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VEGFA-VEGFR Pathway Blockade Inhibits Tumor-Induced Regulatory T-cell Proliferation in Colorectal Cancer

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Abstract

Multitarget antiangiogenic tyrosine kinase inhibitors (TKI) have been shown to reduce regulatory T cells (Treg) in tumor-bearing animals and patients with metastatic renal carcinomas. However, a direct role of the VEGF-A/VEGFR pathway inhibition in this phenomenon is a matter of debate and molecular mechanisms leading to Treg modulation in this setting have not been explored to date. Treg proportion, number, and proliferation were analyzed by flow cytometry in peripheral blood of patients with metastatic colorectal cancer (mCRC) treated with bevacizumab, a monoclonal antibody targeting specifically VEGF-A, and in colon cancer–bearing mice (CT26) treated with drugs targeting the VEGF/VEGFR axis. The direct impact of VEGF-A on Treg induction was assessed together with specific blockade of different isoforms of VEGFRs that may be involved. In CT26-bearing mice, anti-VEGF antibody and sunitinib treatments reduced Treg but masitinib, a TKI not targeting VEGFR, did not. Targeting VEGF-A/VEGFR axis seems sufficient to affect Treg percentages, without any changes in their function. Similarly, bevacizumab inhibited Treg accumulation in peripheral blood of patients with mCRCs. *In vitro*, Treg expressing VEGFR from tumor-bearing mice directly proliferated in response to VEGF-A. Anti-VEGF-A treatment decreased Treg proliferation in mice as well as in patients with mCRCs. VEGFR-2- but not VEGFR-1–specific blockade led to the same results. We identified a novel mechanism of tumor escape by which VEGF-A directly triggers Treg proliferation. This proliferation is inhibited by VEGF-A/VEGFR-2 blockade. Anti-VEGF-A therapies also have immunologic effects that may be used with a therapeutic goal in the future. *Cancer Res; 73(2); 539–49. ©2012 AACR.*

Introduction

More than 1 million new cases of colorectal cancer (CRC) are diagnosed each year worldwide. Disease-specific mortality has decreased significantly in the past 2 decades with the introduction of new chemotherapeutic agents such as oxaliplatin and irinotecan, and, more recently, targeted therapies such as bevacizumab, an anti-VEGF-A monoclonal antibody (1).

The immune system seems to play a role in both the occurrence and the progression of CRCs. Elevated numbers of intratumoral cytotoxic and memory T cells ($CD8^+$ and $CD45RO^+$, respectively) have been shown to correlate with an aggressive phenotype reflected by tumor microinvasive status (2). This infiltration may also be a major predictor of tumor recurrence and patient survival (3). The association between $CD45RO^+$ cell infiltration and overall survival has been confirmed in stage I to IV CRCs (4). Tumors can subvert the immune system, however, notably by inducing immunoregulatory cells such as regulatory T cells (Treg).

Tregs, characterized by CD4, CD25, and Foxp3 expression, inhibit the development of an effective immune response especially against cancer or infectious agents (5). In healthy individuals, they are able to prevent autoimmune disease. In patients with cancer, Tregs expand in peripheral blood and tumor tissue. Strong Treg infiltration of tumors is generally associated with poor clinical outcome (5). Elevated blood and tumor Treg numbers have also been described in CRCs. Although the prognostic impact of Treg in CRCs is still a matter of debate, elevated peritumoral numbers of $Foxp3^+$ Treg cells are associated with advanced-stage tumors and poorer overall survival (6). In murine models of CRCs, systemic removal of Tregs using anti-CD25 antibody results in tumor rejection and in improved vaccine-induced antitumor T-cell responses (7). In addition, Treg depletion from peripheral blood of patients with CRCs unmasks $CD4^+$ and $CD8^+$ T-cell responses against tumor-associated antigens *in vitro* (8, 9). These observations suggest that Tregs could hamper the development of an effective antitumor immune response and that Treg modulation in patients with CRCs

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might boost antitumor immunity or the response to immunotherapy.

Recently, sunitinib, a multitarget tyrosine kinase inhibitor (TKI) that selectively blocks VEGF receptors 1, 2, and 3 (VEGFR-1, -2, -3), platelet-derived growth factor receptors α and β (PDGFRα and β), stem cell factor receptor (c-kit), and Flt3, has been shown to reduce Treg numbers in tumor-bearing mice and in patients with metastatic renal carcinoma (10–13). However, it is unclear whether (i) these observations are limited to patients with renal cancer and (ii) the effects of this multitarget molecule on Tregs result directly from VEGF-A/VEGFR axis inhibition or through another signaling mechanism. If an indirect action through VEGF-induced immature dendritic cells (DC) has been suggested (14, 15), the direct effect of VEGF-A on Treg has never been studied. Moreover, data on anti-VEGF-A therapy and Treg in patients with cancer are scarce and discordant (16, 17). Because bevacizumab has been approved for first- and second-line treatment of metastatic CRCs (mCRC; ref. 18), we analyzed the impact of specific VEGF-A/VEGFR blockade on Treg in a mouse model of CRCs and in patients with mCRCs and the potential direct effect of VEGF-A on tumor-induced Tregs.

Materials and Methods

Mice, tumors, and treatment

Six- to 8-week-old female Balb/c mice were purchased from Charles River Laboratories and kept in specific-pathogen-free conditions at the INSERM U970 animal facility. Experiments respected institutional guidelines and were approved by the Maisons-Alfort Veterinary School ethics committee. CT26, an *N*-nitroso-*N*-methylurethane–induced undifferentiated colon carcinoma cell line, was obtained from American Type Culture Collection. CT26 cells (2×10^5) were injected subcutaneously at day 0 in the right flank of Balb/c mice. Absence of mycoplasma was checked every 2 months, and cells were authenticated by their ability to grow in immunocompetent mice as described in the literature. Tumor growth was monitored twice a week using a caliper. Treatments were started when the tumors reached 9 to 10 mm^2. Sunitinib (SU11248, Sutent) and masitinib (4-(4-methylpiperazin-1-ylmethyl)-*N*-[4-methyl-3-(4-pyridin-3ylthiazol-2-ylamino) phenyllbenzamide-mesylate methane sulfonic acid salt) were given by oral gavage at 40 mg/kg daily and 30 mg/kg twice a day, respectively. Sunitinib and masitinib were dissolved in dimethyl sulfoxide (DMSO) and diluted in PBS before administration. Control mice received DMSO diluted in PBS. In this study, masitinib was used as a negative control to probe the VEGFR inhibition of sunitinib. Masitinib is a highly selective TKI that targets a limited number of key kinases involved in various cancers and inflammatory diseases, including c-kit, PDGFR, and Lyn but not VEGFR (19). Masitinib is currently the most selective TKI in clinical development or already approved to date (20, 21). An anti-mouse VEGF-A antibody (B20-4.1.1) was administered intraperitoneally (i.p.) at 5 mg/kg twice a week. Anti-mouse VEGFR-1 (MF1) or anti-mouse VEGFR-2 (DC101) antibodies (800 μg per mouse) were injected i.p. 3 times a week. Control mice received mouse serum. Sunitinib was kindly provided by Pfizer and anti-mouse VEGF antibody by Genentech (22), Masitinib by AB Science SA and anti-VEGFR-1 and anti-VEGFR-2 antibodies by ImClone.

