The Journal of Immunology

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J Immunol 2012; 189:2151-2158; Prepublished online 23 July 2012;
doi: 10.4049/jimmunol.1103741
http://www.jimmunol.org/content/189/5/2151

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Inhibition of Mechanistic Target of Rapamycin Promotes Dendritic Cell Activation and Enhances Therapeutic Autologous Vaccination in Mice

Eyal Amiel,* Bart Everts,† Tori C. Freitas,* Irah L. King,* Jonathan D. Curtis,† Erika L. Pearce,† and Edward J. Pearce†

Dendritic cells (DCs) are potent inducers of T cell immunity, and autologous DC vaccination holds promise for the treatment of cancers and chronic infectious diseases. In practice, however, therapeutic vaccines of this type have had mixed success. In this article, we show that brief exposure to inhibitors of mechanistic target of rapamycin (mTOR) in DCs during the period that they are responding to TLR agonists makes them particularly potent activators of naive CD8$^+$ T cells and able to enhance control of B16 melanoma in a therapeutic autologous vaccination model in the mouse. The improved performance of DCs in which mTOR has been inhibited is correlated with an extended life span after activation and prolonged, increased expression of costimulatory molecules. Therapeutic autologous vaccination with DCs treated with TLR agonists plus the mTOR inhibitor rapamycin results in improved generation of Ag-specific CD8$^+$ T cells in vivo and improved antitumor immunity compared with that observed with DCs treated with TLR agonists alone. These findings define mTOR as a molecular target for augmenting DC survival and activation, and document a novel pharmacologic approach for enhancing the efficacy of therapeutic autologous DC vaccination. ***The Journal of Immunology*****, 2012, 189: 2151–2158.**

Dendritic cells (DCs) are professional APCs responsible for initiating adaptive immune responses (1, 2). They can be generated from precursor cells in vitro and are of great interest for their potential use in autologous vaccine therapies for cancer and chronic infectious diseases (3, 4). Autologous DC vaccines have exhibited limited success clinically (3, 4), and the short life span of activated DCs is recognized as one obstacle to this promising therapeutic approach (5, 6). Although genetic approaches to modulating DC life span and function can enhance DC vaccine potency in animal tumor models (5, 7, 8), pharmacological approaches for improving DC immune activity in the context of vaccine therapies are desirable for reasons of clinical feasibility.

One of the central nutrient sensing pathways, controlling a diverse array of cellular responses including cell activation, metabolism, and survival, is governed by mechanistic target of rapamycin (mTOR) (9–12). mTOR is activated by the PI3K/Akt signaling pathway, which is downstream of a number of growth factor receptors, as well as TLRs, and we have previously reported that this signaling axis is critically involved in orchestrating the metabolic demands necessary for DC activation (13). Inhibition of mTOR by rapamycin, a macrolide product of the bacterium *Streptomyces hygroscopicus*, is widely reported to extend the life span of eukaryotic cells and organisms (14–16). Based on this evidence, we hypothesized that mTOR could play a regulatory role in controlling DC life span and activation after TLR stimulation.

We decided to directly examine the role of mTOR in DCs during the activation process after exposure to TLR agonists. We have found that inhibiting mTOR during activation considerably extends the life span of DCs, and this is accompanied by enhanced and prolonged costimulatory molecule expression after activation. These phenotypes effectively increase the window of time during which DCs are able to interact with and stimulate Ag-specific T cell responses. Consistent with their increased life span and prolonged activation kinetics, DCs activated in the presence of mTOR inhibitors induce enhanced primary Ag-specific CD8$^+$ T cell responses and stronger and more effective antitumor responses in a therapeutic vaccination model. These findings suggest a novel approach for potentiating the efficacy of autologous DC vaccination for the therapeutic treatment of cancers.

Materials and Methods

Mice and reagents

C57BL/6 mice were purchased from The Jackson Laboratory, and rederived stocks were maintained at the Trudeau Institute under specific pathogen-free conditions under protocols approved by the Institutional Animal Care and Use Committee.

LPS (*Escherichia coli* serotype 0111:B4) was from Sigma-Aldrich and was used at 100 ng/ml. Pam2CSK4 (1 μg/ml), R848 (1 μg/ml), CpG (250 ng/ml), and rapamycin (100 nM) were purchased from Invivogen. KU 0063794 (KU; 100 nM) was purchased from Tocris Biochemicals. All Abs for FACS analysis were from BD Bioscience except for anti-CD11c and -CD40, which were purchased from eBioscience. OVA (250 μg/ml endotoxin-free egg white) was prepared in our laboratory. Kb-OVA tetramers were produced by the Molecular Biology Core Facility at the Trudeau Institute.

*Trudeau Institute, Saranac Lake, NY 12983; and †Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110

Received for publication December 23, 2011. Accepted for publication June 20, 2012.

This work was supported by National Institutes of Health Grant AI053825 (to E.J.P.), National Institutes of Health Institutional Postdoctoral Training Award AI049823 (to E.A.), National Institutes of Health Grant CA158823 (to E.L.P), and the Trudeau Institute.

