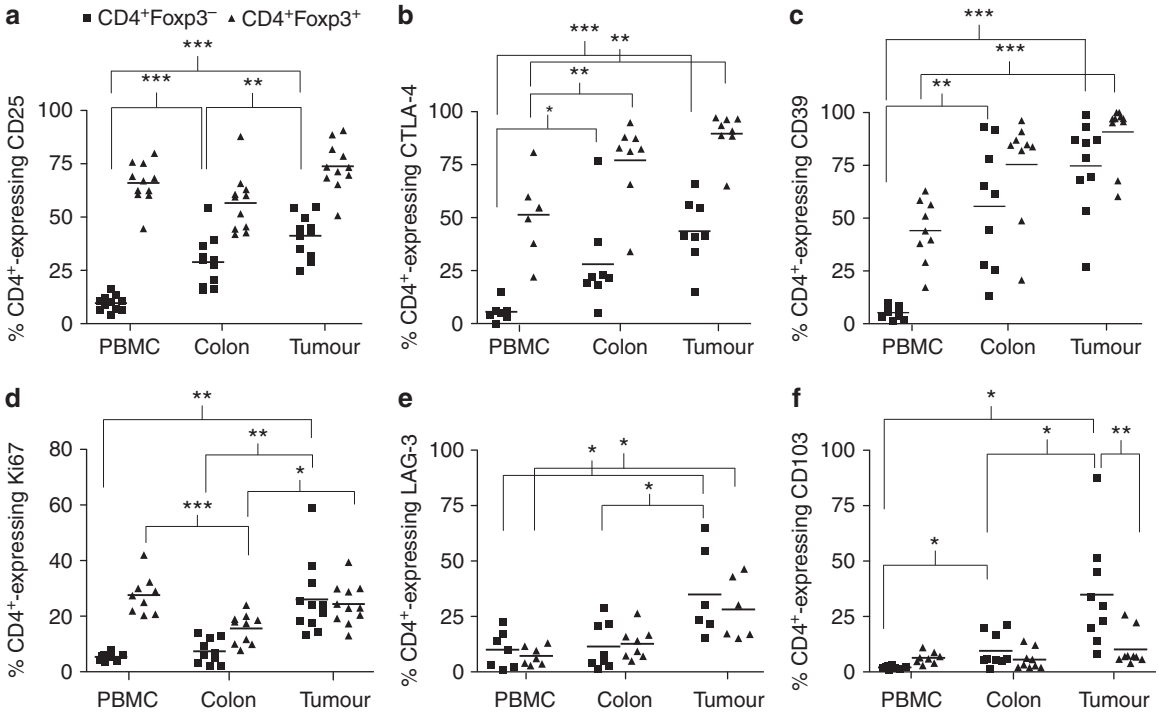

Figure 2 *Ex vivo* phenotypic analysis of CD4$^+$Foxp3$^+$ and CD4$^+$Foxp3$^-$ T cells in different compartments. Compiled flow cytometry data are shown for expression of (**a**) CD25, (**b**) cytotoxic T-lymphocyte antigen 4 (CTLA-4), (**c**) CD39, (**d**) Ki67, (**e**) LAG-3 and (**f**) CD103 among CD4$^+$Foxp3$^+$ Tregs (▲) and CD4$^+$Foxp3$^-$ T cells (■) in matched blood (peripheral blood mononuclear cells (PBMCs)), unaffected colon, and tumor samples. Foxp3, forkhead box P3. Significant differences are indicated: *$P<0.05$, **$P<0.01$, and ***$P<0.001$. Representative flow cytometry plots depicting the gating strategy are shown in **Supplementary Figure S2** online.

Table 1 Phenotypic overview of CD4$^+$Foxp3$^{+/-}$ T cells

		CTLA-4	*LAP*	*LAG-3*	*CD39*	*CD103*	*Ki67*	*CD25*	*Helios*	*ICOS*
Tumor	CD4$^+$FoxP3$^-$	+ +	+ +	+ +	+ + +	+ +	+ +	+ +	−	+
	CD4$^+$FoxP3$^+$	+ + +	+	+	+ + +	+	+ +	+ + +	+ + +	+ + +
Colon	CD4$^+$FoxP3$^-$	+ +	+	−	+ + +	+	−	+	−	+
	CD4$^+$FoxP3$^+$	+ + +	+	−	+ + +	−	+	+ +	+ +	+ +
PBMC	CD4$^+$FoxP3$^-$	−	−	−	−	−	−	−	−	+
	CD4$^+$FoxP3$^+$	+ +	−	−	+ +	−	+ +	+ +	+ + +	+ +

Abbreviations: CTLA-4, cytotoxic T-lymphocyte antigen 4; Foxp3, forkhead box P3; LAG-3, lymphocyte activation gene-3; LAP, latency-associated peptide; ICOS, inducible T-cell costimulator; PBMC, peripheral blood mononuclear cell.
−, Average expression <10% of cells.
+, Average expression 10–24.9% of cells.
+ +, Average expression 25–49.9% of cells.
+ + +, Average expression >50% of cells.

pattern of expression to CD25 (blood 5.54 ± 1.77% vs. colon 28.03 ± 7.66%, $P=0.024$; blood vs. tumor 43.61 ± 5.51%, $P=0.0004$). There was also a significant increase in CTLA-4 expression on intratumoral Foxp3$^+$ Tregs (blood 51.34 ± 6.91% vs. colon 77.08 ± 6.83%, $P=0.0052$; blood vs. tumor 89.60 ± 3.72%, $P=0.0002$).