Patients

Peripheral blood was obtained from patients with mCRCs receiving first-line bevacizumab plus chemotherapy or chemotherapy alone according to the referent physician's choice in the Gastroenterology Division of Georges Pompidou European Hospital (Paris, France) between March 2011 and March 2012. Blood was collected before the first treatment cycle (D0) and after the second cycle of treatment (D28). The study was approved by the local ethics committee, and all the patients gave their written informed consent before blood sampling. Patients exhibiting lymphopenia before the first cycle of treatment has been excluded. Clinical response has been evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST v1.1).

Flow cytometry

Cell surface staining of murine splenocytes and tumor-infiltrating cells used anti-mouse CD4 (RM4-5; Biolegend), anti-mouse CD25 (PC61; eBioscience), anti-mouse VEGFR-2 (89B3A5; Biolegend), and anti-mouse VEGFR-1 (141522; R&D Systems). For human studies, peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood on Ficoll-Hypaque gradients, as previously described (12). PBMCs from patients with mCRCs were stained with anti-human CD4 (OKT4; Biolegend), anti-human CD25 (BC96; eBioscience), and anti-human CD127 (eBioRDR5; eBioscience). Intracellular stainings for Foxp3 (using anti-mouse and anti-human Foxp3; FJK-16 and PHC101, respectively; eBioscience) or Ki67 (B56; BD Biosciences) were conducted after fixation and permeabilization with the reagents of the Foxp3 staining set (eBioscience). Isotype controls were run in parallel. Dead cells were excluded by using the Live/Dead Fixable Aqua Dead Cell Kit (Invitrogen). Treg proportion was assessed for all patients, but Ki67 staining was conducted only on patients included after October 2011. Stained cells were analyzed with a LSRII cytofluorometer and FACS Diva (Becton Dickinson) and Flow-Jo Software (TreeStar).

Bromodeoxyuridine labeling

After the first anti-VEGF injection, mice received 1 mg of bromodeoxyuridine (BrdUrd) solution twice a day for 2 days. Two hours after the last BrdUrd injection, the mice were killed and the spleens removed. BrdUrd was detected by flow cytometry as previously described (23).

Analysis of Treg function

After 2 weeks of treatment with sunitinib or anti-VEGF, mice were killed and their spleens removed. Tregs were purified with the Regulatory T cell Isolation Kit (Miltenyi Biotec). Purity of $CD4^+/CD25^+$ (Treg) and $CD4^+/CD25^-$ (Tconv) cells was typically above 95%. $CD4^+CD25^+$ T-cell suppressive function was studied as previously described (24). Briefly, 5×10^4 Tconvs were cultured with 1×10^5

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irradiated $CD4^+$-depleted splenocytes in the presence of anti-CD3 (145-2C11, 1 μg/mL). Tregs were added at different Treg/Tconv ratios (1:1, 1:5, 1:25). One μCi of ^{3}H-thymidine was added for the last 18 hours. ^{3}H-thymidine incorporation was analyzed using a β-scintillation counter (Perkin-Elmer).

VEGF-induced proliferation

Splenocytes from CT26 tumor–bearing mice were used. Tregs were isolated by using the Regulatory T cell Isolation Kit (Miltenyi Biotec) or cell sorting of $CD4^+CD25^+$ cells after CD4 enrichment ($CD4^+$ T cell isolation kit II, Miltenyi Biotec). Purity after cell sorting was around 99%. Purified Tregs (5×10^4) were cultured in the presence of plate-bound anti-CD3 (10 μg/mL) and interleukin (IL)-2 (100 U/mL) for 4 days. Murine VEGF (Miltenyi Biotec) was added at 50 ng/mL. Treg proliferation was assessed by measuring ^{3}H-thymidine incorporation during the last 18 hours of culture.

VEGF assay

VEGF serum concentrations in CT26 tumor–bearing mice and tumor-free mice were evaluated with an ELISA method (mouse VEGF DuoSet, R&D Systems). In humans, VEGF levels have been determined in plasma using an ELISA method (Human VEGF Quantikine Elisa kit, R&D systems).

Statistical analyses

Results are expressed as means ± SEM or ranges, as appropriate. The Mann–Whitney test was used to compare 2 groups. The Kruskal–Wallis test was used to compare 3 or more groups. The Wilcoxon-matched pairs test was used to analyze the effects of treatment between D0 and D28 in patients with mCRCs. Statistical significance was determined with Prism software (GraphPad Software, Inc). Significance was assumed at $P < 0.05$.

Results

VEGF-A/VEGFR pathway blockade is sufficient to inhibit Treg accumulation

We took advantage of a murine model of colorectal carcinoma to analyze the impact of specific VEGF-A/VEGFR blockade on modulation of Treg accumulation. CT26 tumor–bearing mice were treated with anti-VEGF-A antibody or different TKIs targeting or not VEGFR. In CT26 tumor–bearing mice, the percentage of Treg ($CD25^+$/$Foxp3^+$) among $CD4^+$ T cells was significantly enhanced in the spleen (Fig. 1A and C) and tumor-draining lymph nodes (not shown). Treatment of CT26 tumor–bearing mice with DMSO or mouse serum did not modulate Treg percentages and numbers as compared with nontreated CT26 tumor–bearing mice (not shown). After 2 weeks of treatment, anti-VEGF-A treatment reduced the percentage and absolute number of Tregs in the spleen to physiologic levels (Fig. 1A and Supplementary Fig. S3A) and in tumor-draining lymph nodes (not shown). Anti-VEGF-A also decreased Treg percentage (not shown) and number in the tumor (2,267 ± 614 Treg/mm^2 with mouse serum; 463 ± 88 Treg/mm^2 with anti-VEGF; Fig. 1B). This decrease was observed as early as 1 week after the beginning of the treatment but reached the statistical significance after 2 weeks and was maintained after 3 weeks (Supplementary Fig. S1). Sunitinib, a TKI targeting VEGFR-1, -2, -3, PDGFR, c-kit, and Flt3, resulted in a similar decrease in Treg proportion and numbers in the spleen (Fig. 1C and not shown) and tumor (2,262 ± 462 Treg/mm^2 with DMSO, 857 ± 173 Treg/mm^2 with sunitinib; Fig. 1D and not shown). Sunitinib and anti-VEGF induced strong antitumor effects in CT26 model (Supplementary Fig. S2A and S2B), but Treg modulation is not caused by the reduction of tumor size as no or only weak correlation between Treg percentage in spleens (Supplementary Fig. S2C and S2D) or tumors (Supplementary Fig. S2E and S2F) and tumor size has been observed. Interestingly, sunitinib or anti-VEGFA did not decrease the absolute number of other cell populations including $CD4^+$ T cells, $CD4^+$ $Foxp3^-$ T cells, or $CD8^+$ T cells in spleens (Supplementary Fig. S3A–S3E) or tumors (Supplementary Fig. S4A–S4D). Thus, both anti-VEGF-A and sunitinib inhibited Treg accumulation in tumor-bearing mice without depleting other T-cell subsets.

