mTOR Inhibition Improves Antitumor Effects of Vaccination with Antigen-Encoding RNA

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Abstract
Vaccination with in vitro transcribed RNA encoding tumor antigens is an emerging approach in cancer immunotherapy. Attempting to further improve RNA vaccine efficacy, we have explored combining RNA with immunomodulators such as rapamycin. Rapamycin, the inhibitor of mTOR, was used originally for immunosuppression. Recent reports in mouse systems, however, suggest that mTOR inhibition may enhance the formation and differentiation of the memory CD8⁺ T-cell pool. Because memory T-cell formation is critical to the outcome of vaccination approaches, we studied the impact of rapamycin on the in vivo primed RNA vaccine-induced immune response using the chicken ovalbumin-expressing B16 melanoma model in C57BL/6 mice. Our data show that treatment with rapamycin at the effector-to-memory transition phase skews the vaccine-induced immune response toward the formation of a quantitatively and qualitatively superior memory pool and results in a better recall response. Tumor-infiltrating immune cells from these mice display a favorable ratio of effector versus suppressor cell populations. Survival of mice treated with the combined regimen of RNA vaccination with rapamycin is significantly longer (91.5 days) than that in the control groups receiving only one of these compounds (32 and 46 days, respectively). Our findings indicate that rapamycin enhances therapeutic efficacy of antigen-specific CD8⁺ T cells induced by RNA vaccination, and we propose further clinical exploration of rapamycin as a component of immunotherapeutic regimens. Cancer Immunol Res; 1(6): 386–92. ©2013 AACR.

Introduction
Direct application of in vitro transcribed antigen-encoding RNA is emerging as a promising approach for antitumor vaccination (1, 2). RNA delivers the complete antigen, is selectively internalized by professional antigen-presenting cells (3), and has an intrinsic adjuvant activity. RNA does not integrate into the genome and with the transient expression of the encoded antigens allows better control of immune responses. The capability of RNA vaccines to confer antitumor immunity has been shown in model systems (4–6). Recombinant RNA is easy to produce in large amounts and high purity by in vitro transcription. These advantages prompted clinical testing of vaccination with antigen-encoding RNA, which has demonstrated excellent safety and provided promising data on immunogenicity in patients with cancer (7).

As reported previously, our group has developed pharmacologically optimized RNA with improved stability and translational performance; the encoded antigen was efficiently presented on both MHC class I and class II molecules (8, 9). Injection of such optimized RNA into lymph nodes propagates a proinflammatory microenvironment, resulting in de novo priming and efficient expansion of antigen-specific CD8⁺ and CD4⁺ T cells (5). When tumor-bearing mice were immunized with this RNA vaccine, potent antitumor immunity was elicited, resulting in significant survival benefit (5). We improved the potency of this vaccine platform several logs by molecularly modifying the RNA backbone; our current strategy is to identify synergistic immunomodulatory compounds for combination therapy (10). As CD8⁺ T memory cells are critical for efficient and sustained immunity against cancer, compounds capable of tuning vaccine-induced memory response are of particular interest.

The mTOR has a pivotal role in the modulation of T-cell immune responses. mTOR inhibition was used as a treatment against allo-reaction reactions in solid organ transplantation and in a number of autoimmune/inflammatory diseases. The recent observations on adoptive T-cell transfer therapy against viral and bacterial infection in mouse model systems (11, 12)
have confirmed mTOR as a potential gatekeeper for the differentiation of memory CD8⁺ T cells. mTOR inhibition by rapamycin has been shown to enhance the differentiation and the quantity of memory CD8⁺ T cells (11). Rao and colleagues proposed that the enhanced memory CD8⁺ T-cell differentiation was due to decreased expression of the transcription factor T-bet and persistent expression of Eomesoderm (13). This observation has generated considerable interest in the use of rapamycin for shaping antigen-specific CD8⁺ T-cell responses.

The objective of the current study was to investigate the effect of rapamycin on T-cell responses induced by intranodal RNA vaccination. To this end, mTOR inhibition in combination with RNA vaccination was assessed for the first time in the de novo priming setting in a mouse tumor treatment model. To our knowledge, this is the first study in which rapamycin effects on immune cells in the tumor microenvironment were investigated.

This report presents an approach for enhancing RNA vaccination with the multifaceted effects of rapamycin on T-cell-mediated immune responses.

Materials and Methods

Animals
Six- to 8-week-old female C57BL/6 mice were purchased from Charles River. Animals were kept in accordance with federal and state policies on animal research at the University of Mainz (Mainz, Germany).

Cells
The B16-F10 melanoma cell line expressing the chicken ovalbumin gene (B16-OVA) was a kind gift from H.J. Schild (Institute for Immunology, Mainz, Germany). Cell banks were created immediately upon receipt of the cell line and early passages (third and fourth) of cells were used for tumor experiments. Cells were tested for Mycoplasma every 3 months. Reauthentication of cells was not performed since receipt.