CD39 is an ectonucleotidase that, together with CD73, drives catabolism of extracellular adenosine triphosphate (ATP), resulting in the generation of adenosine, an immunosuppressive molecule often elevated in cancer tissue.[31] Both molecules are often expressed on Foxp3$^+$ T cells, and generation of adenosine by these cells is one of the means by which intratumoral Tregs may enact their suppressive function.[31] We found that Foxp3$^+$ Tregs displayed markedly increased expression of CD39 in healthy colon and tumor (blood 44.10 ± 4.94% vs. colon 75.42 ± 8.16%, $P=0.0003$; blood vs. tumor 90.83 ± 4.53%, $P<0.0001$; **Figure 2c**). However, as with CTLA-4, a marked and significant increase in CD39 expression was also observed among intratumoral Foxp3$^-$ T cells (blood 5.24 ± 0.99% vs. colon 55.58 ± 9.85%, $P=0.0011$; blood vs. tumor 74.8 ± 6.79%, $P<0.0001$).

Table 2 Phenotypic overview of $CD4^+ICOS^{+/-}$ T cells

		Foxp3	*LAG-3*	*CD39*	*CD103*	*Ki67*	*CD25*	*Helios*	*LAP*
Tumor	$CD4^+ICOS^-$	−	++	+++	++	++	++	−	++
	$CD4^+ICOS^+$	++	+	+++	+	++	++	++	+
Colon	$CD4^+ICOS^-$	−	+	++	−	−	+	−	+
	$CD4^+ICOS^+$	++	+	+++	−	+	+	+	+
PBMC	$CD4^+ICOS^-$	−	−	−	−	−	−	−	−
	$CD4^+ICOS^+$	++	−	++	−	+	++	++	−

Abbreviations: Foxp3, forkhead box P3; LAG-3, lymphocyte activation gene-3; LAP, latency-associated peptide; ICOS, inducible T-cell costimulator; PBMC, peripheral blood mononuclear cell.
−, Average expression <10% of cells.
+, Average expression 10–24.9% of cells.
++, Average expression 25–49.9% of cells.
+++, Average expression >50% of cells.

Table 3 Phenotypic overview of $CD4^+LAP^{+/-}$ T cells

	CTLA-4	*Foxp3*	*LAG-3*	*CD39*	*CD103*	*Ki67*	*CD25*	*ICOS*
Tumor								
$CD4^+LAP^-$	+++	++	−	+++	+	++	−	+
$CD4^+LAP^+$	+++	+	+++	+++	++	++	+++	+
Colon								
$CD4^+LAP^-$	++	+	−	++	−	+	−	−
$CD4^+LAP^+$	++	+	−	+++	−	+	+++	+
PBMC								
$CD4^+LAP^-$	+	−	−	−	−	−	−	+
$CD4^+LAP^+$	+	−	−	−	−	+	+++	++

Abbreviations: CTLA-4, cytotoxic T-lymphocyte antigen 4; Foxp3, forkhead box P3; LAG-3, lymphocyte activation gene-3; LAP, latency-associated peptide; ICOS, inducible T-cell costimulator; PBMC, peripheral blood mononuclear cell.
−, Average expression <10% of cells.
+, Average expression 10–24.9% of cells.
++, Average expression 25–49.9% of cells.
+++, Average expression >50% of cells.

Both $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ tumor-infiltrating T cells express the phenotypic markers LAG-3 and CD103 associated with regulatory cells

The marked increase in CD39, together with CD25 and CTLA-4, in the $Foxp3^-$ population suggests a possible regulatory role for these cells within the tumor environment. Accordingly, we examined other markers associated with regulatory function, including LAG-3, described recently as selectively denoting human Tr1-type Tregs.[32–34] Although expression of LAG-3 among $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells was comparable (**Figure 2e**), intratumoral levels were markedly increased on both $CD4^+Foxp3^-$ TILs (blood $10.0 \pm 3.1\%$ and colon $11.44 \pm 3.74\%$ vs. tumor $34.92 \pm 8.18\%$, $P = 0.023$) and $CD4^+Foxp3^+$ Tregs (blood $7.24 \pm 1.56\%$ and colon $12.69 \pm 2.49\%$ vs. tumor $28.18 \pm 5.68\%$, $P = 0.02$). Almost all $CD4^+LAG\text{-}3^+$ T cells were $CD25^+$ (**Figure 3c**).

The αE integrin, CD103, binds to the receptor E-cadherin and is thought to play a role in T cell retention within the gut;[35] it has also been shown to denote populations of highly suppressive $CD25^+$ and $CD25^-$ T cells.[36] In the cohort of patients studied here, CD103 expression was elevated in colorectal tumors, particularly within the $CD4^+Foxp3^-$ population (blood $2.08 \pm 0.22\%$ vs. colon $9.54 \pm 2.49\%$, $P = 0.022$; blood and colon vs. tumor $34.83 \pm 8.06\%$, $P = 0.01$; **Figure 2f**). Recent reports claim that CD103 expression is significantly increased on CRC-resident Tregs compared with unaffected colon;[37] however, despite a trend, we did not find this to be the case (colon $5.56 \pm 1.50\%$ vs. tumor $10.19 \pm 2.66\%$, $P = 0.13$).

In summary, we observed that a significant proportion of intratumoral $CD4^+Foxp3^-$ cells demonstrated a unique phenotype characterized by high expression levels of molecules predominantly associated with Tregs, including CD25, CD39, CTLA-4, LAG-3, CD39, and CD103 (summarized in **Figure 4** and **Tables 1–3**). This finding led us to investigate the function of these $CD4^+Foxp3^-$ cells and, specifically, to determine whether they could contribute to intratumoral immunosuppression.