Address correspondence and reprint requests to Dr. E.J. Pearce, Department of Pathology and Immunology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8118, St. Louis, MO 63110. E-mail address: edwardpearce@path.wustl.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: 7-AAD, 7-amino-actinomycin D; DC, dendritic cell; LN, lymph node; Luc^{hp}, luciferase shRNA construct; mTOR, mechanistic target of rapamycin; mTOR^{hp}, mTOR shRNA construct; shRNA, short hairpin RNA.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1103741

Mouse DC culture, retroviral transduction, purification, and activation

Bone marrow-derived DCs were generated as described previously (17). In brief, bone marrow cells were differentiated in the presence of GM-CSF (20 ng/ml) in complete DC media (RPMI-1640 containing 10% FCS, 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine) for 6 d. Retroviral transduction of DCs was accomplished as described previously (18). Sequences for luciferase or mTOR short hairpin RNAs (shRNAs) were obtained from Open Biosystems and cloned into MSCV-LTRmir30-PI8 retroviral vectors. Recombinant retroviruses were obtained after the transfection of 293T packaging cells with the use of Lipofectamine (Invitrogen); retrovirus-containing supernatants were collected 48 h after transfection and used for spin infection (2500 rpm, 2 h) of day 2 and 3 bone marrow DC cultures in 6-well plates. After 6 d in culture with GM-CSF, DCs were harvested, and transduction efficiency was assessed by human CD8 expression via FACS; typical transduction rates were ~80–90%. On day 6 of culture, DCs were washed in complete DC media and were pulsed as indicated with media alone, rapamycin, LPS, or rapamycin + LPS. Where applicable, cells were pulsed with 250 μg/ml OVA. For retroviral transduction assays, DCs were purified from cultures using $CD11c^+$ or human $CD8^+$ selection (as indicated) with MACS bead sorting (Miltenyi Biotec) according to the manufacturer's protocol. For IL-10R blocking studies, anti–IL-10R mAb 1B1.3A and control Ab (clone HRPN) were purchased from BioXCell.

Human DC culture

Human myeloid DCs were isolated from human blood using $MACS^+$ selection beads. In brief, filters from hospital blood donors were generously donated by the Champlain Valley Physicians Hospital Medical Center (Plattsburgh, NY). Blood cells were obtained by reverse flushing filters in sterile HBSS. PBMCs were obtained by centrifugation of blood samples over Ficoll-Paque Plus (density 1.077 g/ml; GE Healthcare). Myeloid DCs were enriched using the BDCA-1 Positive Selection DC Isolation Kit (Miltenyi Bioscience) per the manufacturer's instructions. Freshly isolated DCs (1×10^5 cells/well in 200 μl) were cultured in complete RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin/streptomycin, and supplemented with 20 ng/ml GM-CSF. DCs were stimulated with 1 μg/ml R848 in the presence or absence of rapamycin or KU (both used at 100 nM). At indicated times, DCs were harvested and analyzed by FACS for maturation markers.

Western blotting

Transduced DCs were MACS purified based on expression of human CD8. Cell lysate preparation, $NaDodSO_4$-PAGE, electrophoretic transfer, immunoblotting, and development with ECL were accomplished as described previously (19).

Cytokine measurements and flow cytometry

DC supernatants were analyzed for IL-12p70, TNF-α, and IL-10 by FACS using the Cytometric Bead Array Mouse Inflammation Kit (BD Bioscience) per the manufacturer's instructions.

Metabolism assays

Glucose and lactate levels in the media after indicated stimulation conditions were measured using the Glucose Assay Kit and Lactate Assay Kit from Eton Bioscience per the manufacturer's instructions.

In vitro T cell responses

For staggered DC activation experiments, DCs were pulsed with indicated treatments for 24 h; then the media were changed to normal growing media (no TLR agonist or mTOR inhibitor). After 24-h activation, DC was changed daily before T cell coculture to prolong survival of activated DCs. DCs were cocultured for 4 d at a 1:5 ratio with CFSE-labeled $CD8^+$ selected OT-I splenocytes (MACS) on days 2, 3, or 4 after initial DC activation.

Tumor challenge experiments

For all tumor studies, mice were challenged with 1×10^5 Ova-expressing B16 melanoma cells intradermally on the peritoneal surface. For therapeutic vaccine studies, mice were challenged with tumor on day 0 and then received autologous DC transfer s.c. in the left footpad on day 3 after tumor challenge. Mice were monitored for tumor growth periodically, and tumor sizes were measured with digital calipers (Fisher Scientific). At time of harvest, mice were sacrificed and tumor was excised with final tumor volumes and weights measured. For analysis of tumor-infiltrating cells, tumors were mechanically dissociated in HBSS with a 1-ml syringe stopper and passed through 70-μm cell strainers to obtain single-cell suspensions of tumor content.

Statistics

For analysis of CFSE-labeled DCs in the draining lymph node (LN), two-way ANOVA was performed on log-transformed data. For analysis of $tetramer^+$ cells in the blood of mice, two-way ANOVA was performed on data that were transformed by $\sqrt{(\text{percent} + 0.5)}$ as appropriate for percentage data with very low values (20). Tumor volumes were analyzed using Student t test on log-transformed values. Tumor weights were first classified by whether they exceeded the lower limit of detection for the assay (10 mg), then were analyzed using Fisher's exact test.

Declaration of ethical compliance with standards for study of human subjects

Human DCs and monocytes were collected from WBCs destined to be discarded after being removed by filtration from blood collected for other purposes. The WBCs were provided to us as samples that were identified by numbers only.

Results

mTOR regulates life span and activation of DCs after stimulation by TLR agonists

DC life span is dramatically reduced after activation by TLR agonists (Fig. 1A) (21, 22). We directly assessed the role of mTOR in DC activation and longevity using retroviral transduction of shRNAs targeting mTOR or luciferase (mTOR shRNA construct [$mTOR^{hp}$] and luciferase shRNA construct [Luc^{hp}], respectively) into bone marrow cells being cultured in GM-CSF (13). Transduced DCs emerged equally well from Luc^{hp} (control) and $mTOR^{hp}$ cultures (Supplemental Fig. 1A), and mTOR protein expression levels were substantially reduced in DCs transduced with $mTOR^{hp}$ (Fig. 1B). Retroviral transduction alone did not induce a mature phenotype in unstimulated DCs (Supplemental Fig. 1B). mTOR signaling, mea-