RNA vaccine and synthetic peptides
The RNA vaccine coding for H2-K⁺-restricted dominant OVA257-264 epitope (SIINFEKL) from chicken Ovalbumin was generated by in vitro transcription as described previously from the plasmid vector pSt1-sec-SIINFEKL-MTDM2hBgUTR-A120 (7, 8). This vector construct features a signal sequence for routing to the endoplasmic reticulum and the MHC class I transmembrane and cytoplasmic domains to improve MHC class I and MHC class II presentation as well as optimized 3′UTR and polyA tail for improved stability and translational efficiency (8, 9). The synthetic SIINFEKL peptide was purchased from Jerini Peptide Technologies.

Immunizations and rapamycin treatment
For intranodal RNA immunization, mice were anesthetized and the inguinal lymph node was surgically exposed. Twenty micrograms of RNA formulated in 10 μL RNase-free water (Ambion) was injected slowly and the wound was closed. In one experiment, mice were boosted on day 35 with s.c. administered SIINFEKL peptide (200 μg) and CpG 1826 (25 μg; MWG Biotech). Rapamycin (Wyeth-Ayerst) was administered intra-peritoneally. Mice were treated either during the effector-to-memory transition phase (day 10–31 post RNA vaccination or d11-33 post tumor implantation) with a daily high dose of 600 μg/kg body weight (11) or during the T-cell expansion phase (day 2–8 in relation to RNA vaccinations) with a low dose of 75 μg/kg body weight (11). Control mice received Phosphal 50 PG (PG50; Phospholipid GmbH).

Tumor experiments
A total of 2 × 10⁵ B16-OVA tumor cells were inoculated s.c. into the flanks of C57BL/6 mice (day 0). Mice received three intranodal RNA immunizations at 3-day intervals. Early tumor models (immunizations on days 3, 6, and 9) as well as advanced tumor models (immunization on days 7, 10, and 13) were explored. Rapamycin was administered daily at the effector-to-memory transition phase starting from day 11 in the early and day 15 in the advanced tumor models. Tumor sizes were measured every 3 days. Mice were sacrificed when the tumor diameter reached 15 mm.

Flow cytometric analysis
All monoclonal antibodies for flow cytometric analysis were purchased from BD Bioscience, except for anti-KLRG1, which was obtained from eBioscience San Diego. Phenotyping of peripheral blood cells and MHC/tetramer-staining were performed as described previously (8). Regulatory T cells (Treg) were detected by using the FoxP3 staining kit (eBioscience) according to the manufacturer’s instructions. Tumor-infiltrating leukocytes (TIL) were obtained by mechanical disruption of the tumor tissue. Phenotyping by flow cytometry was performed with the following markers after gating CD45⁻ leukocytes: myeloid-derived suppressor cells (MDSC; CD11b⁺, Gr1low/high), Treg (CD4⁺, CD25⁺, FoxP3⁺), NK cells (CD3⁻, NK1.1⁻), CD8⁺ T cells (CD3⁺, CD8⁺), and SIINFEKL⁺ CD8⁺ T cells (CD3⁺, CD8⁺, tetramer⁺).

Flow cytometric data were acquired on a FACSCalibur and FACS Canto analytic flow cytometers (BD Bioscience) and analyzed by FlowJo software (Tree Star).

Statistical analysis
Statistical analysis was performed by GraphPad Prism 5 software employing unpaired two-tailed Student t test, one-way ANOVA test with Bonferroni comparison post-test, and Log rank (Mantel–Cox) test. Values of P < 0.05 were considered statistically significant. Data are presented as mean ± SEM.

Results and Discussion
RNA vaccination combined with mTOR inhibition enhances differentiation of antigen-specific CD8⁺ T cells
Once a CD8⁺ T-cell is activated by antigen recognition, it proliferates and differentiates into an effector cell. Peaking of
the CD8⁺ T-cell response is followed by the contraction phase, when most of the effector cells die and the remaining become long-lived memory cells (14). High-dose rapamycin administered during the contraction period had been shown in an LCMV infection model to facilitate transition of adoptively transferred effector CD8⁺ T cells to memory T cells, whereas its administration in the priming phase was observed to deplete CD8⁺ T cells (11).

On the basis of those findings, we studied immune responses induced by intranodal RNA vaccination in conjunction with mTOR inhibition applied during the contraction phase. Naïve mice received repetitive intranodal immunizations (days 0, 3, and 6) with RNA coding for the SIINFEKL epitope of chicken ovalbumin (SIINFEKL-RNA) followed by rapamycin treatment (days 10–31; Fig. 1A, left). We monitored the phenotype of antigen-specific CD8⁺ T cells in the peripheral blood using markers of memory CD8⁺ T cells such as CD127 (also known as interleukin-7 receptor; ref. 15) and the inhibitory killer cell lectin–like receptor G1 (KLRG1; ref. 15).

