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Regulating Mammalian Target of Rapamycin To Tune Vaccination-Induced CD8+ T Cell Responses for Tumor Immunity

Qingsheng Li,* Rajesh Rao,* Joseph Vazzana,* Peter Goedegebuure,† Kunle Odunsi,‡ William Gillanders,‡ and Protul A. Shrikant*

Vaccine strategies aimed at generating CD8+ T cell memory responses are likely to show augmented efficacy against chronic challenges like tumor. The abundance in variety of memory CD8+ T cells behooves development of vaccine strategies that generate distinct memory responses and evaluate them for tumor efficacy. In this study, we demonstrate the ability of a variety of rapamycin treatment regimens to regulate virus vaccination-induced CD8+ T cell memory responses and tumor efficacy. Strikingly, a short course of high-dose, but not low-dose, rapamycin treatment transiently blocks viral vaccination-induced mammalian target of rapamycin activity in CD8+ T cells favoring persistence and Ag-recall responses over type 1 effector maturation; however, prolonged high-dose rapamycin administration abrogated memory responses. Furthermore, a short course of high-dose rapamycin treatment generated CD8+ T cell memory responses that were independent of IL-15 and IL-7 and were programmed early for sustenance and greater tumor efficacy. These results demonstrate the impact a regimen of rapamycin treatment has on vaccine-induced CD8+ T cell responses and indicates that judicious application of rapamycin can augment vaccine efficacy for chronic challenges. The Journal of Immunology, 2012, 188: 3080–3087.

CD8+ T cells play an important role in host defense against intracellular infections and cancer. Their inherent ability to localize, recognize, and kill affected cells, as well as form memory, makes them an excellent target for vaccination against tumors and intracellular infections (1–3). On the basis of our understanding of CD8+ T cell responses to infectious agents, several vaccination strategies have been designed to generate type 1 effector responses, and they have been evaluated for tumor efficacy (4, 5). Although viral vaccination can successfully produce tumor-Ag–specific CD8+ T cell clonal expansion and type 1 effector maturation, their antitumor efficacy has been less than desirable (6–8). It has been suggested that the generation of CD8+ T cell memory responses may be required for protection against indolent challenges like chronic infections and/or cancer (9, 10). Recent advances have identified the role of various cell extrinsic factors in generating a wide variety of CD8+ T cell memory responses (11, 12), although vaccine strategies to produce varying CD8+ memory responses and their efficacy to indolent challenges like cancer remain uncharacterized. Nevertheless, considerable interest has arisen in applying new mechanistic insights regulating CD8+ T cell memory generation toward the development of vaccines against tumor and intracellular infections.

The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase that integrates cell extrinsic signals to control several key functions like transcription, translation, cell size, division, and migration (13). The activation of the mTOR kinase causes phosphorylation of the downstream targets S6 kinase/S6 and 4E-BP1, which mediate protein translation, cell cycle, and growth (14). Early studies had identified the mTORC1-specific inhibitor rapamycin as an immunosuppressive agent, and it was thus used to treat allograft rejection reactions encountered during solid organ transplantation (15). However, owing to its modest efficacy, it is currently used mainly in combination with cyclosporine A and corticosteroids (16). Previously, it was demonstrated that rapamycin treatment induced CD4+ T cell anergy, Foxp3+ regulatory cells, and/or deviation to the type 2 effector phenotype, thus causing immune regulation (17–19). Recent studies have uncovered a novel role for mTORC1 in determining functional fates of both CD4+ and CD8+ T cells and the ability of rapamycin treatment to facilitate transition of effector CD8+ T cell responses to memory (20–23). Strikingly, the ability of in vivo rapamycin administration to augment CD8+ T cell memory responses to viral challenge was cell autonomous (21) and was mediated by a shift from a T-bet+ to an a Eomesodermin-dominated transcription program (23). Although it was previously reported that a rapamycin regimen can affect virus-induced CD8+ T cell memory response (21), the study was not designed to characterize the cellular mechanisms underpinning the impact that the dose and duration of rapamycin treatment had on vaccine-induced CD8+ T cell responses. Moreover, the ability of rapamycin-mediated CD8+ memory responses to affect tumor growth was not tested. Because rapamycin administration can result in tolerance (17, 24), it is imperative that careful studies to understand the impact of rapamycin treatment on vaccine-induced CD8+ T cell responses be conducted prior to further exploration in the clinic.
A vaccination strategy that can consistently generate tumor-Ag-specific CD8+ T cell responses of required quality, magnitude, and duration is highly desirable, and exploiting emerging information on the central role of mTOR in regulating Ag-specific CD8+ T cell responses is particularly attractive owing to ease of translation. In this study, by monitoring vaccine-induced CD8+ T cells, we characterize the impact of the dose and duration of rapamycin treatment on the quantity and quality of CD8+ memory responses induced by viral vaccination and their ability to afford durable tumor protection.