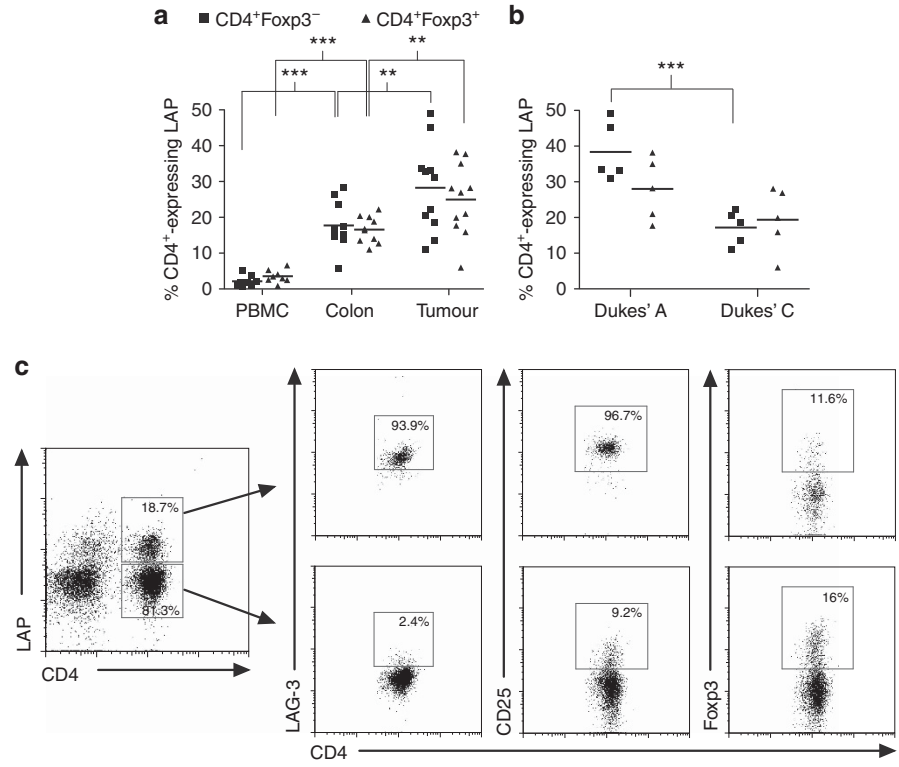

Figure 3 CD4$^+$ T cells expressing latency-associated peptide (LAP) and lymphocyte activation gene-3 (LAG-3), but not forkhead box P3 (Foxp3), are enriched in colorectal tumors. (**a**, **b**) Expression of LAP on CD4$^+$Foxp3$^+$ Tregs (▲) and CD4$^+$Foxp3$^-$ T cells (■) from (**a**) matched blood, unaffected colon, and tumor samples, with the latter stratified as (**b**) Dukes' grade A (early) and Dukes' grade C (advanced) tumors. Significant differences are indicated: $^{**}P<0.01$ and $^{***}P<0.001$. (**c**) Representative phenotypic analysis of CD4$^+$LAP$^+$ and CD4$^+$LAP$^-$ tumor-infiltrating lymphocytes (TILs), showing expression profiles for LAG-3, CD25, and Foxp3.

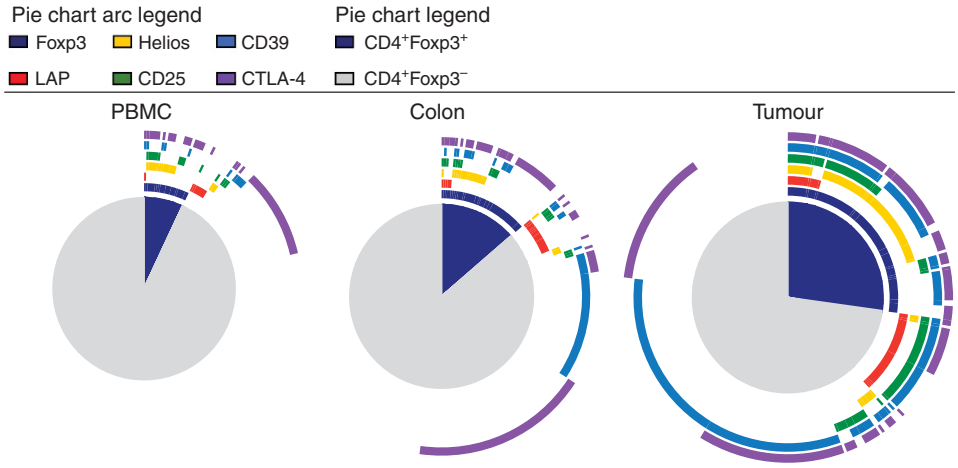

Figure 4 Concatenated *ex vivo* phenotypic analysis of CD4$^+$Foxp3$^+$ and CD4$^+$Foxp3$^-$ cells in different compartments. Pie charts show the relative proportion of Foxp3$^+$ (blue) and Foxp3$^-$ (gray) CD4$^+$ T cells in each compartment; pie chart arcs show expression of the indicated markers in these subsets. CTLA-4, cytotoxic T-lymphocyte antigen 4; Foxp3, forkhead box P3; LAP, latency-associated peptide; PBMC, peripheral blood mononuclear cell.

CD4$^+$LAP$^+$ TILs act as a major regulatory T-cell subset via the production of IL-10 and TGF-β

The latency-associated peptide (LAP) has recently been described as a marker of Tregs in human peripheral blood that are distinct from conventional Foxp3$^+$ Tregs.[38] We examined LAP expression on CD4$^+$Foxp3$^+$ and CD4$^+$Foxp3$^-$ T cells from blood, healthy colon, and tumor samples. Although comparable levels were observed in peripheral blood, LAP expression was markedly increased on both Foxp3$^+$ T cells (blood 3.56 ± 0.63% vs. colon 16.59 ± 1.18%, $P<0.0001$; blood vs. tumor 24.96 ± 3.01%, $P=0.0007$) and Foxp3$^-$ T cells (blood 2.15 ± 0.56% vs. colon 17.72 ± 2.13%, $P=0.0001$; blood

