Rapamycin impairs antitumor CD8⁺ T cell responses and vaccine-induced tumor eradication

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ABSTRACT

The metabolic sensor mTOR broadly regulates cell growth and division in cancer cells, leading to a significant focus on studies of rapamycin and its analogs as candidate anti-cancer drugs. However, mTOR inhibitors have failed to produce useful clinical efficacy, potentially because mTOR is also critical in T cells implicated in immune surveillance. Indeed, recent studies using rapamycin have demonstrated the important role of mTOR in differentiation and induction of the CD8⁺ memory in T cell responses associated with antitumor properties. In this study, we demonstrate that rapamycin harms antitumor immune responses mediated by T cells in the setting of cancer vaccine therapy. Specifically, we analyzed how rapamycin affects the anti-tumor efficacy of a human papilloma virus E7 peptide vaccine (CyaA-E7) capable of eradicating tumors in the TC-1 mouse model of cervical cancer. In animals vaccinated with CyaA-E7, rapamycin administration completely abolished recruitment of CD8⁺ T-cells into TC-1 tumors along with the ability of the vaccine to reduce infiltration of T regulatory cells and myeloid-derived suppressor cells. Moreover, rapamycin completely abolished vaccine-induced cytotoxic T cell responses and therapeutic activity. Taken together, our results demonstrate the powerful effects of mTOR inhibition in abolishing T cell-mediated antitumor immune responses essential for the therapeutic efficacy of cancer vaccines.
INTRODUCTION

Mammalian target of rapamycin (mTOR) is a key kinase that regulates broad aspects of cellular functions (metabolism, protein synthesis, cell growth, proliferation, survival, apoptosis, autophagy) (1, 2) in response to environmental signals, including growth factors, cytokines and oxygen (3). Growing evidence implicated mTOR signaling pathways in numerous diseases, including cancer (1). Indeed, mTOR pathways are highly activated in various types of cancer: laryngeal, cervical, hepatocellular and renal cell carcinomas and gastric, colorectal, prostate and breast cancers (4-6). These observations have urged preclinical studies and clinical trials to use rapamycin or its analogs to inhibit mTOR activation as an anti-cancer therapy. Preclinical studies gave promising results (7), and numerous clinical trials are currently ongoing. In 2007 and 2009, the FDA approved the use of rapamycin and two of its analogs (Temsirolimus and Everolimus) for the treatment of advanced renal cell carcinoma (RCC) (8, 9). However, clinical studies gave modest overall clinical results, leading to disease stabilization rather than tumor regression (5, 10), thus prompting its combination with chemotherapies (7) or immunotherapies. This is due to the fact that mTOR is also a central regulator of the immune system (11), particularly in the differentiation of T-cells. mTOR controls, indeed, the differentiation of naïve CD4+ T-cells into Th1, Th2 or Th17 (12, 13) and naïve CD8+ T-cell into effector cells (14). Araki et al. have shown that treatment with rapamycin during acute infection with LCMV enhanced the virus–specific CD8+ T-cells and favored the CD8+ memory response in mice and macaques (14). Other studies demonstrated the beneficial effect of rapamycin treatment on the vaccine-induced anti-tumoral response in mice (15) and the vaccine-induced anti-viral responses in macaques (16).

Human papilloma viruses (HPVs) cause proliferative lesions in infected skin and squamous mucosa, resulting in hyperplasias, papillomas and condylomas. Most HPV infections are
transient, but in some patients, persistent mucosal lesions due to high-risk HPVs, particularly in the ano-genital tract and the oropharynx, can progress into in situ or invasive carcinomas (17), making cervical carcinoma the second most frequent gynecologic cancer in women.

Our team has developed a new vaccine candidate, the CyaA-vector carrying the E7 oncoprotein from HPV-16, currently under clinical trials and able to induce complete tumor eradication in a mouse model of HPV-related carcinoma (18), although its therapeutic efficacy against large tumors is strongly reduced. However, combining the CyaA-E7 vaccine with different adjuvants, such as CpG-B, strongly enhances the regression of large tumors but still does not induce complete eradication (19).

Therefore, strategies to enhance the therapeutic efficacy of the vaccine-induced immune response against large tumors are still needed. The recent data showing the capacity of rapamycin to promote memory CD8+ T-cell responses (14, 15) suggested that rapamycin may enhance the CyaA-E7 vaccine-induced tumor-specific CD8+ T-cell responses and tumor regression. To address this issue, tumor-bearing mice were vaccinated with CyaA-E7 and received chronic or short-course rapamycin treatments. We evaluated the tumor growth, T-cell responses, and the recruitment of lymphocytes and myeloid-derived suppressor cells (MDSC) to the tumor site. Surprisingly, we observed that rapamycin treatment induces a strong, dose-dependent inhibition of the vaccine-induced cytotoxic CD8+ T-cell recruitment to the tumor site and reduction in Treg infiltration and an overall decrease in tumor control.

This study strongly supports the conclusion that, in our model, treatment with rapamycin is detrimental to the induction of anti-tumoral immune responses. Our data could explain the low efficacy of rapamycin in cancer patients and thus the influence of mTOR inhibitors on anti-tumor immunity deserves careful investigation both in experimental models and in patients.
MATERIALS AND METHODS

Mice and tumors

Specific pathogen-free six-week-old female C57BL/6 mice were purchased from Charles River and were kept in the Pasteur Institute animal facilities under pathogen-free conditions with water and food ad libitum. All the in vivo experiments were carried out in compliance with French and European laws and regulations. TC-1-tumor cells expressing HPV-16 E6 and E7 proteins (20) and that were derived from primary mouse lung epithelial cells were obtained from the American Type Culture Collection LGC (Promochem). TC-1 cells were injected subcutaneously into the shaved left flank of C57BL/6 mice (6x10^5 TC-1 cells/mouse). The tumor size represents the average of two perpendicular diameters (millimeters) and was measured with a digital caliper (Mitutoyo).