Materials and Methods

Mice and reagents

The C57BL/6 (B6) mice and CD8+ TCR transgenic mice with Thy1.1 congenic marker (OT-1) were bred and housed at Roswell Park Cancer Institute. Act-OVA B6 mice (ACTB-OVA) were purchased from The Jackson Laboratory (Bar Harbor, ME) (25). The IL-15-deficient B6 (B6–IL-15−/−) mice were purchased from Taconic (Germantown, NY). All animals were used according to the Institutional Animal Care and Use Committee guidelines of Roswell Park Cancer Institute. Rapamycin was purchased from ChemieTek (Indianapolis, IN). The rapamycin was diluted with PBS and used in vivo by i.p. injection at 0.075 mg/kg/d or 0.75 mg/kg/d. Phorbol ester PMA, ionomycin, and Brefeldin A were purchased from Sigma-Aldrich (St. Louis, MO).

Adoptive transfer and virus immunization

Purified naïve OT-1 cells (2 × 106) labeled with or without 5 μM CFSE (Invitrogen) were (i.v.) adoptively transferred into syngeneic B6 recipients. B6 recipients were immunized with recombinant poxvirus expressing (Invitrogen) were (i.v.) adoptively transferred into syngeneic B6 recipients. All viruses were a kind gift from the National Cancer Institute. Act-OVA B6 mice (ACTB-OVA) were purchased from The Jackson Laboratory (Bar Harbor, ME) (25). The IL-15-deficient B6 (B6–IL-15−/−) mice were purchased from Taconic (Germantown, NY). All animals were used according to the Institutional Animal Care and Use Committee guidelines of Roswell Park Cancer Institute. Rapamycin was purchased from ChemieTek (Indianapolis, IN). The rapamycin was diluted with PBS and used in vivo by i.p. injection at 0.075 mg/kg/d or 0.75 mg/kg/d. Phorbol ester PMA, ionomycin, and Brefeldin A were purchased from Sigma-Aldrich (St. Louis, MO).

Abs and flow cytometry

All Abs used for flow cytometry were purchased from BD Pharmingen, except anti–IL-7Rα (A7R34), anti-Eomesodermin (Eomes, DanilMag), anti–T-bet (eBio4B10), and anti-Granzyme B (16G6), which came from eBioscience; Annexin V-FLICA and propidium iodide (PI) were obtained from Invitrogen. Anti–IL-7Rα (BD Biosciences) was determined after a 5-h Ag restimulation. Single-cell suspensions from splens were analyzed by flow cytometry. Donor OT-1 cells were detected as CD8+ and Thy1.1 double positive and gated for further analysis. LSR II and FACScalibur (Becton Dickinson) were used for flow cytometry event collection, and events were analyzed with FlowJo (Tree Star) and CellQuest software (BD Biosciences).

Evaluation of in vivo OT-1 cell responses

Mice were sacrificed, and the total number of adoptively transferred OT-1 cells (CD8a+Thy1.1+) was detected by flow cytometry; numbers were calculated by multiplying the total cell counts by the percentage of CD8a+Thy1.1+ T cells. The phenotype and CFSE analysis was performed on OT-1 gated cells (>10,000 events).