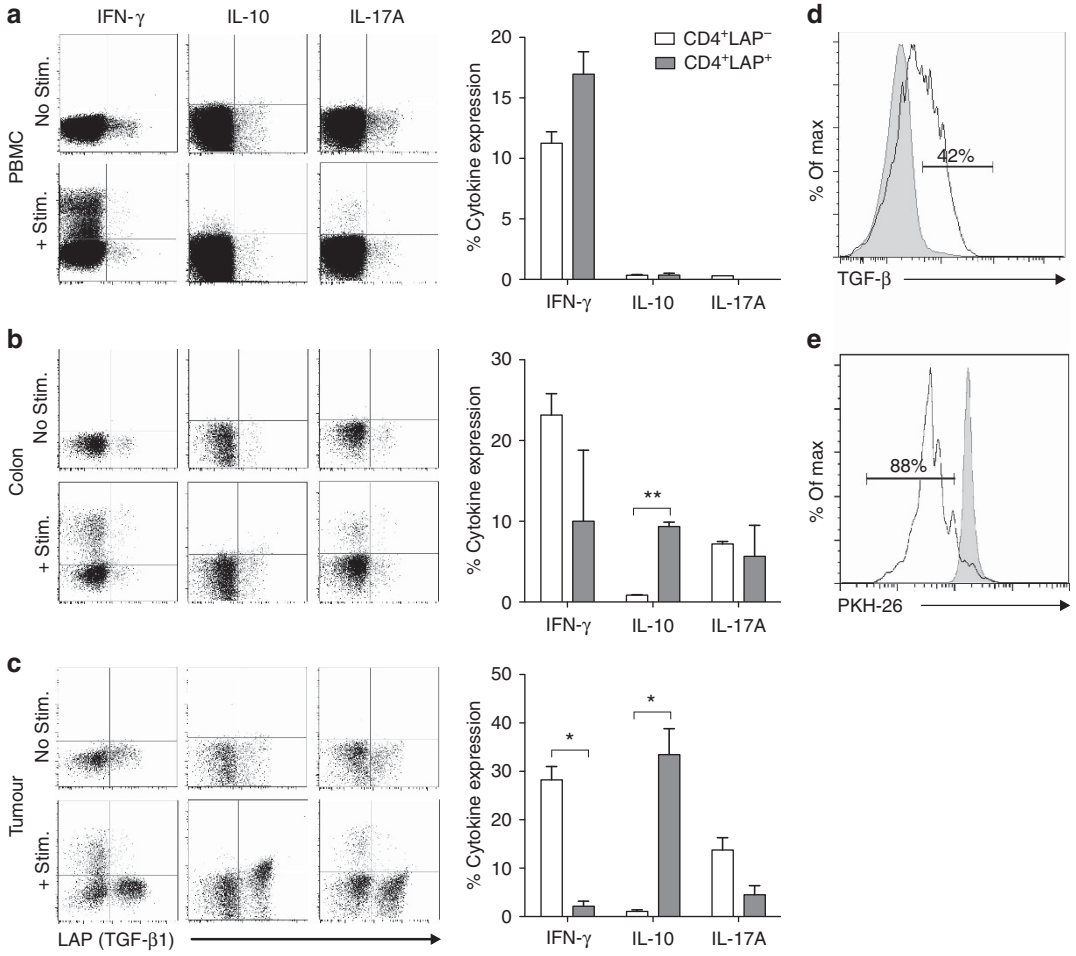

Figure 5 $CD4^+LAP^+$ tumor-infiltrating lymphocytes (TILs) produce immunosuppressive cytokines. Intracellular cytokine staining of $CD4^+LAP^{+/-}$ T cells, with and without phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation, from three matched (**a**) peripheral blood mononuclear cell (PBMC), (**b**) unaffected colon, and (**c**) tumor samples (2 Dukes' B, 1 Dukes' C). Representative fluorescence-activated cell sorting (FACS) plots for interleukin (IL)-17A, interferon-γ (IFN-γ), and IL-10 are shown and significant differences are indicated: $^*P<0.05$ and $^{**}P<0.01$. (**d**) Representative staining for active transforming growth factor-β (TGF-β) expression on unstimulated $CD4^+LAP^+$ TILs (black line) and $CD4^+LAP^-$ TILs (gray shading) isolated from a Dukes' C tumor. (**e**) $CD4^+LAP^+$ TILs (black line), isolated from a Dukes' C tumor, were stained with PKH-26 and stimulated with αCD3/28 beads; proliferation *in vitro* was measured over 72 h. Gray shading indicates the PKH-26-positive control (unstimulated effector T cells). LAP, latency-associated peptide.

vs. tumor $28.24 \pm 3.69\%$, $P = 0.0007$) in healthy colon and tumor samples (**Figure 3a** and **Supplementary Figure S6A**). This marked enrichment of $CD4^+LAP^+$ T cells was most striking in early-stage Dukes' A tumors (**Figure 3b**), although a trend for more $CD4^+Foxp3^-LAP^+$ T cells present in the periphery of patients with more advanced tumors was also noted (**Supplementary Figure S7**), as previously described.[39] The majority of $CD4^+LAP^+$ TILs coexpressed LAG-3, CD25 (**Figure 3c** and **Table 3**), and PD-1 (programmed cell death protein 1; data not shown); in contrast, $<10\%$ of the corresponding $CD4^+LAP^-$ cells expressed LAG-3 and CD25. Of note, however, most $CD4^+LAP^+$ TILs ($> 85\%$) did not express Foxp3. Collectively, these observations demonstrate that the $CD4^+LAP^+$ T cell subset is largely distinct from conventional $Foxp3^+$ Tregs and significantly enriched in tumors compared with blood (**Figure 4**).