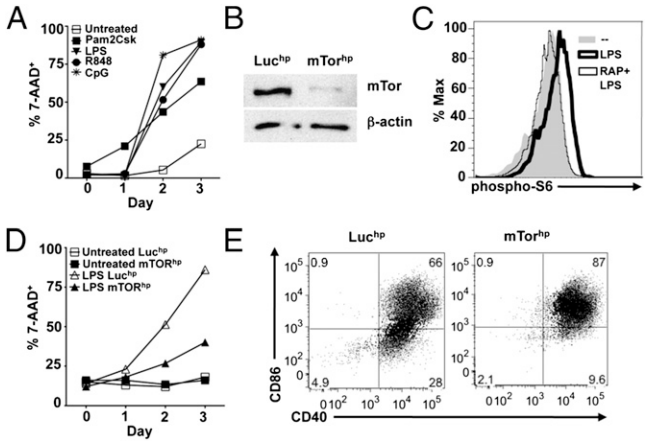

FIGURE 1. Inhibition of mTOR expression prolongs DC life span and promotes expression of costimulatory molecules CD40 and CD86. (**A**) DCs were pulsed with Pam2CSK4, LPS, R848, or CpG for 6 h and then washed and cultured in complete medium. Cell viability was monitored daily by FACS analysis of 7-AAD staining of $CD11c^+$ cells. (**B**) Western blot for mTOR and β-actin protein in luciferase shRNA (Luc^{hp}) or mTOR shRNA ($mTOR^{hp}$)-transduced DCs. (**C**) DCs were stimulated with media alone, LPS, or rapamycin + LPS for 30 min. Cells were subsequently fixed and stained for phosphorylated S6 protein as a molecular readout for mTOR activation and analyzed by FACS. (**D**) Luc^{hp} and $mTOR^{hp}$-transduced DCs were left untreated or stimulated with LPS and monitored daily for cell viability by analysis of 7-AAD staining of $CD11c^+$ cells. (**E**) Luc^{hp} or $mTOR^{hp}$ DCs were cultured with or without LPS for 24 h and analyzed by FACS for CD40 and CD86 expression. All graphs in this figure represent mean values of replicate wells; all experiments were performed at least twice with similar results.

sured as phosphorylation of one of its targets, S6 kinase, is activated by stimulation with LPS and inhibited by rapamycin (Fig. 1C) (23). We therefore asked whether mTOR knockdown would affect the life span, or ability to become activated, of DCs responding to the TLR4 agonist LPS. We observed that mTOR deficiency attenuated cell death induced by LPS activation (Fig. 1D), indicating that TLR-driven mTOR signaling is an important determinant of DC life span after activation. Furthermore, mTOR knockdown resulted in higher percentages of LPS-stimulated DCs becoming $CD40^+$ $CD86^+$ (Fig. 1E), demonstrating that TLR-driven mTOR signaling negatively regulates costimulatory molecule expression after LPS activation. This was not a reflection of $CD40^+$ $CD86^-$ cells being less viable than $CD40^+$ $CD86^+$ cells, because we were unable to detect differences in 7-amino-actinomycin D (7-AAD) staining in these two populations (Supplemental Fig. 1C).

We next tested whether treatment of DCs with rapamycin, a clinically used inhibitor of mTOR signaling (24), or the synthetic ATP-competitive inhibitor of mTOR, KU, could recapitulate the phenotypes observed in DCs transduced with $mTOR^{hp}$. Consistent with the outcome of the $mTOR^{hp}$ experiments, mTOR inhibitors prolonged DC life span after activation with LPS (Fig. 2A) or with agonists for TLR2, TLR7/8, and TLR9 (data not shown). Rapamycin and KU treatment alone did not impact the viability of unstimulated DCs (data not shown). In addition, mTOR inhibition by rapamycin or KU during LPS stimulation resulted in an increase in the percentage of DCs expressing CD40 and CD86 (Fig. 2B), and prolonged the expression of these costimulatory molecules in response to LPS (Fig. 2C). mTOR inhibitors were observed to similarly stabilize the expression of CD80, MHC class I, and MHC class II on LPS-activated DCs (data not shown).

mTOR functions through two, functionally distinct, signaling complexes: mTORC1, which is sensitive to direct disruption by rapamycin treatment, and mTORC2, which is insensitive to the direct inhibitory effects of rapamycin but can be regulated by mTORC1 activity in some contexts (25, 26). mTORC1 signaling regulates protein translation through its interaction with p70S6 kinase and 4E-BP1, whereas mTORC2 signaling is thought to be involved in regulating cytoskeleton dynamics and negatively regulating Akt signaling via phosphorylation of Ser^{473} (25, 27, 28). We observed that activation-induced Akt Ser^{473} phosphorylation was inhibited by both rapamycin and KU (Supplemental Fig. 1D). Therefore, our experiments are unable to distinguish between the role of mTORC1 versus mTORC2 because both of the inhibitors that we used reduce mTORC2-mediated Akt phosphorylation.

Consistent with previous reports, mTOR inhibitors did not negatively affect the production of the proinflammatory cytokines IL-12p70 and TNF-α by LPS-activated DCs, but did inhibit IL-10 production (Fig. 2D) (29, 30). We found that DCs pulsed with OVA in the presence of LPS and rapamycin were highly competent to process and present Ag to $CD8^+$ and $CD4^+$ T cells in vitro (Supplemental Fig. 1E, 1F). Thus, mTOR inhibition in DCs during TLR stimulation protects them from activation-associated cell death and allows them to retain an activated phenotype for prolonged periods without compromising proinflammatory cytokine production or their ability to stimulate T cells in vitro. These effects could not be explained by the observed reduction in IL-10 production, because inhibition of IL-10 signaling using anti–IL-10R Ab did not recapitulate the effects of mTOR inhibition on either life span or the duration of costimulatory molecule expression (Supplemental Fig. 2A, 2B).