Reagents

The synthetic peptide E7_{49-57} \text{RAHYNIVTF}, corresponding to the HPV16-E7 H2D^b restricted epitope (21) was purchased from Polypeptide (Strasbourg, France). The detoxified CyaA of *Bordetella pertussis* carrying a truncated form of the E7 protein (CyaA-E7) was prepared as previously described (18, 19). CpG-B 1826 (5-TCCATGACGTTCTGACGTT-3) was synthesized by Proligo, and mixed with 60 µg N-[1-(2,3-dioleoyloxy)propyl]-NNN-trimethylammoniummethyl sulfate (DOTAP; Roche). Rapamycin was purchased from Sigma-Aldrich and injected intraperitoneally (i.p.) (750 or 75 µg/kg/day) in a total volume of 150 µL (vs. PBS in control mice), for 14 or 22 days, depending on the experimental protocol.

Cell isolation

Peripheral lymph nodes from naive mice (LN N; maxillary, axillary and inguinal) or inguinal tumor-draining LN (dLN) were harvested, mechanically disrupted and filtered to obtain single-cell suspensions. Spleens from both naïve (Sp N) and tumor-bearing mice (Sp T) were
harvested, treated for 20 minutes with 400 U/mL collagenase D and 50 µg/mL DNase I (Boehringer Mannheim), mechanically disrupted and filtered to obtain single-cell suspensions. The tumors were harvested and dissociated using the gentleMACS dissociator (program mTumor-01). The tumors were then incubated for 45 minutes with 400 U/mL collagenase D and 50 µg/mL DNase I and filtered to obtain single-cell suspensions.

**Flow cytometry analysis**

The following monoclonal antibodies (mAbs) were used: APC- or APCeF780-conjugated anti-CD3e (clone 145-2C11), pacific-blue-conjugated anti-CD4 (clone RM4-5) and PerCP-Cy5.5-conjugated anti-CD8a (clone 53-6.7), together with APC-conjugated anti-CD44 (clone IM7), anti-CD45RB (clone C363.16A), APCeF780-conjugated CD45.2 (clone 104), PE-Cy7-conjugated anti-CD19 (clone 6D5), anti-CD62L (clone: MEL-14), anti-CD69 (clone HI.2F), PE-conjugated anti-CD39 (clone 24DMS1), anti-CD73 (clone TY/11.8), anti-GITR (clone DTA-1) and anti-PD-1 (clone J43). For MDSC staining, we used PerCPCy5.5-conjugated anti-CD49b (cloneDX5), biotine-conjugated anti-F4/80 (clone BM8) followed by BV605-conjugated streptavidin, eF450-conjugated anti-CD11c (clone N418), together with a cocktail mix of mAbs from eBiolegend (PE-conjugated anti-CD11b (clone M1/70), and APC-conjugated anti-Gr1 (clone RB6-8C5)). These mAbs were purchased from BD Pharmingen or eBioscience. PE-conjugated H-2D³/E749-57 tetramers were obtained from MBL International Corporation. The pacific orange-coupled Live/dead® fixable aqua dead cell stain kit (Invitrogen, molecular probes®) was used to stain dead cells, according to the manufacturer’s protocol before fixing cells for intracellular staining. Foxp3⁺ cells were detected by intracellular staining with FITC-conjugated anti-Foxp3 mAb (FJK-16), according to the manufacturer’s protocol (eBioscience). The cells were acquired on a CyAn (Coultronics) or LSRFortessa™ (BD) flow cytometers and analyzed with the FlowJo software (Tree Star).
**In vivo killing assay**

For CTL priming, the mice were i.v. immunized with the CyaA-E7 (50 µg/mouse) together with CpG-B-DOTAP (30 µg and 60 µg/mouse, respectively). Seven days after immunization, naive syngenic splenocytes were pulsed with the E7_{49-57} peptide (30 µg/mL, 30 min, 37°C), washed extensively and labeled with a high concentration (2.5 µM) of carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA). The non-pulsed control population was labeled with a low concentration (0.45 µM) of CFSE. CFSE^{high}- and CFSE^{low}-labeled cells were mixed in a 1:1 ratio (5 × 10^6 cells of each population) and i.v. injected into mice. Spleen cells were collected 20-24 h later, washed and resuspended in FACS buffer. The number of CFSE-positive cells remaining in the spleen after 20-24 h was determined by FACS. The percentage of specific lysis was calculated as follows: % specific lysis = 100 – [100 × (% CFSE^{high} immunized mice/% CFSE^{low} immunized mice)/(% CFSE^{high} naive mouse/% CFSE^{low} naive mouse)].

**IFNγ ELISPOT assays.**

Mice were i.v. immunized with the CyaA-E7 and CpG-B-DOTAP as described above. Eight days after immunization, cells were stimulated *in vitro* for the production of IFNγ. Briefly, multiscreen filtration plates (96 wells; Millipore, Molshein, France) were pre-coated with the anti-mouse IFNγ capture antibody (IFNγ ELISpot pair, Diaclone, Besançon, France), overnight at 4°C. Then the plates were washed and blocked with complete medium containing 10% FCS. Eight days after immunization, 10^6 spleen cells were isolated from the mice, added to the wells and incubated for 18 hours with or without the E7_{49-57} peptide (30 µg/mL; 37°C). The plates were revealed by incubation with the biotinylated anti-mouse IFNγ detection antibody (IFNγ ELISpot pair, Diaclone) for 90 min at 37°C, followed by incubation with streptavidin-alkaline phosphatase (BD, PharMingen; 1 hour, 37°C). Finally, spots were revealed using the ready-to-use BCIP/NBT substrate (Sigma-Aldrich, France). The number of
IFNγ-producing cells were determined by counting the number of spot-forming cells in each well (Bioreader 5000, Karben, Germany), and the results were calculated as follows: total number of spot-forming cells after stimulation with E7 – number of spot-forming cells without stimulation for each mouse and expressed as number of spot-forming cells / 10⁶.