Tumor challenge and survival

Intact B6 (n = 10) mice challenged with EL4 thymoma or EG.7-OVA thymoma cells (28) administered i.p. (5 × 106 cells per mouse) on day −10 were adoptively transferred with naive OT-1 cells (2 × 106) on day −2. In some experiments, B6 mice were challenged with EG.7 tumor cells administered i.p. (5 × 106 cells per mouse) on day −10 without OT-1 cell transfer, and challenged mice were immunized with s.c. injection of Tricom (2 × 107 PFU) or empty control virus (2 × 107 PFU) on day 0. Rapamycin (0.075 mg/kg/d or 0.75 mg/kg/d) was given by i.p. injection from day 0 to 7 or day 0 to 39 and monitored thereafter for tumor growth and morbidity. To demonstrate durable protection by programmed OT-1 cells, they were isolated by anti-CD90.1 MicroBeads on a MiniMACS Separator (Miltenyi Biotec) on day 8 from various animals, and 1 × 106 OT-1 cells (thy1.1+) were adoptively transferred into 10-d inoculated EG.7 tumor-bearing hosts. Tumor-free survival was monitored.

Evaluation of in vivo OT-1 cell responses

To study the impact of varying rapamycin treatment on viral vaccine-induced CD8+ T cell responses, we monitored adoptively transferred naive TCR transgenic CD8+ T cells (OT-1) in thy 1.1 congenic B6 recipients. The OT-1 recipients were typically vaccinated on day 0 with canary poxvirus expressing chicken OVA-mLFA-3/miCAM/mB7.1 (Tricom) or control virus (no Ag) (26), and some recipients were given the mTOR inhibitor rapamycin at a dose of either 0.075 mg/kg/d (lo) or 0.75 mg/kg/d (hi) from day 0 to 7 every day. The lymph node and spleen cells from animals treated with Tricom or Tricom plus rapamycin at either dose were stained, gated for CD8+Thy1.1+ and analyzed by flow cytometry on day 4. The intracytoplasmic phosphorylation of ribosomal protein S6 (pS6), an mTOR kinase target (29), was increased in Tricom-immunized animals (Fig. 1A). Surprisingly, low doses of rapamycin treatment produced no reduction in pS6, but substantial decreases were noted in OT-1 cells derived from animals treated with high doses of rapamycin (Fig. 1A). In congruence, high-dose rapamycin treatment dampened activation and resulted in CD44 and CD122 lo, but CD62L and IL-7Rα hi (Fig. 1A), proliferation, as well as lower CFSE dilution (Fig. 1B) and clonal expansion (Fig. 1C, 1D); in contrast, animals receiving low-dose rapamycin showed only marginal reduction in OT-1 activation (surface phenotype CD44hi, CD21lo, CD62Lhi, IL-7Rαlo, and CD122hi) and proliferation (CFSE dilution), but produced enhanced clonal expansion (Fig. 1A–D). The OT-1 responses detected in vaccinated mice were Ag specific, as control virus failed to increase the frequency and number of OT-1 cells (data not shown). The reduction in pS6 and dampened OT-1 responses by high-dose rapamycin was transient, as withdrawal of rapamycin on day 8 rapidly restored mTOR activity along with their activation phenotype, proliferation, and clonal expansion (Supplemental Fig. 1A–D). The ability of low-dose rapamycin treatment to produce higher clonal expansion, along with reduced cell proliferation of viral vaccine-induced OT-1 responses on day 4 (Fig. 1B–D, CFSE dilution and numbers of OT-1 cells), led us to test the impact of rapamycin dose on virus vaccine-induced CD8+ T cell apoptosis by evaluating the Annexin V-FLICA/PI staining and cytoplasmic expression of Bcl-2 protein by OT-1 cells. As anticipated, the addition of rapamycin at either dose dramatically decreased cell death (Annexin V-FLICA/PI–OT-1 cells) (Fig. 2A), with concomitant increases in Bcl-2 expression in a dose-dependent manner (Fig. 2B). Thus, the ability of low-dose rapamycin treatment to enhance vaccine-induced CD8+ T cell expansion was due in part to increased Bcl-2 expression and reduction in apoptotic cell death (Fig. 2A, 2B), but not enhanced proliferation (CFSE dilution) (Fig. 1B). Because the relative expression levels of the transcriptional factors T-bet and Eomesodermin determine the functional fate of CD8+ T cells (30), we next evaluated the impact of rapamycin dose on expression of T-bet and/or Eomes by intracytoplasmic staining and flow cytometry of OT-1 cells on days 4 and 8 after vaccination. As shown in Fig. 3, high-dose rapamycin reduced expression of T-bet and IFN-γ, but produced only a marginal change in Granzyme B and Eomes expression on day 4 after transfer. Of interest, low-dose rapamycin showed no reduction in T-bet and IFN-γ expression relative to Tricom alone, which is in agreement with the levels of CD122 expression (Fig. 1A), as CD122 is a direct T-bet target (30). Similar
to our observed rebound in early pS6 and CD8+ T cell responses after cessation of rapamycin treatment, the levels of T-bet and IFN-γ expression were rapidly restored in OT-1 cells obtained from animals treated with high doses of rapamycin; in fact, in viral-vaccinated animals on day 8 after transfer, the IFN-γ and Granzyme B expression was greater in animals treated with high doses of rapamycin than in those treated with low doses or those untreated with rapamycin (Supplemental Fig. 2). Therefore, the inhibition of mTOR activity, activation, proliferation, and effector maturation by rapamycin treatment is dose dependent and produces distinct regulation of vaccine-induced early CD8+ T cell responses.