A recent report indicated that, consistent with our findings in mouse BMDCs, rapamycin is able to augment costimulatory molecule expression in human myeloid DCs (29). We examined this directly and found that treatment with rapamycin or KU allowed enhanced costimulatory molecule expression in response to stimulation with the TLR agonist R848 in human myeloid DCs (Fig. 3A). Moreover, enhanced expression of CD40 and CD86 was prolonged as in the mouse DCs when mTOR was inhibited (Fig. 3B). Unlike our results with murine BMDCs, we did not observe the same

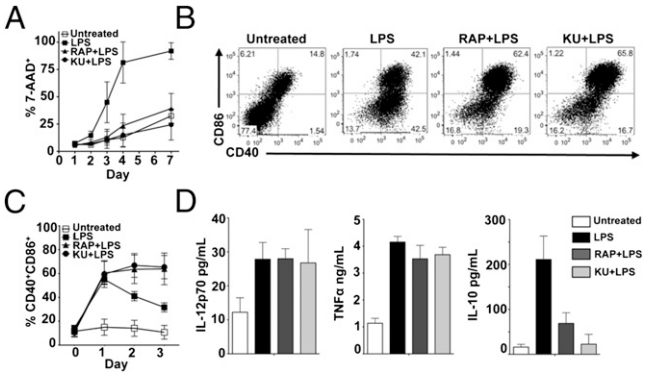

FIGURE 2. Pharmacological inhibition of mTOR augments DC life span and costimulatory molecule expression. (**A**) DCs were stimulated with LPS in the presence or absence of rapamycin (RAP) or KU. Cells were monitored daily for cell viability as described for Fig. 1. Data are presented as mean ± SD of four independent experiments. (**B**) DCs were unstimulated or stimulated with LPS, RAP + LPS, or KU + LPS and analyzed by FACS 24 h later for CD40 and CD86 expression. Data are representative of more than four independent experiments. (**C**) DCs were treated as in (B) and analyzed daily by FACS for CD40 and CD86 expression gated on live $CD11c^+$ cells. Data are presented as mean ± SD of four independent experiments. (**D**) DCs were treated as indicated and supernatants collected 24 h later for analysis by Cytometric Bead Array for IL-12p70, TNF-α, and IL-10. Data are presented as mean ± SD of two independent experiments.

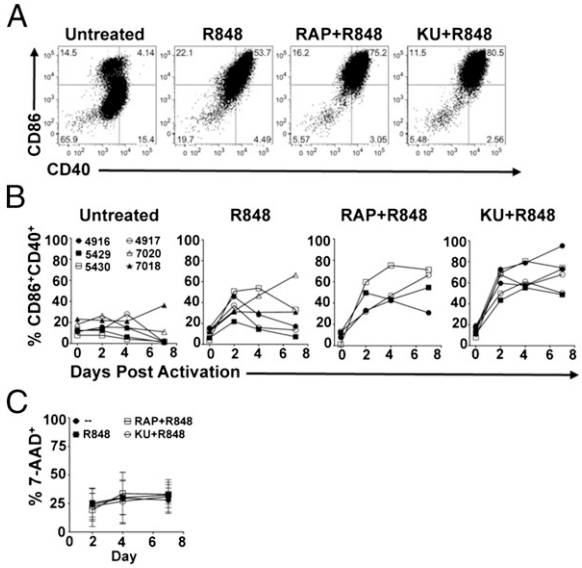

FIGURE 3. Pharmacological inhibition of mTOR augments the duration of costimulatory molecule expression in human myeloid DCs. (**A**) Human myeloid DCs were either unstimulated or stimulated with R848 in the presence or absence of rapamycin (RAP) or KU. CD40 and CD86 expression were analyzed by FACS. Day 4 costimulatory molecule expression from a representative donor (5430) is depicted. (**B**) DCs were treated as in (A) and analyzed daily by FACS for CD40 and CD86 expression. (**C**) DCs were treated as in (A) and analyzed for viability at indicated times by FACS analysis of 7-AAD staining. For each treatment, data from four to six individual donors is depicted.

activation-associated cell death in human myeloid DCs as a result of TLR stimulation (Fig. 3C). However, these findings demonstrate that the enhancing effects of mTOR inhibition on the stability of costimulatory molecule expression observed in mouse DCs is recapitulated in human myeloid DCs, and that this phenotype is not intrinsically dependent on prolonging postactivation survival in these cells.

mTOR promotes commitment to glycolytic metabolism after exposure to LPS in mouse but not human DCs

We recently showed that the metabolism of mouse DCs switches away from oxidative phosphorylation toward aerobic glycolysis after activation by TLR agonists (13). We found that the death of DCs after stimulation with TLR agonists is, in part, due to the fact that they are glucose dependent and able to rapidly exhaust available glucose, and perhaps other nutrients, in tissue culture medium (13). Consequently, daily feeding with glucose in vitro is able to extend the life span of activated DCs (13). Because high levels of glucose consumption generally correlate with shorter life span, and mTOR is documented to control the induction of glycolytic metabolism (31, 32), we reasoned that mTOR inhibitors might extend the life span of activated DCs by limiting their dependence on glucose. Consistent with this, we found that LPS-induced increases in glucose consumption and the production of lactate (the end product of glycolysis) 48 h after activation were profoundly diminished by mTOR inhibitors (Fig. 4A). In contrast, neither the use of glucose nor the production of lactate by human myeloid DCs were affected by exposure to TLR agonists (Fig. 4B), and mTOR inhibitors had no measurable effects on either of these metabolic parameters. These findings may help explain the differences in survival of activated mouse versus human DCs in our system (Fig. 2A versus 3C).