**Statistical analysis**

Prism software (GraphPad Software, Inc.) was used to calculate the statistical significance for the differences in the particular measurements between the groups. Two-tailed unpaired tests (Mann-Whitney test) and the Mantel–Cox Logrank test were used; P values lower than 0.05 were considered statistically significant.
RESULTS

Chronic treatment with rapamycin inhibits CyaA-E7 vaccine-induced tumor regression

To evaluate the impact of rapamycin treatment on the tumor growth and immune response after the tumor graft and therapeutic vaccination, TC-1 tumor-bearing mice were vaccinated with CyaA-E7 adjuvanted with CpG-B on day 14 after the tumor graft, alone or in combination with rapamycin treatments. We first used a high dose of rapamycin (750 µg/kg/day; daily i.p. injections), either from days 0 to 22 or from days 14 to 22 after the tumor graft. As previously shown (19), CyaA-E7 and CpG induced full tumor eradication in 42% of the vaccinated mice and significantly enhanced their survival (Fig. 1A and B). Given alone, both chronic and short-term rapamycin treatments slightly delayed the tumor growth and significantly enhanced the survival of non-vaccinated mice (Fig. 1A and B). However, when given in combination with CyaA-E7 vaccination, the rapamycin treatment completely abrogated the therapeutic effect of CyaA-E7 on tumor growth. Indeed, no tumor regression was observed in vaccinated mice after either regimen of rapamycin. These data clearly show that high dose rapamycin treatments fully abrogate vaccine-induced tumor regression.

Treatment with rapamycin inhibits lymphocyte recruitment to tumor-draining LN and the tumor site and abrogates the therapeutic effect of CyaA-E7

We have previously shown that CyaA-E7 with CpG-B induces antigen (Ag)-specific CD8⁺ T-cells as soon as seven days after vaccination (19). Because the rapamycin treatment completely abrogated the vaccine’s effect on tumor growth, we analyzed its impact on the T-cell compartment in lymphoid tissues and tumors (the FACS gating strategy is shown in Supplementary Fig. S1).

Increased numbers of total and CD45⁺ cells were observed in tumor-draining lymph nodes (dLN), but these numbers were dramatically reduced in mice receiving chronic rapamycin treatments, with or without vaccination. The short-term rapamycin treatment only slightly
reduced the cell numbers in the dLN (Fig. 2A). The rapamycin treatments did not induce any changes in T and B cell percentages compared with the untreated tumor-bearing mice. While vaccination by CyaA-E7 and CpG increased the numbers of total cells and total CD45$^+$ cells in the spleen, chronic rapamycin treatment reduced the cell numbers when given alone and completely inhibited the vaccine’s effect (Supplementary Fig. S2A). However, the rapamycin treatments did not change the B and T-cell percentages in the spleen.

Increased numbers of CD45$^+$ tumor-infiltrating cells were detected in vaccinated untreated mice (Fig. 2B, upper left panel). However, a massive reduction in the total cell numbers (Supplementary Figure S2C), CD45$^+$ tumor-infiltrating cells and of all lymphocyte subsets (Fig. 2B) was observed after the chronic rapamycin treatment (d0-22) (with or without vaccination), while the short-term rapamycin treatment (d14-22) reduced only the number of total cells and of CD45$^+$ tumor-infiltrating cells (Supplementary Figure S2C and Fig. 2B). The CyaA-E7 vaccination increased both the CD8$^+$ T-cell and CD4$^+$ Foxp3$^-$ T effector cell (Teff) recruitment to the tumor site while reducing the CD4$^+$ Foxp3$^+$ regulatory T-cell (Treg) accumulation, demonstrating the ability of this vaccine to induce a more effective anti-tumoral response. In contrast, both the chronic and short-course rapamycin treatments dramatically inhibited the CD8$^+$ T-cell and Teff recruitment to tumors in non-vaccinated mice (Fig. 2B). This inhibition by rapamycin was even greater in the vaccinated mice. Both rapamycin treatments also inhibited the reduction of Treg infiltration in tumors that was observed after CyaA-E7 vaccination. These results show that high doses of rapamycin impede lymphocyte recruitment to tumor-dLN as well as the CD8$^+$ T-cell and CD4$^+$ Teff recruitment to the tumor site. They also demonstrate that rapamycin inhibits both the tumor- and vaccine-induced recruitment of CD8$^+$ T-cells to the tumor site.

We also analyzed the effect of rapamycin on the recruitment of myeloid-derived suppressor cells (MDSC), a population of immature myeloid cells that were shown to accumulate in
lymphoid tissues of tumor-bearing mice and in the blood of cancer patients (22). We found that the numbers and percentages of MDSC (the gating strategy is shown in Supplementary Fig. S3) were increased in the spleen (Fig. 3A) and LN (Fig. 3B) from tumor-bearing mice, and that CyaA-E7 increased their recruitment, particularly in the spleen. However, both rapamycin treatments strongly inhibited their accumulation (Fig. 3A-B, left panels). In contrast, while CyaA-E7 reduced the accumulation of MDSC in the tumor, rapamycin completely inhibited its effect (Fig. 3C). Furthermore, the short treatment also reversed CyaA-E7’s inhibition and enhanced MDSC recruitment to tumor site.

