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Cancer Research

Rapalogs Efficacy Relies on the Modulation of Antitumor T-cell Immunity

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Abstract

The rapalogs everolimus and temsirolimus that inhibit mTOR signaling are used as antiproliferative drugs in several cancers. Here we investigated the influence of rapalogs-mediated immune modulation on their antitumor efficacy. Studies in metastatic renal cell carcinoma patients showed that everolimus promoted high expansion of $FoxP_3^+Helios^+Ki67^+$ regulatory CD4 T cells (T_{regs}). In these patients, rapalogs strongly enhanced the suppressive functions of T_{regs}, mainly in a contact-dependent manner. Paradoxically, a concurrent activation of spontaneous tumor-specific Th1 immunity also occurred. Furthermore, a high rate of $Eomes^+CD8^+$ T cells was detected in patients after a long-term mTOR inhibition. We found that early changes in the T_{regs}/antitumor Th1 balance can differentially shape the treatment efficacy. Patients presenting a shift toward decreased T_{regs} levels and high expansion of antitumor Th1 cells showed better clinical responses. Studies conducted in tumor-bearing mice confirmed the deleterious effect of rapalogs-induced T_{regs} via a mechanism involving the inhibition of antitumor T-cell immunity. Consequently, the combination of temsirolimus plus CCR4 antagonist, a receptor highly expressed on rapalogs-exposed T_{regs}, was more effective than monotherapy. Altogether, our results describe for the first time a dual impact of host adaptive antitumor T-cell immunity on the clinical effectiveness of rapalogs and prompt their association with immunotherapies. *Cancer Res; 76(14); 4100–12. ©2016 AACR.*

Introduction

mTOR protein is a conserved serine/threonine kinase involved in the regulation of cell growth, metabolism, and apoptosis (1). It exerts its physiologic functions through two distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2) downstream of the PI3K/AKT pathway (1). Oncogenic activation of mTOR signaling induces several processes required for the growth, survival, and proliferation of cancer cells (2). Thus, mTOR inhibition has gained great interest in cancer therapy and many rapamycin analogs (rapalogs) are now being used in clinical settings (3). Everolimus and temsirolimus are two rapalogs approved for breast cancer, neuroendocrine carcinoma treatments, and relapsing metastatic renal cell carcinoma (mRCC) patients (4–7).

mTOR also represents a key regulator of immune responses. Notably, this pathway is determinant for the differentiation, homeostasis, and functional regulation of both CD4 and CD8 T-cell subsets (8). The lack of mTOR in naïve CD4 T cells has been shown to promote preferentially forkhead box transcription factor ($FoxP_3^+$) regulatory T cells (T_{regs}) to the detriment of Th1, Th2, or Th17 differentiation (9, 10). In solid organ transplantation, rapalogs promote T_{regs} induction and create an immunosuppressive environment required to prevent from graft rejection (11, 12). Interestingly, it has been recently reported that organ transplant recipients treated with rapalogs have a lower risk of developing cancer, suggesting an impact of mTOR inhibition on antitumor immune responses (13). Indeed, recent immunologic studies showed that blocking mTOR signaling can also promote memory T-cell functions and tumor immunity in animal models (14–16). However, the rapalogs-mediated modulation of antitumor T-cell immunity and its impact on treatment efficacy have not been investigated in patients with cancer.

On the basis of the critical role played by adaptive T-cell immunity in cancer (17, 18), we hypothesized that anticancer rapalogs could promote suppressive T_{regs}, which in turn could

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be detrimental for host antitumor T-cell immunity. In this regard, we recently described a striking modulation of T-cell responses in a mRCC patient treated with everolimus (19). This patient presented at the time of disease control a strong antitumor Th1 response, which was completely lost upon disease progression when high T_{regs} expansion occurred.

Here, we studied the modulation of both T_{regs} and antitumor T-cell responses in a cohort of mRCC patients treated with everolimus. The influence of immune modulation on treatment efficacy was investigated in our cohort and confirmed in mouse tumor models.

Patients and Methods

Patients and sample collections

mRCC patients treated with everolimus were enrolled after the signature of informed consent at the University Hospital Minjoz (Besançon, France) between November 2011 and January 2015. Everolimus was administrated 10 mg daily, or 5 mg daily when occurrence of adverse events. Blood samples were collected at baseline and every 2 months. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll-Hyperpaque gradient (Sigma-Aldrich) and frozen until use. Disease was classified as defined by Heng and colleagues (20) and evaluation of response was performed according to RECIST.

Monitoring of T_{regs} and telomerase-specific Th1 responses in mRCC patients

T_{regs} staining protocol is detailed in Supplementary Materials section. Samples were acquired on a FACSCanto II (BD Biosciences) and analyzed with the Diva or FlowJo softwares. Antitumor Th1 responses were assessed after *in vitro* stimulation of PBMC with a mixture of HLA-DR–restricted peptides derived from telomerase (TERT; 5 μg/mL) during 7 days (21, 22). The presence of specific T cells was measured by IFNγ-ELISPOT Assay (Diaclone; ref. 21). Spot-forming cells were counted using the C.T.L. Immunospot System (Cellular Technology Ltd). The number of specific T cells expressed as spot-forming cells per 10^5 cells was calculated after subtracting negative control values (background). Responses were positive when IFNγ spots number was higher than 10 and more than twice the background.

T_{regs} suppressive assay

T_{regs} functions were evaluated in a CellTrace 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled T-cell Proliferation Assay (Invitrogen). Briefly, 5×10^5 fresh allogenic T cells from healthy donors labeled with CFSE were cocultured for 3 days at 1:2 ratio with freshly sorted T_{regs} from healthy donors or patients, or at 1:1 ratio with sorted T_{regs} from *in vitro* culture in the presence of coated anti-CD3 (2.5 μg/mL) and soluble anti-CD28 (5 μg/mL) antibodies (BD Biosciences). Cytokines production was measured by ELISA (Diaclone). Proliferation suppression assays were also performed using transwell columns (Merck Millipore) to separate 3×10^5 T_{regs} (top chambers) from 3×10^5 allogenic T cells (bottom chambers) in the presence of soluble anti-CD3 (5 μg/mL) and anti-CD28 (5 μg/mL) antibodies (BD Biosciences).

Tumor cell lines

The murine RCC RENCA and the melanoma-B16F10 cells transfected with ovalbumin (B16-OVA) were kindly provided by E. Tartour (INSERM U970). The murine mammary carcinoma cell line 4T1 was kindly provided by Dr. Apetoh (INSERM U866, Dijon, France). All cells were periodically authenticated by morphologic and histologic inspection, and animal grafting for assessing their ability to grow. Cells were regularly tested for mycoplasma using Myco Alert Kit (Lonza).

Mice

Female C57BL/6NCrl and BALB/cAnCrl mice, 6 to 8 weeks old, were purchased from Charles River Laboratories and housed under pathogen-free conditions. FoxP3-eGFP and DEREG transgenic mice (23) were kindly provided by Dr. Perruche (INSERM UMR1098, Besançon, France). All experimental studies were approved by the local ethics committee (#58) and the French Ministry of Higher Education and Research and were conducted in accordance with the European Union's Directive 2010/63.

Tumor challenge and treatment

BALB/cAnCrl mice were subcutaneously injected with 5×10^5 RENCA or 10^5 4T1 cells in 100 μL of saline buffer in the abdominal flank or in the mammary zone, respectively. C57BL/6NCrl, $FoxP_3$-eGFP, or DEREG mice were subcutaneously injected with 2×10^5 B16-OVA cells in 100 μL of saline buffer in the abdominal flank. Tumor growth was monitored every 2 to 3 days and mice were euthanized when tumor mass reached 300 mm^2. When tumors reach 20 mm^2, mice were treated either with 2 mg/kg of temsirolimus intraperitoneally every 3 days or with everolimus administrated orally everyday by gavage at 0.65 mg/kg. The rapalogs were used at concentrations based on the study of their pharmacokinetics in patients (24). Mice from control groups were injected with the solvent used to dissolve drugs. Rapamycin (Sigma-Aldrich) was administrated intraperitoneally at 75 μg/kg/day. The CCR4 antagonist (AF399/420/18 025) provided by Dr. Bayry (INSERM U872) was injected intraperitoneally at 1.5 μg/3 days.

