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ORIGINAL RESEARCH

Regulatory T cells with multiple suppressive and potentially pro-tumor activities accumulate in human colorectal cancer

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ABSTRACT

Tregs can contribute to tumor progression by suppressing antitumor immunity. Exceptionally, in human colorectal cancer (CRC), Tregs are thought to exert beneficial roles in controlling pro-tumor chronic inflammation. The goal of our study was to characterize CRC-infiltrating Tregs at multiple levels, by phenotypical, molecular and functional evaluation of Tregs from the tumor site, compared to non-tumoral mucosa and peripheral blood of CRC patients. The frequency of Tregs was higher in mucosa than in blood, and further significantly increased in tumor. *Ex vivo*, those Tregs suppressed the proliferation of tumor-infiltrating $CD8^+$ and $CD4^+$ T cells. A differential compartmentalization was detected between $Helios^{high}$ and $Helios^{low}$ Treg subsets (thymus-derived versus peripherally induced): while $Helios^{low}$ Tregs were enriched in both sites, only $Helios^{high}$ Tregs accumulated significantly and specifically in tumors, displayed a highly demethylated TSDR region and contained high proportions of cells expressing CD39 and OX40, markers of activation and suppression. Besides the suppression of T cells, Tregs may contribute to CRC progression also through releasing IL-17, or differentiating into Tfr cells that potentially antagonize a protective Tfh response, events that were both detected in tumor-associated Tregs. Overall, our data indicate that Treg accumulation may contribute through multiple mechanisms to CRC establishment and progression.

Abbreviations: CRC, colorectal cancer; CT, center of the tumor; HCC, hepatocellular carcinoma; IM, invasive margin; NT, non-tumor; PB, peripheral blood; PBMC, peripheral blood mononuclear cells; SNP, single nucleotide polymorphism; T, tumor; Tconv, conventional T cell; Tfh, T follicular helper; Tfr, T follicular regulatory; Th17, T helper 17; Treg, regulatory T cell; TSDR, Treg-specific demethylated region

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CD39; colorectal cancer; helios; OX40; Tfh; T follicular regulatory; Th17; tregs

Introduction

An appropriate balance between effector and regulatory activities ensures mounting of protective immune responses while limiting tissue damage. Regulatory T cells (Tregs), a $CD4^+$ T cell subset specialized in immune suppression, crucially maintain immune system homeostasis in physiological conditions. However, in chronic inflammatory diseases and cancer, the effector/regulatory equilibrium may be persistently perturbed, finally leading to a failed eradication of the primary injury. Indeed, in a variety of experimental tumor models and human cancers, the relative proportion of Tregs, with respect to effector T cells, is significantly increased at the tumor site and correlate with an adverse prognosis. We have recently shown that, in human liver carcinogenesis, Treg expansion is an early event, occurring already in a premalignant condition, i.e. cirrhosis. Such expanded Tregs display a phenotype ($Helios^{high}$, $OX40^+$, $CD39^+$) that is suggestive of a strong immune suppressive activity *in vivo*.[1]

Contrary to the majority of cancer types, in colorectal cancer (CRC), a high Treg density at the tumor site (evaluated by immunohistochemistry) has been associated to a better outcome.[2-4] This finding led to hypothesize that, in CRC, Treg-mediated suppression of chronic inflammation may prevail over the control of antitumor immunity.[3,5] However, other data strongly suggest that Tregs do not inhibit but rather promote pro-tumor Th17 responses,[6] and actively suppress tumor-specific $CD8^+$ T cell activation in CRC patients.[7-9]

Besides $CD8^+$ T cell infiltration,[10] the occurrence of ectopic lymphoid tissue, often localized at the invasive cancer front, has a favorable prognostic impact for early stage CRC.[11] Specialized Treg subsets, such as T follicular regulatory cells (Tfr), are devoted to antagonize follicular responses.[12] The goal of our study was to characterize at several levels (phenotypical, functional and epigenetic) the Tregs infiltrating human CRC specimens, in order to assess whether they exerted suppressive

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function *ex vivo*, presented a Helios^{high}, OX40^{+}, CD39^{+} phenotype and displayed Th17-like or Tfr properties.

Results

Suppressive Tregs accumulate at tumor site in CRC patients

First, we estimated the frequency of Tregs, identified as CD127^{low}FOXP3^{+}/CD4^{+} T cells, in peripheral blood (PB), tumor fragments (T) and specimens of normal colonic mucosa (NT) of CRC samples, through flow cytometry. Confirming previous results (mostly obtained through the analysis of CD25^{+} and/or FOXP3^{+} cells),[7,9,3] we observed that Treg proportion slightly increased in NT, compared to PB, but was massively expanded in T, compared to both PB and NT (Fig. 1A–B). *Ex vivo* Treg depletion resulted in an enhanced proliferation, following polyclonal stimulation, of both conventional T cells (CD4^{+}FOXP3^{-}, Tconvs) and CD8^{+} T cells from PB, NT and T (Fig. 1C–D); notably, the extent of the rescue in CD8^{+} and Tconv proliferation, following Treg depletion, was significantly enhanced in T samples (Fig. 1E), suggesting that the relative impact of Treg suppression was more relevant in the tumor microenvironment, likely due to the higher Treg proportions at that site.

However, we did not detect in our cohort any significant difference in T-derived (T-)Treg frequency in CRC patients with different stage (Fig. S1A) or grade (Fig. S1B). As a surrogate indicator for tumor outcome, we evaluated the CD3^{+} cell density in the center of the tumor (CT) and at the invasive margin (IM), previously recognized as a strong predictor of favorable prognosis in CRC.[10] In line with published data,[10] CD3^{+} cell densities in IM and CT, as evaluated by immunohistochemistry, showed positive correlation (Fig. S2A), and both tended to decrease with stage (Fig. S2B). When patients were stratified according to high/low CD3^{+} cell density at IM or CT, we could observe that Treg frequency paralleled, despite not significantly, the CD3^{+} scoring in NT or T sites (Fig. S1C–D).