To confirm that VEGF-A/VEGFR pathway inhibition impeded Treg accumulation in tumor-bearing mice, we used masitinib as a negative control, as it is a TKI close to sunitinib that targets c-kit, PDGFR, Fak-kinases but not VEGFR (19). Contrary to sunitinib and anti-VEGF-A, masitinib did not reduce Treg proportion or numbers in the spleen (Fig. 1E) or tumor (Fig. 1F).

Thus, specific VEGF-A/VEGFR(-1,-2) inhibition was sufficient to prevent tumor-induced Treg accumulation in animals spleens and tumors.

Bevacizumab reduces Treg proportion in peripheral blood of mCRC patients

To confirm that these observations were also relevant in human, we first quantified Tregs in peripheral blood of patients with mCRCs. As previously described (6), Treg percentages were higher in patients with mCRCs than in healthy volunteers (HV; Fig. 2A). This difference could not be related to age difference between healthy volunteers and patients with mCRCs (51.05 ± 13 and 66.7 ± 11.5 years old, respectively) as we and others have not observed an age-related effect on Treg proportion (ref. 6 and not shown). After 2 cycles of bevacizumab plus chemotherapy, blood Treg percentage was significantly reduced (day 0: 5.94 ± 0.5 vs. day 28: 4.14 ± 0.41, $P = 0.0039$). As the standard first-line treatment for mCRC is chemotherapy, with or without bevacizumab, we were unable to study patients treated with bevacizumab alone. We thus studied patients treated with chemotherapy alone as controls. It is of note that no major differences between patients treated with chemotherapy associated with bevacizumab or chemotherapy alone have been observed (Supplementary Table S1). Interestingly, patients treated with 2 cycles of chemotherapy alone showed no significant decrease in their peripheral blood Treg percentages (day 0: 6.4 ± 1.09 vs. day 28: 5.85 ± 0.97, $P = 0.19$), suggesting that the decrease observed in patients treated with chemotherapy plus bevacizumab was due to bevacizumab (Fig. 2B and C). Chemotherapy alone or associated with

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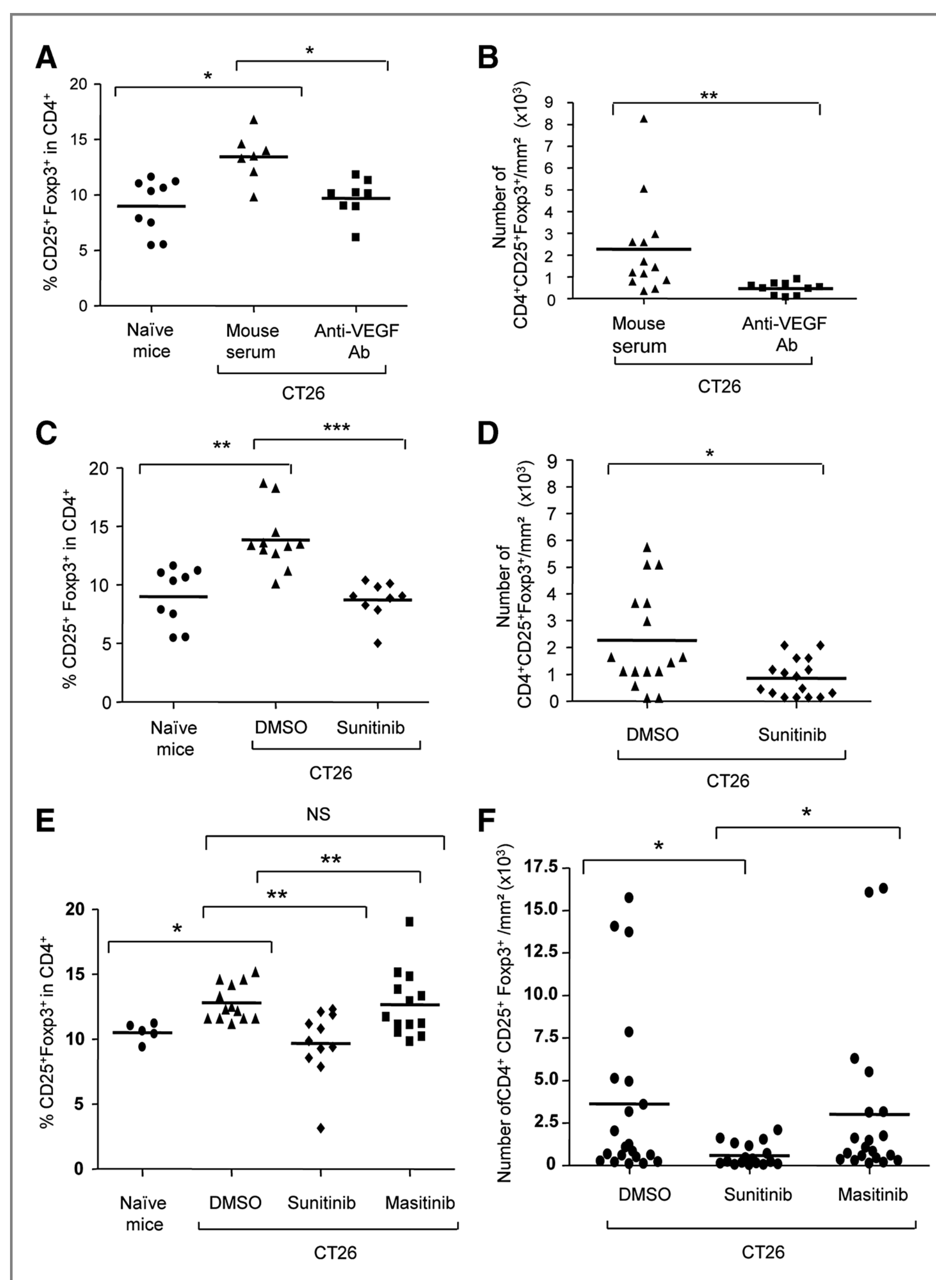

Figure 1. Anti-VEGF-A and sunitinib treatments significantly reduce spleen and tumor Treg proportions and numbers in a mouse model of CRCs. A and B, Tregs are significantly reduced in the spleen and tumors after anti-VEGF-A treatment. Balb/c mice were injected subcutaneously with 2×10^5 CT26 tumor cells. Treatment was started when the tumors reached 9 to 10 mm^2. Anti-VEGF-A (100 μg/mouse twice a week) or mouse serum were administered i.p. After 2 weeks of treatment, mice were sacrificed. Splenocytes and tumor-infiltrating lymphocytes were stained for $CD4^+CD25^+Foxp3^+$ Tregs. A, the graphs depict the percentage of $CD25^+/Foxp3^+$ cells among total $CD4^+$ T cells in spleens in 2 independent experiments with 3 to 5 mice per group. B, in tumors, Treg numbers per mm^2 are shown from pooled results of 2 experiments with 5 to 7 mice per group. This experiment was carried out at least 4 times with similar results. C and D, Treg proportions and numbers are significantly reduced in spleens and tumors after sunitinib treatment. Same experimental setting as in A and B, but Treg staining was conducted after 2 weeks of sunitinib or DMSO treatment. C, the graphs depict the percentage of $CD25^+/Foxp3^+$ cells among total $CD4^+$ T cells in spleens in 2 independent experiments with 4 to 6 mice per group. D, in tumors, Treg numbers per mm^2 are shown from pooled results of 2 experiments with 8 mice per group. These experiments were carried out at least 4 times with similar results. E and F, same experimental setting as in A and B, but mice received sunitinib (40 mg/kg), masitinib (30 mg/kg twice daily), or DMSO (as a control). Mice were studied after 2 weeks of treatment. Treg staining was conducted on splenocytes (E) or tumor-infiltrating cells (F). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

bevacizumab did not result in lymphopenia (Supplementary Fig. S5A) and did not affect the percentages of $CD4^+$ and $CD8^+$ T-cell subsets (Supplementary Fig. S5B and S5C).