In vivo T-cell depletion experiments

To study the implication of immune cells on the antitumor effect of rapalogs, mice were injected intraperitoneally before tumor graft then every 2 weeks with 200 μg of monoclonal-depleting antibodies (mAb). Anti-CD4 (clone GK1.5), CD8 (2.43), and CD25 (PC61.5) antibodies or isotype controls were purchased from BioXcell. To deplete T_{regs}, mice were injected intraperitoneally twice (day −4 and day 0) before tumor graft with 250 μg of PC61.5 mAb (BioXcell). DEREG mice were injected intraperitoneally with 80 μg/kg of diphtheria toxin (Sigma-Aldrich) to deplete T_{regs}. Depletion efficiency was checked in the blood.

Assessment of OVA-specific T-cell responses

The ovalbumin-specific T cells were analyzed *ex vivo* in splenocytes and in tumor-infiltrating lymphocytes (TIL). TILs were recovered after tumor treatment with DNAse, hyaluronidase, and collagenase (Sigma-Aldrich). The $OVA_{257–264}$ (SIINFEKL, SL8) K^b-Dextramer (Immudex) staining was used to quantify OVA-specific CD8 T cells. Functionality of $OVA_{257–264}$-specific CD8 T cells was analyzed by IFNγ-ELISPOT on spleen-isolated $CD8^+$ T cells (Miltenyi Biotec; ref. 25). For anti-OVA CD4 T-cell responses, spleen-isolated $CD4^+$ T cells were cocultured in presence of

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dendritic cells loaded with the OVA protein (10 μg/mL; Sigma-Aldrich) and T-cell reactivity was analyzed by IFNγ-ELISPOT. Functional analysis of $CD4^+$ TILs was performed by cocultured TILs in presence of the OVA protein or of a nonantigen-specific stimulation with PMA/ionomycin. $CD4^+$ TILs reactivity was evaluated by using intracytoplasmic IFNγ staining.

Statistical analysis

Data are presented as means ± SEM. Statistical comparison between groups was based on Student *t* test using Prism 6 GraphPad Software. *P* values lower than 0.05 (*) were considered significant. Data cutoff for survival analysis was January 7, 2015. To determine the impact of the everolimus-mediated immune modulation on survival, we used a model based on the normalized variation after 2 months of both immune variables T_{reg} (ΔT_{reg}) and anti-TERT Th1 (Δanti-TERT Th1; Supplementary Materials section). Mice and patients' survival was estimated using the Kaplan–Meier method. The log-rank tests were used to compare survival distribution. The exponential regression model was used to fit the experimental data of the tumor growth (Supplementary Materials section).

Results

Everolimus treatment promotes expansion of highly suppressive $FoxP_3^+$ T_{regs} in mRCC patients

A prospective immunomonitoring study was conducted in 23 mRCC patients treated with everolimus. The patients' main characteristics are depicted in Supplementary Table S1. The monitoring of $FoxP_3^+$ T_{regs} was performed within blood at baseline and every 2 months (Supplementary Fig. S1). We observed that both percentage and absolute number of T_{regs} gradually increased (at least >20%) after treatment in 21 of 23 patients (91.3%) compared with baseline (Fig. 1A and B; 3.5% vs. 6.5%, $P = 0.0002$ and 46 vs. 75×10^6 T_{regs}/L, $P = 0.0006$, respectively, between baseline and 6 months). In 7 patients, a first drop of T_{regs} levels was observed before a subsequent increase. T_{regs} presented the phenotype of natural T_{regs} (nT_{regs}): $CD25^{hi}CD127^{lo}FoxP_3^+Helios^+$ (26) and expressed CTLA-4 and ICOS (Fig. 1C). Furthermore, a higher expression of Ki67 in T_{regs} was detected after everolimus treatment, suggesting a proliferation of this population *in vivo* (Fig. 1D). The analysis of total blood lymphocytes showed a relative stability of these cells during treatment; however, an increase of total $CD4^+$ T cells was observed, which could be associated to T_{regs} expansion (Supplementary Fig. S2).

T_{regs} function of the patients was then evaluated by analyzing their ability to inhibit allogenic T-cell proliferation *in vitro*. As compared with T_{regs} of healthy donors, sorted T_{regs} of patients exerted a higher inhibition of T-cell proliferation. Interestingly, inhibition of T-cell proliferation was greatly increased in presence of T_{regs} isolated after everolimus as compared with the baseline (Fig. 1E and F). These results showed that everolimus promotes expansion of highly suppressive T_{regs} in mRCC patients.

Rapalogs-exposed T_{regs} mediate contact-dependent T-cell suppression *in vitro*

To confirm the ability of rapalogs to promote highly functional T_{regs}, we isolated T_{regs} from PBMCs of healthy donors cultured 10 days in presence or absence of everolimus or temsirolimus (Fig 2A). We showed that rapalogs effectively blocked the phosphorylation of S6 ribosomal protein (ser235) but not Akt (ser473), the downstream targets of mTORC1 and mTORC2, respectively (Fig. 2B and C).

As compared with nonexposed T_{regs}, rapalogs-exposed T_{regs} strongly inhibited allogenic T-cell proliferation (Fig. 2D and E) and decreased the effector cell production of IL2 and IFNγ (Fig. 2F). To further dissect how rapalogs-exposed T_{regs} exerted their suppressive activity, we first measured the inhibitory cytokines IL10 and TGFβ1 in the supernatants from T-cell suppressive assays. No significant production of these cytokines was observed (not shown). Although these T_{regs} highly expressed CTLA-4, ICOS, GITR, CD39, and CCR4 (Fig. 2G), the addition of blocking antibodies against these membrane receptors during T-cell stimulation did not affect the suppressive functions of these T_{regs} (not shown). Finally, we performed the same suppressive assays as before but using a transwell between rapalogs-exposed T_{regs} and effector T cells. As shown in Fig. 2H and I, the inhibition of T-cell proliferation was radically impaired when T_{regs} were separated from stimulated allogenic T cells. Similarly, the production of IL2 and IFNγ was totally recovered in absence of T_{regs}–T-cell contact (Fig. 2J). Thus, rapalogs-exposed T_{regs} preferentially exert inhibitory activity in a cell contact–dependent manner.

Increase of spontaneous TERT-specific Th1 response and $Eomes^+$ CD8 T cells after everolimus treatment

Concurrent to T_{regs} monitoring, the spontaneous tumor-specific Th1 response was evaluated in this cohort. To this end, we performed an IFNγ-ELISPOT to measure the lymphocytes reactivity of patients to TERT, a shared tumor antigen overexpressed in RCC (19, 27). At baseline, 11 of 23 patients' PBMCs (47.8%) demonstrated a spontaneous anti-TERT Th1 response and this frequency was increased to 17 of 23 patients (73.9%) 2 months after the beginning of treatment, suggesting the *de novo* activation of anti-TERT Th1 cells in 6 patients (Fig. 3A). Furthermore, we showed that the magnitude of this response was generally higher after treatment (42 vs. 105 anti-TERT IFNγ spots/10^5 cells, $P = 0.01$; Fig. 3B). Thus, everolimus treatment favored a higher tumor-specific Th1 immunity.