Next, we stimulated TILs polyclonally with phorbol 12-myristate 13-acetate and ionomycin. $CD4^+LAP^+$ T cells isolated from peripheral blood produced no IL-10 or IL-17A (**Figure 5a**), whereas in the colon, the comparable cell subset produced significantly more IL-10 than $CD4^+LAP^-$ T cells (**Figure 5b**). The largest amounts of IL-10 were produced by $CD4^+LAP^+$ T cells isolated from the tumor, whereas minimal IL-17A and no interferon-γ induction was observed among these cells (**Figure 5c**). $CD4^+LAP^+$ T cells also stained for membrane-bound active TGF-β (**Figure 5d**), indicating that LAP expression denotes populations of cells that produce TGF-β, consistent with previous reports.[40] Together, these data support the premise that LAP expression denotes a population of intratumoral $CD4^+Foxp3^-$ cells with considerable immunosuppressive potential.

To assess the functional significance of LAP expression, $CD4^+LAP^+$ and $CD4^+LAP^-$ T cells were purified from

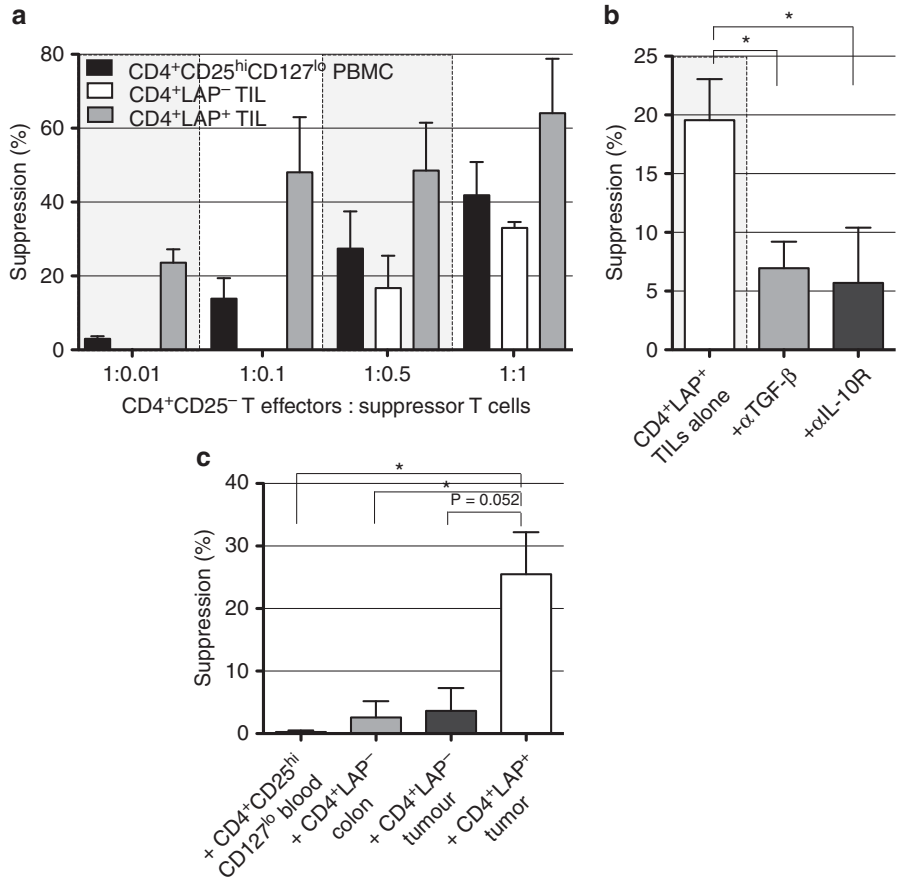

Figure 6 $CD4^+LAP^+$ tumor-infiltrating lymphocytes (TILs) potently suppress the proliferation of $CD4^+CD25^-$ T effector cells. (**a**) $CD4^+CD25^{hi}CD127^{lo}$ T cells were fluorescence-activated cell sorting (FACS) purified from peripheral blood mononuclear cells (PBMCs), together with $CD4^+LAP^+$ and $CD4^+LAP^-$ T cells from the colorectal tumor of the same patient (see **Supplementary Figure S4** online), and coincubated with autologous $CD4^+CD25^-$ T effector cells at the indicated ratios ($1 = 4 \times 10^4$ cells). Suppression indicates the percent reduction in proliferation of activated autologous effector T cells over a period of 72 h. Data are inclusive of four independent experiments (2 Dukes' B, 2 Dukes' C tumors). (**b**) Addition of an anti-transforming growth factor-β (αTGF-β) blocking antibody or an anti-interleukin-10R (αIL-10R) blocking antibody partially restored proliferation of effector T cells cultured in an E:T ratio of 25:1. Data are inclusive of three independent experiments (1 Dukes' A, 1 Dukes' B, 1 Dukes' C tumor). (**c**) A transwell assay was established, whereby carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled $CD4^+CD25^-$ effector T cells were stimulated with anti-CD3/CD28 beads, and the indicated cell subset isolated from blood, colon, or tumor was added to the top insert of the transwell. Cells were left for 72 h before analyzing for CFSE dilution among the stimulated effector T cells. Results indicate the reduction in the proportion of cells that have undergone proliferation in comparison with control wells containing stimulated effectors alone (% suppression). Data are inclusive of two independent experiments (1 Dukes' A, 1 Dukes' B tumor). Significant differences are indicated: $^*P < 0.05$. LAP, latency-associated peptide.