Thus, these data suggest that in patients with mCRCs, neutralization of VEGF-A could also induce a decrease in Treg proportion.

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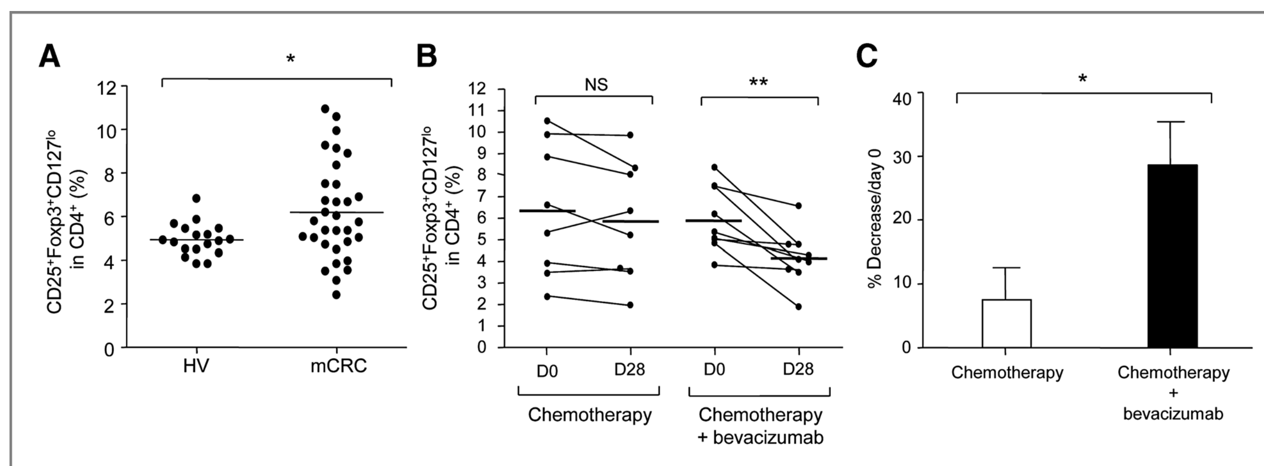

Figure 2. Chemotherapy combined with bevacizumab but not chemotherapy alone reduces Treg proportion in peripheral blood of patients with mCRCs. A, Treg proportion is enhanced in peripheral blood of patients with mCRCs compared with healthy volunteers. The Treg proportion ($CD25^+Foxp3^+CD127^{lo}$ cells among $CD4^+$ cells) was analyzed in the peripheral blood of healthy volunteers (HV) and patients with mCRCs at diagnosis. B and C, Treg proportion was monitored in peripheral blood of patients with mCRCs before (day 0) and after 2 cycles of treatment (day 28) with bevacizumab plus chemotherapy ($n = 9$) or chemotherapy alone ($n = 8$). The proportion of $CD25^+Foxp3^+CD127^{lo}$ among $CD4^+$ cells was shown for each patient before (D0) and after 2 cycles of treatment (D28; B). **, $P < 0.01$. The percentage decrease in Treg was calculated as (% Treg at day 0 − % Treg at day 28)/% Treg at day 0 (C). *, $P < 0.05$.

VEGF-A/VEGFR blockade does not affect suppressive function of Treg

We next determined whether blocking VEGF-A/VEGFR pathway using anti-VEGF-A or a TKI targeting VEGFR such as sunitinib modulated Treg function. Purified Tregs from the spleens of CT26 tumor–bearing mice treated with sunitinib, anti-VEGF-A mAb, or vehicle were cocultured at different ratios with conventional $CD4^+$ $CD25^-$ T cells stimulated by irradiated $CD4^+$-depleted splenocytes and anti-CD3 as previously described (24). Proliferation was assessed in terms of ^{3}H-thymidine incorporation. Tregs from both sunitinib- and anti-VEGF-A–treated mice maintained their capacity to inhibit conventional T-cell proliferation (Fig. 3A) and IFN-γ secretion (stimulated Tconv: 1,744 ± 30 pg/mL; stimulated T conv + Treg: 425 ± 99 pg/mL; stimulated Tconv + Treg sunitinib: 357 ± 89 pg/mL; stimulated Tconv + Treg anti-VEGF-A Ab: 121 ± 100 pg/mL; Fig. 3B). Likewise, conventional $CD4^+$ $Foxp3^-$ T cells are not decreased by sunitinib or anti-VEGF treatments (Supplementary Figs. S3D and S4C). Susceptibility of conventional T cells to Treg suppression was unaffected by these treatments (data not shown). Thus, VEGF-A/VEGF-R(-1,-2) pathway blockade by anti-VEGF-A or sunitinib depleted Tregs in the spleen and tumor but did not modulate their function.

VEGF-A directly induces Treg proliferation in tumor-bearing mice

Treg accumulation in tumor-bearing mice could occur through different mechanisms, such as expansion of preexisting Tregs, conversion of conventional naïve or memory $CD4^+$ T cells into Tregs, and migration or preferential survival of Tregs (25). Although VEGF-A can induce Treg differentiation by generating immature DCs (14, 15, 26), it is also an anti-apoptotic and a proliferation factor for several cell subtypes, including endothelial cells and tumor cells (27). Whether VEGF-A secreted by tumor cells can directly act on Treg is unclear. *In vitro*, CT26 cells were able to secrete high levels of VEGF-A (332.5 pg/mL ± 10.4 per 2.10^4 cells after 72 hours). To examine the effect of VEGF-A on Treg in tumor-bearing mice, we first measured blood VEGF-A levels in tumor-bearing mice and healthy controls. Serum VEGF-A levels were indeed enhanced in CT26 tumor–bearing mice (Fig. 4A). Proliferation of splenic Treg (measured using the cell-cycle marker Ki67) but not conventional T cells was enhanced in CT26-bearing mice as compared with healthy controls (Fig. 4B and Supplementary Fig. S6A). Anti-VEGF-A treatment of tumor-bearing animals reduced the percentage of $Ki67^+$ (proliferating) Tregs as compared with animals receiving mouse serum (Fig. 4C). This was confirmed by the injection of BrdUrd, a nucleoside analogue that is selectively incorporated by dividing cells. This decrease is observed as early as day 7 after the beginning of the treatment but reached significance after 2 weeks (not shown). After only one injection of anti-VEGF-A, the proportion of $BrdUrd^+$ Tregs was significantly reduced (Fig. 4D). Similar results were observed with sunitinib (data not shown). Interestingly, anti-VEGFA has no effect on proliferation of conventional T cells (Supplementary Fig. S6A). As previously described (28), only $CD44^{hi}$ memory Treg proliferated in tumor-bearing animals. Proliferation of this Treg subset is decreased after anti-VEGF-A treatment (Fig. 4E). Thus, VEGF-A appeared to induce Treg proliferation in tumor-bearing mice.