T-Tregs are mostly Helios^{high} and carry demethylated TSDR

The human Treg pool contains several subsets with distinct proliferative and suppressive potentialities,[14] and Treg heterogeneity has been found also in pre-malignant and malignant

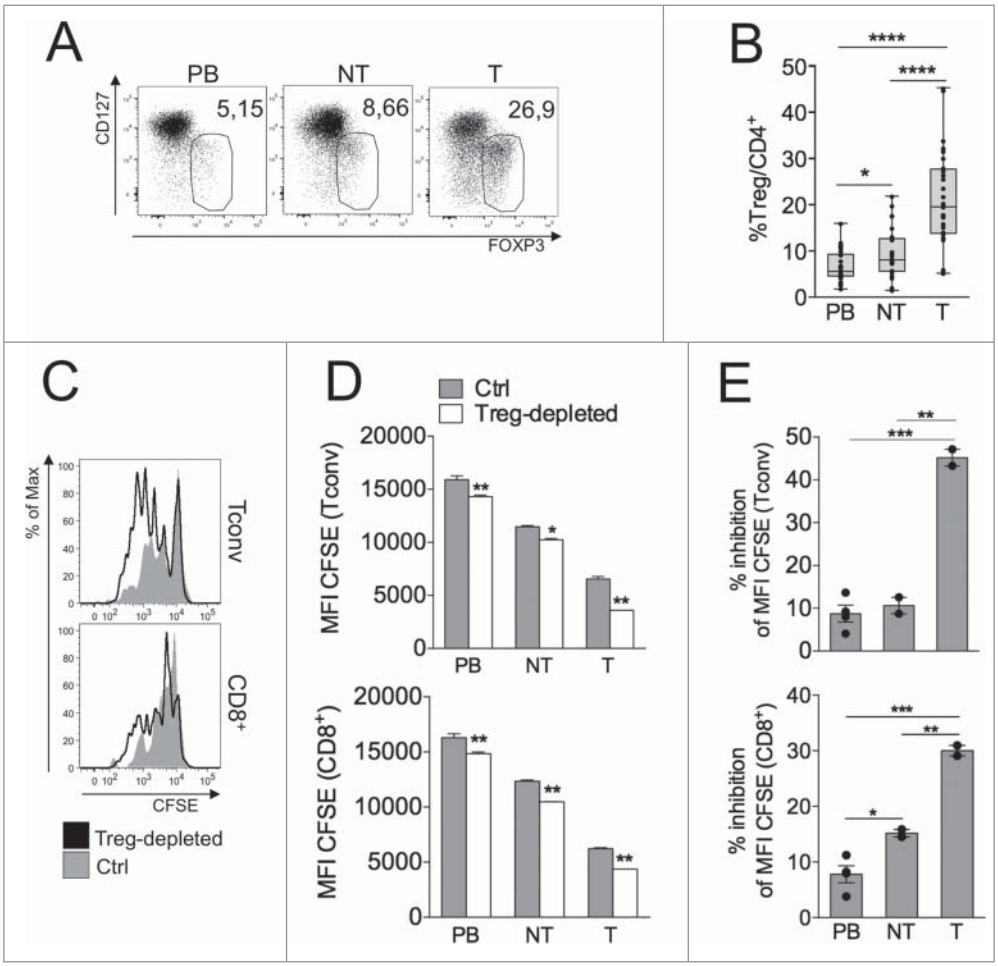

Figure 1. Suppressive Tregs accumulate at T site in CRC patients. (A–B) Representative CD127/FOXP3 staining (A) and percentages of Tregs (B) in gated CD4^{+} T cells in PB, NT and T samples of CRC patients (n = 31). $^{*}p < 0.05$, $^{****}p < 0.001$, by Wilcoxon matched-pairs test, two-tailed. In all figures, Tregs and Tconvs have been identified as FOXP3^{+} CD127^{low} or FOXP3^{-}, respectively, within the CD14^{-} CD16^{-} CD56^{-} CD19^{-} viability dye^{-} CD4^{+} gate. (C–E) CFSE profile overlay (C), mean fluorescence intensity (MFI) of CFSE (D), and percentage of inhibition of CFSE dilution (E) in gated Tconvs (CD4^{+}FOXP3^{-}) or CD8^{+} T cells from Treg-depleted or total (Ctrl) mononuclear cells, obtained from a representative CRC patient. The experiment was repeated thrice with cells of individual patients giving similar results. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, by Student *t* test, unpaired.

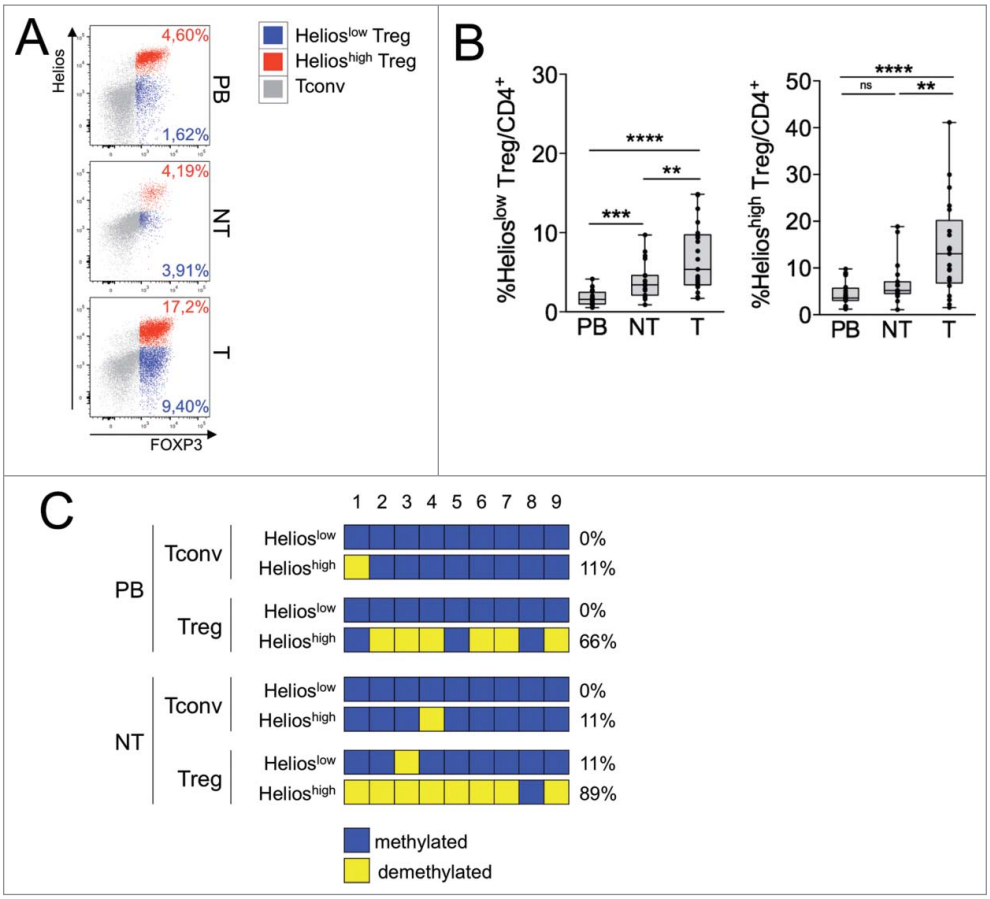

Figure 2. Helios^{high}, epigenetically stable, Tregs specifically increase in tumor. (A–B) Representative Helios/FOXP3 staining in overlaid Helios^{high} Tregs (red), Helios^{low} Tregs (blue) and Tconvs (gray) (A) and percentages of Helios^{low} and Helios^{high} Tregs in gated CD4^{+} T cells (B) in PB, NT and T of CRC patients (n = 21). $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$, by Wilcoxon matched-pairs test, two-tailed. ns, not significant. (C) The indicated subsets were FACS-sorted from PB and NT samples of a CRC patient and TSDR analysis was conducted as detailed in the Patients and Methods section. We could not perform TSDR analysis from any T specimen due to a very low yield of recovered cells. Color-coding represents the methylation status; numbers on the top indicate the sequential position of each CpG residue; percentages on the right refer to the frequency of demethylated sites.