mTOR is a negative regulator of the ability of DCs to activate T cells

The increased duration of costimulatory molecule expression in DCs activated in the presence of mTOR inhibitors indicated that cells treated in this way might be able to continue to activate T cells at times when DCs activated in the absence of mTOR inhibitors are no longer able to do so. To directly test this, we stimulated DCs with LPS and OVA in the presence or absence of mTOR inhibitors, and initiated cocultures with $CD8^+$ OVA-specific OT-I cells on day 2, 3, or 4 after activation; DCs were thoroughly washed to remove drugs, Ag, and TLR agonists before addition to these cocultures, and equal numbers of DCs were added to each coculture condition. To focus this analysis on the survival-independent effects of mTOR inhibition, we performed daily media changes of DCs before coculture with T cells, which minimized cell death (Supplemental Fig. 2C); this is consistent with previous work from our laboratory demonstrating that media supplementation can extend the life span of LPS-activated DCs (13). As anticipated, the ability

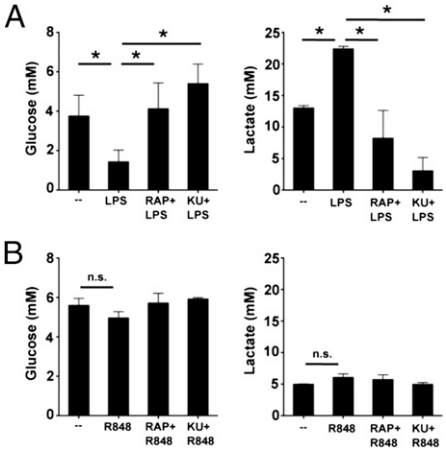

FIGURE 4. mTOR inhibition affects activation-induced metabolic changes in mouse but not human DCs. (**A**) Mouse BMDCs were either left unstimulated or treated with LPS in the presence or absence of rapamycin or KU as indicated, and supernatants were collected 48 h later for analysis of glucose concentration (*left*) and lactate concentration (*right*). (**B**) Human myeloid DCs were either left unstimulated or treated with R848 in the presence or absence of rapamycin or KU as indicated, and supernatants were collected 48 h later for analysis of glucose concentration (*left*) and lactate concentration (*right*). Asterisks indicate statistically significant differences between groups ($p < 0.05$). For all graphs, data represent the mean ± SD of data from at least three individual mice or donors.

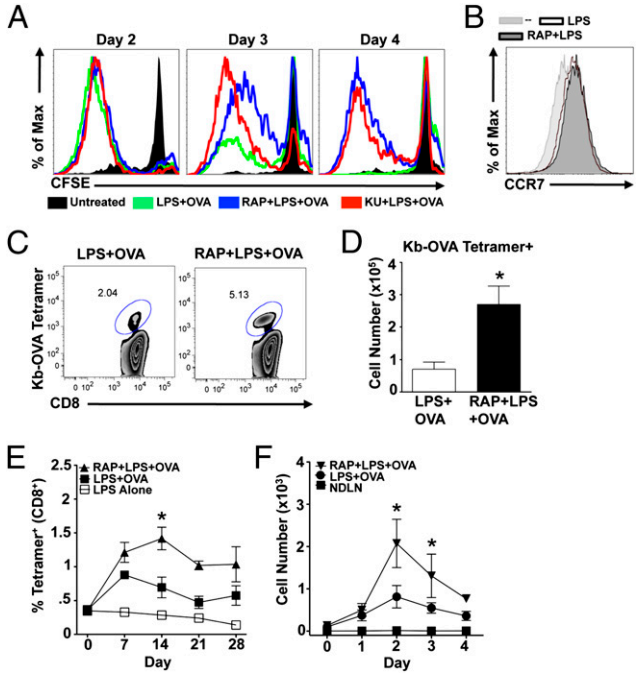

FIGURE 5. mTOR inhibition in DCs improves their ability to stimulate CD8 T cell responses. (**A**) DCs were treated as indicated for 24 h, after which cells were washed and replaced with normal media. Two, 3, or 4 d after activation, DCs were cocultured at a 1:5 ratio with CFSE-labeled OT-I $CD8^+$ T cells for 4 d. T cell proliferation was determined by CFSE dilution within $CD8^+$ cell population. Data are representative of three independent experiments. (**B**) DCs were treated as indicated for 24 h and stained for CCR7 expression. Data are representative of two independent experiments. (**C**) Ten mice per group were immunized s.c. with DCs stimulated in vitro for 6 h with LPS, LPS plus OVA, or rapamycin (RAP) plus LPS plus OVA. Seven days later, draining (popliteal) LNs were harvested and frequencies of Kb-OVA $tetramer^+$ $CD8^+$ cells were determined. FACS plots represent concatenated data from all 10 individual mice per group. Data are representative of more than three individual experiments. (**D**) Total numbers of $tetramer^+$ cells from (C) were calculated. Asterisk indicates statistically significant differences between mice immunized with RAP-treated DCs and normally activated DCs ($p < 0.05$). (**E**) Mice were immunized as in (C) and bled weekly for 1 mo thereafter. The frequencies of Kb-OVA $tetramer^+$, $CD44^+$, $CD8^+$ cells at different times after immunization are shown. Asterisk indicates statistically significant differences between mice immunized with RAP-treated DC and normally activated DCs ($p < 0.05$). (**F**) CFSE-labeled DCs were treated as indicated and injected s.c. into mice. On indicated days, the total number of $CFSE^+$ $CD11c^+$ DCs within draining or nondraining LNs (NDLN) were calculated and are displayed (n = 3–5 per group per day). Asterisks indicate statistically significant differences between RAP-treated and control DC groups ($p < 0.05$). Data are representative of three individual experiments.

of control DCs activated with LPS and OVA to stimulate T cells deteriorated over time after activation (Fig. 5A). In contrast, DCs activated in the presence of mTOR inhibitors, which were as capable as control DCs of stimulating T cell proliferation at day 2, retained their ability to stimulate T cells even at day 4 after DC activation (Fig. 5A). Thus, the ability of DCs to remain activated and capable of stimulating T cells after exposure to Ag and TLR agonists is markedly enhanced by the inhibition of mTOR.