High-dose rapamycin enhances vaccination-induced CD8+ T cell memory responses

Instructions provided early during Ag stimulation produce a programmed long-term CD8+ T cell response (31). To determine whether the rapamycin dose-dependent effects on vaccine-induced CD8+ T cells also regulated long-term responses, we evaluated OT-1 persistence until day 40 after vaccination and tested their ability to mount Ag-recall responses (rechallenge on day 40–43). The persistence of viral vaccine-induced OT-1 responses was significantly enhanced by rapamycin treatment in a dose-dependent manner. The short duration of high-dose rapamycin treatment produced greater OT-1 cell persistence than did a lower dose of rapamycin treatment (Fig. 4A, 4B). Surprisingly, the surface phenotype of the OT-1 cells in all treatment groups was similar: CD44hi, CD62Lhi, and IL-7Rαhi, except for the expression of T-bet and CD122 (Fig. 4C). In contrast to OT-1 cells from Tricom-vaccinated mice, OT-1 cells from mice vaccinated with Tricom plus rapamycin demonstrated heightened Ag-recall responses 3 d after rechallenge (day 43 absolute OT-1 number, as well as production of IFN-γ and Granzyme B), with high-dose rapamycin treatment showing significantly greater memory response (Fig. 4D). It was also noted that although both low- and high-dose rapamycin treatment augmented Tricom vaccine-mediated Ag-specific recall response, the fold expansion observed with high-dose rapamycin treatment was inferior to that of low-dose rapamycin treatment (Fig. 4D, recall response), which may reflect the differences in the type of CD8+ memory generated (11). These observations indicate that regulation of vaccine-induced mTOR activity by dose of rapamycin administered can produce quantitatively and qualitatively distinct CD8+ T cell memory responses.

Persistent inhibition of mTOR suppressed CD8+ memory responses

Previously, persistent low-dose rapamycin treatment has been shown to enhance virus-specific CD8+ T cell memory responses (21). On the basis of our observations above, we reasoned that persistent dampening of vaccine-induced activation, proliferation, and effector maturation would further enhance CD8+ T cell memory responses. To test this notion, we extended the high-dose rapamycin treatment from day 0 to 39 and evaluated its impact on long-term OT-1 responses. Surprisingly, prolonged high-dose (0.75 mg/kg/d) rapamycin administration abrogated OT-1 persistence (day 40) produced by short-course high-dose rapamycin treatment (Fig. 5A). Notably, the low frequency of OT-1 cells in day 0–39 rapamycin-treated animals produced a weak Ag-recall response on day 43, albeit with high IFN-γ but no Granzyme B expression (Fig. 5B, right)
This finding showed that long-term mTOR blockade was not suitable for generating robust memory CD8+ T cell responses and emphasized the importance of mTOR reinduction for enhanced memory responses with both IFN-γ and Granzyme B production.