liver tissue microenvironment.[1] Therefore, we performed multiparameter flow cytometry to better characterize Treg diversity in NT and T specimens from CRC patients. First, we focused on the transcription factor Helios, which has been associated to the thymic origin, to a stronger suppressive function [15] and to a more stable regulatory program [16] maintained by epigenetic events, i.e., demethylation in the Treg-specific demethylated region (TSDR) of the *FOXP3* locus.[1] The frequency of Helios^{high} Tregs significantly increased only in T (Fig. 2A–B), in line with data in hepatocellular carcinoma (HCC).[1] Conversely, the proportion of Helios^{low} Tregs accumulated in both NT and T, compared to PB, possibly as a physiological consequence of colonic Treg induction in response to food and microbial antigens.[17] To support the relationship between Helios expression and regulatory stability, we analyzed the level of TSDR methylation in highly purified Helios^{high} or Helios^{low} Tregs or Tconvs isolated from the PB or NT specimen (but not from T specimen, because of the paucity of lymphocytes extracted from the T district that did not allow these assays requiring very high number of cells). Notably, Helios^{high}, but not Helios^{low}, Tregs from different sites displayed a highly demethylated TSDR (Fig. 2C), a strong indicator of a committed regulatory program also in tumors.[18]

OX40 expression supports Treg survival and proliferation in CRC

Our previous data revealed that HCC-infiltrating Tregs expressed high level of OX40, an event that was related to a superior immune suppressive function.[1] Based on the observation of a pivotal role of OX40 in supporting Treg fitness in experimental models of gut inflammation,[19,20] we investigated whether Treg expansion in CRC was related to higher levels of OX40 expression. Indeed, we found that the frequency of both OX40^{+} Tconvs and Tregs significantly increased in NT and much more in T samples (Fig. 3A–B); in addition, OX40 was always more represented in Tregs than in Tconvs (Fig. 3A–B), even though the extent of OX40 expression in the two subsets showed a positive correlation in all districts (Fig. 3D). Looking within the Treg gate, OX40 was upregulated in NT and T samples in both Helios^{low} and Helios^{high} Tregs and the two events were positively associated, despite it was always higher on the Helios^{high} counterpart (Fig. 3C and E).

To better evaluate the net impact of OX40 stimulation on the whole CRC-infiltrating T cells, we stimulated *in vitro* mononuclear cells extracted from T specimens of CRC patients in the presence of crosslinked rOX40L: our results indicated that OX40 engagement promoted the proliferation

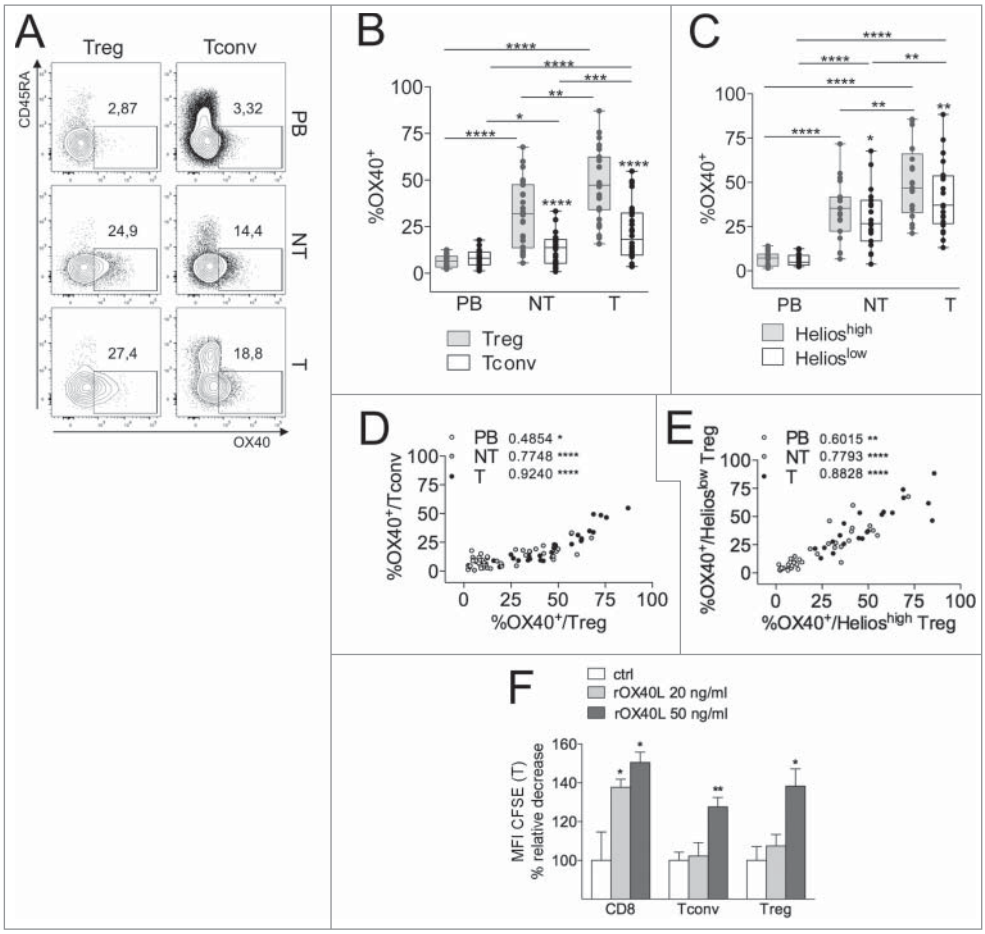

Figure 3. T-Tregs express OX40, conveying proliferative signal, at highest levels. (A–C) Representative CD45RA/OX40 staining (A) and $OX40^+$ percentages in gated Tregs and Tconvs (n = 29) (B) or $Helios^{high}$ vs. $Helios^{low}$ Tregs (n = 20) (C) from PB, NT and T of CRC patients. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$, by Wilcoxon matched-pairs test, two-tailed. (D–E) Spearman correlations between the percentages of $OX40^+$ Tconvs and Tregs (D) and of $OX40^+$ $Helios^{high}$ or $Helios^{low}$ Tregs (E). $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.001$. (F) Mononuclear cells, obtained from the T sample of a representative CRC patient, were stimulated in the presence of scaled concentrations of rOX40L, and proliferation was estimated in gated $CD8^+$, Tconvs ($CD4^+FOXP3^-$) and Tregs ($CD4^+FOXP3^+$), in terms of the relative change in CFSE MFI. The experiment was independently repeated in two patients giving similar results. $^*p < 0.05$, $^{**}p < 0.01$, by Student *t* test, unpaired, compared to ctrl.

not only of $CD8^+$ cells and Tconvs (in line with the well-known costimulatory activity of this molecule on effector T cells), [21] but also of Tregs (Fig. 3F), in line with data obtained in HCC,[1] suggesting that OX40 triggering may concomitantly promote both effector and regulatory activities in the tumor microenvironment.