We further assessed whether the respective subpopulations of naïve (T_{NAIVE}: $CD8^+CD45RO^-CD62L^+CD127^+$), central ($T_{CM}$: $CD8^+CD45RO^+CD62L^+CD127^+$) or effector memory ($T_{EM}$: $CD8^+CD45RO^+CD62L^-CD127^+$) CD8 T cells were also impacted by everolimus treatment (Supplementary Fig. S1). No significant modulation was observed prior and after treatment (Fig. 3C). Furthermore, we analyzed the expression of the transcription factor Eomesodermin (Eomes), a key driver of memory T-cell differentiation (28), in CD8 T cells prior and after treatment. As depicted in Fig. 3D, after a long-term everolimus exposure (> 6 months), a higher percentage of $Eomes^+CD8^+$ T cells was detected in patients. Although no modulation of $CD8^+CD45RO^+/CD8^+CD45RO^-$ ratio was observed (Fig 3E), the $CD8^+CD45RO^+/T_{regs}$ ratio significantly decreased after treatment (Fig. 3F), suggesting a negative impact of T_{regs} induced following everolimus treatment on memory CD8 T cells.

Influence of immune modulation on everolimus efficacy in mRCC patients

We next addressed the effect of this immune modulation on everolimus clinical efficacy. At the time of this analysis, treatment was ongoing for 3 patients, 1 stopped for toxicity reasons and 19 patients had disease progression. At the time of disease

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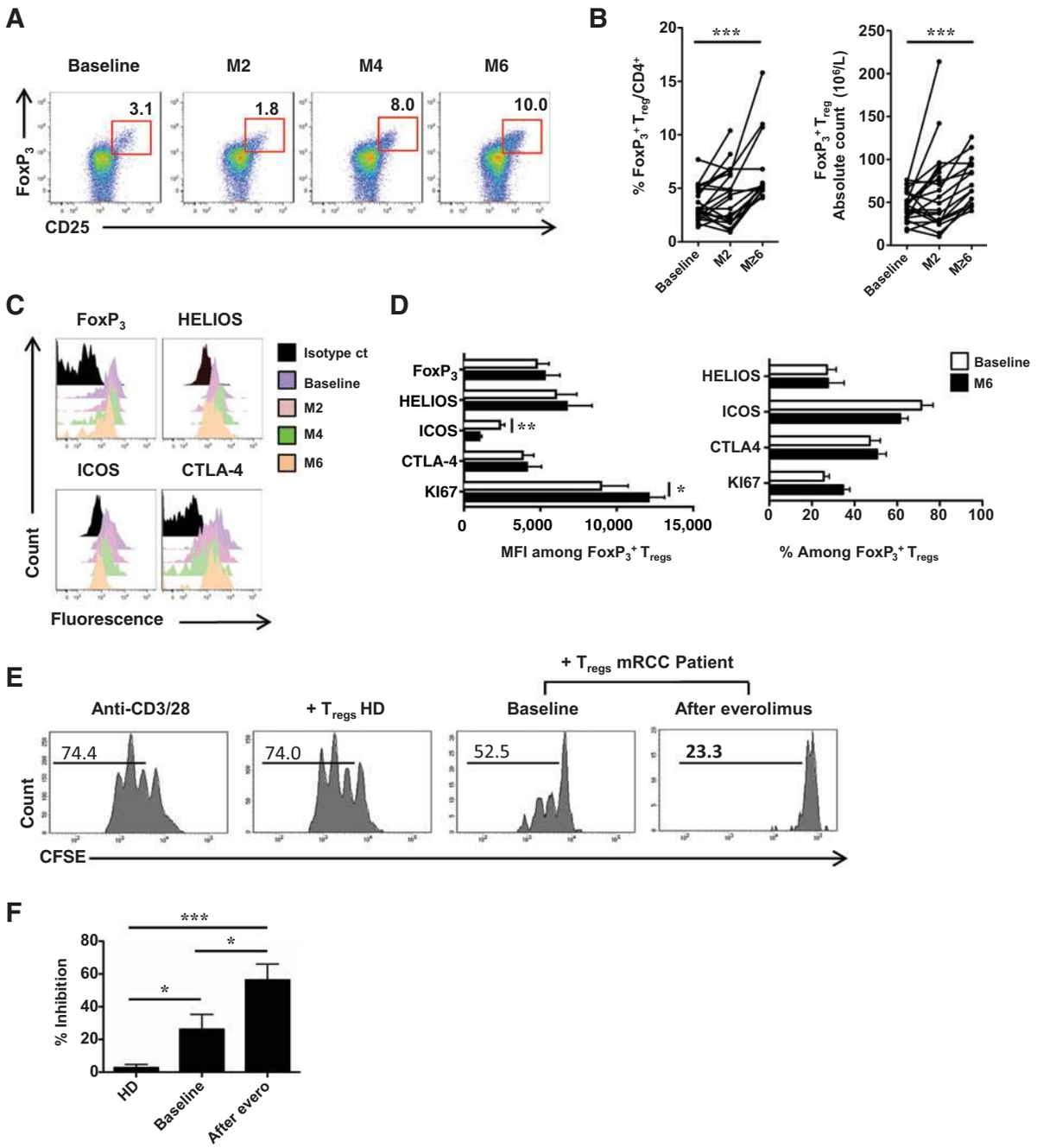

Figure 1.

Everolimus (evero) induces high immunosuppressive T_{regs} in mRCC patients. $FoxP_3^+$ T_{reg} cells were monitored ($n = 23$). A, representative plots of T_{regs}. B, T_{regs} evolution upon everolimus; percentage (left) and absolute number (right). C, representative T_{regs} phenotype analysis. D, mean fluorescence intensity (MFI) and percentage of T_{regs} markers. E, analysis of CFSE dilution in activated $CD3^+$ T cells cocultured with T_{regs}. F, percentage of inhibition of T-cell proliferation by T_{regs} ($n = 4$/group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student t test).

progression, the majority of patients (17/19) had a marked increase of circulating T_{regs} (Fig. 4A). This was associated with a loss of the anti-TERT Th1 responses (10/13; Fig. 4B). Accordingly, the anti-TERT Th1/T_{regs} ratio significantly decreased when disease progressed under everolimus treatment (Fig. 4C). The antiviral T-cell responses measured at the same time were slightly reduced but remained present in most patients (Supplementary Fig. S3). The everolimus blood concentration (EBC) was fairly similar among patients with a median EBC of 10.3 μg/L (range, 3.90–53.70 μg/L; Fig. 4D). We showed that both T_{regs} and anti-TERT Th1