**Dose of rapamycin treatment determines IL-15 requirement for CD8+ T cell memory**

The common γ-chain cytokine IL-15 has been shown to be crucial for CD8+ T cell memory generation (32). Previously, we had reported the ability of rapamycin to augment lymphopenia-induced CD8+ T memory responses in an IL-15-independent manner (27). Because the dose of rapamycin treatment differentially affected T-bet and CD122 expression by OT-1 cells (Fig. 1A, Fig. 3), we predicted that rapamycin in a dose-dependent manner generates qualitatively distinct CD8+ memory responses in terms of their dependence on cell extrinsic factors like IL-15. In agreement with previous reports, OT-1 cells transferred into IL-15−/− hosts and vaccinated with Tricom failed to produce persistent responses at day 40 (Fig. 6A) and generated poor memory responses (Fig. 6B). It is interesting that, rapamycin treatment rescued Tricom-induced OT-1 cells in IL-15−/− recipients in terms of both persistence (Fig. 6A, day 40) and Ag recall (Fig. 6B, day 43). However, only the high dose of rapamycin, which blocked mTOR at early time-points (Tricom+Rapa [hi]. Tricom+Rapa [lo]), was able to produce CD8+ memory in a cell-autonomous manner. It was noted, though, that no significant differences in effector molecule expression, IFN-γ and Granzyme B, was observed during Ag recall with either dose of rapamycin used (Fig. 6B, right panel). Furthermore, the memory responses observed with high-dose rapamycin treatment were also maintained in the presence of IL-7 blockade in vivo (Supplemental Fig. 3), although the persistence was slightly reduced. This observation implies that short but complete mTOR blockade facilitates transition of effector cells to memory and rapamycin dose variation affects the dependence on cell extrinsic factors for CD8+ memory generation.

**FIGURE 3.** Vaccine-induced T-bet expression and effector function were inhibited in response to high-dose rapamycin treatment. B6 recipients receiving OT-1 cells were daily injected with either no, low-dose, or high-dose rapamycin, as indicated above. Spleen cells harvested on day 4 after the start of rapamycin treatment were stained on the surface and intracellularly and then gated and analyzed by flow cytometry for T-bet, Eomes, IFN-γ, and Granzyme B. Data representing three experiments with identical outcomes are shown.

**FIGURE 4.** Transient mTOR blockade enhances vaccine-induced CD8+ T cell memory. Recipients of OT-1 cells were challenged with Tricom or control virus on day 0 followed by no, low-dose (0.075 mg/kg/d), or high-dose (0.75 mg/kg/d) rapamycin treatment every day from day 0 to 7. The spleen cells were harvested, stained, and evaluated on day 40 for persistence and on day 43 for Ag-recall responses (3 d postimmunization). (A) The percentage of OT-1+ (CD8α+Thy1.1+) cells on day 40. (B) The absolute numbers of OT-1 cells on day 40. (C) The expression of CD44, CD62L, IL-7Rα, CD122, and T-bet on day 40. (D) Left panel, The absolute numbers of OT-1 cells on day 43. The numbers in parentheses indicate the fold increase in OT-1 postimmunization. Right panels, The expression of IFN-γ and Granzyme B on gated OT-1 cells. Values represent means plus or minus SD of three independent experiments. *p < 0.05, **p < 0.01.
Ag-induced CD8+ T cell responses, as animals with non–antigen-expressing virus plus high-dose rapamycin treatment showed no survival benefits (data not shown). In congruence with the loss of CD8+ T cell memory due to longer duration of high-dose rapamycin administration, we also failed to observe increased survival (Fig. 7B). The observed impact of rapamycin dose on tumor immunity was identical when the modality was tested in a fully syngeneic (Actin-OVA transgenic mouse) tumor challenge model without adoptive transfer of TCR transgenic CD8+ (OT-1) cells (Fig. 7C). Although some differences in the extent of tumor-free

FIGURE 5. Persistent mTOR inhibition abrogates vaccine-induced CD8+ T cell responses. Naive OT-1 cell recipients were challenged with Tricom (2 × 10^7 PFU) or control virus at day 0. Rapamycin (high-dose, 0.75 mg/kg/d) was administered from day 0 to 7 or day 0 to 39. Spleen cells harvested on day 40, persistence, or day 43 (3 d postimmunization), Ag-recall response, were stained, gated, and evaluated by flow cytometry. (A) The absolute number of OT-1 cells, day 40. (B) The absolute number of OT-1 cells and IFN-γ and Granzyme B expression, day 43. The numbers in parentheses indicate the fold increase in OT-1 numbers postimmunization. All data are representative of two independently performed experiments. *p < 0.05, **p < 0.01.

