Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function

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Naive CD8+ T cells rely upon oxidation of fatty acids as a primary source of energy. After antigen encounter, T cells shift to a glycolytic metabolism to sustain effector function. It is unclear, however, whether changes in glucose metabolism ultimately influence the ability of activated T cells to become long-lived memory cells. We used a fluorescent glucose analog, 2-NBDG, to quantify glucose uptake in activated CD8+ T cells. We found that cells exhibiting limited glucose incorporation had a molecular profile characteristic of memory precursor cells and an increased capacity to enter the memory pool compared with cells taking up high amounts of glucose. Accordingly, enforcing glycolytic metabolism by overexpressing the glycolytic enzyme phosphoglycerate mutase-1 severely impaired the ability of CD8+ T cells to form long-term memory. Conversely, activation of CD8+ T cells in the presence of an inhibitor of glycolysis, 2-deoxyglucose, enhanced the generation of memory cells and antitumor functionality. Our data indicate that augmenting glycolytic flux drives CD8+ T cells toward a terminally differentiated state, while its inhibition preserves the formation of long-lived memory CD8+ T cells. These results have important implications for improving the efficacy of T cell–based therapies against chronic infectious diseases and cancer.

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Introduction

CD8+ T cells play an important role in the adaptive immune response to intracellular pathogens and cancer (1, 2). After stimulation with cognate antigen, CD8+ naive T cells (Tns) clonally expand and differentiate into effector T cells (Teffs) and distinct memory T cell subsets, including stem cell memory T cells (Tscms), central memory T cells (Tcms), and effector memory T cells (Temss) (3). These subsets can be identified by distinct cell surface marker expression and gene expression profiles that enable their functional specialization (3). Preclinical studies using adoptive transfer of purified CD8+ T cell populations have revealed that less-differentiated Tscms and Tcms can mediate enhanced antitumor (4, 5) and antiviral (6) responses compared with cells taking up high amounts of glucose. Accordingly, enforcing glycolytic metabolism by overexpressing the glycolytic enzyme phosphoglycerate mutase-1 severely impaired the ability of CD8+ T cells to form long-term memory. Conversely, activation of CD8+ T cells in the presence of an inhibitor of glycolysis, 2-deoxyglucose, enhanced the generation of memory cells and antitumor functionality. Our data indicate that augmenting glycolytic flux drives CD8+ T cells toward a terminally differentiated state, while its inhibition preserves the formation of long-lived memory CD8+ T cells. These results have important implications for improving the efficacy of T cell–based therapies against chronic infectious diseases and cancer.

Naive CD8+ T cells rely upon oxidation of fatty acids as a primary source of energy. After antigen encounter, T cells shift to a glycolytic metabolism to sustain effector function. It is unclear, however, whether changes in glucose metabolism ultimately influence the ability of activated T cells to become long-lived memory cells. We used a fluorescent glucose analog, 2-NBDG, to quantify glucose uptake in activated CD8+ T cells. We found that cells exhibiting limited glucose incorporation had a molecular profile characteristic of memory precursor cells and an increased capacity to enter the memory pool compared with cells taking up high amounts of glucose. Accordingly, enforcing glycolytic metabolism by overexpressing the glycolytic enzyme phosphoglycerate mutase-1 severely impaired the ability of CD8+ T cells to form long-term memory. Conversely, activation of CD8+ T cells in the presence of an inhibitor of glycolysis, 2-deoxyglucose, enhanced the generation of memory cells and antitumor functionality. Our data indicate that augmenting glycolytic flux drives CD8+ T cells toward a terminally differentiated state, while its inhibition preserves the formation of long-lived memory CD8+ T cells. These results have important implications for improving the efficacy of T cell–based therapies against chronic infectious diseases and cancer.

Authorship note: Nicholas P. Restifo and Luca Gattinoni contributed equally to this work.

Conflict of interest: Edward D. Karoly and Robert P. Mohney are employees and shareholders of Metabolon Incorporated.

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Research article

Results

Metabolic reprogramming upon CD8+ T cell differentiation. Activation of CD8+ T cells is accompanied by effector differentiation and the loss of memory potential in the majority of cells. To explore the metabolic changes that occur during this process, we first evaluated the gene expression of key rate-limiting enzymes involved in FAO and glycolysis, such as Cpt1a and Hk2, in Tns after stimulation with antibodies specific to CD3 and CD28. Tns displayed high amounts of Cpt1a, but these levels rapidly declined within hours of activation (Figure 1A). In sharp contrast, Hk2 was profoundly upregulated after anti-CD3/CD28 stimulation (Figure 1A). In addition, numerous other genes regulating glucose metabolism, including several glycolytic enzymes and the glucose and lactate/pyruvate transporters, were increased upon activation and effector differentiation (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI69589DS1).

To determine whether these changes in gene expression were associated with modifications of cellular metabolism, we evaluated the metabolome of Tns and Teffs using a variety of platforms, including gas chromatography–mass spectrometry (GC/MS) with and without EI. We stimulated pmel-1 splenocytes with cognate antigen gp100 and IL-2 to induce effector differentiation (26) and isolated T cells with low and high 2-NBDG uptake (2-NBDGlo and 2-NBDGhi, respectively) by flow cytometry sorting (Figure 2A). Uptake of radiolabeled 2DG confirmed that 2-NBDG uptake discriminated cells based on their capacity to incorporate glucose (Figure 2B). 2-NBDGhi T cells had increased mRNA levels of solute carrier family 2 (Slc2a1) and Slc2a3, which encodes the glucose transporter Glut-1) and Hk2 compared with 2-NBDGlo cells (Supplemental Figure 3), which suggests that this fluorescent glucose analog could efficiently distinguish cells with differential glucose metabolism. Furthermore, cells labeling intensely with 2-NBDG displayed greater ECAR than did their 2-NBDGlo counterparts (Figure 2C), indicating that 2-NBDG uptake reflects functional differences in glycolytic metabolism.