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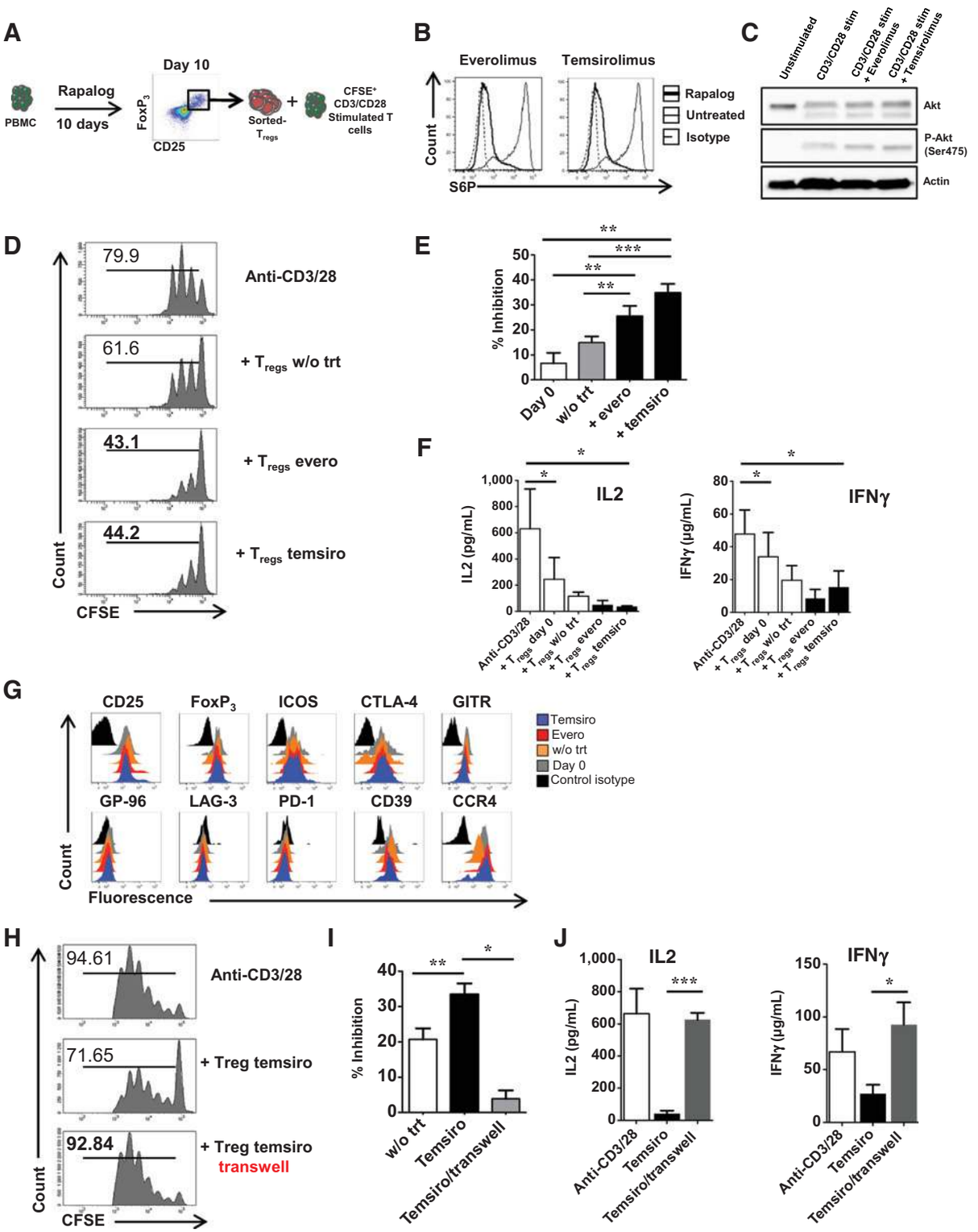

Figure 2.

In vitro analysis of rapalogs-exposed T_{regs} suppressive functions. A, protocol scheme with everolimus (evero; 100 ng/mL/day) or temsirolimus (temsiro; 500 ng/mL/3 days). After 24 hours of rapalogs exposure, PBMCs were stimulated for 30 minutes with anti-CD3 (5 μg/mL) and anti-CD28 (5 μg/mL) and assessed for pS6 (mTORC1; B) expression by phospho-flow cytometry and pAkt Ser473 (mTORC2; C) expression by Western blotting. D, CFSE dilution in activated $CD3^+$ T cells cocultured with rapalogs-exposed T_{regs}. Results from one representative donor. E, percentage of inhibition of T-cell proliferation by T_{regs} ($n = 10$). F, IL2 and IFNγ production ($n = 4$). G, representative T_{regs} phenotype analysis. H, CFSE dilution in $CD3^+$ T cocultured with rapalogs-exposed T_{regs} in transwell assay. Results from one representative donor. I, percentage of inhibition of T-cell proliferation by T_{regs} ($n = 3$). J, IL2 and IFNγ production ($n = 5$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student *t* test).

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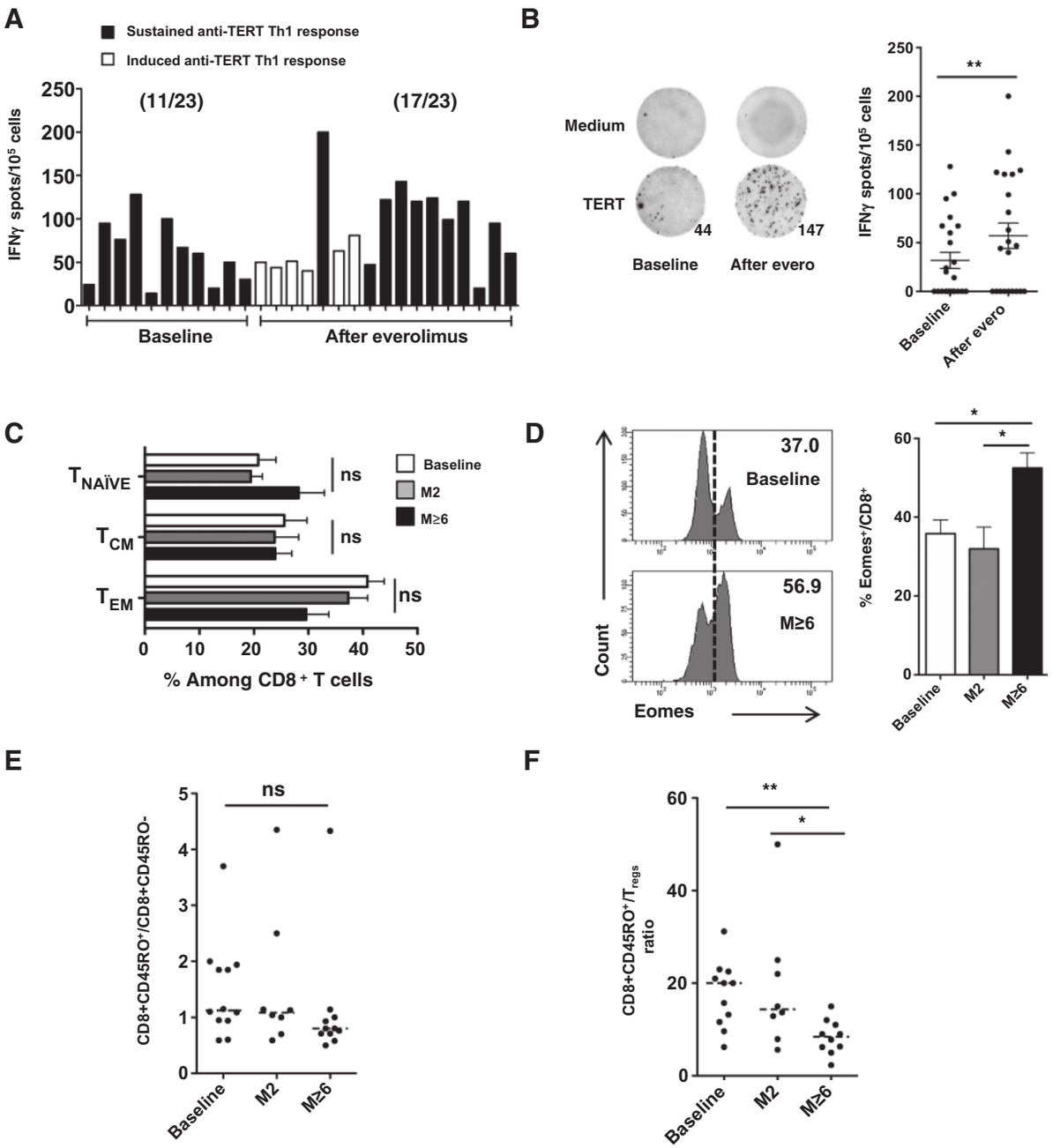

Figure 3.