FIGURE 6. Rapamycin promotes IL-15–independent CD8+ T cell memory in a dose-dependent manner. WT or IL-15−/− OT-1 recipients immunized with Tricom or control virus were treated with either no, low-dose (0.075 mg/kg/d), or high-dose rapamycin (0.75 mg/kg/d) from day 0 to 7. Spleen cells harvested on day 40, persistence, or day 43, Ag-recall, were stained, gated, and evaluated by flow cytometry. (A) The absolute numbers of OT-1 cells on day 40. (B) Ag-recall response of OT-1 cells on day 43. Left panel, The absolute number of OT-1 cells. The numbers in parentheses indicate the fold increase in OT-1 numbers postimmunization. Right panel, The expression of IFN-γ+ and Granzyme B+ by gated OT-1 cells. The error bars are SD of values obtained from three animals per group, and a representative of two independent experiments with identical outcomes is shown. *p < 0.05, **p < 0.01, ***p < 0.001.
FIGURE 7. A short course of high-dose rapamycin treatment enhances antitumor vaccine efficacy. B6 mice challenged with 3 × 10^6 EG.7 by i.p. injection on day −10 received 2 × 10^6 naive OT-1 cells on day −1 followed by s.c. injection of Tricom (dose as indicated above) or control virus on day 0. (A) Vaccinated animals were treated either with rapamycin from day 0 to 7 at low dose (0.075 mg/kg/d) or at high dose (0.75 mg/kg/d) (A) or with rapamycin from day 0 to 7 or 0 to 39 at high dose (0.75 mg/kg/d) (B). (C) Act-OVA B6 mice were inoculated with 3 × 10^6 EG.7 by i.p. injection on day −10 and treated with Tricom and rapamycin (dose and duration as indicated above). (A–C) Survival curve of tumor-free animals. The results of (A) and (B) are the summation from three independent experiments with 10 mice per experimental group per experiment. The results of (C) are the summation of two independent experiments with 10 mice per experimental group. The outcomes of all experiments were similar.

Survival were observed, the overall conclusions reached were identical, thus demonstrating the benefit of transient mTOR inhibition by high-dose rapamycin treatment for viral vaccine-induced CD8^+ T cell memory responses and tumor efficacy. To confirm that rapamycin induced early changes—on day 8, in CD8^+ T cells it was responsible for the augmented CD8^+ memory and tumor efficacy—we isolated OT-1 cells (Thy1.1^+) on day 8 from animals treated with either Tricom alone or Tricom plus high-dose rapamycin (day 0–7); equal numbers of purified OT-1 cells were retransferred into day −10 EG.7 or EL-4 (non-Ag parental tumor) tumor-bearing recipients, and their impact on tumor growth/survival was monitored. Remarkably, the OT-1 cells obtained from mice treated with Tricom and high-dose rapamycin (day 0–7) produced greater (60% EG.7; but not EL4) tumor-free survival (Fig. 8A) and demonstrated greater memory functions (Fig. 8B). These findings indicate that a short course of high-dose rapamycin treatment programs vaccine-induced CD8^+ T cells early for cell-autonomous memory and tumor efficacy.

Discussion

The paradigm-shifting observations that the mTOR inhibitor rapamycin can augment CD8^+ T cell memory responses to lymphophytic choriomeningitis virus (LCMV) infection by acting directly on CD8^+ T cells in vivo (21) and the central role of mTOR in regulating expression of master transcriptional factors—T-bet for type 1 effector functions and Eomesodermin for memory (23)—have generated considerable interest in the use of rapamycin or mTOR inhibitors for generating memory CD8^+ T cell responses. In this article, by characterizing the impact of the rapamycin treatment regimen on vaccine-induced CD8^+ T cell activation, expansion, and differentiation for type 1 effector and/or memory responses and relating these observations to their ability to promote efficacy against a syngeneic murine tumor, we provide new information to validate the use of rapamycin in vaccination. Our findings demonstrate that a short course of high-dose rapamycin treatment transiently blocks viral vaccination-induced mTOR activity to dampen CD8^+ T cell activation, proliferation, and effector differentiation. Strikingly, a longer course of rapamycin treatment abrogated the enhanced viral vaccination-induced CD8^+ memory generation, persistence, and Ag recall generated by transient mTOR blockade. Although the phenotypes of Ag-specific CD8^+ memory responses produced by vaccination alone or by vaccination plus rapamycin appeared to be identical, they were quantitatively and qualitatively distinct and demonstrated different tumor efficacy. These findings lend support to the notion that memory, rather than effector CD8^+ T cells, is desirable for tumor efficacy.

