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Friends Not Foes: CTLA-4 Blockade and mTOR Inhibition Cooperate during CD8+ T Cell Priming To Promote Memory Formation and Metabolic Readiness

Virginia A. Pedicord,*1 Justin R. Cross, † Welby Montalvo-Ortiz,*2 Martin L. Miller,‡,3 and James P. Allison*•,2

During primary Ag encounter, T cells receive numerous positive and negative signals that control their proliferation, function, and differentiation, but how these signals are integrated to modulate T cell memory has not been fully characterized. In these studies, we demonstrate that combining seemingly opposite signals, CTLA-4 blockade and rapamycin-mediated mammalian target of rapamycin inhibition, during in vivo T cell priming leads to both an increase in the frequency of memory CD8+ T cells and improved memory responses to tumors and bacterial challenges. This enhanced efficacy corresponds to increased early expansion and memory precursor differentiation of CD8+ T cells and increased mitochondrial biogenesis and spare respiratory capacity in memory CD8+ T cells in mice treated with anti–CTLA-4 and rapamycin during immunization. Collectively, these results reveal that mammalian target of rapamycin inhibition cooperates with rather than antagonizes blockade of CTLA-4, promoting unrestrained effector function and proliferation, and an optimal metabolic program for CD8+ T cell memory. The Journal of Immunology, 2015, 194: 000–000.

 Through profound Ag-responsive proliferation, secretion of effector cytokines, and direct killing of target cells, CD8+ T cells play a vital role in multiple aspects of host protection, including resistance to pathogenic infections and tumorigenesis. The destructive potential of these cells is kept in check, to some degree, by expression of a potent negative regulator of T cell responses, CTLA-4, early after activation, and Ab blockade of this molecule has been shown to augment primary and memory T cell responses (1–3). In addition to CTLA-4 expression, early CD8+ T cell survival and proliferation are highly influenced by the presence of various cytokine and inflammatory cues, which also help to direct differentiation of short-lived effector cells (SLECs) or memory precursor effector cells (4, 5). This environmental modulation of CD8+ T cell differentiation is accomplished, in part, through signaling via the mammalian target of rapamycin (mTOR). Initially, inhibiting the mTOR pathway downstream of cytokine and costimulatory signals was shown to antagonize recall Ag responsiveness and promote anergy in CD4+ T cells (6, 7); however, more recent studies indicate that low doses of rapamycin can actually increase memory precursor effector cell frequency and the subsequent pool of long-lived memory CD8+ T cells (8).

Although they both result in increased CD8+ T cell memory, mTOR inhibition and CTLA-4 blockade appear to have divergent effects on CD8+ T cell proliferation and phenotype. It has been proposed that anti–CTLA-4 in CD4+ T cells allows unrestrained Ag-driven clonal expansion and cell-cycle progression and that this leads to improved secondary Ag responses (6). This likely results, at least to some extent, from increased costimulatory CD28 signaling upon blockade of higher-affinity binding of CTLA-4 to the shared CD80/C211 ligands, CD80 and CD86 (9–11).

In our previous studies, we showed that a single dose of anti–CTLA-4 during T cell priming also results in improved expansion of Ag-specific CD8+ T cells and a larger pool of functional effector memory phenotype (CD62Llow, KLRG1low, IL-7Rαlow) cells producing IFN-γ and TNF-α (1). In addition, when memory CD8+ T cells from different priming conditions were adoptively transferred in equal numbers to naive recipients, cells previously treated with anti–CTLA-4 were more effective at clearing subsequent bacterial challenges on a per-cell basis. In contrast, in studies of mTOR inhibition-mediated enhancement of CD8+ T cell memory, low doses of the mTOR inhibitor rapamycin administered during T cell priming did not lead to increased expansion but decreased contraction of Ag-specific CD8+ T cells, and this corresponded to an increased frequency of KLRG1low, IL-7Rαhigh, memory precursor phenotype cells
that were predominantly CD62Lhigh (8). Although mTOR activation has been shown to play an important role in protein synthesis and cell-cycle control after T cell activation, it has been proposed that the improved differentiation of memory cells that results from blocking mTOR can be attributed to alterations in mTOR-regulated T cell metabolism (8, 12). In addition, the mTOR inhibitor used in these studies, rapamycin, preferentially blocks signaling through TORC1, as opposed to TORC2 (13), and TORC1 and TORC2 have recently been shown to play distinct roles in effector and regulatory CD4+ T cell differentiation (14, 15). Although subsequent increases in long-term memory CD8+ T cells after rapamycin treatment have also been shown to yield potent antiviral responses, it has not been clearly established how rapamycin prompts beneficial changes in CD8+ T cell memory function.