Monitoring of anti-TERT Th1 cells and CD8 T cells in mRCC patients treated with everolimus (evero). Spontaneous anti-TERT Th1 and CD8 T cells were monitored ($n = 23$). A, frequency of patients with spontaneous anti-TERT Th1 response. B, representative IFNγ spots wells (left) and number of IFNγ-producing anti-TERT Th1 cells (right). C, percentage of naïve ($T_{NAÏVE}$: $CD8^+CD45RO^-CD62L^+CD127^+$), central ($T_{CM}$: $CD8^+CD45RO^+CD62L^+CD127^+$), and effector memory ($T_{EM}$: $CD8^+CD45RO^+CD62L^-CD127^+$) CD8 T cells. D, representative plots of $CD8^+Eomes^+$ T cells (left) and $CD8^+Eomes^+$ evolution (right). $CD8^+CD45RO^+/CD8^+CD45RO^-$ T (E) cells and $CD8^+CD45RO^+$ (F) T cells/T_{regs} ratio evolution. *, $P < 0.05$; **, $P < 0.01$ (Student t test). ns, not significant.

cells modulation were not directly influenced by EBC (Fig. 4E). This minimized a potential role of differential drug exposure.

To investigate the influence of the immune modulation on survival, we used a model taking into account the early variation (between baseline and 2 months) of both T_{regs} and anti-TERT Th1 cells to classify patients into three immune groups (Fig. 4F). In patients belonging to group 1 (Δ^{pos}), T_{regs} and anti-TERT Th1 cells evolved toward the same direction (growth or decline; $n = 6$). Group 2 (Δ^{null}) represents patients for whom the two immune parameters are rather stable through time or that the T_{regs} or

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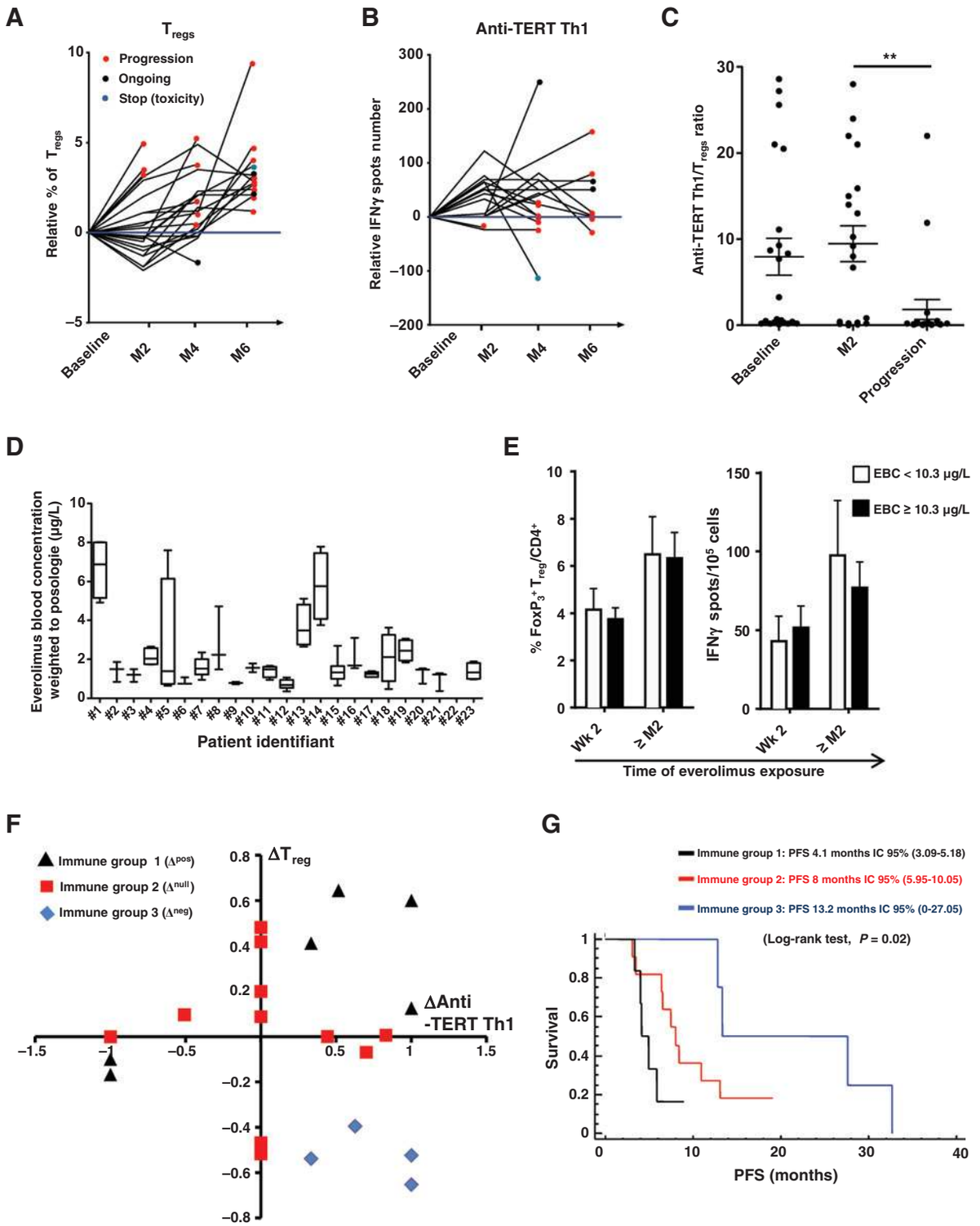

Figure 4.

Influence of immune modulation on patients' survival. T_{regs} (A) and anti-TERT Th1 (B) cells variations. C, anti-TERT Th1/T_{regs} ratio upon everolimus. D, EBC weighted to posology. E, correlation between EBC and T_{regs} (left) or IFNγ-producing anti-TERT Th1 cells (right) at week 2 and month 2 of treatment. Patients ($n = 21$) are classified into three immune groups according to their early (between baseline and 2 months) variation rate of T_{regs} and anti-TERT Th1 response. F, patients' distribution in each group. Symbols represent individual patient. G, Kaplan–Meier curves for progression-free survival (PFS; log-rank test). **, $P < 0.01$ (Student t test).

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anti-TERT Th1 variation was insignificant ($n = 11$). In the third group (Δ^{neg}), the T_{regs} values of all patients ($n = 4$) decreased, whereas the anti-TERT Th1 values greatly increased. The patients belonging to the group 3 showed a longer progression-free survival (PFS; 13.2 months) than in the others groups (4.1 and 8 months for group 1 and group 2, respectively, $P = 0.02$; Fig. 4G). However, this early immune modulation had no significant impact on overall survival (not shown). Similar results were observed when immune parameters were calculated by taking into account the possible fluctuations in the total lymphocytes count into a distribution in two instead of three groups (Supplementary Fig. S4). Similar observations supporting these results were noticed in patients with neuroendocrine tumors treated with everolimus, in whom the survival correlated with a T_{regs}/anti-TERT Th1 modulation (Supplementary Fig. S4).

Likewise, when focusing on $CD8^+$ T cells, an increase of memory $CD8^+$ T cells was observed in mRCC patients belonging to the group 3 (where T_{regs} decreased early after treatment) as compared with the two other groups (Supplementary Fig. S5). Thus, a shift toward T_{regs} decrease and high expansion of antitumor Th1 immunity improves the everolimus treatment effectiveness.