colorectal tumor samples by FACS (**Supplementary Figure S6C**). Stimulation of the $CD4^+LAP^+$ population *in vitro* with αCD3/CD28 beads revealed that nearly 90% of these cells were capable of rapid cell division (**Figure 5e**); this stands in marked contrast to the anergic phenotype described for conventional Tregs *in vitro*,[41] despite the high degree of turnover seen *in vivo*.[42] The ability of $CD4^+LAP^+$ and $CD4^+LAP^-$ T cells to inhibit effector $CD4^+$ T cell proliferation *in vitro* was assessed using standard suppressor assays and compared with conventional Tregs, which were sorted as $CD4^+CD25^{hi}CD127^{lo}$ T cells (Foxp3 expression >90%; **Supplementary Figure S6B**). Autologous peripheral blood mononuclear cell (PBMC)-derived $CD4^+CD25^-$ T cells (Foxp3 expression <5%) labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) were used as targets in these suppression assays, and co-cultured with isolated TIL subsets at ratios of 1:1, 1:0.5, 1:0.1, and 1:0.01 (**Figure 6a**). A direct comparison of $CD4^+LAP^+$ T cells, $CD4^+LAP^-$ T cells, and conventional Tregs demonstrated that LAP^+ TILs were able to suppress target cell proliferation ~50-fold more potently than either of the other two subsets. Interestingly, the $CD4^+LAP^-$ population could still suppress at higher ratios, most likely because of the presence of $Foxp3^+$ Tregs. The suppressive effects of $CD4^+LAP^+$ TILs were mediated by both TGF-β and IL-10, as addition of antibodies to block either cytokine partially restored effector T-cell proliferation levels ($CD4^+LAP^+$ TILs alone 19.6 ± 3.5%, + anti-TGF-β 6.9 ± 2.3%, $P = 0.040$; + anti-IL-10R 5.7 ± 4.7%, $P = 0.041$; **Figure 6b**). Even when $CD4^+LAP^+$ TILs were separated from CFSE-labeled responder T cells by 0.4 μm transwell inserts, significant levels of suppression were still observed ($CD4^+LAP^+$ TILs 25.5 ± 6.7% vs. $CD4^+LAP^-$ TILs 3.7 ± 3.7%, $P = 0.052$; vs. $CD4^+LAP^-$ Colon 2.6 ± 2.6%, $P = 0.043$; vs. $CD4^+CD25^{hi}CD127^{lo}$ blood 0.3 ± 0.3%, $P = 0.032$; **Figure 6c**).

This finding is consistent with a role for immunosuppressive cytokines in mediating the inhibitory effects of $CD4^+LAP^+$ T cells. Thus, tumor-derived $CD4^+$ T cells expressing LAP are highly immunosuppressive and represent a major regulatory T cell population among TILs.

DISCUSSION

Adaptive immune responses can control tumors, as illustrated by successful vaccination and adoptive immunotherapy studies.[43] However, many immunotherapy trials have shown poor response rates, potentially because of the inhibition of tumor-specific responses by immune regulatory networks. A large body of data now associate increased frequencies of $CD4^+Foxp3^+$ T cells (Tregs) with a range of tumors.[14] Furthermore, Treg accumulation is linked with poor outcome in several malignancies, including ovarian cancer[44] and CRC.[16] Previously, we showed that antitumor $CD4^+$ T cell responses were reduced in patients with CRC before resection of the tumor; this phenomenon was related to Treg frequencies in peripheral blood, defined initially by high levels of CD25 expression on $CD4^+$ T cells,[15] and more recently by expression of the transcription factor Foxp3.[16]

Despite substantial evidence indicating that regulatory T cells impinge upon antitumor immune responses in patients with cancer,[10] many studies in humans have focused largely on blood-derived Tregs. This raises the question of whether intratumoral Tregs carry out the same function as those in blood. Indeed, the role of colorectal tumor-infiltrating Tregs in particular has been widely debated.[45] Aberrant intratumoral expression of Foxp3, which may not denote a homogenous population of suppressive T cells, has been mooted as a reason why Treg numbers seem to be high in early tumors with a better prognosis.[29,46,47] This apparent contradiction may be resolved by ongoing clinical trials that deplete $Foxp3^+$ Tregs in CRC patients as a potential adjuvant to antitumor T cell stimulation. Nonetheless, detailed insights into the complexity and function of colorectal tumor-infiltrating T cell subsets are essential to guide the rational manipulation of T cell responses for the purpose of optimizing antitumor immunity.

Access to matched blood, healthy colon, and colorectal tumor samples from multiple CRC patients enabled us to conduct a detailed phenotypic comparison of $CD4^+$ T cells derived from these different compartments. The relative proportion of $CD4^+$ T cells that expressed Foxp3, conventionally classified as Tregs, was significantly higher within tumors compared with healthy colon. Expression of Helios was confined to this $Foxp3^+$ population, which was also highly enriched for ICOS-expressing cells. However, an extensive comparison of $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ populations revealed that the expression of other markers classically associated with Tregs in peripheral blood, including CD25, CTLA-4, CD39, and CD127, was less specific for intratumoral $Foxp3^+$ cells. These molecules are upregulated on effector memory T cells during antigen-driven activation. Strikingly, however, these intratumoral $CD4^+Foxp3^-$ cells did not express the activation marker ICOS, thereby suggesting that their unique phenotypic profile was not solely the result of T cell activation.

Expression of the ectonucleotidase CD39 suggested that intratumoral $CD4^+Foxp3^-$ T cells might exert a regulatory role. This was further borne out by analyses of LAP and LAG-3 expression. Suppressive T cells that express LAP but not Foxp3 have previously been identified in humans.[38] In addition, T cell expression of the CD4 homolog and negative regulator, LAG-3, has been shown to maintain tolerance to self and tumor antigens by directly regulating $CD8^+$ T cell effector function;[48,49] synergy between LAG-3 with PD-1 on TILs has also been shown to promote tumoral immune escape in a number of tumor models.[33] We detected a large proportion of $CD4^+$ T cells that expressed LAP, part of the membrane-bound latent TGF-β complex; increased proportions of $CD4^+LAP^+$ T cells among peripheral blood and tumor-infiltrating lymphocytes have recently been described, but the functional analysis of these cells was limited to blood-derived samples.[39] In agreement with this work, we found LAP was only expressed on very small populations of T cells in the peripheral blood, although this did increase with advancing tumor stage. T cell expression of LAP was strongly associated with the tumor microenvironment, particularly among early-stage Dukes' A tumors. In the case of these tumors, LAP^+Foxp3^- T cells outnumbered both $Foxp3^+Helios^+$ and $Foxp3^+Helios^-$ cells, implying that at this stage in cancer progression, LAP^+Foxp3^- T cells are more prevalent than both naturally occurring and induced $Foxp3^+$ Tregs. These $CD4^+LAP^+$ cells coexpressed LAG-3 in the tumor, but only a minority expressed Foxp3, consistent with descriptions of $LAG\text{-}3^+$ T cells that infiltrate Hodgkin's lymphomas and bear suppressive activity without Foxp3 expression.[50]