In this study, we analyzed endogenous memory CD8+ T cells that arise in vivo in the presence of both mTOR inhibition and CTLA-4 blockade during immunization with a model Ag. In the context of these apparently opposing signals, we observe both an increased reservoir of long-lived Ag-specific CD8+ T cells and improved memory efficacy against reinfection and tumorigenesis. This is associated with an early increase in CD8+ T cell expansion and differentiation toward a memory precursor phenotype. We also discovered an enhanced fatty acid metabolism transcriptional profile and increased mitochondrial biogenesis, and memory CD8+ T cells from mice immunized in the presence of anti–CTLA-4 and rapamycin exhibited significant increases in respiratory capacity and effector cytokine production. Taken together, these results indicate that modulating the environmental cues of primary T cell activation, namely, through inhibition of both mTOR signaling and CTLA-4–mediated negative regulation, allows fine-tuning of memory programming to promote an expanded pool of memory CD8+ T cells metabolically capable of more efficiently responding to reactivation.

Materials and Methods

Mice

Mouse work was performed in accordance with institutional guidelines and animal protocols approved by the Institutional Animal Care and Use Committee at Memorial Sloan Kettering Cancer Center. All mice were maintained in a specific pathogen-free facility according to National Institutes of Health Animal Care guidelines. C57BL/6J (B6) mice, congenic CD45.1<sup>+</sup> B6.SJL mice, and OVA-specific OTI TCR transgenic mice were purchased from The Jackson Laboratory. Immunodeficient Rag2<sup>−/−</sup> mice were purchased from Taconic Farms. OTI mice were crossed to Rag2<sup>−/−</sup> and B6.SJL mice to generate mice harboring only congenically marked Ag-specific OTI T cells.

Blockade of CTLA-4 and rapamycin administration

Approximately 2 h before primary infection (immunization), mice were injected i.p. with 200 μg anti-mouse CTLA-4 (clone 9H10) or hamster IgG isotype control (both from BioXCell) diluted in sterile PBS. Rapamycin (Sigma-Aldrich) was dissolved in ethanol to a concentration of 2 mM and diluted in sterile PBS for i.p. injection at a dose of 75 μg/kg/day for 10 d, days 1 to 8 postimmunization (p.i.).

Bacteria

Recombinant OVA-expressing Listeria monocytogenes (LM-OVA) was provided by Dr. H. Shen (University of Pennsylvania, Philadelphia, PA). Bioluminescent L. monocytogenes (in this article referred to as luciferase-expressing L. monocytogenes [LM-lux]) were acquired from Caliper Life Sciences (Xen32). Bacteria were cultured in Bacto BHI broth (BD Biosciences). Bacteria were grown to log phase (A<sub>600</sub> of 0.08–0.13) and resuspended in PBS before i.v. injection into lateral tail veins. For immunization, mice were injected with a low dose, 5 × 10<sup>3</sup> CFU/mouse. At least 7 d p.i., mice were rechallenged with a high dose of Listeria i.v. injection. At 24 h postinfection, spleens were harvested, weighed, and dissociated in PBS containing 0.1% Triton X-100 (Roche). Numbers of bacterial CFUs were determined by plating serial dilutions on BHI agar plates containing 2 μg/ml erythromycin (Sigma-Aldrich). Alternatively, ventral imaging of LM-lux-rechallenged mice was conducted at numerous time points postinfection using an IVIS-200 bioluminescence imaging system (Xenogen), and luminescence was quantified using corresponding Living Image software (Xenogen) to determine the kinetic of bacterial proliferation and clearance.

Tumors

EG.7 tumor cells (EL4 cells expressing OVA) were purchased from ATCC (CRL-2113), grown to 70% confluence, and cryopreserved in individual aliquots. For each experiment, an aliquot from this initial bank was thawed and passaged once before injection. At least 7 wk after immunization with LM-OVA, mice were challenged with 5 × 10<sup>5</sup> (for tumor growth and survival) or 2.5 × 10<sup>6</sup> (for tumor and tumor-draining lymph node analysis) EG.7 cells by intradermal (i.d.) injection in the shaved right flank. Tumor size and animal health were monitored at least three times per week, and animals were euthanized upon signs of illness or above-threshold tumor size. For analysis of tumor-associated lymphocytes, tumor-challenged mice were euthanized 12 d after EG.7 injection, and tumors and draining inguinal lymph nodes were harvested.