OX40 signal has been shown to impart a survival advantage to T cells, and especially Tregs, under different T cell-depleting regimens.[20,22] Consistent with this idea, we could observe that CRC patients previously treated with neoadjuvant chemotherapy showed higher frequencies of total Tregs and especially of $OX40^+$ Tregs, but not of $OX40^+$ Tconvs, compared to matched controls for tumor stage and location (Fig. S3). Overall these data support the notion that OX40 upregulation may sustain T-Treg expansion in CRC, especially of the $Helios^{high}$ Treg subpopulation.

Both genetic and environmental factors contribute to determining CD39 expression

CD39, an ectonucleotidase that degrades extracellular ATP, is constitutively expressed by Tregs and plays a well-recognized role in mediating Treg suppressive function. CD39 expression level in humans may be determined by both genetic and microenvironmental factors,[23] and the tumor milieu favors the expansion of $CD39^{high}$ Tregs.[1,24] We stratified the CRC patients according to their genotype at the *ENTPD1* single nucleotide polymorphism (SNP, rs10748643) that is linked to CD39 expression level,[23,25] and indeed we found that, in PB, this genetic factor correlated with CD39 expression in Tconvs and even more in Tregs (Fig. 4A). The analysis of $CD39^{high}$ cells also in NT and T samples showed that (i) in all genotypes and all districts, Tregs contained more $CD39^{high}$ cells than Tconvs; (ii) in both Tconvs and Tregs, $CD39^{high}$ proportions increased in NT and even more in T compared to respective PB, only in AA and AG genotypes and not in the GG group; (iii) in both Tconvs and Tregs, $CD39^{high}$ proportions increased in AG and even more in GG compared to AA genotype, only in PB and NT and not in the T site (Fig. 4B). Most of $CD39^{high}$ cells were contained in the $Helios^{high}$ subset and included the majority of $OX40^+$ cells; importantly, $Helios^{high}$ $CD39^{high}$ $OX40^+$ triple-positive cells, which were always more frequent in Tregs than Tconvs, gradually increased according to genotype and to district, reaching a maximum in T samples of GG patients (Fig. 4C). Overall, these data demonstrate that both genetic and microenvironmental factors contribute to set CD39 expression,

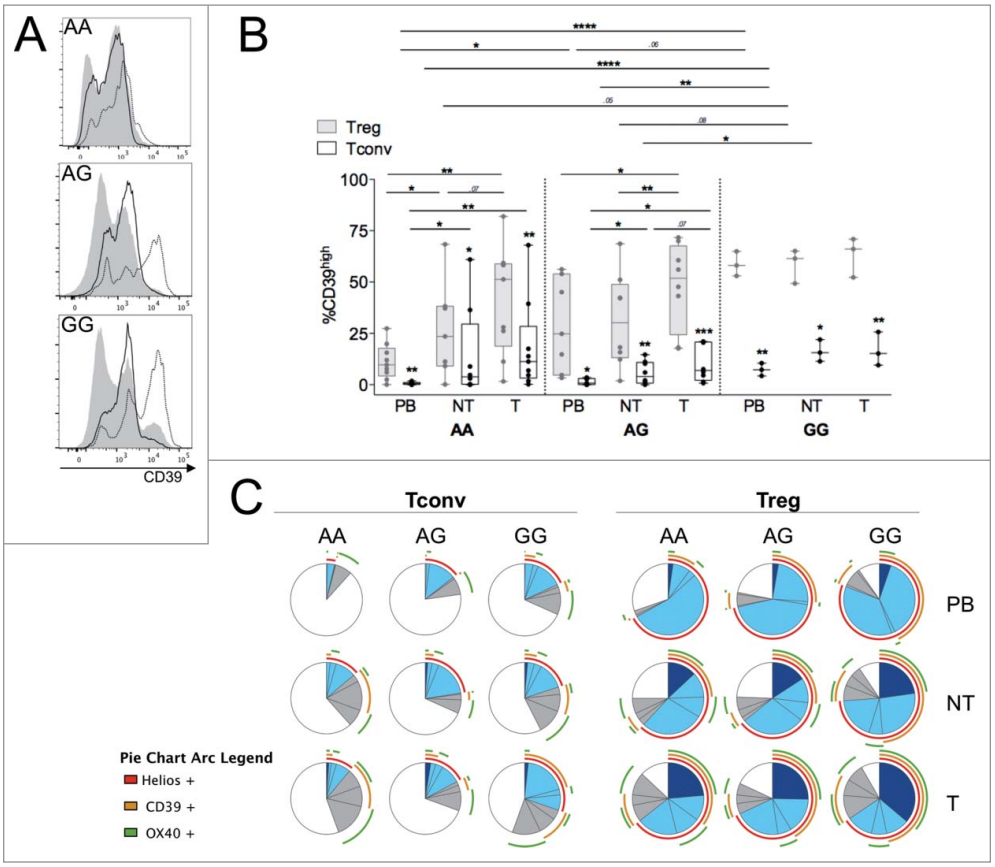

Figure 4. Both genetic and microenvironmental factors contribute to set high CD39 levels in T-Tregs. (A) Representative histogram overlay of CD39 profile in total lymphocytes (gray areas), gated Tconvs (black lines) or gated Tregs (dotted lines) in the PB of CRC patients, on the basis of their genotype at the SNP rs10748643 (AA, AG or GG). (B) Percentages of CD39^{high} cells in gated Tregs and Tconvs from PB, NT and T samples of CRC patients, stratified into AA (n = 9), AG (n = 9) or GG (n = 3) genotypes. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$, by Student *t* test, paired, between Tregs and Tconvs, by Wilcoxon matched-pairs test between different districts, and by Student *t* test, unpaired, between different genotypes. (C) Concatenated analysis of Helios, CD39 and OX40 expression in different subpopulations (Tregs and Tconvs), compartments (PB, NT and T) and genotypes (AA, AG and GG). Each pie represents grouped data from seven (AA), eight (AG) or two (GG) patients.

being the GG genotype or the T district sufficient to determine the highest level of CD39^{high} Treg frequency.

IL-17-producing Tconvs and Tregs accumulate at tumor site and mainly within the Helios^{low} subsets

In line with the idea that the colonic environment promotes the polarization of Th17 cells and Th17-like Tregs,[26] we found that both Tregs and Tconvs released IL-17 *ex vivo* both in NT and even more in T samples, compared to PB; of note, the frequency of IL-17-producing cells was always higher in Tregs than in Tconvs, supporting the notion of a Treg preferential propensity to Th17 polarization [14,27] (Fig. 5A). The frequency of IL-17-producing Tregs and Tconvs showed a positive reciprocal correlation in PB and T, suggesting that the two subsets may be under the control of similar stimuli (Fig. 5B). Recent data demonstrate that Tregs contribute to the pro-tumor Th17 polarization via an extrinsic mechanism, i.e., IL-2 sequestration: [6] accordingly, we could observe a significant positive association between Treg and Th17 percentages selectively in T specimens (Fig. 5C).