These observations clearly show that rapamycin not only inhibited the recruitment of CD8^+ T-cells and CD4^+ Teffs to the tumor site, but also enhanced the accumulation of MDSC known to inhibit the cytotoxic CD8^+ T-cell response (23), thus favoring the escape from anti-tumor immune responses.

**Rapamycin inhibits the vaccine-induced reduced activation of tumor-infiltrating Tregs**

Araki et al. showed that chronic rapamycin treatment increases the expression of CD62L and other memory T-cell markers (14). Therefore, we analyzed the changes in the expression of various markers on T-cells after vaccination and rapamycin treatment. We found that, while vaccination with CyaA-E7 had no effect on these markers, the rapamycin treatments slightly reduced the CD44 expression on CD8^+ T-cells (Supplementary Fig. S4A and B) and CD4^+ Teffs (data not shown) in the spleen and LN and increased the expression of CD73. Surprisingly, rapamycin increased the CD62L expression on CD4^+ Teffs but not CD8^+ T-cells in the LN (data not shown and Supplementary Fig. S4, respectively). The chronic rapamycin treatment decreased the CD62L expression on CD8^+ T-cells in the spleen, while the short-term treatment increased this expression only in vaccinated mice (Supplementary Fig. S4). Overall, these results suggest that the rapamycin treatments did not enhance the memory
phenotype of CD8\(^+\) T-cells in lymphoid tissues in our model but rather reduced their activation as assessed by CD44 expression.

We have previously shown that tumor-infiltrating CD4\(^+\) T-cells, especially Tregs, are highly activated with a significantly increased expression of CD44, CD69 and PD-1 (an inducer of T-cell tolerance and a marker for T-cell exhaustion (24)) compared with dLN or spleen Tregs (25), they lose CD62L expression and acquire an effector/memory phenotype (CD44\(^{\text{high}}\) CD62L\(^{-}\)). Similar results were obtained here for tumor-infiltrating CD8\(^+\) T-cells with an increased CD44 and PD-1 expression (Fig. 4C-D) and decreased CD62L expression (Supplementary Fig. S4C) compared with lymphoid organs (Supplementary Fig. S4A-B), demonstrating their strong activation and effector/memory phenotype.

CyaA-E7 vaccination had no effect on the phenotype of tumor-infiltrating CD4\(^+\) Teffs and CD8\(^+\) T-cells (Supplementary Fig. S5A and S4C, respectively) but significantly reduced CD44 expression and increased GITR expression on Tregs (Fig. 4A-B). Because GITR is a co-stimulatory molecule for Teff and an inhibitor of Treg suppressor activity (26), CyaA-E7 vaccination seems to also reduce Treg’s suppressive function. Vaccination with CyaA-E7 did not impact the other markers on tumor-infiltrating T-cells (Supplementary Fig. S4C and S5).

In addition, the chronic rapamycin treatment increased the expression of CD73 on tumor-infiltrating CD8\(^+\) T-cells and Teffs in non-vaccinated mice (Fig. 4C-D and Supplementary S5A, respectively) and of CD44 and PD-1 on tumor-infiltrating Tregs and inhibited the CyaA-E7 effect on GITR expression by Tregs (Fig. 4A-B). The GITR expression on Tregs from the spleen and LN was also reduced after rapamycin treatment (data not shown). The short-term rapamycin treatment only slightly increased PD-1 expression but strongly inhibited the vaccine-induced increase in GITR expression on tumor-infiltrating Tregs (Fig. 4A-B) and
had no impact on tumor-infiltrating CD4+ Teffs (Supplementary Fig. S5A) or CD8+ T-cells (Supplementary Fig. S4C).

Taken together, these results indicate that CyaA-E7 vaccination activated the CD8+ T-cell responses and dampened the suppressive function of Tregs in tumors. Moreover, both rapamycin treatments inhibited these effects and increased the expression of suppressive molecules on tumor-infiltrating effector CD4+ and CD8+ T-cells.

**Low-dose rapamycin treatment impedes lymphocyte recruitment to the tumor site and tumor-draining LN**

In contrast to previous studies (14, 15, 27), our results show that treatment with a high dose of rapamycin (750 µg/kg/day) is detrimental for T-cell responses and tumor regression in our model. Li et al. have demonstrated that short-term low-dose rapamycin treatments (75 µg/kg/day) increased the Ag-specific CD8+ T-cell early response (15). We therefore analyzed whether a lower dose of rapamycin would have a positive impact on the CyaA-E7-vaccine-induced T-cell responses. On day 22 post-tumor graft, both the short-term and chronic treatments of rapamycin at low dose decreased the numbers of total and CD45+ cells (but not the percentages of lymphocytes) in the spleen, dLN and tumors and inhibited the CyaA-E7-induced recruitment (Supplementary Fig. S6A-C). The chronic rapamycin treatment strongly reduced the increased percentage of CD8+ T-cells and CD4+ Teffs that were observed in tumor-bearing mice vaccinated with CyaA-E7 (Supplementary Fig. S6C). Rapamycin also reduced the percentage of CD4+ T within the CD45+ cells without affecting the proportions of Tregs and CD4+ Teffs. In contrast, the short-term rapamycin treatment had a weaker effect on the infiltration of total CD4+ or CD8+ T-cells (Supplementary Fig. S6C). These data show that although the low-dose rapamycin treatments had a reduced inhibitory effect on tumor-infiltrating T-cells compared with high dose, they had no beneficial impact on
CD8\(^+\) T-cell infiltration and increased the expression of CD44 and PD-1 on tumor-infiltrating Tregs (Supplementary Fig. S7).