Flow cytometry

Typically, cells were stained for surface protein markers for 20–30 min on ice in wash buffer (PBS with 2% FCS and 0.1% NaN<sub>3</sub>) using fluorophore-conjugated Abs against indicated surface proteins. For staining of nuclear Foxp3, T-bet, and eomesodomin, cells were fixed, permeabilized, and stained with corresponding Abs (all from eBioscience).

Tetramer staining

SINFEKL peptide (OVA257–264) was synthesized and HPLC purified by the Molecular Biology Proteomics Facility of the University of Oklahoma Health Sciences Center. Peptide/MHC tetramers were produced by I. Leiner in the Memorial Sloan Kettering Cancer Center Tetramer Core Facility. Cells were stained with tetramer-PE in the presence of anti–CD8α-Pacific Blue (clone 53-6.7, eBioscience) and other surface marker Abs for 1 h on ice. Stained cells were analyzed by flow cytometry.

Cytokine production

T cells were restimulated ex vivo with 1 μg/ml SINFEKL peptide at 37°C. For intracellular cytokine staining, cells were restimulated for 4 h in the presence of monensin or brefeldin A. Cells were stained for surface markers, fixed, and permeabilized (BD Cytofix/Cytoperm), and Ab stained for intracellular IFN-γ and TNF-α (eBioscience) before analysis by flow cytometry.

Cell sorting and adoptive transfer

CD8+ T cells from OTI TCR transgenic mice were isolated for adoptive transfer using MACS CD8<sup>+</sup> T Cell Isolation Kit for mouse (Miltenyi). Memory OTI CD8<sup>+</sup> T cells were sorted electronically by FACS after staining with Abs against CD8α, CD45.1, and CD44 (eBioscience). Purity of isolated cells was confirmed on aliquots of cells by postsort flow cytometry, and sorted cells were counted using a hemacytometer or Guava PCA/ViaCount cell counting system (Millipore) before use in further assays.

mRNA microarray data processing

FACS-sorted memory CD8<sup>+</sup> cells from different treatment conditions were centrifuged and lysed in Tri-Reagent (Sigma-Aldrich) for RNA isolation via manufacturer’s instructions. Transcript profiling was performed with the Illumina Mouse WG-6 V2 R3 microarray platform, and data were processed with BeadStudio software. Probe intensities were log2 scaled, quantile normalized, and averaged across genes. Two-tailed Student t tests were performed between the anti–CTLA-4 and rapamycin treatment group and the control Ig group at each time point (five biological replicates at day 5 and three biological replicates at day 3), and the expression changes between the treatment groups were calculated from the average gene expression level. These data have been deposited in National Center for Biotechnology Information’s Gene Expression Omnibus and are accessible through Gene Expression Omnibus Series accession number GSE63022 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63022).

Gene set enrichment analysis

Normalized gene-level microarray data were analyzed with the gene set enrichment analysis (GSEA) command line software tool version 2 (16).
The KEGG pathway gene set from the curated gene set C2 (v4.0) was selected as gene set database, and the experimental treatment conditions (anti–CTLA-4 and rapamycin versus control Ig at day 5) were used as phenotype vector. Default parameters were applied (1000 permutations on phenotype, 15 minimum set, 500 maximum set, weighted scoring scheme, and t test metrics).

**Mitochondrial stress assays**

Oxygen consumption and extracellular acidification were measured in real-time on FACS-sorted memory CD8+ T cells using a XF-96 Extracellular Flux Analyzer with XF Cell Mito Stress Test Kit reagents (Seahorse Bioscience). Memory CD8+ T cells were adhered to assay plates with Cell-Tak (BD) and incubated in the absence of CO2 in assay medium (nonbuffered DMEM containing 10 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate) for 30 min before the assay. Mitochondrial inhibitors were added to achieve final concentrations of 1 μM oligomycin, 3 μM fluoro-carbonyl cyanide phenylhydrazone, and 1 μM each antimycin A and rotenone at indicated time points.

**Real-time PCR**

Isolated RNA from sorted memory cells (see earlier) was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) with prescribed protocol. Real-time PCR was performed on resulting cDNA using TaqMan primers for mouse CO1, GLUT1, HIF1α, and HK2 with GAPDH and HPRT as endogenous controls with TaqMan Universal Master Mix according to manufacturer’s instructions on an ABI 7500 real-time PCR thermal cycler (all from Applied Biosystems). For quantifying mitochondrial/nuclear DNA ratios, real-time PCR was performed on isolated genomic DNA using the phenol-chloroform method and primers for NDUFV1 and CO1 as previously described (17–19).

**Statistical analyses**

Statistical analyses were performed in GraphPad Prism software with the tests indicated in each figure legend. A p value < 0.05 was considered significant.