DCs in which mTOR is inhibited have an enhanced capacity to induce therapeutic CD8 T cell responses in vivo

The ability of mTOR inhibition to prolong DC activation and T cell stimulatory capacity after maturation in vitro suggested that inhibition of DC mTOR signaling might enhance the ability of these cells to induce T cell responses in vivo. To test this possibility, we stimulated DCs with LPS, LPS plus OVA, or rapamycin plus LPS plus OVA for 6 h, then washed them extensively and injected them s.c. into mice. Endogenous OVA-specific $CD8^+$ T cell responses in draining LNs were monitored by tetramer staining 7 d after DC transfer. Rapamycin-treated DCs were not impaired in their ability to migrate to LN draining sites of injection (data not shown and discussed later in this article), consistent with the fact that they increased expression of CCR7 in response to LPS equivalently to DCs that were stimulated with LPS in the absence of rapamycin (Fig. 5B). The addition of rapamycin to DCs during the time that they were pulsed with LPS plus OVA enabled them to induce stronger immune responses as measured by LN expansion (Supplemental Fig. 3A) and the frequency (Fig. 5C) and number (Fig. 5D) of Kb-OVA $tetramer^+$ $CD8^+$ T cells in reactive LNs. We also detected Ag-specific $CD8^+$ T cells in blood and spleen of immunized mice, and these cells were present in higher frequencies in the mice that received rapamycin-treated DCs (Supplemental Fig. 3B, 3C). We observed that frequencies of circulating Ag-specific $CD8^+$ T cells increased between days 7 and 14 postimmunization with rapamycin-treated DCs, whereas contraction of the Ag-specific population occurred during this period in mice immunized with DCs that had not been treated with rapamycin (Fig. 5E). In addition, we observed higher numbers of injected rapamycin-treated DCs than control DCs in reactive LN draining sites of immunization on days 3 and 4 after DC transfer (Fig. 5F), suggesting that the in vitro survival advantage conferred by mTOR inhibition (Fig. 2A) may also be at play in vivo. Based on in vitro data (Fig. 5A), we would expect the rapamycin-treated DCs persisting at days 3 and 4 to be able to continue to activate T cells in the in vivo setting.

Our principal focus was on the ability of mTOR inhibition to augment DC activity during the primary T cell response after autologous DC transfer. However, we were also interested in testing whether vaccination with DCs activated in the presence of mTOR inhibitors could induce the development of a population of Ag-specific memory $CD8^+$ T cells. Immunization with DCs pulsed with LPS plus OVA or with rapamycin plus LPS plus OVA resulted in the establishment of robust memory $CD8^+$ T cell populations that, 4–5 wk after priming, were highly capable of responding to challenge infection with OVA-expressing *Listeria monocytogenes* (Supplemental Fig. 4A), or of mediating protection against challenge with OVA-expressing B16 melanoma cells (Supplemental Fig. 4B, 4C). Taken together, our data demonstrate that DCs activated in the presence of rapamycin are capable of inducing enhanced CD8 T cell primary expansion and contraction during the primary phase of the response, and the establishment of a population of memory $CD8^+$ T cells that can be recalled by re-exposure to Ag.

Therapeutic autologous DC vaccination has significant promise for cancer treatment (33). We reasoned that the advantages conferred to DCs by mTOR inhibition might enhance their ability to induce therapeutic antitumor immunity. To test this hypothesis, we intradermally inoculated mice with OVA-expressing B16 melanoma cells. Three days later, we vaccinated tumor recipients with DCs that had been pulsed in vitro for 6 h with LPS, with LPS plus OVA, or with rapamycin plus LPS plus OVA. Mice that received the rapamycin-treated DCs after tumor inoculation had increased frequencies of OVA-specific $CD8^+$ T cells in the blood 1 wk after DC vaccination compared with mice that received DCs that had not been treated with rapamycin (data not shown), indicating that the in vivo benefit conferred by rapamycin-treated DCs is not sensitive to systemic tumor-mediated immune suppression. Consistent with a protective role for $CD8^+$ T cells in this system, the kinetics of tumor growth were substantially delayed in mice vaccinated with DCs stimulated with LPS plus OVA (Fig. 6A). Most importantly, there was a highly significant reduction in tumor burden, obvious macroscopically (Fig. 6B) and as measured by tumor volume (Fig. 6C) and mass (Fig. 6D) at the time of sacrifice in mice that received the rapamycin-treated DCs. As anticipated, regardless of whether they were treated with rapamycin, DCs pulsed with LPS plus OVA conferred significant therapeutic advantage over the control treatment of DCs that had been pulsed with LPS without Ag. The improved therapeutic antitumor effect of immunization with rapamycin-treated DCs was associated with a doubling in the frequency of Ag-specific $CD8^+$ tumor-infiltrating lymphocytes observed in single-cell suspensions of harvested tumors (Fig. 6E). These data provide evidence that mTOR inhibition in DCs during in vitro stimulation with Ag and TLR agonists before transfer into a recipient can significantly improve the subsequent in vivo therapeutic potential of these cells.