We tested whether Th17-polarizing cytokines were differentially released in NT or T samples. To this aim, we dosed cytokine amounts in NT- and T-conditioned medium (CM), and found that IL-1β, but not IL-6 or IL-23p19, was significantly increased in CM from T compared to NT samples (Fig. 5D). When the number of NT- or T-infiltrating cells was enough to perform further analyses (in a limited number of patients), we showed a trend toward the co-expression of IL-17 and CD39 in T-Tregs (Fig. 5E), confirming previous observations in the literature about the role for CD39 signal in promoting Th17 polarization.[28] Conversely, IL-17 and Helios expression tended to be mutually exclusive (Fig. 5F, left), according to the intrinsic stability of Helios^{high} Tregs [1,16,29] and to the segregation between Helios and RORγt expression in gut Tregs.[30] OX40 expression was similarly distributed in IL-17-positive and -negative cells (Fig. 5F, right).

Follicular Tconvs and Tregs accumulate at both NT and T sites

Finally, we analyzed the proportion of Tfh (BCL6^{+}CXCR5^{+}FOXP3^{-}) and Tfr (BCL6^{+} CXCR5^{+}FOXP3^{+}) cells in all districts. Both cell types accumulated in NT and T compared to PB; only in NT, Tfr frequency outnumbered Tfh percentage (Fig. 6A–B). Both Tfr and Tfh cells roughly contained similar levels of Helios and IL-17 but lower levels of CD39 and higher levels of OX40 than total Tregs or Tconvs respectively (Fig. 6C). The latter observation is in line with data confirming a role for OX40/OX40L axis in T follicular cell polarization.[31] Overall, these results suggest that, at NT and more at T site,

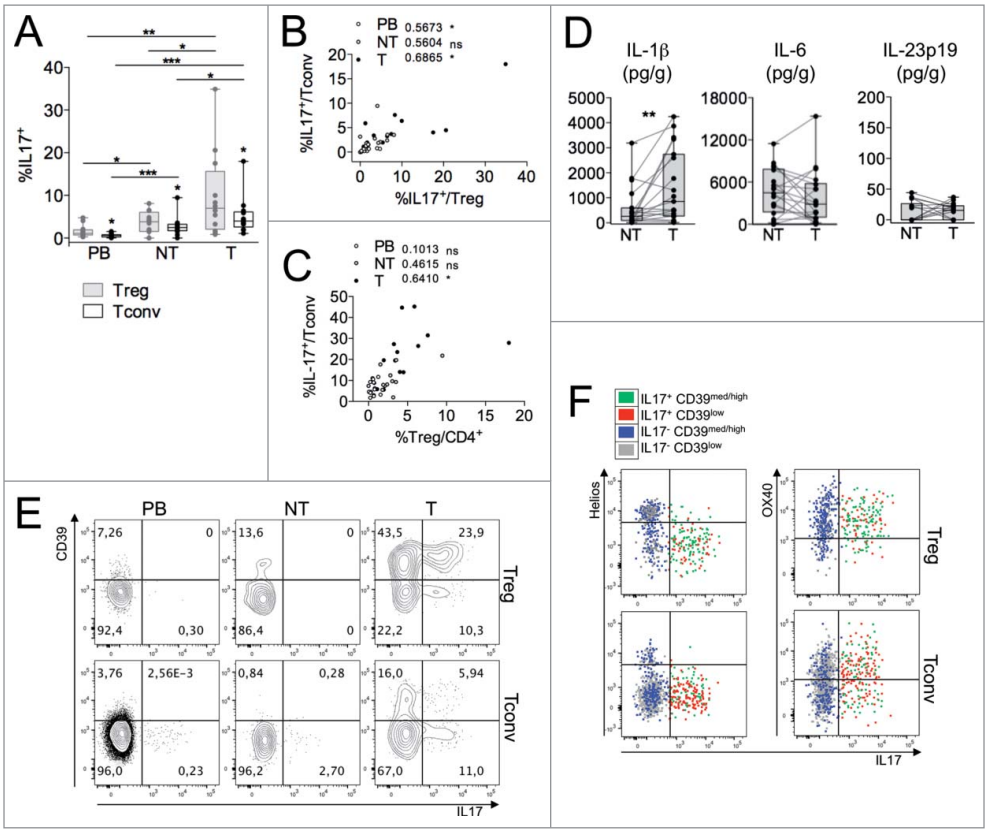

Figure 5. Th17-like Tregs, contained in the CD39^{high}Helios^{low} subset, accumulate at T site. (A) Percentages of IL-17-producing Tregs and Tconvs in different compartments from CRC patients (n = 14). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, by Student *t* test, paired, between Tregs and Tconvs, or by Wilcoxon matched-pairs test between different districts. (B–C) Spearman correlations between the percentages of IL-17^{+} Tconvs and Tregs (B) and of IL-17^{+} Tconvs respect to total Tregs (C). $^{*}p < 0.05$; ns, not significant. (D) Cytokine amounts (estimated as the pg released in 18 h per gram of tissue) in NT- compared to T-CM from CRC patients (n = 19). $^{**}p < 0.01$, by Wilcoxon matched-pairs test. (E) Representative IL-17 versus CD39 expression in Tregs or Tconvs from the indicated districts. (F) Representative IL-17 vs. Helios (left) or OX40 (right) expression in T-Treg and T-Tconv subsets distinguished on the basis of CD39 and IL-17 expression. Data shown are representative from one out of four patients tested.

multiple subpopulations of Tregs accumulate, which may display shared as well as unique features, and may concur to promote immune suppression and tumor progression through different mechanisms.

Discussion

Among many cancer types, CRC is considered tightly linked to inflammatory and immune events, from several points of view. Indeed, a well-established link between CRC and chronic inflammation, sustained by tumor cell-extrinsic as well as -intrinsic pathways, has been recognized in multiple settings. In particular, the IL-23/IL-17 axis has been linked to worse outcome in CRC patients [32] and has been recognized to drive colorectal carcinogenesis in experimental models,[33] through a variety of mechanisms that include a direct promotion of enterocyte proliferation by IL-17.[34] Conversely, CD8^{+} T cell infiltration has been recognized as a major prognostic factor in CRC prognosis,[10] an event probably favored by follicular helper response and tertiary lymphoid structure formation,[11,35] indicating the attempts of protective immune-surveillance to counteract tumor progression.