**Results**

Combining anti–CTLA-4 with rapamycin during CD8+ T cell priming improves memory responses

It has previously been proposed that mTOR inhibition may negate the effects of CTLA-4 blockade in terms of T cell recall responses (6). To evaluate the functional consequences on T cell memory of adding mTOR inhibition to CTLA-4 blockade during T cell priming, we immunized wild-type C57BL/6 mice with a low dose of LM-OVA in the presence of a single dose of anti–CTLA-4 and/or 10 daily, low-dose rapamycin injections and waited at least 7 wk to allow for complete clearance of the bacteria and true memory T cell differentiation. Immunized animals were then challenged with EL4 tumor cells that also express the OVA Ag (EG.7 cells) to assess memory-mediated, Ag-specific, antitumor responses. Tumor growth and animal survival were tracked for at least 100 d. Mice that received both anti–CTLA-4 and rapamycin during T cell priming exhibited a significant decrease or delay in tumor growth and a significant increase in tumor-free survival when compared with mice that were immunized in the presence of an isotype control or either of the single treatments, anti–CTLA-4 or rapamycin alone (Fig. 1A). Analysis of PBLs collected from these mice before tumor challenge showed a significant increase in the frequency of Ag-specific CD8+ T cells present in animals inoculated in the presence of both CTLA-4 blockade and rapamycin (Fig. 1B), and this increased frequency negatively correlated with early tumor burden (Fig. 1C). These data indicate that the combination of anti–CTLA-4 and rapamycin during T cell activation leads to a greater prevalence of Ag-specific memory CD8+ T cells and improved memory responses to Ag rechallenge.
signifying a supportive rather than antagonistic role for rapamycin with CTLA-4 inhibition.

To investigate memory responses in a different physiological setting, we next examined bacteria clearance upon high-dose rechallenges with *Listeria*. To track the kinetics of bacteria proliferation and elimination, we immunized mice with a low dose of LM-lux in the presence of CTLA-4 blockade and rapamycin and then challenged immunized mice 7 wk later with a high dose of the same bacteria. In vivo luminescence was then measured at numerous time points postinfection. Mice that were immunized in combination with anti–CTLA-4 and rapamycin displayed decreased bacterial burden and more rapid control of LM-lux titer in comparison with mice that received either agent alone or the isotype control (Fig. 2A, 2B). Similar results were seen in ex vivo quantification of bacterial burden when mice previously immunized with LM-OVA were later rechallenged with the same bacteria (Fig. 2C). Collectively, these data show that T cell priming in the presence of both CTLA-4 blockade and mTOR inhibition results in more timely and efficient memory responses upon Ag re-encounter.

**CTLA-4 blockade and mTOR inhibition increase effector cytokine-producing memory CD8+ T cells**

To understand potential contributors to this efficient memory functional phenotype, we next examined factors associated with improved memory responses in mice immunized with the dual anti–CTLA-4 and rapamycin regimens. Although in all treatment conditions overall T cell composition was not significantly altered, including absolute number of proliferating polyclonal effector CD8+ and CD4+ T cells and frequency of T regulatory cells (Tregs) (Supplemental Fig. 1), early expansion of OVA-specific CD8+ T cells was significantly increased with CTLA-4 blockade (Supplemental Fig. 1), early expansion of OVA-specific CD8+ T cells was significantly increased with CTLA-4 blockade and rapamycin and this corresponded to decreased tumor mass (Fig. 3B). This increased frequency was not present in lymphocytes from mice that received anti–CTLA-4 alone or the isotype control, but mice that received rapamycin alone also harbored an increased frequency of effector cytokine-producing cells at 12 d after tumor challenge. Similar increases in Ag-specific IFN-γ- and TNF-α-producing CD8+ T cells were also present in the spleens of mice reinfected with a high dose of LM-OVA after being previously immunized in the context of CTLA-4 blockade and mTOR inhibition (Fig. 3A, bottom panels). Contrary to previous studies suggesting that rapamycin promotes T cell anergy (7), these data indicate that priming of CD8+ T cells in the presence of anti–CTLA-4 and rapamycin results in an increased frequency of Ag-specific memory CD8+ T cells readily capable of producing effector cytokines.