We then tested the capacity of VEGF-A to directly induce Treg proliferation. Treg proliferation, assessed by measuring ^{3}H-thymidine incorporation, was induced when anti-CD3 was combined with IL-2, but not with anti-CD3 alone, as previously reported (29). Interestingly, Treg proliferation was further enhanced when VEGF-A was added (Fig. 4F), suggesting that VEGF-A can directly enhance mitogenic-induced Treg proliferation. *In vitro* studies showed that VEGF-A did not improve Treg survival (data not shown).

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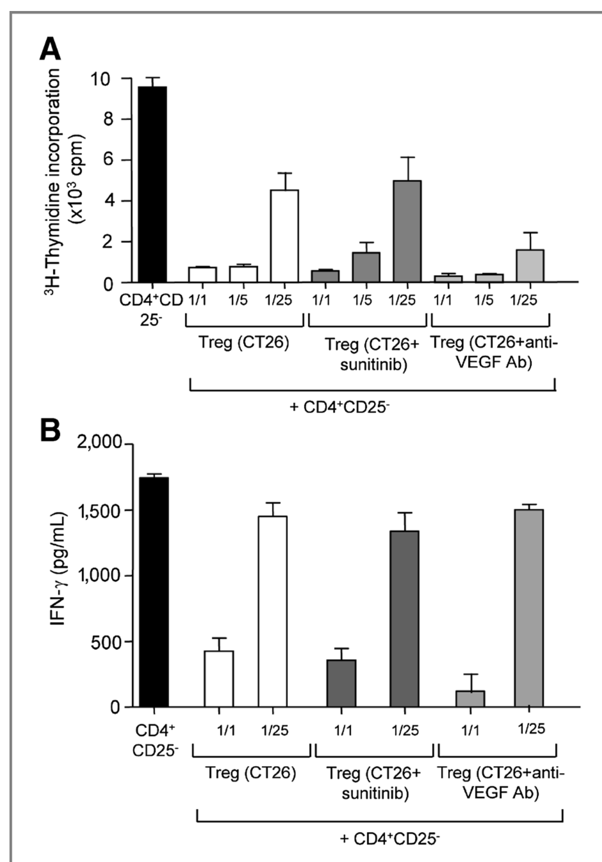

Figure 3. Treg function is not modulated by anti-VEGF-A or sunitinib treatment. A and B, same experimental setting as in Fig. 1. Tregs purified from splenocytes were cultured with conventional $CD4^+CD25^-$ T cells as described in Materials and Methods. A, proliferation was assessed by measuring ^{3}H-thymidine incorporation. In controls, Tregs from tumor-bearing mice = 93 cpm, Tregs from tumor-bearing mice treated with sunitinib = 112 cpm, Tregs from tumor-bearing mice treated with anti-VEGF = 65 cpm. B, IFN-γ production was analyzed in the culture supernatant.

Altogether, these results suggest that Treg proliferation observed in tumor-bearing mice may be induced, at least in part, by a direct action of VEGF-A, whereas VEGF-A inhibition blocks this Treg proliferation.

Treg proliferation is dependent on VEGFR-2 expression by Treg

In healthy mice, only a small proportion of Tregs expressed VEGFR-1 or -2, whereas expression of both VEGFR-1 and -2 was enhanced in tumor-bearing mice (Fig. 5A). To determine whether tumor-induced Treg proliferation was dependent on VEGFR-1 and/or -2, we administered anti-VEGFR-1 or -2 antibodies for 2 weeks to mice with tumors. Anti-VEGFR-2 but not anti-VEGFR-1 treatment decreased Treg proportion in spleen (Fig. 5B). Furthermore, anti-VEGFR-1 treatment had no effect on Treg proliferation assessed by Ki67 expression (Fig. 5C, left), whereas anti-VEGFR-2 treatment inhibited it (Fig. 5C, right).

Thus, VEGFR-2, but not VEGFR-1, appears to be involved in VEGF-A–induced Treg expansion in this mouse model of CRCs.

Bevacizumab reduces Treg proliferative status in peripheral blood of mCRC patients

Then, we tried to confirm results observed in the CT26 mouse model in patients with mCRCs. We first showed that plasma VEGF level in patients with mCRCs was enhanced compared with healthy volunteers (Fig. 6A). Then, we found that in patients with mCRC, the proportion of cycling Tregs but not conventional T cells was significantly enhanced as compared with healthy volunteers (Fig. 6B and Supplementary Fig. S6B). After 2 cycles of treatment, the percentage of $Ki67^+$ Treg in peripheral blood seems to be reduced in patients treated with chemotherapy plus bevacizumab (Fig. 6C) but not in patients treated with chemotherapy alone (Fig. 6D). Interestingly, bevacizumab associated with chemotherapy has no impact on proliferation of conventional T cells (Supplementary Fig. S6C).

Thus, these results suggest that in patients with mCRCs, neutralization of VEGF-A might decrease Treg proliferation in peripheral blood.

Altogether, these results suggest that specific VEGF-A/VEGFR-2 inhibition could prevent VEGF-A–induced Treg proliferation resulting in a Treg decrease in a mouse model of CRCs and in patients with mCRCs.

Discussion

Antiangiogenic molecules, especially those targeting VEGF-A signaling, have recently been shown to modulate Tregs. In physiologic conditions in naïve mice, sunitinib has been reported to decrease Treg percentages (30) but this was not confirmed by other groups (10). We found that sunitinib and anti-VEGF-A treatment did not reduce Treg percentages in naïve mice (data not shown). In contrast, sunitinib and sorafenib, another TKI targeting the VEGFR family, have been shown to reduce Treg frequency in tumor-bearing animals and patients with metastatic renal cancer (10–13, 31–34). However, these TKIs have multiple targets such as c-Kit and PDGFR that can have a direct impact on immune cells such as DC, natural killer (NK), and others (35, 36). The specific role of targeting the VEGF/VEGFR pathway has thus not been clearly shown with these works. More recently, 2 studies suggested an impact of a specific VEGF/VEGFR blockade on Tregs. First, anti-VEGF-A treatment has been suggested in a single immunofluorescence study of a mouse model of breast cancer to decrease tumor infiltrating Tregs (37). Second, administration of an adenoviral vector expressing soluble VEGFR-1/-2 has also been described to reduce tumor Treg proportion in a mouse model of CRCs (38). However, these data are both preliminary and scarce and as soluble VEGFR-1/-2 could also block VEGF-B and placenta growth factor (PlGF), it is difficult to conclude about the specific role of VEGF-A blockade on Treg. Here, in a mouse model of CRC, we show that sunitinib and anti-VEGF-A reduce the proportion and number of Tregs in tumor and spleen without reducing other T-cell subset numbers. However, masitinib, a TKI that does not target the VEGF-A/VEGFR axis, but targets c-Kit and PDGFR did not deplete Treg in our model.