Despite a slight but non-significant increase in the expression of CD62L on the dLN CD8\(^+\) T-cells (Supplementary Fig. S8), the low-dose rapamycin treatment weakly impacted the activation status of tumor-infiltrating CD4\(^+\) Teffs (Supplementary Fig. S7A) and CD8\(^+\) T-cells in the tumors and lymphoid tissues (Supplementary Fig. S7C and S8, respectively), confirming the overall observations made with the high-dose rapamycin treatments.

**Low-dose rapamycin treatments inhibit the therapeutic effect of CyaA-E7 vaccination on tumor regression**

We then determined whether the low-dose rapamycin treatment was beneficial for tumor growth control. However, it had no significant effect on the growth of TC-1 tumors in non-vaccinated mice but strongly inhibited the CyaA-E7-induced tumor regression (Fig. 5A). In the chronically-treated group, only 17\% of the mice rejected their tumor vs. 50\% in the untreated mice. Both rapamycin regimens also significantly reduced the vaccine-induced enhanced survival (Fig. 5B). Taken together, these results clearly show that even low-dose rapamycin treatment negatively regulates the T-cell infiltration and response to tumor. Given these results and according to the data from Li et al who showed that an early seven-day treatment with a high dose of rapamycin strongly enhances the anti-tumor CD8 T-cell memory response and tumor regression (15), we also tested the effect of a short treatment with rapamycin from days 0 to 7 after TC-1 graft on the tumor growth in vaccinated and non-vaccinated mice. This short, early treatment with either high or low dose of rapamycin had no effect on the CyaA-E7-induced tumor regression (Supplementary Fig. S9), suggesting that the effect of the treatment with rapamycin from day 0 to 7 does not last long enough to impact the therapeutic activity of the CyaA-E7 vaccine given on day 14.
Rapamycin inhibits the antigen-specific CTL responses in a dose-dependent manner

In contrast to previous studies (14, 15, 27), our results show that long and short rapamycin treatments have detrimental effects on tumor control and vaccine-induced anti-tumoral responses. We therefore determined whether rapamycin also has an effect on the induction of Ag-specific cytotoxic T-cell responses *in vivo*. Mice were immunized on day 0 with CyaA-E7/CpG-B and received daily injections of rapamycin (75 or 750 µg/kg/day) from day -1 until day 8. The induction of CTL responses and IFNγ-producing cells was assayed on day 8 by *in vivo* killing and ELISPOT assays, respectively (*Fig. 6A-B*). The CyaA-E7 vaccine induced a significant killing of E7-loaded targets that was strongly inhibited by both doses of rapamycin (*Fig. 6A*). Rapamycin also reduced the numbers of IFNγ-producing cells in a dose-dependent manner (*Fig. 6B*). We also analyzed the numbers of E7-specific CD8^{+} T-cells in the spleen 7 days after immunization. As expected, CyaA-E7 vaccination induced E7-specific CD8^{+} T-cell responses (19) and rapamycin treatments significantly reduced them in a dose-dependent manner (*Fig. 6C*). We also analyzed rapamycin’s effect on OVA-specific CTL responses and found a similar dose-dependent inhibition of the antigen-specific CTL response after vaccination with OVA/CpG (*Supplementary Fig. S10*). Because Araki et al showed that rapamycin favors memory CD8^{+} T-cell responses after vaccination (14), we assayed the CD8 T-cell response 25 days after immunization, and found inhibitory effects of rapamycin treatment on CTL responses and on the number of IFNγ-producing cells, consistent with our results on day 8 (*Fig. 6D-E*). In contrast, the effect of rapamycin treatments on the percentages of E7-specific CD8^{+} T-cells was not significant on day 25 (*Fig. 6F*). These data demonstrate that rapamycin inhibits the induction of Ag-specific cytotoxic CD8^{+} T-cell responses and seem to have a long-term effect on CTL function but not on the numbers of Ag-specific CD8^{+} T-cells.
DISCUSSION

Chemotherapeutic agents are widely used for cancer treatment alone or in combination with other treatments. In addition to their well-known beneficial effect against tumors, some chemotherapeutics impact the immune system. Cyclophosphamide induces the type-I IFN-associated inflammatory response (28), Treg depletion (29) and enhances the therapeutic effect of vaccination (19). Anthracyclines induce the recruitment of a subset of myeloid cells that are particularly efficient at presenting tumor-cell Ag to CD8^+ T-cells in the tumor (30). However, these agents have an ambivalent impact on the immune system: gemcitabine and 5-fluorouracil (5FU) induce massive tumor cell apoptosis and the selective depletion of MDSCs in the spleen and tumor microenvironment (31, 32). However, they enhance tumor growth by strongly activating the inflammasome in MDSCs (33). Therefore, strategies using different combinations, such as chemotherapy and immunotherapy, but also rapamycin and immunotherapy, are being evaluated. Recent studies indeed showed that rapamycin enhances the memory CD8^+ T-cell differentiation and response to viral vaccines in mouse models of chronic infections and tumors (14-16, 27). Therefore, here we analyzed whether rapamycin enhances the effect of our CyaA-E7 vaccine on tumor-specific CD8^+ T-cell responses and tumor eradication in a mouse model of HPV-related tumors. To address this question, we vaccinated TC-1-bearing mice with the CyaA-E7 and treated them with either low or high doses of rapamycin (75 or 750 µg/kg/day) using two different administration regimens (days 0 to 22 after tumor graft or days 14 to 22). Surprisingly, we found that rapamycin completely inhibits the CyaA-E7-induced CD8^+ T-cell recruitment to the tumor site and tumor regression in a dose-dependent manner. Similarly, rapamycin abrogates the induction of Ag-specific CTL responses. Furthermore, rapamycin represses the CyaA-E7-induced reduction in Treg activation and recruitment to the tumor site, clearly showing that, in our model, treatment with rapamycin is harmful to the vaccine-induced anti-tumor immune responses.
mTOR is a microenvironment sensor that plays a major role in many diseases including cancer where its pathways are highly activated (1). Therefore, new cancer therapies are being evaluated using rapamycin or its analogs to inhibit mTOR activation. However, despite some promising results, particularly in the treatment of RCC (8, 9) and mantle-cell lymphoma (34), the overall clinical results are not conclusive and show that rapamycin and its analogs lead to disease stabilization rather than cancer eradication in patients. This could be because rapamycin inhibits cancer cell proliferation (35) but does not induce cell death.