**Combination treatment enhances both early expansion and memory cell differentiation**

To determine how adding mTOR inhibition to CTLA-4 blockade affects memory CD8+ T cell differentiation, we also sampled PBLs from mice at various time points after immunization and...
examined CD8+ T cell phenotypes. Mice that received rapamycin during T cell priming displayed a significant early increase in the frequency of KLRG1 low, IL-7Rα high, Ag-specific CD8+ T cells (Fig. 4A). This phenotype has been described to be associated with memory precursor CD8+ T cells (4, 20) and recapitulates memory precursor increases previously seen with low doses of rapamycin alone (8). Although this increased frequency of memory precursor cells was present both in mice that received rapamycin alone and mice that received anti–CTLA-4 with rapamycin, the greatest early expansion of tetramer+ CD8+ T cells was observed in mice that received anti–CTLA-4 (Supplemental Fig. 2), and the combination of both anti–CTLA-4 and rapamycin resulted in the largest long-term pool of Ag-specific memory cells (Fig. 4B). In total, these findings indicate that the robust CD8+ T cell expansion elicited by anti–CTLA-4 and enhanced memory precursor differentiation elicited by rapamycin, along with increased effector cytokine production, may contribute to the efficient antitumor and antimicrobial memory responses that we observe in mice immunized in the presence of CTLA-4–blocking Abs and this mTOR inhibitor.

With mounting evidence of enhanced CD8+ T cell memory formation and function, we hypothesized that CTLA-4 blockade and rapamycin might be influencing early memory differentiation. Because the transcription factors T-bet and eomesodermin (eomes) have been strongly implicated in CD8+ T cell memory (4, 21), we inspected the expression of these transcription factors by performing flow cytometry on CD8+ T cells shortly after primary LM-OVA infection in the presence of anti–CTLA-4 and rapamycin. Congenically marked, OVA-specific TCR transgenic CD8+ T cells (OTI cells) were adoptively transferred to wild-type mice 1 d before immunization to track Ag-specific cells. At 3 d after activation, OTI cells primed in the context of CTLA-4 blockade and rapamycin showed a marked decrease in the frequency of T-bet–expressing cells compared with either anti–CTLA-4 or rapamycin alone or the isotype control (Fig. 5A, 5B). The presence of both T-bet and eomes is essential for memory CD8+ T cell maintenance and function (21); however, a gradient of T-bet expression is created by the inflammatory environment shortly after infection, with higher levels of T-bet expression promoting differentiation of SLECs and lower levels of T-bet promoting memory CD8+ T cell differentiation (4, 22). Although a previous study showed increased expression of eomes during homeostatic proliferation in response to rapamycin alone (23), the early frequency of eomes-expressing cells was not significantly affected by any of our treatments (Fig. 5A, 5B). Together with the decreased expression of T-bet, this may signify an enhanced tip in the balance of these two transcription factors toward a memory precursor-promoting transcriptional program in CD8+ T cells primed in the presence of both CTLA-4 blockade and rapamycin.

Rapamycin and CTLA-4 blockade fine-tune the memory CD8+ T cell metabolic profile

To identify other factors involved in the improved CD8+ T cell memory function established by priming in combination with CTLA-4 blockade and mTOR inhibition, we extended our expression profiling by performing transcriptional microarray analyses. Ag-specific CD8+ T cells from mice immunized with LM-OVA were sorted at days 5 and 35 p.i., and mRNA was isolated for transcriptional microarray analysis.
further transcriptional characterization. Although comparisons of gene expression at the two time points examined yielded many changes previously described to be associated with CD8⁺ T cell activation and memory differentiation (Fig. 6A) (24), GSEA using KEGG pathways strongly indicated the presence of early expression changes in lipid metabolism in cells immunized in the presence of anti–CTLA-4 and rapamycin (Fig. 6B). Strikingly, in addition to enrichment observed in ribosomal, cell-cycle, and protein synthesis pathways, of 15 enriched pathways identified, 4 pathways were categorized to be involved in lipid metabolism, including the oxidative phosphorylation (OXPHOS) pathway (Fig. 6B). The GSEA enrichment profile of OXPHOS-associated genes at day 5 p.i. pointed to a number of genes differentially regulated in this pathway (Fig. 6C, 6D). Metabolic changes regulated by the mTOR pathway have recently been described to be important for memory CD8⁺ T cell differentiation and function (8, 12, 25), and our data corroborate a role for fatty acid metabolism in the combination anti–CTLA-4/rapamycin–mediated enhancement of memory CD8⁺ T cell formation and functional efficacy.