T-cell subsets depletion differentially shapes the antitumor effect of rapalogs *in vivo*

To analyze more extensively the role of T cells during rapalogs treatment, we performed *in vivo* T-cell depletion experiments in B16-OVA–bearing mice treated with rapalogs. We showed that CD8 T-cell depletion significantly reduced the antitumor efficacy of temsirolimus or everolimus against B16-OVA ($P < 0.05$; Fig. 5A). In contrast to CD8 depletion, a strong inhibition of B16-OVA growth was observed in mice lacking CD4 T cells before rapalogs administration ($P < 0.001$; Fig. 5B). Furthermore, a loss of rapamycin or temsirolimus efficacy was showed in B16-OVA–bearing mice when both T-cell subsets were removed together (Supplementary Fig S6). Similar experiments were also performed in renal carcinoma RENCA and mammary carcinoma 4T1 models. However, the depletion of T cells in these models had a low impact on treatment efficacy (Supplementary Fig. S6). The results in B16-OVA model supposed a deleterious effect of CD4 T cells especially T_{regs} during rapalogs treatment. So we assessed whether these drugs could promote T_{regs} expansion in B16-OVA–bearing mice. An early decrease of blood T_{regs} levels was observed in half rapalog-treated mice corresponding to what was observed in patients (Fig. 5C). However, a nonsignificant increase of T_{regs} in spleen and tumor was observed (Fig. 5D). As tumor growth naturally induces T_{regs}, we estimated the T_{regs} /tumor size ratio and showed that this ratio was highly increased in mice after treatment, both in tumor and spleen (Fig. 5E). Thus, like in human, rapalogs treatment promotes T_{regs} induction in tumor-bearing mice.

The presence of T_{regs} *in vivo* altered the efficacy of rapalogs via the inhibition of antitumor T-cell immunity

To study the role exerted by T_{regs} during rapalogs treatment in the B16-OVA tumor model, we used DEREG mice, which allow to selectively deplete T_{regs} after injection of diphtheria toxin (Fig. 6A). A strong tumor regression occurred in mice treated with temsirolimus followed by diphtheria toxin injection. This regression occurred at day 30, corresponding to T_{regs} elimination *in vivo* 5 days after toxin injection (Fig. 6B and C). This temporary T_{regs} depletion significantly increased the survival of mice treated with temsirolimus as compared with control mice (Fig. 6D).

Furthermore, we showed that T_{regs} ablation during temsirolimus treatment induced a higher expansion of functional anti-OVA CD8 T cells in the spleen and the tumor (Fig. 6E and F). This was also associated with the stimulation of potent IFNγ-producing anti-OVA CD4 T cells in mice (Fig. 6G–I). These results suggest that the rapalogs-induced T_{regs} abrogate antitumor T-cell functions *in vivo*. Accordingly, we evaluated *in vivo* the combination of rapalogs with therapeutic agents that deplete T_{regs} or block their suppressive functions (29). First, we showed that the anti-CD25 mAb (clone PC61.5; ref. 30) used to deplete T_{regs} in B16-OVA–bearing mice prior to everolimus treatment induced a stronger inhibition of tumor growth than everolimus alone (Fig. 7A and B). Because high level of CCR4 expression was found on rapalogs-exposed T_{regs} (Fig. 2G), we next combined temsirolimus with CCR4 antagonist, a competitive class of T_{reg} inhibitor (25). As depicted in Fig. 7C and D, this association efficiently delayed the B16-OVA growth and increased mice survival. Furthermore, mice treated with the temsirolimus plus CCR4 antagonist showed a significant decrease of T_{regs} associated with a high number of anti-OVA CD8 T cells within the TILs (Fig. 7E and F). Altogether, these results highlighted the interest to combine T_{regs} inhibition with anticancer rapalogs.

Discussion

The rapalogs everolimus and temsirolimus are two mTOR inhibitors approved as antiproliferative drugs in several cancers such as RCC (4, 5). On the basis of the critical role of mTOR on T-cell activation, the same drugs are also used in organ transplantation as immune suppressor agents (31). In this study, we reported that anticancer rapalogs induce striking modulation of host antitumor T-cell immunity, which in turn shapes the treatment efficacy.

We showed that everolimus promotes an expansion of $FoxP_3^+$ T_{regs} in mRCC patients. This T_{regs} increase started mostly 2 months after the beginning of treatment and remained high in most patients. T_{regs} induced after everolimus were $Helios^+$, suggesting that they arise from the nT_{reg} pool and proliferated *in vivo* according to the Ki67 expression (26, 32). Furthermore, everolimus exposure strongly increases patients' T_{regs} suppressive functions. Indeed, rapalogs-exposed T_{regs} highly suppress allogenic T-cell proliferation and Th1 cytokines production *in vitro*. Although the precise mechanism of suppression required future investigations, rapalogs-exposed T_{regs} preferentially exerted a cell-contact immunosuppression as also described for nT_{regs} (26).

Very few studies have investigated the modulation and function of T_{regs} in cancer patients treated with rapalogs. A preliminary study reported a significant increase of $FoxP_3^+$ T_{regs} in 7 mRCC patients treated with temsirolimus (33). One previous study did not find any modulation of T_{regs} after rapalog treatment but T_{regs} were monitored only once at 1 month after the beginning of treatment (34). However, an increase of T_{regs} was also reported in prostate cancer patients treated with everolimus (35). Thus, like in organ transplantation, mTOR inhibition increases T_{regs} number and their suppressive functions in cancer patients (12, 36).

The antitumor CD4 Th1 immunity was concurrently explored in mRCC patients. To this end, we tested the reactivity of patients'

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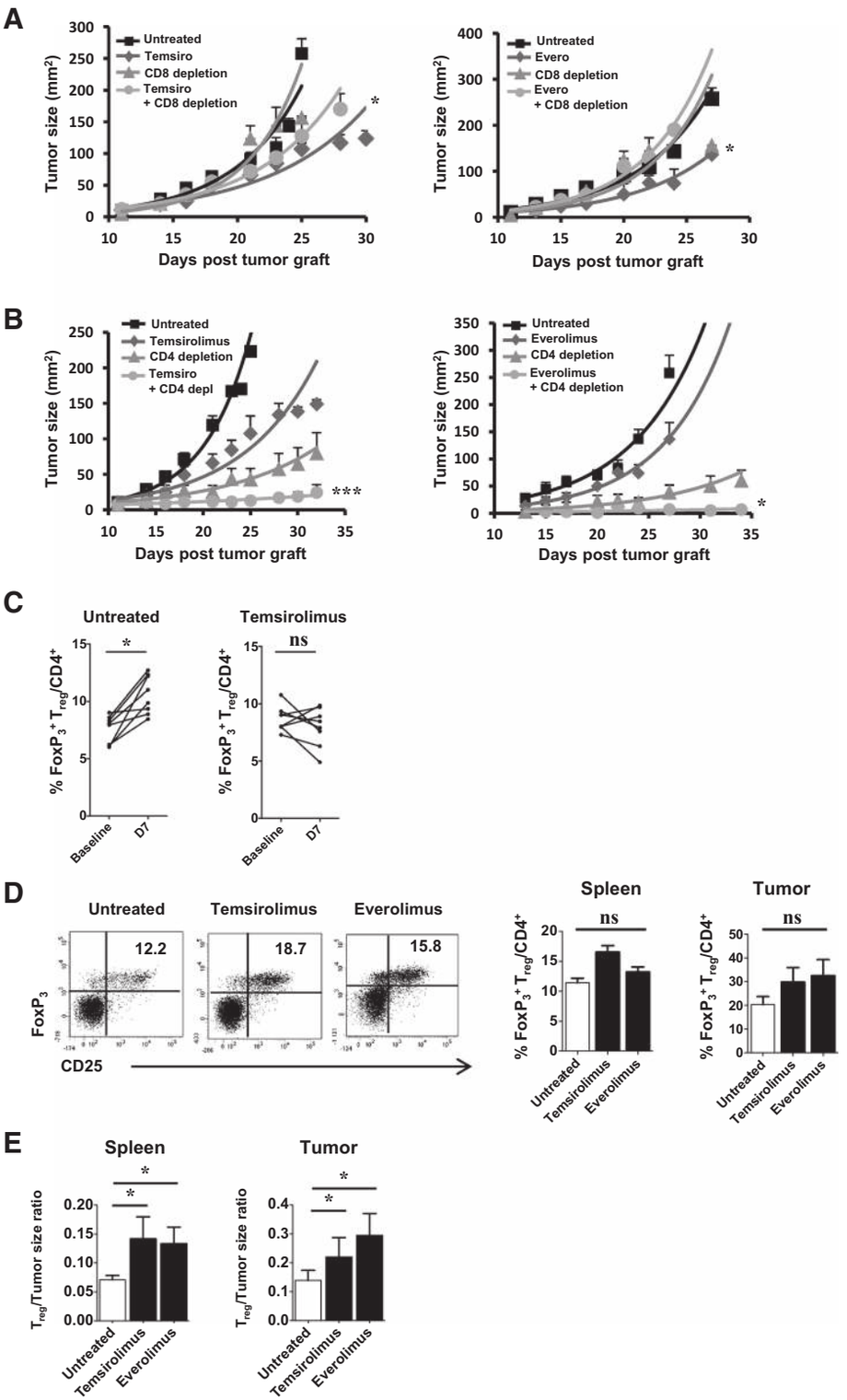

Figure 5.