Importantly, rapamycin and its analogs are also widely used as immunosuppressive agents, especially in transplant patients. By inhibiting mTOR, rapamycin inhibits the transduction of microenvironmental signals, including cytokines, and abrogates cell proliferation and protein synthesis in immune cells, thus inhibiting immune responses (11). Rapamycin was first used as an immunosuppressive drug in combination with cyclosporine and was shown to decrease the risk of post-transplant malignancies (36, 37) due to its properties as an inhibitor of cancer cell proliferation, leading to its use in cancer therapies.

In addition to blocking the cytokine-induced immune response, rapamycin also regulates T-cell differentiation. Indeed, mTOR controls the differentiation of naïve CD4+ T-cells into Th1, Th2 or Th17 cells (38), while its inhibition favors Treg development (11, 39). However, less is known concerning the role of mTOR in the differentiation and immune response of CD8+ T-cells. Surprisingly, recent studies showed that mTOR inhibition by rapamycin enhances the CD8+ memory T-cell response in mice or macaques after viral infection and/or vaccine-induced anti-viral responses (14, 16). Indeed, Araki et al. showed that prolonged treatment with low-dose rapamycin (75 µg/kg/day) enhances the numbers of Ag-specific virus CD8+ T-cells and the functional qualities of memory response, while a short-term treatment favors memory T-cell differentiation (14). They also showed that prolonged treatment with a higher dose of rapamycin (600 µg/kg/day) enhanced the memory CD8+ T-cell response after...
vaccination (14). A short-term treatment with a high dose of rapamycin (750 µg/kg/day) also favored the induction of an Ag-specific memory CD8⁺ T-cell response (15) and was more efficient than a low-dose regimen (75 µg/kg/day). Temsirolimus, an analog of rapamycin, also promoted the vaccine-induced eradication of established tumors (27). However, none of these studies analyzed the impact of rapamycin on the T-cell responses and recruitment in lymphoid organs and more importantly at the tumor site. Similarly, the impact of rapamycin on the anti-tumoral responses in patients has not been reported. Therefore, in the present study we asked whether the rapamycin treatment of tumor-bearing mice affects the vaccine-induced tumor regression and T-cell response and recruitment to the tumor site and lymphoid organs.

In our TC-1 model, CyaA-E7 vaccination induced complete tumor regression in 50% of the mice. To evaluate the impact of rapamycin on vaccination, we selected two doses of rapamycin, 750 and 75 µg/kg/day, administered as a chronic or short-term treatment, according to previous studies. Surprisingly, both regimens completely abrogated the CyaA-E7-induced tumor eradication, while a short early treatment from day 0 to 7 with either dose had no effect. Moreover, while CyaA-E7 increased the numbers of tumor-specific CD8⁺ T-cells as previously demonstrated (19), 8 days after immunization, rapamycin completely inhibited CyaA-E7’s effect. On day 25, the inhibitory effect of rapamycin was still significant on CTL responses, but not on the percentage of E7-specific T-cells, in contrast to the results obtained by Araki et al. However, another study obtained results similar to ours, showing that the rapamycin-induced memory response was impaired in mice and memory CTL were unable to reject a tumor challenge in mouse models of melanoma and breast cancer (40).

Furthermore, rapamycin decreased the numbers of CD45⁺ cells in the spleen, dLN and tumor. Since rapamycin inhibits the proliferation of CD4⁺ and CD8⁺ T-cells and/or dendritic cells (11, 14, 27) and also regulates T-cell migration (11), we suggest that in our model, rapamycin impedes T-cell recruitment and expansion both in the lymphoid tissues and at the tumor site.
Previous studies have suggested that rapamycin favors the differentiation of memory CD8+ T-cells and increases the expression of memory markers, including CD62L and CD44. Our present results contradict these observations. Indeed, we did not observe any significant effect of the rapamycin treatments on CD62L expression on CD8+ T-cells in the lymphoid organs or at the tumor site. Moreover, the high-dose rapamycin treatments even reduced the CD44 expression on CD8+ T-cells in the lymphoid organs. These data clearly show that in our model, rapamycin does not enhance memory cell differentiation and function either.

Although we used the same protocols and doses of rapamycin as in the studies mentioned above, our results are in disagreement with their conclusions. These differences could be explained by the use of different antigens, infection and tumor models, by the type of vaccine used and by the route of infection. Indeed, both, the LCMV virus used in one study (15) and the OVA-expressing poxvirus used in another study (14), induce strong and persistent CD8+ T-cell responses and inducing their differentiation into memory CD8+ T-cells by rapamycin treatment could be beneficial. On the contrary, although E7-expressing TC-1-cells induce CD8+ T-cell response, it remains low/weak as compared to the one induced by viruses or OVA. Furthermore, in the study by Araki et al, they used virus infection while we used tumor inoculation. Moreover, the vaccines used in these two studies were replicating and thus persisted longer than the vaccine used in our model. Although the CyaA-E7 delivers the E7-antigen to dendritic cells and induces an efficient and therapeutic E7-specific CD8+ T-cell response (19), this response remains low compared with the CTL responses induced by viral infection and does not persist for more than few weeks (1.16 ± 0.17% of E7-specific CD8+ T-cells on day 8 vs. 0.165 ± 0.047% on day 25 after vaccination). Finally, we used the sc route of injection for tumor inoculation while Li et al used a systemic route (i.p) (16) thus inducing a different and stronger immune response. Besides, we also showed that rapamycin inhibits the OVA-specific CTL response in mice immunized with OVA/CpG, in a dose-dependent
manner. Moreover, the study by Ferrer et clearly showed that rapamycin has different effects on the OVA-specific CD8+ T-cell response induced in mice either by infection with OVA-expressing bacteria or by OVA-expressing skin-graft. Indeed, rapamycin augmented the CD8+ T-cell response only when induced by infection but not in the context of a transplant (41).