Our results demonstrate the ability of rapamycin to regulate vaccine-induced CD8^+ T cell mTOR activity in a dose-dependent manner and provide evidence of the impact differential mTOR inhibition has on early activation phenotype, proliferation/cell death for clonal expansion, and T-bet–mediated differentiation for type 1 effector functions. Although some of these findings

FIGURE 8. A short course of high-dose rapamycin treatment programs vaccine-induced CD8^+ T cells for durable antitumor response. The congenic OT-1^+ cells Thy1.1^+ (1 × 10^5) isolated on day 8 from animals treated with either Tricom alone or Tricom plus rapamycin (day 0–7 at 0.75 mg/kg/d) were adoptively transferred into recipients bearing day 10 EL4 (non-Ag control) or EG.7 tumors (A) or intact B6 mice (B). (A) Tumor-free survival. (B) Persistence, day 40, and Ag-recall responses, day 43. Left panel, The absolute number of OT-1^+ cells on day 40. Right panel, The absolute number of OT-1^+ cells on day 43 after rechallenge with Ag (IFA/OVA) on day 40. The numbers in parentheses indicate the fold increase in OT-1 numbers postimmunization. The results shown are representative of two independent experiments with identical outcomes. *p < 0.05.
confirm previously noted changes in the clonal pool of Ag-specific CD8+ T cells generated in response to LCMV infection (21), the insights provided by characterizing cellular and molecular mechanisms underpinning the impact of dose and duration of rapamycin treatment on vaccine-induced CD8+ T cell responses will allow judicious use of rapamycin treatment in a preclinical model and facilitate future clinical translation. The ability of low-dose rapamycin treatment to significantly augment vaccine-induced clonal expansion can be largely attributed to its reduction in apoptosis, perhaps by augmenting Bcl-2 expression, a finding in agreement with previous studies that show the ability of rapamycin (10 μM) to reduce apoptosis of TH2 type cells owing to decreased activation of capase 3 and 9, as well as Bim and Bid expression, and simultaneously causing increased Bcl-XL expression (33). The mechanisms for this effect remain unclear, but it can be conjectured that rapamycin treatment may reduce phosphorylation of p53, which in turn dampens the upregulation of proapoptotic protein of Bax and/or caspase activation for reduced apoptotic death (34, 35). Because high-dose rapamycin treatment reduced vaccine-induced OT-1 clonal expansion, our results demonstrate that the relative balance between proliferation and cell death is regulated by transient mTOR inhibition, thereby determining the extent of clonal expansion achieved by immunization. Strikingly, withdrawal of rapamycin treatment on day 7 led to a rapid restoration of mTOR activity and resumption of cell proliferation, which produced considerable clonal expansion by day 8 in animals treated by high-dose rapamycin. Although these observations seem to contradict previous studies (21), we contend that the differences may be due to differences in experimental conditions, such as the strength of Ag signal, LCMV versus Tricom, and the characterization of blood rather than splenocytes and lymph node cells, which we have evaluated. It is also possible that the Tricom and LCMV infections produce varying levels of CD8+ T cell proliferation for clonal expansion and that a high dose of rapamycin shows greater ability to block a higher mTOR activation state and favor transition of CD8+ T cells for memory, which is consistent with the role of mTOR in instructional activation and proliferation of CD8+ T cells (17, 36, 37). The new information on rapamycin dose-dependent impact on vaccine-induced clonal expansion by distinct cellular and molecular pathways is insightful and will be useful in designing preclinical studies for evaluating early responses and their predictive value in future clinical trials.

Analysis of memory CD8+ T cell formation after challenge vaccine plus low- or high-dose rapamycin supports the following conclusions. First, the extent of mTOR inhibition produced by different doses of rapamycin administered after viral vaccination causes a qualitative shift in CD8+ memory responses. The observed difference in CD122 and T-bet expression on day 40 by OT-1 cells in vaccinated animals treated with varying doses of rapamycin suggests that a transient early blockade of mTOR activity programs memory generation by cell-intrinsic mechanisms. Second, the dose of rapamycin determines whether extrinsic cytokine IL-15 and/or IL-7 is required for vaccine-induced memory CD8+ T cells. The ability of high-dose rapamycin treatment to “rescue” the loss of CD8+ T cell memory responses in the absence of IL-15 or of functional IL-7 indicates the cell-intrinsic mechanisms of high-dose rapamycin–induced memory CD8+ T cell generation. It is noteworthy that recovery of mTOR activity was necessary for the persistence of CD8+ T cells (Supplemental Fig. 1A), as long-term high-dose rapamycin fails to overcome the defects in memory CD8+ T cell generation in the absence of functional IL-7 and/or IL-15 signaling (27). These results suggest that rapamycin treatment-mediated transient mTOR blockade promotes transition of vaccine-induced effector cells for memory.