In vivo T-cell depletion impacts on rapalogs treatment efficacy. B16-OVA–bearing C57BL/6 mice ($n = 5$/group) depleted with anti-CD8 (A) or anti-CD4 (B) mAbs injections were treated with rapalogs. Control mice received solvent and isotype control mAb. Tumor growth rate are shown. The symbols represent the evolution of mean ± SEM tumor size and the lines are the exponential regression model fitting the mean tumor size. C and D, $FoxP_3^+$ T_{regs} percentage in the blood of B16-OVA–bearing mice at baseline and 7 days after the beginning of temsirolimus treatment ($n = 10$/group; C) and at day 25 in the spleen and tumor, representative dot plots (D). E, T_{regs}/tumor size ratio in the spleen (left) and in the tumor (right). Results represent at least three independent experiments. *, $P < 0.05$ (Student *t* test). Evero, everolimus; temsiro, temsirolimus. ns, not significant.

T cells against MHC class II-restricted peptides derived from TERT (21, 22). We showed that everolimus treatment stimulated and sustained spontaneous anti-TERT Th1 response. Furthermore, an increase in the magnitude of this response was observed after treatment. So, dual modulations of host antitumor CD4 T-cell responses can occur during everolimus treatment. One plausible explanation of the stimulation of tumor-specific CD4 T cells may be related to the ability of mTOR inhibition to promote

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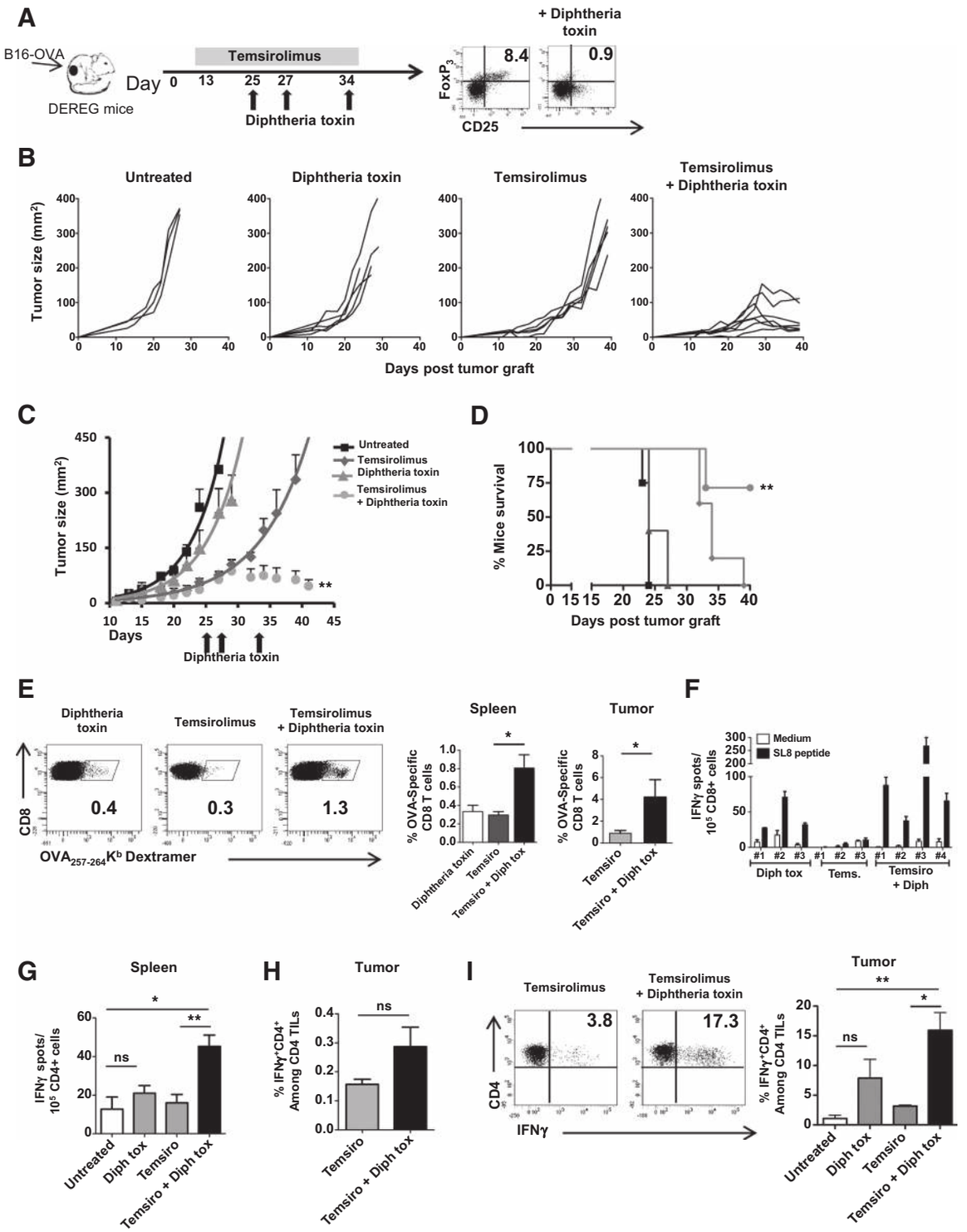

Figure 6.

Effects of conditional T_{regs} removal during rapalogs treatment. DEREG mice ($n = 4$/group) were grafted with B16-OVA and then treated or not with temsirolimus (temsiro). A, diphtheria toxin injections (80 μg/kg) and example of T_{regs} depletion at sacrifice. B and C, tumor growth (B) and comparison of tumor growth rate (C). The regression model was not applicable for the group treated by temsirolimus + Diphtheria toxin (Diph tox) over the 30th day. D, Kaplan–Meier survival curves (log-rank test). E, $OVA_{257-264}$ K^b-dextramer staining in spleen and TILs at day 35. Representative splenocytes dot plots and percentage of $OVA_{257-264}$-specific $CD8^+$ T cells. Functional analysis of $OVA_{257-264}$-specific $CD8^+$ (F) and $CD4^+$ (G) T cells measured *ex vivo* in the spleen by IFNγ-ELISPOT at day 35. H, $CD4^+$ T cells reactivity was analyzed in the tumor by IFNγ intracellular staining after OVA stimulation (H) or PMA/ionomycine stimulation with representative dot plots and percentage of $IFN\gamma^+$ $CD4^+$ TILs (I). Experiments were reproduced three times. *, $P < 0.05$; **, $P < 0.01$ (Student *t* test).