In cancer patients, the dose and length of administration of rapamycin/analogs vary in each study and depending on the cancer type. The weekly i.v. administration of temsirolimus (25 mg) (8) or the daily oral administration of everolimus (10 mg) are given to treat RCC (42), whereas 175 mg temsirolimus for three weeks followed by a 75 mg weekly administration is used to treat mantle cell lymphoma (34). In contrast, in transplant patients, the dosing of rapamycin and its analogs is significantly lower than in cancer patients: 2-5 mg rapamycin given orally daily (43) or 0.75-1 mg everolimus given orally every 12 hours (44). Here, we tested different schedules and doses of rapamycin, a high dose, close to that used in cancer patients, and a low dose, close to that used in transplant patients, and we show that, overall, all of the tested regimens had a negative impact on T-cell recruitment and activation and on tumor control.

It is now well established that the type and frequency of T-cells infiltrating the tumor site play a major role in disease control in lung (45), breast (46) and colorectal (47) cancers. Indeed, patients with higher infiltration of CD8+ T-cells with cytotoxic activity at the tumor site have increased disease-free survival rates compared with patients with poor CD8+ T-cell infiltration (47), clearly showing the importance of lymphocyte recruitment to tumors (48), while Treg infiltration in ovarian and breast cancers is a bad prognosis for patients (49, 50). To our knowledge, our study, based on an extensive analysis of the T-cell compartment in the spleen, dLN and within tumors, using a wide panel of surface molecules, is the first demonstration that rapamycin inhibits T-cell recruitment and expansion in lymphoid tissues and at the tumor site while favoring Treg recruitment and activation in tumors. Rapamycin
treatment also inhibits the induction of antigen-specific cytotoxic CD8+ T-cell responses, thus completely abrogating the vaccine-induced tumor eradication. Furthermore, rapamycin favors the intra-tumoral recruitment of MDSC, known to inhibit cytotoxic CD8+ T-cells (23), thus further contributing to the suppression of the anti-tumor immune response.

Therefore, we clearly establish that although rapamycin might act directly on tumor cells and inhibit their proliferation, in our experimental model, rapamycin also inhibits T-cell recruitment to the tumor site and the induction of anti-tumor immune response by the CyaA-E7 vaccine. These data could explain why rapamycin or its analogs gave only modest clinical results in cancer patients. Thus, the influence of mTOR inhibitors on anti-tumor immunity deserves careful investigation in patients.
AUTHORS’ CONTRIBUTIONS

N.C. contributed to the design of the project, performed the research and wrote the manuscript. C.F B.D., M.O. and A.T. performed research. D.L. provided the CyaA-E7 vaccine. C.L. conceived the project and wrote the manuscript. We thank M. Davi for expert technical assistance in preparing the CyaA-E7 protein.
REFERENCES


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FIGURE LEGENDS

Figure 1. Rapamycin treatment inhibits the tumor regression that is induced by CyaA-E7 vaccination

(A-B) C57BL/6 mice were grafted with 6x10⁵ TC-1 cells on day 0. Mice received daily i.p. injections of rapamycin (750 µg/kg/day) from day 0 to day 22 (A, middle panels) or day 14 to day 22 (A, bottom panels) or of PBS (A, top panels) with (A, right panels) or without (A, left panels) i.v. vaccination with CyaA-E7 (50 µg/mouse) and CpG-B/DOTAP on day 14. (B) Percent survival is shown. The results represent the cumulative data from two independent experiments (n=12 mice per group). *p<0.05 ** p<0.05 ***p<0.001 Mantel–Cox Logrank test.

Figure 2: Rapamycin treatments inhibit lymphocyte recruitment to tumor-draining LN and tumor site and abrogates the therapeutic activity of CyaA-E7.

C57BL/6 mice were grafted with 6x10⁵ TC-1 cells on day 0. The mice received daily i.p. injections of either rapamycin or PBS from day 0 to day 22 or day 14 to day 22, with or without vaccination with CyaA-E7 and CpG-B/DOTAP on day 14. On day 22 after the tumor graft, the mice were killed, and cell suspensions were prepared from the dLN and the tumor and analyzed by flow cytometry. The peripheral LN from naïve mice were processed similarly and used as controls. (A-B) The total number of CD45⁺ cells that were isolated from the LN (A) and the tumor (B) are shown in the top left panels. The total numbers of lymphocyte subsets (top right panels), the percentages of lymphocyte subsets within the CD45⁺ cells (bottom left panels) and the CD4⁺ Foxp3⁺ Tregs and CD4⁺ Foxp3⁻ Teffs within the total CD3⁺ CD4⁺ cells (bottom right panels) are shown. The results represent the
cumulative data from four independent experiments (mean ± SEM; n=6-8 mice per group).

*p<0.05, **p<0.01 and *** p<0.001 Mann-Whitney test.