In this scenario, Tregs have been proposed to potentially exert dual functions, depending on whether their suppressive activity mainly impacts on IL-17-dominated responses rather than on CD8^{+} T cell-mediated immune-surveillance.[5] The observed association between FOXP3^{+} cell density at tumor site and favorable prognosis[4] has led to the conclusion that, in CRC, the dominant Treg activity was the suppression of protumor inflammation, mostly driven by IL-17 axis.[3,5] However, other data indicate that the actual roles of Tregs in CRC may be addressed to the promotion of tumor progression through several mechanisms.[6-9,13,36]

Collectively, our results delineate a network of diverse Treg subsets populating the CRC microenvironment. First, in line with other studies (estimating Treg proportions through flow cytometry or immunohistochemistry),[7,9] we provide evidence that the frequency of Tregs is increased at tumor site in CRC patients similarly to what observed in other tumor types, a data suggesting that CRC may take advantage from creating a favorable milieu for the accumulation of Tregs. Importantly, circulating Tregs have been demonstrated to perform efficient suppression of antitumor immunity in CRC patients.[7-9] Accordingly, our results show that, in the context of CRC, Treg depletion *ex vivo* unleashes effector T cell proliferation in T specimens to a relatively higher extent compared with NT and PB, supporting the idea that a high proportion of suppressive Tregs are recruited into the tumor district. In addition, we report that, in CRC, T-Tregs are enriched in Helios^{high}, CD39^{high} and OX40^{+} cells, phenotype compatible with a strong suppressive activity, a high proliferative potential and a stable regulatory program. The proportion of the triple positive subset

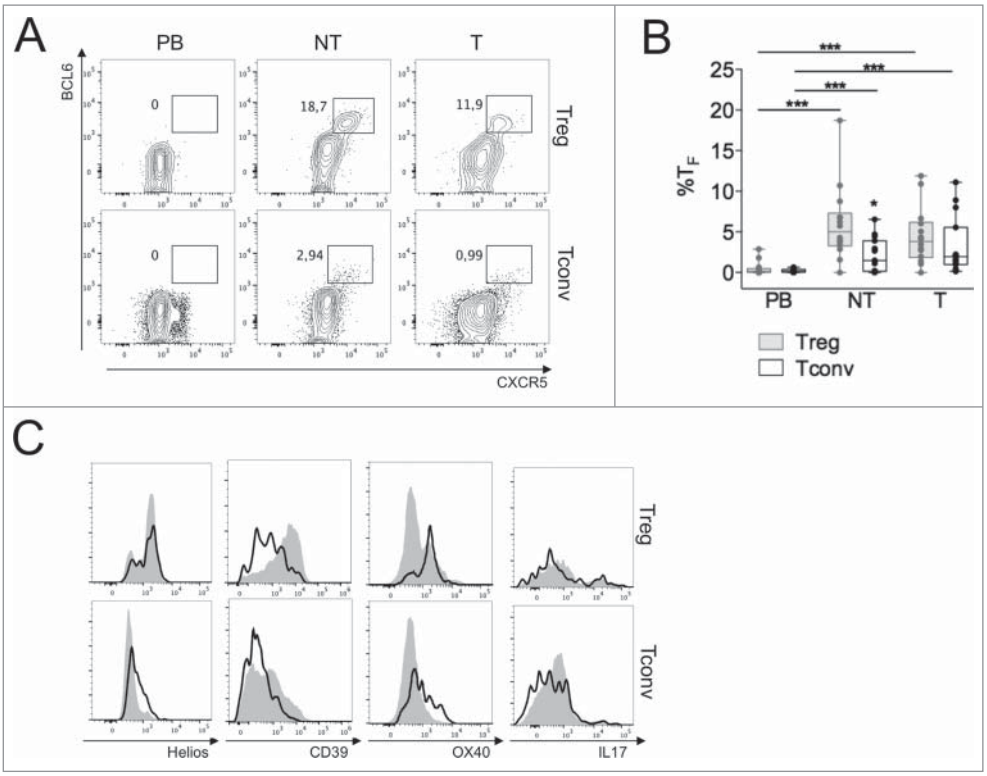

Figure 6. Tfh and Tfr, enriched in the CD39^{low}OX40^{+} subset, expand in both NT and T compartments. (A–B) Representative CXCR5/BCL6 staining (A) and percentage of BCL6^{+}CXCR5^{+} follicular cells (B) in gated Tregs and Tconvs from PB, NT and T of CRC patients (n = 16). $^{*}p < 0.05$, $^{***}p < 0.005$, by Student *t* test, paired, between Tregs and Tconvs, or by Wilcoxon matched-pairs test between different districts. (C) Overlay of the indicated markers in the total Treg or Tconv pool (gray areas) compared to the T follicular subpopulation (black lines), in a representative T sample from three patients analyzed.

depended not only on the location (being maximal in the tumor) but also on genetic factors: indeed, we found that, in line with data in the literature,[23,25] the baseline CD39 expression level was determined by a genetic polymorphism in the *ENTPD1* gene. Notably, a key role of CD39, expressed by Tregs, in suppressing the protective antitumor immunity, has been demonstrated in a mouse model of metastatic CRC.[37] The highly committed regulatory identity of T-Tregs is evident also from the epigenetic characterization of the TSDR region and from the functional evaluation of T-Tregs suppression (by depletion experiments) directly *ex vivo*. We could not assess the suppressive capacity of total T-Tregs, of single T-Treg subsets, or of the CD39^{+} Tconv population emerging in the colon, by performing standard co-culture suppression assays, due to insufficient cell numbers recovered from NT and T specimens. However, it is conceivable that diverse mechanisms cooperate in ensuring proper Treg suppression in a certain context,[38] while CD39 expression may not be sufficient to impart regulatory activity into FOXP3^{-} cells, according to data in the literature.[39]

Taken together, these data speak in favor of a tumor-specific expansion of strongly suppressive Helios^{high}CD39^{high}OX40^{+} Tregs exhibiting a highly demethylated TSDR, committed to the control of antitumor immunity, thus potentially playing pro-tumor roles also in this context. Helios, associated to a demethylated TSDR and originally recognized as thymus-derived (t)Treg-specific,[40] was subsequently detected also in peripherally derived (p)Tregs in certain conditions,[41] and more recent studies have underscored a tight link between Helios expression/TSDR demethylation and Treg function/epigenetic stability, independently from Treg origin.[15,16,42-45] Overall, while the Helios^{low} TSDR-methylated cells virtually contains only pTregs, especially in the colonic microenvironment exposed to commensal microbiota,[17] Helios^{high} TSDR-demethylated Tregs may indeed encompass a mixture of tTregs and of "stabilized" pTregs.[44]

The finding that both NT- and T-Treg frequencies tended to parallel the CD3^{+} density (that was in turn directly correlated with an improved survival) [10] suggests that the expansion of suppressive Tregs may be a wide-ranging and early event in colorectal carcinogenesis, and that Tregs seem to limit rather than completely suppressing antitumor immunity, as the result of the physiological Treg commitment to establish the immune homeostasis. Consistent with this hypothesis, CD8^{+} T cell-dependent antitumor immunity might be more efficient in fighting tumor without the parallel limitation by suppressive Tregs.[46] However, the estimation of total CD3^{+} density should be interpreted taking into consideration that also Tregs are included in that population, and that such analysis cannot discriminate between selective Treg expansion and co-recruitment of Tregs and Tconvs/CD8^{+} T cells.