Overall, the data presented here reveal the presence of a potent suppressive $CD4^+Foxp3^-$ T-cell population within the colorectal tumor regulatory landscape. These $CD4^+LAP^+$ T cells, which coexpress CTLA-4, LAG-3, PD-1, CD39, CD25, CD103, and Ki67, appear to be highly activated, divide easily both *in vivo* and *in vitro*, and are distinct from the regulatory populations found in healthy colon and peripheral blood. A recent paper by Donkor *et al.*[51] demonstrated that tumor growth and metastasis could be blocked by increased cytotoxic T cell activity, but that the failure of immune protection involved TGF-β-mediated suppression of tumor antigen-specific T-cell responses. Most compellingly, the major source of TGF-β was the T cells themselves, rather than either the tumor cells or $CD4^+Foxp3^+$ Tregs. We have identified a major regulatory subset of TILs that does not require Foxp3 for suppression, but instead expresses TGF-β and IL-10, the two key regulatory cytokines. Functional analyses revealed that both these cytokines play a substantial role in the immunosuppressive capabilities of tumor-derived $CD4^+LAP^+$ T cells, exerting suppressive activities *in vitro* that were at least 50-fold more potent than "conventional" $CD4^+CD25^{hi}CD127^{lo}$ Tregs. Although preventing cell contact between peripheral blood-derived $Foxp3^+$ Tregs and responder T cells abrogates their suppressive function (**Figure 6c** and as previously

Table 4 Characteristics of the colorectal cancer patients included in this study

	Male	*Female*
n	26	14
Age (range)	69 (38–86)	77 (49–88)
Tumor location (%)		
Ascending	2 (8)	7 (50)
Transverse	0 (0)	1 (7)
Descending	1 (4)	1 (7)
Sigmoid	8 (31)	4 (29)
Rectum	15 (58)	1 (7)
TNM stage, 5th edition (%)		
T1	3 (12)	2 (14)
T2	8 (31)	3 (21)
T3	13 (50)	7 (50)
T4	2 (8)	2 (14)
(Lymph node spread)		
N0	18 (69)	8 (57)
N1	6 (23)	2 (14)
N2	2 (8)	4 (29)
Dukes' stage (%)		
A	10 (38)	3 (21)
B	8 (31)	5 (36)
C1	8 (31)	3 (21)
C2	0 (0)	3 (21)
D	0 (0)	0 (0)

TNM, tumor node metastasis.

described[52]), no such effect was observed with $CD4^+LAP^+$ TILs; hence, it is the production and release of inhibitory cytokines that mediates suppression by this T cell subset rather than cell contact-dependent mechanisms. In addition, the lack of cell death found upon stimulation of CFSE-labeled effector T cells over the course of a 72-h *in vitro* suppression assay argues against cytotoxicity as a major mechanism of suppression by $CD4^+LAP^+$ TILs (data not shown). Tumor-infiltrating $CD4^+LAP^+$ T cells therefore represent a subset functionally distinct from $Foxp3^+$ Tregs derived from peripheral blood.[39]

The limited numbers of $CD4^+LAP^+$ T cells isolated from small colorectal cancer specimens prohibited an investigation of the effects of this suppressive T cell subset on $CD8^+$ CTL. However, as both IL-10 and TGF-β have been shown to limit the cytotoxic activity of $CD8^+$ CTL, it is likely that $CD4^+LAP^+$ T cells also suppress this important antitumor effector function. Overall, the data presented in this study indicate that the $CD4^+LAP^+$ T-cell population likely controls antitumor immune responses in the local environment, even in the face of systemic antitumor responses present during the early stages of colorectal tumor development. Thus, therapeutic strategies that aim to overcome Treg activity as a means of enhancing antitumor immune responses need to take into account this novel intratumoral subset of highly suppressive $CD4^+Foxp3^-$ T cells.

METHODS

Sample groups. Peripheral blood, colorectal tumor, and paired background (unaffected) colon specimens were obtained from 40 patients undergoing primary tumor resection for colorectal adenocarcinoma at the University Hospital of Wales, Cardiff. Patient characteristics are shown in **Table 4**. Blood samples were collected no more than 7 days before surgery. Autologous colon samples were cut from a macroscopically normal section of the excised tissue, at least 10 cm from the tumor. All fresh tumor samples were derived from the luminal aspect of the specimen, so as not to interfere with the deep part of the tumor required for histopathological staging. Peripheral blood samples from age-matched non-tumor-bearing donors were used as controls. Informed consent was obtained from all participants. The Bro Taf Local Research Ethics Committee granted ethical approval for this study.

Lymphocyte extraction. PBMCs were isolated by centrifugation over Lymphoprep (Axis-Shield, Dundee, Scotland, UK). Background colon and tumor specimens were transported and washed in extraction medium comprising Iscove's modified Dulbecco's medium supplemented with penicillin, streptomycin, and L-glutamine (Gibco, Paisley, UK), 2% human AB serum (Welsh Blood Service, Pontyclun, UK), $20\,\mu g\,ml^{-1}$ gentamicin (Invitrogen, Paisley, UK), and $2\,\mu g\,ml^{-1}$ fungizone (Invitrogen). Within 30 min of resection from a patient, samples were minced with blades in a Petri dish and forced through 70 μm cell strainers to collect a single-cell suspension. In no instances were collagenase or DNase treatments used. Cells were centrifuged twice in extraction medium, then lymphocytes were isolated over a discontinuous Ficoll gradient as described previously.[53] The lymphocyte layer was aspirated and washed a further two times before use.