**Figure 3. Rapamycin treatment favors MDSC recruitment and accumulation in the tumor.**

C57BL/6 mice were grafted with 6x10⁵ TC-1 cells on day 0. The mice received daily i.p. injections of either rapamycin (750 µg/Kg/day) or PBS from day 0 to day 22 or day 14 to day 22, with or without vaccination with CyaA-E7 and CpG-B/DOTAP on day 14. On day 22 after the tumor graft, the mice were killed, and cell suspensions were prepared from the spleen, dLN and tumor and analyzed by flow cytometry. The spleens and peripheral LN from naïve mice were processed similarly and used as controls. The numbers (upper left panels) and the percentages of total MDSC (CD11b⁺ Gr1⁺) within the CD45⁺ cells (upper right panels) are shown for the spleen (A), LN (B) and tumor (C). The results represent the cumulative data from two independent experiments (mean ± SEM; n=4 mice per group).

*p<0.05, **p<0.01 and *** p<0.001 Mann-Whitney test.

**Figure 4. Rapamycin treatment enhances the immunosuppressive phenotype of tumor-infiltrating T-cells.**

C57BL/6 mice were grafted with 6x10⁵ TC-1 cells on day 0. The mice received daily i.p. injections of either rapamycin or PBS from day 0 to day 22 or day 14 to day 22, with or without vaccination with CyaA-E7 and CpG-B/DOTAP on day 14. On day 22 after the tumor graft, the mice were killed, and the tumors were processed to obtain cell suspensions. The cells were then analyzed by flow cytometry. The surface expression of CD44, PD-1 and GITR on tumor-infiltrating CD4⁺ Foxp3⁺ Tregs and of CD44, PD-1 and CD73 on tumor-infiltrating CD8⁺ T-cells are shown in A-B and C-D, respectively. (A and C). The expression of these
molecules on tumor-infiltrating Tregs from mice treated daily with rapamycin from day 0 to 22 (with or without vaccination) (left panels) and mice treated daily with rapamycin from day 14 to 22 (right panels) is shown. The graphs in the left panels show the expression in the rapamycin-treated (days 0-22) unvaccinated mice (filled red lines) and the rapamycin-treated (days 0-22) vaccinated mice (orange lines), while the right panels show the expression in the rapamycin-treated (days 14-22) unvaccinated mice (filled blue lines) and the rapamycin-treated (days 14-22) vaccinated mice (green lines). In the right and left panels, the PBS-treated unvaccinated mice are represented by filled grey lines and the PBS-treated and vaccinated mice with black lines. The isotype controls are shown in the dashed lines. The data from one representative experiment are shown. (B and D) Geometric Mean Fluorescence Intensity (MFI ± SEM of CD44, PD-1 and GITR on Tregs and CD44, PD-1 and CD73 on CD8+ T-cells isolated from tumors are shown (mean results from three independent experiments are shown (n=6 mice per group). *p<0.05, **p<0.01 and *** p<0.001 Mann-Whitney test.

Figure 5. Low-dose rapamycin treatments reduce the anti-tumor therapeutic effect of the CyaA-E7 vaccine

(A-B) C57BL/6 mice were grafted with 6x10⁵ TC-1 cells on day 0. The mice received daily i.p. injections of rapamycin (75 µg/kg/day) from day 0 to day 22 (A, middle panels) or day 14 to day 22 (A, bottom panels) or of PBS (A, top panels) with (A, right panels) or without (A, left panels) i.v. vaccination with CyaA-E7 (50 µg/mouse) and CpG-B/DOTAP on day 14. (B) Percent survival is shown. The results represent the cumulative data from two independent experiments (n=12 mice per group). *p<0.05 ** p<0.05 ***p<0.001 Mantel–Cox Logrank test.
Figure 6. Rapamycin inhibits the antigen-specific CTL responses in a dose-dependent manner

(A-F) C57BL/6 mice were i.v. immunized (on day 0) with CyaA-E7 (50 µg/mouse), adjuvanted with CpG-B/DOTAP or were left untreated. The immunized mice received daily i.p injections of either rapamycin from day -1 to day 7 (75 or 750 µg/kg/day; “Rapa Low” and “Rapa High”, respectively) or PBS. The CTL responses were assayed by in vivo killing with target splenocytes pulsed with the E7 peptide RAHYNIVTF on day 8 (A) and 25 (D). IFNγ production by splenocytes after in vitro stimulation with the E7 peptide was determined by ELISPOT, on days 8 (B) and 25 (E). The percentages of E7-tetramer-specific CD8⁺ T-cells were also analyzed in the spleen by flow cytometry on days 8 (C) and 25 (F). The results represent the cumulative data from three independent experiments (n=11-13 mice/group in A-E and 5-6 mice/group in F). *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 Mann-Whitney test.
Figure 1

A

PBS
0/12 (0%)

CyaA-E7/CpG
5/12 (42%)

Rapa d0-22
0/12 (0%)

Rapa d0-22 + CyaA-E7/CpG
0/12 (0%)

Rapa d14-22
0/12 (0%)

Rapa d14-22 + CyaA-E7/CpG
0/12 (0%)

B

Percent survival

Days

Days

Days

Days

PBS
CyaA-E7/CpG
Rapa d0-22
Rapa d0-22 + CyaA-E7/CpG
Rapa d14-22
Rapa d14-22 + CyaA-E7/CpG

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A LN

B Tumor
Figure 3

A Spleen

B LN

C Tumor
Figure 4

A  
**CD4^+ Foxp3^+ Tregs**

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C

**CD8^+ T Cells**

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**CD44**

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Figure 5

A

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B

- PBS
- CyaA-E7/CpG
- Rapa d0-22
- Rapa d0-22 + CyaA-E7/CpG
- Rapa d14-22
- Rapa d14-22 + CyaA-E7/CpG

Percent survival

- ***
- *
- ns
Figure 6
Rapamycin impairs antitumor CD8+ T cell responses and vaccine-induced tumor eradication

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