This conclusion may appear discordant with data arising from the analysis of Treg infiltration in CRC samples, indicating a favorable impact of FOXP3^{+} cell infiltration on prognosis.[4] However, it may be argued that the absolute Treg counts simply follow and reflect the extent of the overall infiltration of effector T cells that actually exert beneficial effects. Though representing a good biomarker for cancer prognosis, the

estimation of Treg density may not be as relevant as supposed in understanding their biological and immunological activities *in vivo*. Rather, Treg numbers may simply reflect the potency of antitumor immunity, as shown in breast cancer,[47] and the relative ratio between Tregs and effector T cells may be more informative than the sole Treg count in elucidating Treg functions.[46] Indeed, a low intraepithelial effector/Treg ratio was associated to a worse prognosis in CRC,[36] an observation that supported a pro-tumor effect by Tregs also in this context. In addition, many pieces of evidence demonstrate that Tregs and Th17 cells do not exert antagonistic activities and rather cooperate in a variety of contexts.[48] In a mouse model of CRC, Tregs have been shown to favor Th17 cell development and tumor progression through the deprivation of IL-2, a cytokine able to inhibit Th17 polarization,[6] data consistent with our observation in human CRC of a direct correlation between the frequency of Th17 cells and the total Treg percentage in the tumor site.

Alternatively, Tregs themselves can switch to an IL-17-producing, Th17-like, phenotype in proper conditions,[49] and human Tregs even represent preferential Th17 precursors compared to other $CD4^+$ T cells.[27] In this context, our data emphasize the heterogeneity of Treg populations with different phenotype and function that can infiltrate the tumor site. Indeed, we also show the accumulation, at the tumor site, of Th17-like Tregs (enriched in the $Helios^{low}CD39^{high}OX40^{+/-}$ subpopulation, i.e., pTregs) that are directly correlated with the percentage of conventional Th17 cells. On the basis of the evidence showing a preferential Th-17 cell development in IL-2 deprivation conditions,[6] it would be of interest to determine if PD-1 expression, previously demonstrated to limit IL-2/STAT-5 signaling in Tregs,[50] may favor Th17-like Treg deviation. Furthermore, a distinct subset of $ROR\gamma t^+$ gut-resident Tregs, controlling colonic inflammation, has been recently identified, supporting the idea that FOXP3 and $ROR\gamma t$ may work not in antagonism but in cooperation at the transcriptional level.[30] A $ROR\gamma t^+$ Treg subpopulation (not always coinciding with IL-17-producing Tregs) has been found expanded in human CRC and murine polyposis, with $ROR\gamma t$ contributing to tumor progression through shifting tumor Treg functions from anti-inflammatory to immune-suppressive.[13] Furthermore, Tregs can directly support tumor angiogenesis via VEGF in mouse cancer models,[51] or can directly promote tissue repair through amphiregulin in a model of lung injury.[52]

Overall, these data indicate that Tregs may contribute to tumor progression not only by exerting immune suppression (by stable suppressive $Helios^{high}CD39^{high}OX40^+$ Tregs exhibiting a highly demethylated TSDR), but also by performing other activities directly regulating tissue functions (by rather unstable $Helios^{low}CD39^{high}OX40^{+/-}$ pTregs presenting a poorly demethylated TSDR region and thus prone to acquire a plastic, Th17-like, phenotype). It would be interesting to examine whether also NT-Tregs show some aberrant features, compared to Tregs from the normal mucosa of healthy individuals or patients with non-tumoral colonic diseases, and play some role in disease evolution. In line with this idea, a high density of $FOXP3^+$ cells in non-tumoral tissue has been associated with a worse prognosis.[4] A dysfunction also in NT-Tregs in CRC patients may have multiple explanations: on the one side, CRC development may condition the immunological scenario also at distant locations in the same organ; on the other side, pre-existing immune dysfunctions in the intestine may contribute to CRC onset/progression.

Another possible mechanism by which Tregs control the tumor-host interaction is the modulation of lymphoid follicle organization at the tumor site. Our data in human CRC samples show that Tfr, mostly contained in the $CD39^{low}OX40^+$ subset, accumulated at NT as well at T sites, thus possibly controlling Tfh response in both contexts. A dynamic analysis of tumor infiltrating "immunome" has revealed a protective role of Tfh response in arranging antitumor response in CRC.[35] In line with this observation, the presence of tertiary lymphoid structures in early stage CRC has revealed a favorable prognostic impact on tumor course,[11] even though the association between lymphoid aggregates (which are mostly extra-tumoral) and prognosis remains controversial.[53] The subset of Tfr cells is specialized in suppressing Tfh responses and inhibiting the organization of efficient lymphoid follicles.[12] Therefore, it is conceivable that Tfr play detrimental roles in tumor progression. Unfortunately, no data are yet available about a prognostic relevance of Tfr in CRC progression. Moreover, Tfr may not only influence follicle organization but also regulate local T cell trafficking and functions. Indeed, in a mouse model of lung cancer, Tregs have been shown to preferentially localize in tertiary lymphoid structures, where they regulate antigen-presenting cell functions and prevent T cell activation.[54]

Our data indicate that both $Helios^{high}$ and $Helios^{low}$ Tregs accumulate at the CRC site but display distinct phenotypes and may possibly establish a division of labor: while the former may be specialized in suppressing antitumor T cells, in favoring Tconv polarization into Th17, and in differentiating into Tfr cells (mostly $OX40^+CD39^-$), the latter (especially $OX40^{+/-}CD39^+$ cells) may be equipped for IL-17 release. In summary, the tumor microenvironment may promote the local accumulation of tTregs as well as pTregs whose functions may converge toward pro-tumorigenic events. Future studies will ascertain whether any of these subpopulations, including the Tfr, can be considered a reliable prognostic marker for cancer progression (in terms of density or better of relative frequency) or even a possible target for cancer immunotherapy.

Because of the wide expression of OX40 on the different human Treg populations analyzed within CRC, our data point to novel roles for OX40 in promoting human Treg expansion and/or survival. Indeed, the observation that OX40 triggering *ex vivo* on tumor-infiltrating cells boosts the proliferation of both effector (Tconv and $CD8^+$ T cell) and regulatory (Treg) arms indicates that the immunotherapeutic efficacy of possible OX40-agonistic compounds [55] may be limited by a concomitant Treg expansion. Also, our data underscore a possible role for OX40 in determining the preferential survival of activated Tregs following neoadjuvant chemotherapy. Accordingly, others have found that the frequency of Tregs expressing markers of activation and suppression increases following biologic therapy or chemoradiotherapy in head and neck cancer [56,57] and in breast cancer [58] patients, and that Tregs are relatively resistant to cisplatin *in vitro* due a higher induction of Bcl-2 and Bcl-xL compared to Tconvs.[57] Notably, OX40 sustains $CD4^+$ T cell survival through the induction of these two anti-apoptotic molecules.[59] Further studies are needed to

ascertain whether such residual immune regulatory activity plays detrimental (suppressing antitumor immunity) or beneficial (controlling systemic inflammation) roles for the host, following oncological therapies.