Discussion

In this article, we describe mTOR inhibition during DC activation as a novel strategy to improve autologous DC vaccination in a murine melanoma tumor model. We demonstrate that pharmacological inhibition of mTOR prolongs the life span of TLR-activated mouse DCs and extends the time period over which they exhibit an activated phenotype. The combined effect of these two processes results in a dramatic difference in outcome. By 2–4 d after activation in the absence of mTOR inhibitors, most DCs are dead, and those that remain alive are no longer expressing high levels of costimulatory molecules. In contrast, by 2–4 d after activation in the presence of mTOR inhibitors, most of the DCs within the starting population are alive and continuing to express high levels of costimulatory molecules. Not surprisingly in light of these findings, DCs activated in the presence of mTOR inhibitors induce larger CD8 T cell responses both in the local lymphoid compartments and systemically when adoptively transferred into naive host animals. This enhanced CD8 T cell response induced by DCs in which mTOR is inhibited correlates with a more pronounced protective effect against an aggressive melanoma in a therapeutic vaccination model. Strikingly, these beneficial effects of mTOR inhibition on DCs are induced by a brief 6-h exposure to mTOR inhibitors at the time of the addition of TLR agonists to these cells. The fact that short-term in vitro pharmacological intervention can have beneficial effects on the kinetics human DC activation status suggests that it may be technically feasible to translate this approach for enhancing autologous DC vaccination into a clinical setting.

We observed identical effects on DC life span, costimulatory molecule expression, and cytokine production with either rapamycin or KU treatment. However, because both rapamycin and KU treatment showed comparable inhibitory effects on the phosphorylation of Akt Ser^{473}, a downstream target of mTORC2 activity, we cannot distinguish from these data which mTOR signaling complex is the predominate effector complex regulating DC activation and life span in our system. Future studies genetically targeting the role

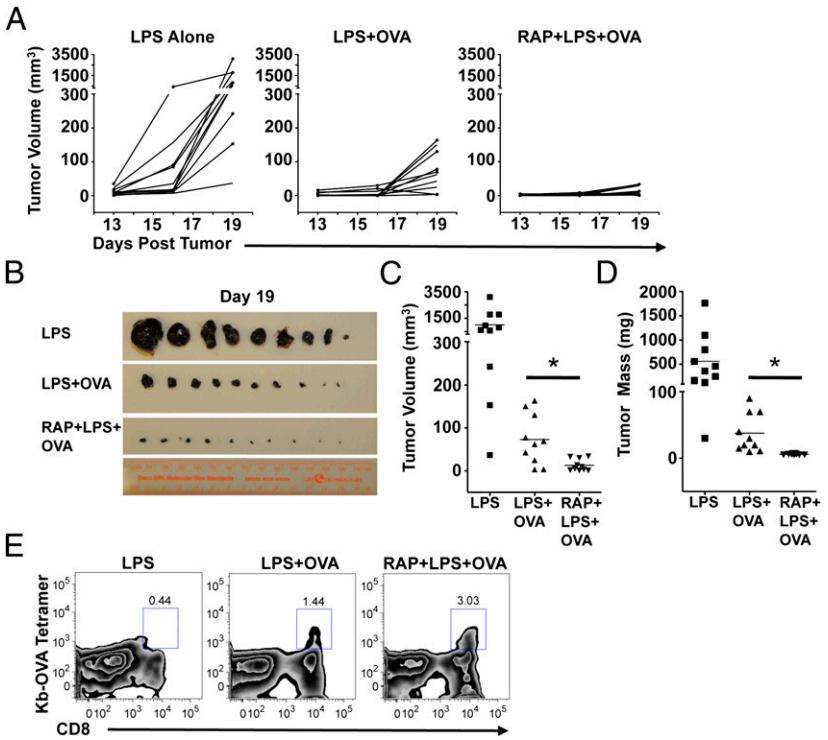

FIGURE 6. Rapamycin enhances the ability of DCs to induce therapeutic antitumor immunity. (**A**) Mice inoculated with tumors on day 0 were each immunized once s.c. with 5×10^5 DCs treated as indicated on day 3 and then monitored every 3 d for tumor growth. Tumor volumes were measured at each time point. The kinetics of tumor growth for each individual mouse in the experiment ($n = 10$ per group) is plotted. Nineteen days after immunization, mice were sacrificed and tumors were excised, photographed (**B**), and tumor volume (**C**) and mass (**D**) were calculated. Data from all individual mice in the experiment are shown, and mean values are illustrated by horizontal bars in (C) and (D). Asterisks show statistically significant differences ($p < 0.05$). (**E**) Tumor single-cell suspensions were analyzed by FACS, and the frequencies of Kb-OVA tetramer$^+$ CD8$^+$ T cells within the CD45$^+$ gates are shown. Data are concatenated from all tumors for each mouse group. All tumor experiments were repeated at least three times with similar results.

of mTORC1 versus mTORC2 in DC activation and survival will be an interesting avenue for further investigation.

Elucidating the role of mTOR during DC activation in response to TLR agonists is complicated by its established role as central mediator of signaling initiated by growth factor receptors, including that for GM-CSF, an important DC differentiation and survival factor (25, 34). Work in human monocyte-derived DCs has demonstrated that mTOR inhibition can disrupt GM-CSF signaling in these cells, inhibiting differentiation and leading to apoptosis (34). Consistent with this, the inclusion of pharmacological inhibitors of mTOR in mouse bone marrow DC cultures did negatively affect the final yield of DCs (data not shown). In contrast, the suppression of mTOR expression by retrovirally introduced mTOR-targeting shRNA did not prevent GM-CSF–driven differentiation of mouse DCs from bone marrow, possibly because the hairpin is not expressed until after the DC precursors have finished proliferating in response to GM-CSF (35). Taken together, these data indicate that mTOR signaling may be important for different functions at different times over the life span of a DC. The fact that mTOR inhibition actually promoted increased DC survival after activation by TLR agonists emphasizes the emerging concept that the effects of mTOR regulation of DC metabolism and growth factor signaling may be highly context specific. Understanding the nuances of the functional role of mTOR in DCs has potential to provide important insights about the underlying cell biology controlling activation and survival signals in these cells.