We found that the frequencies (Fig. 1A, right) and absolute numbers (Supplementary Fig. S1A) of RNA vaccine-induced antigen-specific CD8⁺ T cells were not altered by rapamycin as comparable with those of the PG50-treated control group (Fig. 1A). When we assessed CD8⁺ T-cell subpopulations, however, we found that a lower frequency of short-lived antigen-specific effector cells (KLRG1⁺) resulted from RNA vaccination in combination with rapamycin (Fig. 1B, left graph). In contrast to this skewing of the effector pool, the frequency (Fig. 1B, right graph) and absolute number (Supplementary Fig. S1B) of the antigen-specific memory CD8⁺ T-cell precursors (KLRG1⁻CD127⁺) were higher in these mice. In the advanced tumor B16-OVA melanoma model, similar results were obtained in the blood and spleen of immunized mice treated with rapamycin (Supplementary Fig. S1C). Moreover, antigen-specific effector T cells of rapamycin-treated mice expressed CD44 at a higher frequency (Fig. 1C), which is known to contribute to the overall cellular fitness and survival of CD8⁺ T cells (15). In line with these findings, CD8⁺ T-cell memory precursors of rapamycin-treated mice were also superior with regard to their proliferation rate in response to boosting with SIINFEKL peptide on day 35 (Fig. 1D and Supplementary Fig. S1D). This was not the case, when a low dose of rapamycin was administered during the priming and the expansion phase (days 2–8) of vaccine-induced antigen-specific immune responses. Therefore, the role of rapamycin in vaccine-induced memory CD8⁺ T cells is the focus of our current studies.
response (Supplementary Fig. S2A). The frequency (Supplementary Fig. S2B) and the absolute number (Supplementary Fig. S2C) of antigen-specific CD8$^+$ T cells were markedly lower on day 10 in the peripheral blood of rapamycin-treated mice. We ruled out a general suppression of the hematopoietic system by rapamycin, as hemogram parameters are not altered with rapamycin treatment (Supplementary Fig. S3). Previous data, which demonstrated that mTOR inhibition may have negative effects on priming by suppressing the in vivo differentiation and antigen presentation capacity of dendritic cells (16), may provide an explanation for this observation. Moreover, it was shown that factors like antigen format and antigen strength influence the outcome of rapamycin in combination with vaccines, suggesting that rapamycin treatment protocols require specific tuning for each vaccine setting (17).

In summary, our data show that targeted modulation with rapamycin treatment during the effector-to-memory transition phase shapes immune responses induced by RNA vaccines toward the formation of a quantitatively and qualitatively superior memory pool of antigen-specific T cells.

**mTOR inhibition augments the therapeutic effect of antitumor RNA vaccination**

Next, we wanted to know whether immunomodulation by rapamycin treatment might enhance the efficacy of RNA vaccination in vivo.

It had been reported that the modest effects of recombinant protein-based tumor antigen vaccines in RENCA renal cell cancer and B16 melanoma models can be augmented by the mTOR inhibitor temsirolimus (18). However, the study used adaptively transferred antigen-specific T cells and therefore only indirect evidence for the modulation of memory T cells was available.

We have reported that intranodal vaccination with SIINFEKL-RNA could elicit strong protective and therapeutic antitumor immune responses in C57BL/6 mice against B16 melanoma cells expressing chicken ovalbumin (B16-OVA; ref. 5). We used this tumor model to assess whether immunomodulation by the combined regimen of RNA vaccine with rapamycin affects the growth kinetics of tumors and the survival of the animals.

Mice were implanted with B16-OVA cells and 3 days later were immunized with SIINFEKL-RNA for three cycles at 3-day intervals (i.e., on days 3, 6, and 9). On day 10, all mice with similar antigen-specific CD8$^+$ T-cell frequency (Supplementary Fig. S4) were divided into two groups for treatment with or without rapamycin during the T-cell effector-to-memory transition phase from days 11 to 33.

Consistent with our previous reports, immunization with the RNA vaccine alone had a significant effect on tumor growth compared with the nonimmunized control groups (Fig. 2A; ref. 5). When rapamycin was added, the growth reduction was even more profound (Fig. 2A). Tumor latency, the period of time required to reach a defined tumor volume (300 mm$^3$; ref. 19), was significantly prolonged from 34 and 32 days in the group that received either RNA vaccination or rapamycin treatment alone, respectively, to 61 days in the group that received both the RNA vaccine and the rapamycin treatment (Fig. 2B and Table 1). The inhibition of tumor growth translated to a significantly longer survival for the mice (Fig. 2C). The combination of RNA vaccination with rapamycin treatment extended the median survival from 32 and 46 days in the rapamycin- or RNA-treated groups, respectively, to 91.5 days in the group treated with the combination (Table 1). In an advanced B16-OVA model in which the RNA immunization was initiated 7 days after tumor inoculation, the superiority of RNA vaccination was confirmed (Fig. 2D).