In a recent study, CD8⁺ T cells exhibited increased mitochondrial biogenesis, fatty acid oxidation, and spare respiratory capacity in vitro in the presence of the memory-inducing cytokine IL-15 (17), and these changes have been shown to contribute to the ability of memory CD8⁺ T cells to rapidly respond to rechallenge (26). Given the differential expression of metabolism genes we encountered in CD8⁺ T cells primed in the presence of anti–CTLA-4 and rapamycin, we next specifically investigated changes in expression of mitochondrial transcription factor A (TFAM) and mitochondrial transporter carnitine palmitoyltransferase 1 (CPT1a). TFAM is crucial for maintenance of

FIGURE 4. CTLA-4 blockade and rapamycin result in increased Ag-specific CD8⁺ T cell expansion and memory precursor frequency. PBLs were isolated from mice previously immunized with LM-OVA in the presence of anti–CTLA-4 and/or rapamycin and analyzed by flow cytometry. Representative (median) plots gated on SIINFEKL tetramer⁺ CD8⁺ T cells are shown for expression at day 7 p.i. of the memory precursor markers KLRG1 and IL-7Rα (A, left panel) and quantified pooled frequencies are shown (A, right panel). Frequency of Ag-specific cells in peripheral blood was determined by SIINFEKL tetramer staining and quantified over three time points with pooled tetramer⁺ frequency data shown for each time point (B). All quantifications were pooled from three independent experiments with n = 7–8 mice per group per experiment and were analyzed by one-way ANOVA. *p ≤ 0.05, ***p ≤ 0.001.
mitochondrial DNA and is a key activator of mitochondrial gene expression (27), whereas CPT1α has been shown to regulate fatty acid oxidation in memory CD8^+ T cell development (17, 28). When we interrogated treated cells by real-time PCR at time points early after activation, we observed significant early increases in CPT1α in mice treated with both CTLA-4 blockade and rapamycin (Supplemental Fig. 3). In contrast, anti–CTLA-4 alone appeared to modestly promote glycolytic respiration based on trends toward increased expression of key components of the glycolysis pathway, including glucose transporter 1, hypoxia-inducible factor 1-α, and hexokinase 2 (Supplemental Fig. 3). Minimal early changes in expression of glycolysis-associated genes are consistent with previous studies of rapamycin treatment alone or CD28-mediated T cell costimulation (29–31).

Although CTLA-4 blockade increases overall proliferation, Ag-activated cells readily proliferate in all treatment conditions at early time points (Supplemental Fig. 2), and the robust metabolic and transcriptional changes known to occur during growth and proliferation could confound or overwhelm our analyses of treatment-induced differences in long-term metabolic potential. We therefore sought to analyze memory CD8^+ T cells by real-time PCR at later times when confounding effects of the proliferative phase, and SLECs, are no longer present. In Ag-specific CD8^+ T cells sorted from mice 50 d after immunization in the presence of anti–CTLA-4 and rapamycin, both Tfam and CPT1α displayed significantly increased relative expression versus cells from mice immunized in the presence of the isotype control (Fig. 7A). Sorted Ag-specific CD8^+ T cells were also probed for evidence of increased mitochondrial biogenesis at this later time point by comparing mitochondrial/nuclear DNA ratios, and combination-treated CD8^+ T cells also exhibited significantly increased mitochondrial/nuclear DNA ratios when compared with anti–CTLA-4- or rapamycin-alone regimens (Fig. 7B).

Because metabolic changes toward increased reliance on OXPHOS have been reported to be associated with increased spare respiratory capacity in memory CD8^+ T cells (17), we next examined the respiratory profiles of Ag-specific cells from mice immunized in the context of the combination treatment. To facilitate sorting of enough cells for the assay, we adaptively transferred OTI cells to wild-type mice 1 d before infection with a low dose of LM-OVA and administration of CTLA-4 blocking Abs, rapamycin, or the combination of the two. OTI cells were then sorted ex vivo during contraction (day 15 p.i.) and memory (day 50 p.i.) phases, and immediately evaluated for real-time extra-cellular acidification rates (ECARs) and oxygen consumption rates (OCR) at baseline and in response to mitochondrial inhibitors. Consistent with a preference for glycolysis in proliferating T cells (32), ECAR, reflecting lactic acid production via glycolysis, was greatest in anti–CTLA-4–treated CD8^+ T cells, and this translated to a lower OCR/ECAR ratio in these cells (Supplemental Fig. 4). This indicates that cells primed in the presence of CTLA-4 blockade alone exhibit an increased reliance on glycolysis. In contrast, sparse respiratory capacity, assessed as the increase in OCR from baseline in response to a mitochondrial uncoupler, was consistently increased in OTI cells sorted from mice that received both anti–CTLA-4 and rapamycin during priming (Fig. 7C). This signifies an increased oxidative potential in Ag-specific CD8^+ T cells from dual-treated mice and translated to higher OCR/ECAR ratios in these cells (Supplemental Fig. 4B). Collectively, these data reveal that priming of CD8^+ T cells in the presence of the combination of CTLA-4 blockade and mTOR inhibition leads to increased mitochondrial biogenesis and spare respiratory capacity during memory differentiation. Taken together, our studies suggest that rapamycin cooperates with CTLA-4 blockade during T cell priming by modulating the metabolic programming of these cells to promote more robust and efficient memory CD8^+ T cell responses.