Flow cytometry. Clusters of antibodies were split into four panels for staining, each comprising a core panel of CD4, CD25, Foxp3, and a viability dye alongside four other monoclonal antibodies (mAbs). This approach allowed for coexpression analysis of a large range of phenotypic markers using a FACSCanto II flow cytometer (BD Biosciences, Oxford, UK). Samples were resuspended in phosphate-buffered saline at a concentration of $2–5 \times 10^6$ cells per ml in 96-well plates (Nunc, Dorset, UK). Cells were initially stained with the amine-reactive viability dye Live/Dead fixable Aqua (Invitrogen) for 15 min in the dark at room temperature. Subsequently, cells were washed twice in FACS buffer (phosphate-buffered saline and 2% bovine serum albumin), and then resuspended in 30 μl FACS buffer for surface marker staining. The directly conjugated mAbs listed in **Supplementary Table S1** were applied in various combinations and allowed to incubate for 20 min in the dark at 4 °C. Following two more wash steps with FACS buffer, cells were permeabilized and fixed using a Fixation/Permeabilization kit (eBioscience, Hatfield, UK), and incubated for 40 min at 4 °C. Cells were then washed using 1 × Permeabilization buffer, and Fc receptors were blocked using rat serum for 15 min at 4 °C. Directly conjugated mAbs specific for intracellular markers (**Supplementary Table S1** online) were then added in various combinations and allowed to incubate for 30 min in the dark at 4 °C. The cells were then washed once with Permeabilization buffer and fixed in phosphate-buffered saline containing 1% paraformaldehyde (Sigma-Aldrich, Dorset, UK). Fixed cells were stored in the dark at 4 °C until acquisition. Data were analyzed using FlowJo software version 9.4 (TreeStar, Ashland, OR) and gates were drawn based on FMO (fluorescence minus one) controls.

In some instances, cells were additionally stimulated with 20 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich) and $1\,\mu g\,ml^{-1}$ ionomycin (Sigma-Aldrich) for 4 h at 37 °C. After 1 h, $1\,\mu l\,ml^{-1}$ GolgiStop (containing monensin; BD Biosciences) was added. Intracellular cytokine staining was conducted using directly conjugated mAbs specific for IL-10, IL-17 A, interferon-γ, and TGF-β (**Supplementary Table S1**).

Cell sorting. For functional studies, PBMCs were sorted into effector ($CD4^+CD25^-$) and regulatory ($CD4^+CD25^{hi}CD127^{lo}$) T-cell

subsets using the mAbs listed above. Various $CD4^+$ T-cell subsets were sorted from colon and tumor specimens using a modified FACSAria II flow cytometer (BD Biosciences). The gating strategy is detailed in **Supplementary Figures S5B and S5C**. Lymphocyte purity in post-sort analyses was always >95%.

^{3}H-thymidine incorporation assays. T cells were plated in triplicate on a 96-well plate (Nunc) and cultured in OpTmizer CTS medium (Invitrogen) supplemented with penicillin, streptomycin, and L-glutamine for 3 days at 37 °C in a 5% CO_2 atmosphere. Stimulation was conducted using Dynabeads Human T-Activator CD3/CD28 (Invitrogen) in a bead to effector cell ratio of 1:2. Irradiated autologous PBMCs were added as necessary to ensure that overall cell numbers in each assay were consistent. Cells were then pulsed with ^{3}H-thymidine (1 μCI per well; GE Healthcare, Little Chalfont, UK) for 6 h and harvested onto filter mats using a TomTec (Hamden, CT) Cell Harvester. Proliferation was measured as a function of tritiated hydrogen incorporation.

CFSE/PKH-26-based suppression assays. The *in vitro* function of actively proliferating FACS-purified T-cell subsets was analyzed by labeling autologous effector $CD4^+CD25^-CD127^{hi}$ T cells with 0.5 μM CFSE (Invitrogen) and potential regulatory subsets with 2 μM PKH-26 (Sigma-Aldrich). To assess the impact of isolated TIL subsets on effector T-cell proliferation, cells were coincubated in 96-well plates at various ratios and stimulated with Dynabeads Human T-Activator CD3/CD28 (Invitrogen) in a bead to effector cell ratio of 1:2. In some instances, blocking antibodies to TGF-β (1D11, final concentration 10 μg ml^{-1}) or IL-10R (polyclonal; R&D Systems, Abingdon, UK, final concentration 20 μg ml^{-1}) were added directly to the cell cultures. Transwell experiments were also performed using 0.4 μm pore-sized inserts in a 96-well permeable support system (Corning, Lowell, MA) to separate isolated T-cell subsets from CFSE-labeled responder cells. After 3 days, cells were harvested and acquired using a FACSCanto II flow cytometer (BD Biosciences). Proliferation of T-cell subsets was analyzed by conventional gating and the division index was calculated using the proliferation platform in FlowJo software version 9.4 (TreeStar) as described previously.[54]

Statistical and graphical analysis. GraphPad Prism Version 5 was used for all statistical analyses. All results are expressed as mean values together with the s.e.m. where appropriate. Paired *t*-tests were used to compare data obtained from matched blood, colon, and tumor tissue from the same patient. Unpaired *t*-tests were used for all other comparisons.

Analysis and presentation of T-cell subset distributions were performed using SPICE version 5.1, downloaded from http://exon.niaid.nih.gov.[55]

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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AUTHOR CONTRIBUTIONS

Research design: M.S., A. Gallimore, and A. Godkin; performed experimental work: M.S., K.L., M.B., K.S., and H.B.; provision of samples: A.C., TH, M.D., S.P., and R.H.; wrote paper: M.S., A. Gallimore, A. Godkin, and D.P.

DISCLOSURE

The authors declare no conflict of interest.

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