Several studies have found that inflammation generates “short-lived” effector CD8+ T cells owing to increased T-bet expression (38–40). In this study, a short course of high-dose rapamycin treatment caused transient reduced T-bet expression without regulation of expression of Eomes. However, the relative ratio of Eomes to T-bet is higher in OT-1 cells from Tricom plus high-dose rapamycin–treated animals (data not shown), which indicates that reduced T-bet expression and/or increased Eomes expression enhances vaccine-induced CD8+ effector cells to survival. Nevertheless, the association of reduced T-bet expression or relative high ratio of Eomes versus T-bet and increases in persistence of OT-1 numbers in high-dose rapamycin–treated hosts suggests that regulation of T-bet and/or Eomes enhances transition of CD8+ effector cells to memory and CD122 expression, thereby facilitating their transition to memory (40). These observations are congruent with the notion that robust activation and effector maturation are detrimental for persistence and memory generation.

Many studies have indicated that early instructions program CD8+ T cells for long-term responses, and our observation that viral vaccination plus high-dose rapamycin–generated CD8+ T cells produced greater tumor efficacy upon retransfer to tumor-bearing untreated (vaccine and/or rapamycin) mice provides further evidence of the inherent programming of CD8+ T cells by day 8, which resulted in enhanced tumor efficacy in the absence of cell-extrinsic conditions. The fact that readaptative transfer of OT-1 cells educated with vaccine plus high-dose rapamycin produced better tumor protection (~30% versus ~60% survivors) points to the potential impact of rapamycin treatment on other cell types that may negatively influence CD8+ T cell tumor efficacy. These insights have important implications for developing new vaccination strategies for cancer and identify the use of mTOR inhibition regimens as an effective means to regulate CD8+ T cell functional fate for immunity.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Dose of rapamycin treatment regulates vaccine induced CD8+ T cell proliferation and clonal expansion. Recipients of CFSE labeled OT-1 T cells (2 x 10^6 cells) on day -1 were immunized with Tricom (2 x 10^7 pfu) on day 0 and treated with no rapamycin (Tricom) or rapamycin at either (0.075mg/Kg/day-Tricom+Rapa (lo)) or (0.75mg/Kg/day-Tricom+Rapa (hi)) from day 0 to 7. Splenocytes harvested on day 8 were subjected to surface and intracellular staining and gated OT-1 cells were analyzed by flow cytometry. A, Surface and intracellular phenotype; B, CFSE dilution; C, Clonal frequency; D, Number of OT-1 cells. The results shown are representative of three independent experiments with similar outcomes. The average values with SD (error bars) obtained from three independent experiments in duplicate are shown.
Supplemental Figure 2. Dose of rapamycin treatment shifts T-bet versus Eomesodermin expression for CD8+ functional maturation. OT-1 recipients immunized with Tricom on day 0 were either untreated or treated with low dose or high dose rapamycin from day 0 – 7. On day 8, spleen derived cells were stained, gated (OT-1-CD8+thy1.1+) and analyzed by flow cytometry. The markers evaluated are as shown under each histogram. The data shown is representative of three independent experiments with similar outcomes.
Supplemental Figure 3. Rapamycin enhanced memory CD8+ T cell responses by vaccination are partially IL-7 dependent. Naïve OT-1 recipients were vaccinated with Tricom on day 0 and treated with either no rapamycin or high dose rapamycin for 8 days (day 0-7). Some vaccinated animals were injected with anti-IL-7Rα blocking antibodies (100mg/twice weekly). Spleen cells harvested were stained and evaluated for frequency of OT-1 cells (numbers above squares) and the fold expansion after re-challenge (numbers in parenthesis) on day 40 (persistence) and day 43 (three days post re-challenge). The results shown are representative of three independent experiments with identical outcomes.