Similar results to those obtained in our mouse colorectal tumor model were obtained in patients with mCRCs. Indeed, high circulating levels of VEGF-A were observed, the Treg proportion was enhanced in the peripheral blood of patients

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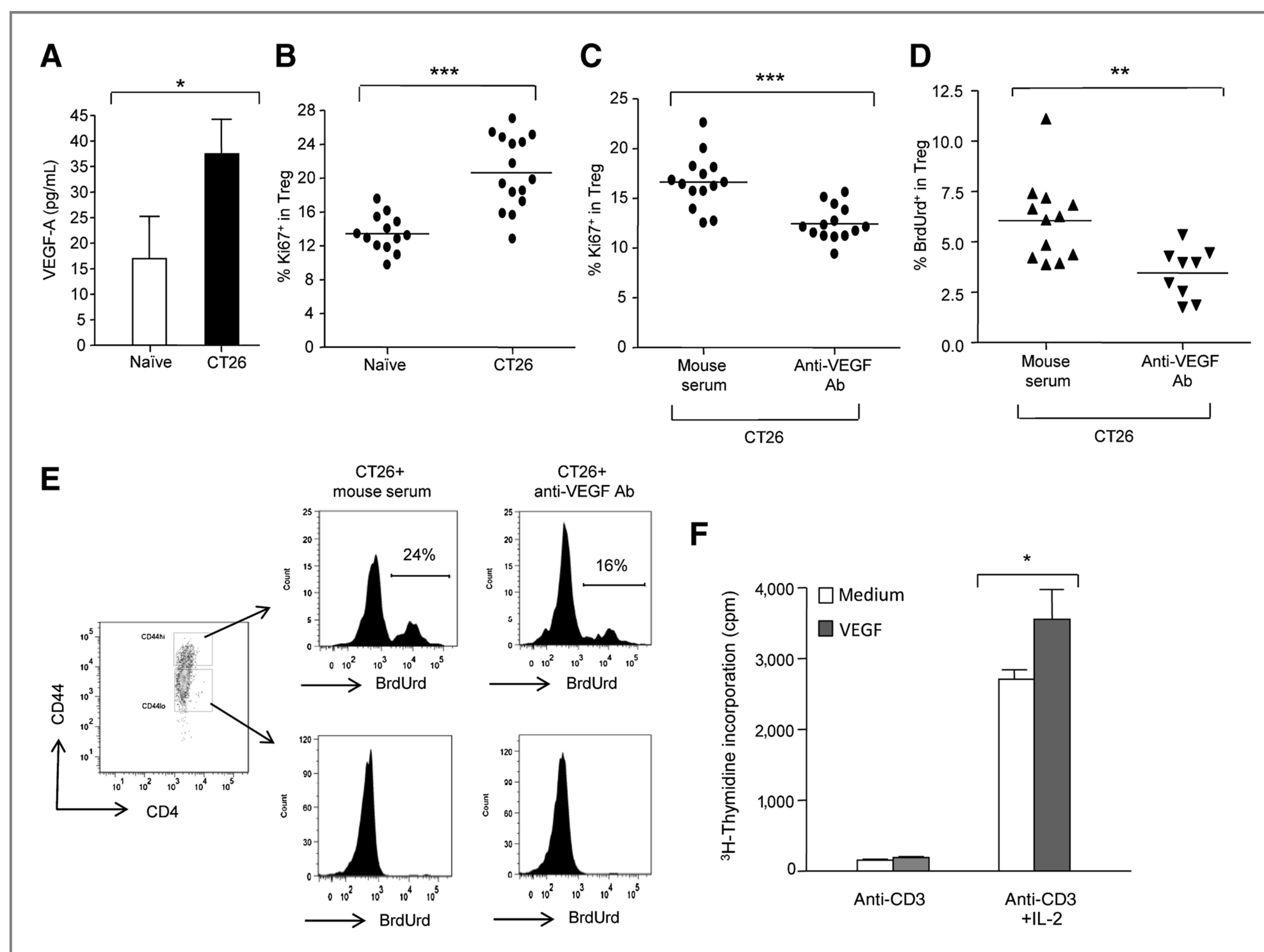

Figure 4. Tregs proliferate in CT26 tumor–bearing mice in response to VEGF-A stimulation. A, elevated serum VEGF-A levels in CT26 tumor–bearing mice. VEGF-A levels were measured by ELISA in serum of naïve and CT26 tumor–bearing mice. B, Treg proliferation is enhanced in CT26 tumor–bearing mice compared with healthy mice. Tregs from splenocytes of naïve and tumor-bearing mice were identified by $CD4^+/CD25^+/Foxp3^+$ staining and analyzed for Ki67 expression. Data pooled from 3 independent experiments with 4 to 5 mice per group are shown. C and D, anti-VEGF-A treatment downregulates Treg proliferation in CT26 tumor–bearing mice. Anti-VEGF-A was administered to CT26 tumor–bearing mice, and Ki67 (C) and BrdUrd incorporation (D) by Treg in spleens was studied. Data pooled from 3 independent experiments with 3 to 5 mice per group are shown. E, anti-VEGF-A Ab decreases $CD44^{hi}$ Treg proliferation. Same experimental setting as in D but showing flow cytometric analysis of BrdUrd incorporation in $CD44^{hi}$ and $CD44^{lo}$ Treg after anti-VEGF-A or mouse serum treatment. F, recombinant mouse VEGF-A directly increases Treg proliferation *in vitro*. Purified Tregs were cultured in the presence of plate-bound anti-CD3 and IL-2, with or without VEGF-A. Proliferation was assessed by measuring ^{3}H-thymidine incorporation. This graph depicts one representative experiment of 3. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

with CRCs, and treatment with bevacizumab reduced it. In a preliminary study, Treg decrease seems to correlate with tumor response to chemotherapy associated with bevacizumab according to RECIST. Tregs might be a useful predictive tool on the efficacy of chemotherapy associated with bevacizumab. However, these results should be confirmed in a larger series of patients.

Altogether, these observations suggest that AA molecules modulate a tumor-related phenomenon and that specific blockade of the VEGF-A/VEGFR axis by an anti-VEGF mAb is sufficient to inhibit Treg accumulation in mouse spleen and tumor in our CRC model and in peripheral blood of patients with mCRCs.