**Discussion**

We hypothesized that adding rapamycin-mediated mTOR inhibition to CTLA-4 blockade during T cell priming would not antagonize but rather enhance memory CD8^+ T cell responses by combining increased memory precursor differentiation with unrestrained early proliferation, respectively. To investigate this hypothesis, we examined the frequency, phenotype, and function of endogenous Ag-specific CD8^+ T cells in vivo in multiple physiologically relevant settings. In addition to bacterial re-infection, we used a tumor system in which only one protein Ag was shared between the immunization and rechallenge conditions. Our studies showed significant increases in memory CD8^+ T cell function in animals immunized in the context of both transient CTLA-4 blockade and mTOR inhibition as indicated by decreased tumor growth, more rapid bacterial clearance, and increased effector cytokine production. This enhanced memory function was indeed associated with increased expansion and memory precursor differentiation, corresponding to an early decrease in T-bet expression. Upon further investigation of the mechanisms behind this cooperative effect, we discovered evidence of metabolic changes in

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**FIGURE 5.** Ag-specific CD8^+ T cells exhibit decreased T-bet expression early after priming in the presence of CTLA-4 blockade and rapamycin. Congenically marked, OVA-specific TCR transgenic CD8^+ T cells (OTI cells) were adoptively transferred to wild-type mice 1 d before immunization with LM-OVA and administration of anti–CTLA-4 and/or rapamycin to track Ag-specific cells. At 3 d after activation, CD8^+ T cells from pooled spleen and lymph nodes were magnetically sorted and analyzed by flow cytometry for expression of the transcription factors T-bet and eomesodermin (eomes). Representative plots are shown of OTI cells gated based on expression of CD8 and the congenic marker CD45.1 (A). Quantified data are shown as mean ± SEM and were pooled from three independent experiments with n = 2–3 mice/group/experiment (B). All quantifications were analyzed by one-way ANOVA. ^p ≤ 0.05.
CD8+ T cells primed in the presence of anti–CTLA-4 and rapamycin, and went on to demonstrate increased mitochondrial biogenesis and respiratory capacity in these cells. These results contradict the notion that mTOR inhibition antagonizes the positive effects of CTLA-4 blockade on CD8+ T cell memory and support the concept of modulating multiple environmental cues during primary activation to achieve optimal long-term memory formation and function.

Recent work characterizing the roles of different metabolic states in T cell development and function suggests that glycolytic respiration is characteristic of effector T cells, whereas fatty acid oxidation is preferentially used by naive and memory CD8+ T cells (17, 33, 34). Although not intensely examined as a monotherapy in our metabolism studies, anti–CTLA-4 treatment alone appears to result in an OCR profile indicative of glycolytic respiration. This is consistent with the Warburg effect observed in proliferating T cells and promoted by CD28-mediated costimulation (35, 36). This push toward aerobic glycolysis could potentially limit the efficacy of anti–CTLA-4 in enhancing CD8+ T cell memory and may also be associated with the strong skewing toward an
effector-memory (CD62L low) phenotype that we observed in our previous studies (1) due to the link between metabolic switching and downregulation of CD62L (37). It therefore seems likely that rapamycin cooperates with CTLA-4 blockade, at least in part, by counterbalancing a cue that favors glycolytic respiration with a metabolic program that better favors memory cell differentiation and function. The corresponding metabolic switch toward fatty acid oxidation and mitochondrial biogenesis may thereby ultimately allow dual-treated memory CD8+ T cells to be better poised for rapid recall responses.

Although our data indicate that the combination of anti–CTLA-4 and rapamycin results in a unique metabolic profile previously associated with CD8+ T cell memory readiness, we can neither prove nor refute a causal role for metabolism in regulating memory maturation, and we cannot exclude the likelihood that other effects of this combination regimen may also be contributing to the efficacy of the memory responses that we observe. Previous studies indicate that rapamycin-mediated inhibition of mTOR signaling results in reduced IL-12–induced IFN-γ production in human T cells (38). In contrast, we see increased production of IFN-γ in recall responses when our combination regimen is administered during immunization; however, we have not extensively explored the effects of combining CTLA-4 blockade with rapamycin on early cytokine cues during CD8+ T cell priming. Because mTOR is a downstream signaling component of several cytokine and growth factor receptors, rapamycin may also serve to modulate T cell responsiveness to the cytokine milieu during activation. Further studies are necessary to dissect the relative contribution of different cytokine signals to the enhanced memory CD8+ T cell responses generated with anti–CTLA-4 and rapamycin. In addition, differential impacts of TORC1 and TORC2 signaling on CD8+ T cell memory (39) may warrant blocking TORC2 in future experiments to determine whether additional enhancements are possible by these means. Finally, although recent work also describes a role for mTOR-regulated metabolic programs in CD4+ effector and Treg differentiation (14, 40), our current studies do not focus on the effects of our combination regimen on CD4+ T cells or their possible role in improved CD8+ T cell memory. We did not, however, observe significant differences between treatment groups in the absolute number of CD4+ T cells or the frequency of Foxp3+ Tregs in our system.