In conclusion, our work underscores the complexity of human CRC-infiltrating Treg subpopulations and proposes that multiple immunosuppressive and pro-carcinogenic pathways may be recognized as novel players in tumor-host interaction and in the development of new prognostic and therapeutic strategies.

Patients and methods

Patients and samples

PB, T and NT (collected from the upstream distal resection margin) specimens were obtained from CRC patients not previously treated with steroid therapies and with no history of inflammatory bowel disease or immunodeficiency. In selected analyses, two patients that had received neoadjuvant chemotherapy (based on capecitabine and/or oxaliplatin, stopped at least 1 mo before surgery) were studied. Patient characteristics are listed in Table 1.

Peripheral blood mononuclear cells (PBMCs) were isolated from the PB of CRC patients by density gradient centrifugation with Lympholyte (Cedarlane) and collected in complete RPMI 1640 medium containing 10% FBS (HyClone GE Healthcare Life Sciences), 2 mM L-glutamine (Sigma-Aldrich), penicillin/streptomycin, nonessential amino acids, and sodium pyruvate (all from EuroClone), and 50 μM 2-mercaptoethanol (Sigma-Aldrich).

Mononuclear cells were extracted from normal mucosa or tumor through a modified published protocol.[60] Briefly, the fragments were minced and incubated at 37°C for 20 min in calcium- and magnesium-free Hanks' buffered saline solution (HBSS) containing 2.5% FBS and 1mM dithiothreitol (Sigma), to remove mucus. The samples were then incubated in 0.75 mM EDTA (Sigma) at 37°C in three rounds of 15 min each, to remove epithelial cells. Afterwards, samples were transferred in GentleMACS C tubes (Miltenyi Biotec) containing calcium- and magnesium-supplemented HBSS with 0.5 mg/mL Collagenase IV (Sigma), 50 ng/mL DNAse I (Worthington), 2% FBS and 10% BSA. Tissue dissociation was made on a GentleMACS Octo Dissociator with Heaters (Miltenyi Biotec), by performing the program "h_tumor 01_03" thrice (with a 10-min incubation at 37°C between the second and the third round). Single cell suspensions were obtained by disrupting the fragments with a syringe plunger over a cell strainer, washing with cold HBSS. Then, cells were pelleted through a 40% isotonic Percoll solution, and finally centrifuged over a Lympholyte density gradient.

Human studies were performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Institutional Ethical Committee (No. 3596). Informed consent was obtained from all patients.

Table 1. Characteristics of all CRC patients included in the study (n = 34 , not treated with any neoadjuvant therapy).

Age (years, mean ± SD)	68 ± 11
Sex	
Male	20 (58.8%)
Female	14 (41.2%)
Stage (AJCC)	
I	6 (17.6%)
II	9 (26.5%)
III	12 (35.3%)
IV	7 (20.6%)
Tumor location	
Cecum	1 (2.9%)
Ascending colon	9 (26.5%)
Transverse colon	2 (5.9%)
Descending colon	1 (2.9%)
Sigmoid colon	13 (38.2%)
Rectum	8 (23.5%)

Flow cytometry

A complete list of Abs used is available as Table S1. Surface staining was performed by incubating the cells with selected Abs at 4°C for 20 min in PBS 2% FBS. Intracellular staining of cytokines and transcription factors was performed in accordance with the manufacturer's instructions for FOXP3 detection (eBioscience). Before IL-17 intracellular staining, cells were stimulated for 4 h at 37°C with Cell Stimulation Cocktail plus protein transport inhibitors (eBioscience), in order to identify the cells polarized *in vivo* into Th17/Th17-like. Dead cells were excluded using Fixable Viability Dye eFluor®780 (eBioscience). Samples were acquired on the BD LSRFortessa cell analyzer (BD Biosciences) and analyzed with FlowJo software, version 10.0.8r1 (Treestar), and Spice software, version 5 (downloaded from http://exon.niaid.nih.gov).

Functional assays in vitro

Tregs were depleted from mononuclear cell preparations of PB, NT and T samples by magnetic separation using CD25 microbeads (Miltenyi Biotec). Treg depletion was checked by flow cytometry and was almost complete in all samples. Then, cells were labeled for 15 min with CFSE (Life Technologies) and stimulated in complete medium for 6 d in the presence of Treg Suppression Inspector microbeads at 1:16 bead/cell ratio (Miltenyi Biotec). CFSE dilution was analyzed by flow cytometry in gated $CD8^+$ T cells or Tconvs (identified as $CD4^+FOXP3^-$ cells). In some experiments, scaled concentrations of crosslinked recombinant OX40L (RnDSystems) were added as previously described.[1]

Cytokine dosage

Tissue-CM was obtained by incubating for 18 h at 37°C small tissue fragments in 1 μL of complete medium per mg of tissue. Cytokine dosage was performed at LaboSpace (Milan, Italy) using AimPlex multiplex kit (YLS Bio).

CD39 SNP genotyping

DNA was extracted from PBMCs according to the manufacturer's protocol (DNeasy blood and tissue kit, Qiagen). Analysis of the SNP (rs10748643) in the *ENTPD1* gene (Chr10:97516764) was performed using a specific TaqMan SNP Genotyping assay

(Applied Biosystems). Real-time PCR was performed on a StepOne system (Applied Biosystem).

Cell sorting and TSDR analysis

$CD4^+$ T cells were enriched from PB and NT mononuclear cells using the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec), and stained with the following antibodies: $CD4^+$ AlexaFluor488, CD127 PECy7, CD19 APCeFluor780, CD56 APCeFluor780, CD16 APCeFluor780, CD14 APCeFluor780 (all from eBioscience); intracellular staining of Foxp3 and Helios was performed in accordance with the manufacturer's instructions (eBioscience) using FOXP3 PerCPCy5.5 and Helios APC. $Helios^{high}$ and $Helios^{low}$ Tregs and Tconvs were sorted using a FACSAria II (Becton Dickinson).

The extent of methylation in the TSDR was analyzed, according to a published protocol,[61] by performing two touchdown nested PCRs and then sequencing the amplified TSDR region. The CpG islands that were analyzed mapped into to the following chromosomal positions: NCBI36:X:49004163-49004190:1 and NCBI36:X:49004227-49004251.

Immunoscoring

Immunohistochemistry was performed on formalin fixed paraffin-embedded tissue samples using an anti-CD3 mAb and the streptavidin-biotin-peroxidase complex method (Vector Laboratories). $CD3^+$ cell density was assessed in line with procedures described in the literature.[10]

Statistical analysis

Statistical analysis was performed using Prism software (version 6, GraphPad). Unpaired Student *t* test, two-tailed, was used to analyze *in vitro* data, while Mann–Whitney test, two-tailed, or Wilcoxon matched-pairs test, two-tailed, was applied to compare groups of *ex vivo* samples. Correlations were calculated using the nonparametric Spearman's correlation test, two-tailed. Every *in vitro* assay was performed in triplicates or quadruplicates when possible. In all graphs, bars show means ±SEM. In all tests, $p < 0.05$ was considered statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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