Tumor-induced Treg expansion is classically explained by 4 possible mechanisms. The first is a preferential Treg recruitment to the tumor: Tregs express receptors for chemokine such as CCR4, CCR5, CXCR4, and CCR10 that could induce their migration toward the tumor (25, 39, 40). Second, naïve and memory conventional T cells can convert into Tregs with the help of immature antigen-presenting cells or myeloid-derived suppressor cells (MDSC; refs. 25, 26, 41). Third, naturally occurring Tregs are more resistant to oxidative stress than conventional T cells, possibly contributing to their survival in stressful tumor environments (42). Finally, Tregs themselves can proliferate. In physiologic settings, Tregs proliferate more strongly than naïve $CD4^+$ T cells (43). We found that $9.7\% \pm 0.7\%$ of Tregs in spleens of tumor-free mice (Fig. 4B) and $13.59\% \pm 1.5\%$ of Tregs from peripheral blood of healthy volunteers (Fig. 6B) expressed the cycling marker Ki67. This physiologic proliferation can be enhanced in various circumstances, including cancer (28). In mouse models of mammary tumors and melanoma, Treg proliferation is higher in draining lymph nodes at the time of tumor

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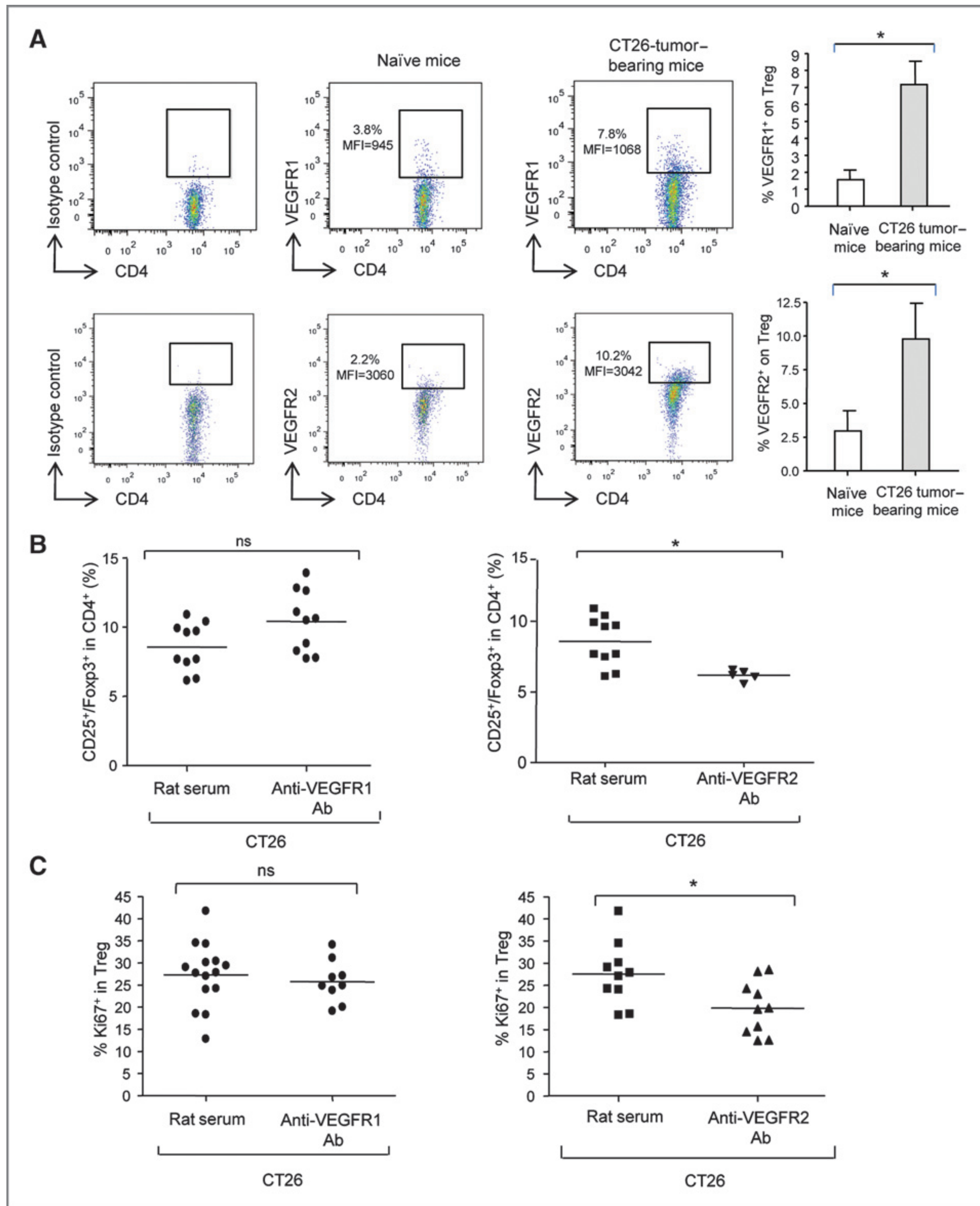

Figure 5. Tregs in tumor-bearing mice express VEGF receptors and their proliferation is VEGFR-2–dependent. A, expression of VEGFR-1 and -2 was analyzed in Tregs obtained from splenocytes of tumor-bearing and tumor-free mice (naïve mice). Dead cells were removed using Live/Dead staining. A representative staining on Treg is shown (left) and pooled results from at least 3 experiments are shown (right). *, $P < 0.05$ in the Mann–Whitney test. B, anti-VEGFR-2 treatment decreases Treg proportion in spleens of CT26 tumor–bearing mice. Balb/c mice were injected subcutaneously with 2×10^5 CT26 tumor cells. Treatment was started when the tumors reached 9 to 10 mm^2. Anti-VEGFR-1, -2 (800 μg/mouse 3 times a week) or rat serum (as control) was administered i.p. Treg proportion in spleens is shown. C, Treg proliferation in tumor-bearing mice depends on VEGFR-2 signaling. Same experimental setting as in B, but Ki67 expression by Treg was analyzed after 2 weeks of treatment. NS, not significant. *, $P < 0.05$.