One of the challenges raised by the findings reported in this article showing adjuvant-like effects of inhibiting mTOR in DCs is to reconcile our data with the expansive literature on the use of mTOR inhibitors to promote immune tolerance. Inhibition of mTOR by rapamycin is reported to support the induction of tolerogenic DCs (36, 37) and to inhibit both Flt3 ligand and GM-CSF–driven DC differentiation in vitro (29, 34, 38, 39). In contrast, our data strongly support an emerging view that, in certain contexts, mTOR inhibitors administered simultaneously with TLR stimulation can enhance the activation of DCs (29, 30). The underlying explanation for this apparent disagreement may lie in the details of how experiments are performed and in the nature of the DCs being tested. Based on the role of mTOR in signaling by a number of growth factors and cytokines (12, 25, 34), we speculate that specific culture conditions during in vitro generation of DCs may determine the effect of mTOR inhibition on these cells. For example, Flt3 ligand-differentiated DCs may respond differently to mTOR inhibitors during TLR activation than GM-CSF–differentiated DCs. We speculate that by restricting mTOR inhibition specifically to the initial phases of TLR activation, we can modulate DC activity without inducing the deleterious effects that may be associated with disrupted growth factor signaling in some DC subtypes.

We previously proposed that the prolonged increase in aerobic glycolysis in activated DCs serves to rapidly generate ATP, whereas conserving the metabolic intermediates such as amino acids and fatty acids that we predicted would be important for the complete enactment of the activation process (13). Nevertheless, we observed that mTOR inhibition in the context of TLR activation potently inhibits metabolic commitment to glycolysis but does not affect TLR-mediated activation, insofar as Ag processing and presentation, IL-12 and TNF-α production, costimulatory molecule expression, cellular migration, and DC:T cell interactions are concerned. Thus, it is clear that TLR agonist-mediated DC activation does not require commitment to aerobic glycolysis when mTOR is inhibited. Indeed, our findings indicate that sustained glycolysis subsequent to TLR activation is associated with rapid cell death, and that restricting glucose consumption by mTOR inhibition prolongs the life span of these cells without compromising their ability to stimulate T cells in vitro or in vivo. A strong association between glucose consumption and cell death is widely reported in the literature, and restricting glucose usage and caloric intake leads to increased cellular and organismal longevity (10, 14, 15, 40–42). Interestingly, the strong metabolic shift to aerobic glycolysis on LPS stimulation was not observed in human myeloid DCs, which may be an important factor in the different survival kinetics of activated human and mouse DCs in our systems. However, mTOR inhibition augmented and prolonged costimulatory molecule expression in both human and mouse DCs, indicating that mTOR may be regulating DC survival and costimulatory

molecule expression by two different mechanisms. The underlying mechanism by which inhibition of mTOR allows DCs to enact their activation program in the absence of a switch to glycolysis as well as the apparent differences in metabolic regulation of DCs between human and mouse are current focuses of research in our laboratory.

One of the intriguing findings of our study is the disparity in sensitivity of IL-10 versus IL-12p70/TNF-α production to mTOR inhibition. The mTOR substrate 4EBP1 drives cap-dependent translation (43–46), so despite the fact that similar observations have been reported previously regarding the specific inhibition of IL-10 production in DCs by mTOR ablation (29, 30), it was surprising to us that although TLR agonist-induced IL-10 production was inhibited by rapamycin, the production of IL-12p70 and TNF-α remained unaffected. This finding raises the possibility that translation of IL-12p70 and TNF-α transcripts can be initiated by cap-independent processes (47). In light of our observations on the effects of mTOR on the surface expression of costimulatory molecules, we speculate that normal cellular dynamics like vesicular trafficking may also have slowed in rapamycin-treated cells. This is consistent with the fact that mTOR controls the expression of genes involved in cholesterol and fatty acid synthesis (32).

There is an ongoing interest in biomedical research in harnessing the immunostimulatory properties of DCs for immune intervention against tumors. The Food and Drug Administration recently approved Provenge, the first autologous cellular immunotherapy for use in cancer patients (48), which marks an important hallmark in advancing the use of cellular immunotherapy strategies in the clinic. Furthermore, DC vaccination strategies have shown significant promise in early-phase clinical trials in human melanoma patients (49). Our studies using a mouse model of melanoma outline a potential new strategy for augmenting DC activation in the context of therapeutic autologous vaccination. Because brief exposure to mTOR inhibitors in vitro enhances the activation phenotype in both mouse and human DCs, we believe that our findings may be of significant relevance to ongoing clinical research aimed at improving the potency of DC therapeutic vaccines.

Our data define mTOR signaling as a new molecular target for augmenting DC survival and activation. We show that mTOR inhibition extends the life span of DCs and enhances the expression of key costimulatory molecules involved in the initiation of adaptive immune responses. Ongoing studies are aimed at further understanding the consequences of mTOR inhibition in the context of the beneficial effects on DCs reported in this study. The fact that mTOR inhibitors, including rapamycin, are approved for use in human patients, and that our approach requires that DCs be exposed to rapamycin only in vitro before transfer into recipients, increases the feasibility of potentially translating this simple strategy to clinical settings. In summary, our studies demonstrate a novel pharmacological approach for temporally extending DC life span, prolonging DC activation, and improving the outcome of autologous DC vaccination for experimentally induced cancer in mice.

Acknowledgments

We thank Brent Berwin and Dick Dutton for tumor cells, Larry Johnson for help with statistical analysis, and the Champlain Valley Physicians Hospital Medical Center for donation of blood filters.

Disclosures

The authors have no financial conflicts of interest.

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