In conclusion, our studies demonstrate that diverse costimulation and metabolic cues during priming contribute to expansion and differentiation of memory CD8+ T cells and that modulating these cues can fine-tune this program to enhance memory efficacy. In particular, we show that combining CTLA-4 blockade with rapamycin concurrent with immunization results in early proliferation and synergistic improvements in spare respiratory capacity and metabolic readiness in long-lived memory CD8+ T cells that correspond to improved recall responses to bacterial infection and cancer. Understanding the mechanisms of action of this and other combination regimens may have important implications for optimizing immunization strategies and cancer immunotherapy.
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Disclosures
J.P.A. is a paid consultant for Bristol-Myers Squibb and is the primary inventor on the patent “Blockade of T lymphocyte down-regulation associated with CTLA-4 signaling.”

References
Figure S1. Immunization in the presence of CTLA-4 blockade and rapamycin does not adversely affect endogenous polyclonal T cell numbers or relative frequency. Wild-type C57BL/6 mice received either control Ig or anti-CTLA-4 with or without rapamycin just prior to i.v. inoculation with 5x10^3 cfu LM-OVA. Daily rapamycin injections were continued through day 8 post-immunization (p.i.). At day 45 p.i., lymphocytes were isolated from spleen and analyzed by flow cytometry. Absolute numbers were calculated based on flow cytometry frequency and total cell counts from each spleen. Data shown were pooled from 3 independent experiments with n=7-8 mice per group per experiment. All quantifications were analyzed by 1-way ANOVA but exhibited no significant differences (p>0.05).
Figure S2. CTLA-4 blockade and rapamycin result in increased antigen-specific CD8+ T cell expansion. Lymphocytes were pooled from spleen and lymph nodes from mice immunized with LM-OVA in the presence of anti-CTLA-4 and/or rapamycin. Isolated cells were counted using an automatic cell counter and analyzed by flow cytometry to calculate absolute number of antigen-specific SIINFEKL tetramer+ CD8+ T cells at indicated time-points (A). Pooled lymphocytes from treated mice were also stained for Ki-67 to determine the frequency of proliferating cells, and representative histograms with corresponding frequencies among gated CD8+ T cells are shown (B). Quantifications in (A) were pooled, and representative plots (B) were from 2 independent experiments with n=2-3 mice per group per time-point per experiment. All quantifications were analyzed by 1-way ANOVA. *, p≤0.05.
Figure S3. Altered metabolism-associated gene expression in antigen-specific CD8+ T cells early after immunization in the presence of CTLA-4 blockade and rapamycin. OVA-specific CD8+ T cells from pooled spleen and lymph nodes were sorted by FACS at 3 or 7 days after immunization in the presence of anti-CTLA-4 and rapamycin or an isotype control, and mRNA was isolated from sorted cells for analysis by real-time PCR. Relative expression of glycolysis-associated genes: glucose transporter 1 (GLUT1), hypoxia-inducible factor 1-alpha (HIF1α), and hexokinase 2 (HK2) along with relative expression of CPT1α and Tfam are shown in representative plots from one of 3 independent experiments with n=5 mice per group per experiment. All quantifications were analyzed by 1-way ANOVA. *, p≤0.05; **, p≤0.01.
Figure S4. Differential preferences for glycolysis and OXPHOS in CD8+ T cells primed in the presence of rapamycin and anti-CTLA-4. OVA-specific OTI cells were adoptively transferred to naive mice 1 day prior to inoculation with LM-OVA in the presence of anti-CTLA-4, rapamycin, and/or an isotype control. OTI cells from pooled spleen and lymph nodes were sorted by FACS at day 15 p.i. to measure extracellular acidification (ECAR) and oxygen consumption rates (OCR) in real time at 3 time-points under basal conditions. Representative data from one of 3 independent experiments with n=5 mice per group are shown. Data were analyzed by 1-way ANOVA. *, p≤0.05; **, p≤0.01; ***, p≤0.001.