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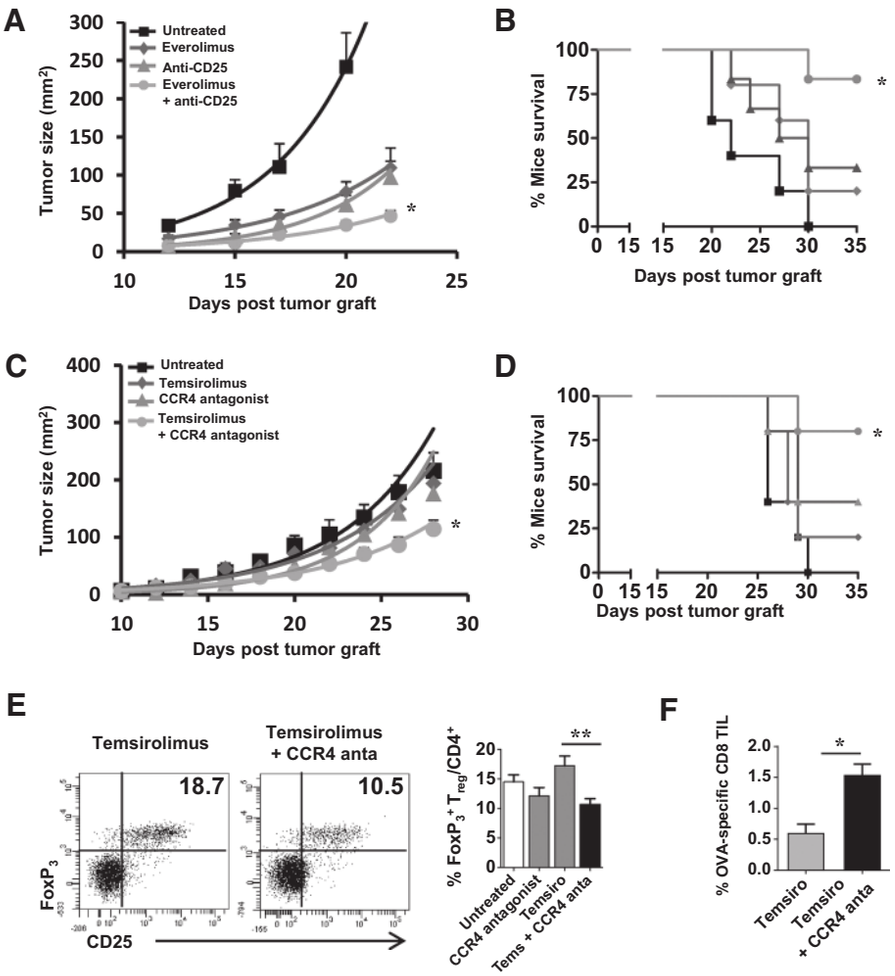

Figure 7.
Combination of rapalogs with anti-CD25 mAb or CCR4 antagonist. $FoxP_3$-eGFP mice ($n = 5$/group) were depleted or not with anti-CD25 mAb and then grafted with B16-OVA tumor. Tumor-bearing mice were treated or not with everolimus (0.65 mg/kg/day). A, the symbols represent the evolution of mean ± SEM tumor size for each group and the lines are the exponential regression model fitting the mean tumor size. Tumor growth rates were compared. B, Kaplan–Meier survival curves (log-rank test). C, B16-OVA–bearing mice were concomitantly or individually treated with temsirolimus (temsiro, Tems) and CCR4 antagonist (1.5 μg/mice) and tumor growth rates were compared. D, Kaplan–Meier survival curves (log-rank test). E and F, $FoxP_3^+$ T_{regs} staining in spleen at day 25 with representative dot plots (E) and percentage $OVA_{257-264}$-specific $CD8^+$ TILs detected by dextramer staining at day 25 (F). $n = 5$ mice/group were used and experiments were reproduced two times. *, $P < 0.05$; **, $P < 0.01$ (Student t test).

autophagy (37). Autophagy has been shown to be critical for the antitumor immune response elicited by dying tumor cells (38). In addition, this process improves antigen processing by MHC class II molecules (39, 40). These results also support our previous observation in one mRCC patient treated with everolimus that presented an amplification of antitumor Th1 cells followed by T_{regs} induction (19). Furthermore, mTOR inhibition has been shown to increase both the quantity and quality of memory T-cell responses (14–16).

An important issue of this study is a potential correlation between patients' survival (PFS) and immune modulation observed after everolimus treatment. At the time of disease progression under everolimus treatment, the majority of patients totally lost the anti-TERT Th1 response in favor to a marked increase of T_{regs}. Accordingly, we observed a high decrease of $CD8^+CD45RO^+/T_{regs}$ and $Th1/T_{regs}$ ratio at the same time. A mathematical model based on the early variation of both T_{regs} and anti-TERT Th1 cells was used to study the relationship between immune modulation and patient's clinical outcome. Our results suggested that an early establishment of a good immune environment toward the decrease of T_{regs} and the increase of antitumor Th1 immunity may enhance everolimus clinical efficacy. However, due to the small number of patients enrolled in this study, our hypothesis deserves further confirmation in a larger cohort of mRCC patients and in other tumors.

To dissect the role of T cells during rapalogs treatment, we used various mouse tumor models. Our choice of models was based on rapalogs indications in renal and breast carcinoma (4, 5, 7) and their current evaluation in melanoma (41). In contrast with that in RENCA and 4T1 tumors, we observed that T-cell subsets can differentially shape the efficacy of rapalogs against B16-OVA tumor growth. While CD8 T-cell depletion reduces rapalogs efficacy on B16-OVA tumor growth, we showed that the removal of CD4 T cells strongly increased the antitumor effect of these drugs. The discrepancy in these tumor models may be related to the difference in the genetic background of the mice. In this regard, RENCA and 4T1 grow in Balb/c mice, a genetic background commonly known to develop a weaker Th1 response than C57BL/6 (42). Furthermore, B16-OVA was previously used in several studies to evaluate the immune responses after mTOR inhibition (43–45).

Because CD4 T-cell depletion increases rapalogs efficacy, we focused our attention on the role of T_{regs} *in vivo*. Like in patients, we showed that everolimus or temsirolimus induced T_{regs} expansion in mice and temporary depletion of these cells during rapalogs treatment in DEREG mice drastically increased treatment efficacy. Interestingly, T_{regs} ablation during rapalogs treatment promotes high expansion of both anti-OVA CD8 and CD4 T cells within the tumor supporting an inhibitory effect of rapalogs-exposed T_{regs} on antitumor T cells *in vivo*. Similar data

have recently reported by Wang and colleagues, using a RENCA expressing CA9 as tumor antigen in Balb/C mice (46). These observations led us to combine rapalogs with strategies that block T_{reg} cells *in vivo* (29). Then we found that rapalogs efficacy was highly improved by combining with an antagonist of CCR4, a CCL17 and CCL22 chemokines receptor (47) highly expressed on rapalog-exposed T_{regs}. This association also promotes a high expansion of anti-OVA $CD8^+$ TILs.

In conclusion, this study clearly indicates that anticancer rapalogs shape the host antitumor T-cell immunity and thereby affect patients' clinical outcome. Because RCC is an immunogenic tumor and is known to respond to immunotherapies (48, 49), we believed that there is strong rational to combine rapalogs with T_{regs} or immune checkpoint blockade to shift host immune responses toward protective antitumor immunity.

Disclosure of Potential Conflicts of Interest

J. Bayry has ownership interest (including patents) in US patent 20110171261 and WO/2009/150433 for CCR4 antagonists. A. Thierry-Vuillemin is a consultant/advisory board member for Novartis and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Beziaud, L. Mansi, P. Ravel, L. Rangan, S. Vrecko, L. Queiroz, E. Curtit, B. Royer, B. Gaugler, J. Bayry, E. Tartour, Y. Godet, O. Adotévi
Writing, review, and/or revision of the manuscript: L. Beziaud, L. Mansi, G. Mouillet, T. Nguyen Tan Hon, E. Curtit, B. Royer, B. Gaugler, E. Tartour, A. Thiery-Vuillemin, X. Pivot, Y. Godet, O. Adotévi
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