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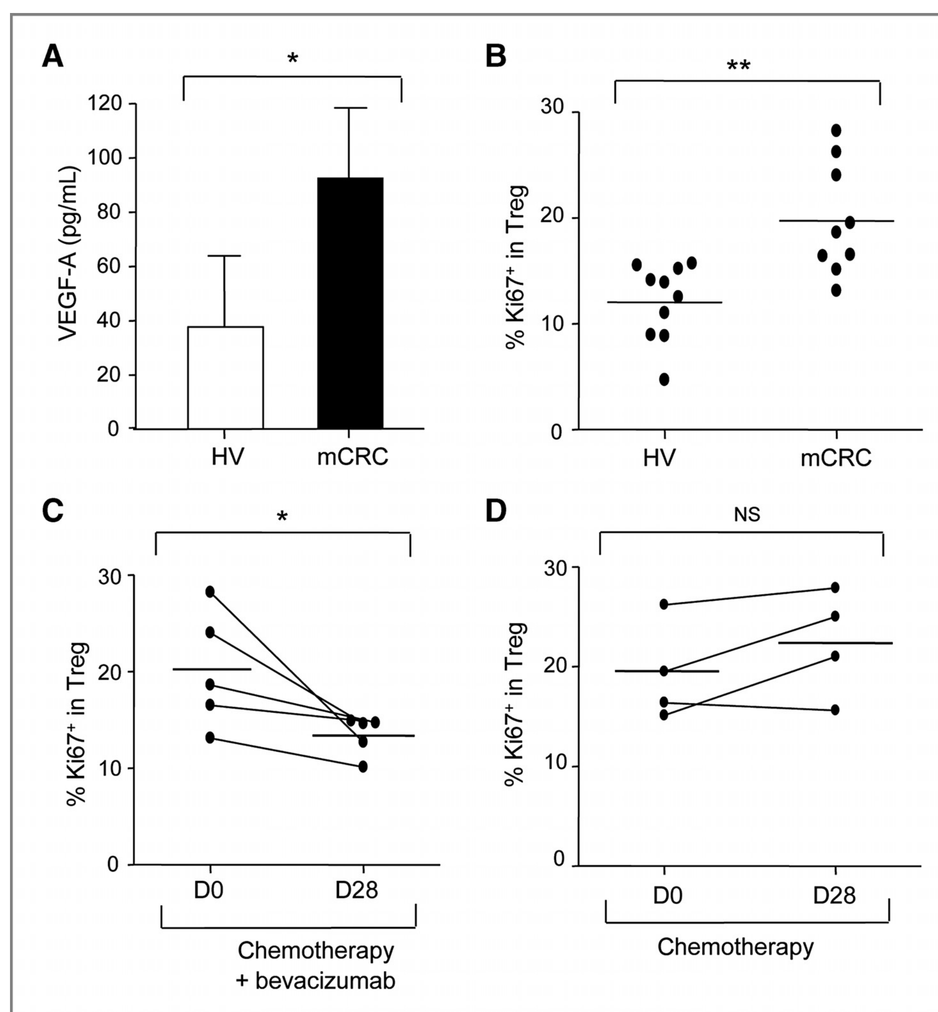

Figure 6. Bevacizumab reduces Treg proliferation in patients with mCRCs. A, elevated plasmatic VEGF-A levels in patients with mCRCs. VEGF-A levels were measured by ELISA in plasma of patients with mCRCs or healthy volunteer (HV). B, Treg proliferation is enhanced in the peripheral blood of patients with mCRCs compared with healthy volunteers. Tregs from peripheral blood of healthy volunteers and patients with CRCs were analyzed for Ki67 expression. *, $P < 0.05$. C and D, bevacizumab reduces Treg proliferation in patients with mCRCs. Ki67 expression was assessed in Tregs from patients with mCRCs treated with chemotherapy + bevacizumab (C) or chemotherapy alone (D) before (D0) and after 2 cycles of treatment (D28). NS, not significant. *, $P < 0.05$.

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emergence than in healthy controls (28). We confirm these data in our colorectal tumor model. Treg proliferation was enhanced in the spleen, both at the time of tumor emergence and also when the tumors were established (>100 mm^2; Fig. 3B). In human, 2 studies have shown an increased proportion of dividing Tregs in tumors or peripheral blood in patients with breast cancer and acute myeloid leukemia (44, 45). We found here that the proportion of Ki67-expressing Tregs was enhanced in peripheral blood of metastatic patients with CRCs as compared with healthy volunteers (Fig. 6B).

As CT26 tumor cells produce VEGF-A (Fig. 3A) and AA molecules targeting VEGF-A inhibit tumor-induced Treg accumulation, we postulated that VEGF-A could be partly responsible for tumor-induced Treg accumulation. VEGF-A acts as a proliferative factor for endothelial and some cancer cells (27). We found that anti-VEGF-A treatment reduced Treg cycling, as assessed by Ki67 expression and BrdUrd incorporation, and that VEGF-A directly enhanced the proliferation of Tregs derived from tumor-bearing mice. To our knowledge, VEGF-A has not previously been described as a proliferative factor for Tregs.

Immature myeloid cells that expand during tumor progression, such as TGFβ-expressing DCs and MDSCs, are generally thought to be responsible for tumor-induced Treg proliferation (26, 41). Here, we describe a novel mechanism whereby VEGF-A, which is probably produced by colorectal tumor cells, directly promotes Treg proliferation. In patients with CRCs, Foxp3 expression in CD4$^+$ T cells decreased after surgery (46). VEGF-A produced by the tumor cells could, at least in part, explain this phenomenon. Antiangiogenic (AA) molecules targeting the VEGFR pathway inhibited tumor-induced Treg accumulation, at least partly, by inhibiting VEGF-induced Treg proliferation.

Little is known on Treg VEGFR-1 and -2 expression. In mice, CD4$^+$ T cells express very low levels of VEGFR unless they are activated with anti-CD3/CD28 (47), and to our knowledge, no specific data on CD4$^+$Foxp3$^+$ Tregs have been published. Suzuki and colleagues have suggested that human Tregs expressed VEGFR-2 in a TGFβ-dependent manner (48). We show here that Tregs express both VEGFR-1 and -2 in tumor-bearing but not in healthy mice. VEGFR-2 is the main mediator of endothelial cell proliferation, whereas VEGFR-1 seems more involved in their migration (49). We extend these observations by showing that VEGF-A–induced Treg proliferation is dependent on Treg VEGFR-2 signaling and not on VEGFR-1 signaling.

Although AA molecules such as sunitinib and anti-VEGF modulate immunosuppressive cells, it could be interesting to determine whether these treatments could restore a specific antitumor immune response. Suppression of tolerogenic pathways induced by the tumor seems to be a key to successful cancer immunotherapy. Several approaches have been developed to modulate Tregs in cancer. Low-dose cyclophosphamide inhibits or depletes Tregs in the context of a tumor and enhances antitumor responses (50). Other strategies using anti-CD25 or anti-GITR antibodies could modulate Treg numbers or function but would also affect activated effector T cells (25). Interestingly, we found that VEGF-A/VEGFR-2 pathway blockade did not deplete all Tregs but simply restored their proportion to physiologic levels. Thus, to combine antiangiogenic molecules targeting VEGF-A/VEGFR with immunotherapeutic approaches might be interesting to target selectively Tregs avoiding the depletion of effector T cells and to minimize the occurrence of autoimmune mediated side effects often associated with a total Treg depletion.

In conclusion, we show that specific inhibition of the VEGF-A/VEGFR axis is sufficient to prevent Treg accumulation in a CRC mouse model and in patients with mCRCs. Treg proliferation was enhanced in tumor-bearing animals and this was, at least in part, due to a direct action of VEGF-A on Tregs via VEGFR-2. VEGF-A/VEGFR-2 blockade by AA molecules inhibited this proliferation. Combining VEGFA/VEGFR-2–targeting AA molecules with immunotherapeutic approaches in the future might thus be relevant in patients with CRCs.

Disclosure of Potential Conflicts of Interest

A.F. Carpentier is a consultant/advisory board member of Roche. J. Taieb has advisory role in Roche and research grant from Roche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Terme, S. Pernot, E. Marcheteau, F. Sandoval, N. Benhamouda, O. Colussi, A.F. Carpentier, E. Tartour, J. Taieb
Writing, review, and/or revision of the manuscript: M. Terme, S. Pernot, A. F. Carpentier, J. Taieb
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Terme
Study supervision: J. Taieb
Inclusion of patients: O. Dubreuil

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