Our findings show that the modulation of RNA vaccination-induced immune responses by rapamycin translates into superior antitumor effects.

**Increased tumor-infiltrating leukocytes and decreased MDSC frequencies are observed after RNA-rapamycin combination therapy**

We investigated the composition of immune effector cell within tumors of treated mice. As described in Fig. 2A, tumors were harvested on day 25 for flow cytometric analysis of TIL.

Tumors of RNA-vaccinated mice contained a significantly higher frequency of total CD8$^+$ T cells as well as of NK cells as compared with those from the untreated or rapamycin-only-treated control mice (Fig. 3A), confirming the capability of RNA vaccine to efficiently induce antitumor immunity (20). Combining RNA vaccination with rapamycin had only a modest added value on the total CD8$^+$ T-cell and NK cell frequencies as compared with RNA vaccination alone. However, the frequency of antigen-specific CD8$^+$ T cells was enhanced in the tumors of mice treated with rapamycin after RNA vaccination (Fig. 3C, left).

Analysis of tumors for infiltration by the two major myeloid-derived suppressor cell (MDSC) populations (Gr1$^{hi}$CD11b$^+$ and Gr1$^{low}$CD11b$^+$ phenotypes) revealed that rapamycin significantly lowers the frequency of these immunosuppressive cell populations in situ in both vaccinated and unvaccinated mice (Fig. 3B). This effect of mTOR inhibition has not been reported so far. The mechanism of this rapamycin effect has not been determined; one possibility is that it may function via rapamycin-mediated inhibition of VEGF production, which is known to have a role in the generation of MDSCs (21, 22).

Rapamycin has been described to promote the expansion of human CD4$^+$FoxP3$^+$ (23, 24). However, we did not observe any significant increase in intratumoral Treg in rapamycin-treated animals as compared with that in the control groups (Fig. 3C, right). Results from another study in the B16 melanoma model indicated that not the absolute number of Treg but the ratio of intratumoral CD8$^+$ T cell to Treg (Teff/Treg) is correlated with therapeutic efficacy (25). We found that tumors from RNA-vaccinated mice displayed a high intratumoral Teff/Treg ratio, which was further improved by mTOR inhibition (Fig. 3D). The high Teff/Treg ratio together with the low numbers of MDSC in the tumor microenvironment may explain the superior control of tumor growth resulting from the combined treatment of RNA vaccination and mTOR inhibition.
In conclusion, this report shows for the first time synergy between rapamycin and intranodal RNA vaccination in de novo priming of the endogenous CD8+ T-cell repertoire resulting in significantly improved outcome in a therapeutic tumor model. We provide direct evidence that mTOR inhibition supports memory T-cell formation and promotes immune-beneficial alterations of the tumor microenvironment.

Given the ease of translation of such a concept, these findings may open up new paths for clinical development of RNA vaccines.

### Table 1. Mean tumor latency and median survival of groups shown in Fig. 2B and Fig. 2C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean tumor latency, d</th>
<th>Median survival, d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.2</td>
<td>23.5</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>32.4</td>
<td>32</td>
</tr>
<tr>
<td>RNA</td>
<td>34.1</td>
<td>46</td>
</tr>
<tr>
<td>RNA + rapamycin</td>
<td>61.5</td>
<td>91.5</td>
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</table>
Disclosure of Potential Conflicts of Interest

S. Kreiter is a consultant/advisor and has ownership interest (including patents) in Ribological GmbH. C. Huber is a cofounder and member of the supervisory board, has an ownership interest (including patents), and is a consultant/advisory board member of BioNTech, and has commercial research support in Cluster individualized immune intervention CI3. U. Sahin is employed on a consulting basis as founder and CEO of BioNTech. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Diken, A. Selmi, S. Attig, J. Diekmann
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Diken, S. Kreiter, F. Vascotto, A. Selmi, S. Attig, J. Diekmann
Writing, review, and/or revision of the manuscript: M. Diken, S. Kreiter, F. Vascotto, S. Attig, C. Huber, O. Türeci, U. Sahin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Selmi, S. Attig
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Acknowledgments

The authors thank M. Holzmann and R. Roth for excellent technical assistance and Dr. John Castle for critical proofreading of the article.

Grant Support

This work was supported by the GO-Bio and CI3 excellence cluster programs of the Federal Ministry of Education and Research (BMBF).

Received April 24, 2013; revised August 22, 2013; accepted September 8, 2013; published OnlineFirst September 20, 2013.

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doi:10.1158/2326-6066.CIR-13-0046

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