Glucose uptake segregates short-lived Teffs from memory T cell subsets. Although these data indicated that activation and effector differentiation of CD8+ T cells was associated with dramatic increases in glycolysis and lactate production, it remained to be determined whether these changes in glucose metabolism could ultimately influence the ability of activated T cells to become long-lived memory cells. To separate activated CD8+ T cells based on intrinsic differences in glycolytic activities, we used a fluorescent glucose analog, 2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose (2-NBDG), which allows for direct quantification of glucose incorporation in living cells by flow cytometry (24, 25). We stimulated pmel-1 splenocytes with cognate antigen gp100 and IL-2 to induce effector differentiation (26) and isolated T cells with low and high 2-NBDG uptake (2-NBDGlo and 2-NBDGhi, respectively) by flow cytometry sorting (Figure 2A). Uptake of radiolabeled 2DG confirmed that 2-NBDG uptake discriminated cells based on their capacity to incorporate glucose (Figure 2B). 2-NBDGhi T cells had increased mRNA levels of solute carrier family 2 (Slc2a1; which encodes the glucose transporter Glut-1) and Hk2 compared with 2-NBDGlo cells (Supplemental Figure 3), which suggests that this fluorescent glucose analog could efficiently distinguish cells with differential glucose metabolism. Furthermore, cells labeling intensely with 2-NBDG displayed greater ECAR than did their 2-NBDGlo counterparts (Figure 2C), indicating that 2-NBDG uptake reflects functional differences in glycolytic metabolism. Interestingly, 2-NBDGhi T cells displayed a higher OCR/ECAR ratio (Figure 2D), which suggests that these cells preferentially used OXPHOS compared with 2-NBDGlo cells. However, this difference in OCR/ECAR ratio was driven by the denominator, as basal OCR was lower in 2-NBDGlo cells (Supplemental Figure 4A). These functional studies indicated that 2-NBDGhi cells have increased energetic demand compared...
with 2-NBDG<sup>lo</sup> cells, which appear more metabolically quiescent. Accordingly, forward scatter (FSC) and side scatter (SSC) measurements of these 2 populations revealed that 2-NBDG<sup>hi</sup> cells were blastic and highly granular, whereas 2-NBDG<sup>lo</sup> cells appeared smaller in size (Supplemental Figure 4B). Notably, these metabolic differences were not merely due to lack of proper Tn stimulation. Indeed, dilution of the cell proliferation dye eFluor670 and measurement of the T cell activation markers CD44, CD25, and CD69 revealed that 2-NBDG<sup>lo</sup> cells had undergone extensive proliferation and activation after priming (Supplemental Figure 5).

To evaluate whether differences in glucose metabolism in CD8<sup>+</sup> T cells are associated with differences in transcriptional regulation of T cell differentiation, we quantified the mRNA levels of key transcription factors and molecules associated with either memory precursors or short-lived, terminally differentiated Teffs in 2-NBDG<sup>hi</sup> and 2-NBDG<sup>lo</sup> cells (8). We found that the Wnt–β-catenin signaling transducers transcription factor 7 (Tcf7), lymphoid enhancer factor 1 (Lef1), and B cell CLL/lymphoma 6 (Bcl6), which promote memory CD8<sup>+</sup> T cell formation (4, 27–29), were significantly overexpressed in 2-NBDG<sup>lo</sup> versus 2-NBDG<sup>hi</sup> CD8<sup>+</sup> T cells (Figure 2E). In contrast, PR domain containing 1, with ZNF domain (Prdm1) — which encodes B lymphocyte–induced maturation protein-1 (Blimp-1), a master regulator of terminal differentiation (30–33) — was enriched in 2-NBDG<sup>hi</sup> cells compared with their 2-NBDG<sup>lo</sup> counterparts (Figure 2E). In addition, perforin (Prf1) and granzyme B (Gzmb), which encode key cytotoxic effector molecules, were also highly expressed in 2-NBDG<sup>lo</sup> cells (Figure 2E). Thus, glucose uptake enabled the segregation of T cells that had molecular profiles associated with Teffs (2-NBDG<sup>hi</sup>) and memory precursor cells (2-NBDG<sup>lo</sup>).

Given these findings, we sought to ascertain whether glucose uptake could also be used to segregate T cells with differential capacity to establish memory in vivo. We adoptively transferred 2-NBDG<sup>hi</sup> and 2-NBDG<sup>lo</sup> cells into wild-type mice, which were then infected with a recombinant strain of vaccinia virus encoding the cognate antigen gp100 (referred to herein as gp100-VV). Strikingly, cells that experienced an increased rate of glucose metabolism exhibited poor engraftment, proliferation, and survival capacities compared with 2-NBDG<sup>lo</sup> cells, which exhibited 10- to 100-fold higher frequency over the time course of infection (Figure 2, F and G). Taken together, these findings indicated that during T cell activation, there is heterogeneous induction of glycolysis: cells that uptake high levels of glucose becoming short-lived Teffs, whereas cells that maintain reduced glucose incorporation preferentially form memory.

Constitutive activation of glycolytic flux limits CD8<sup>+</sup> T cell memory development. To determine whether high glycolytic flux is merely...
associated with cells with an impaired capacity to become memory or, rather, is causally related to the observed defect in CD8+ T cell memory formation, we used a genetic approach to enforce glycolytic metabolism. We transduced CD8+ T cells with the glycolytic enzyme Pgam1, which has been shown to enhance glycolytic flux (34, 35) and modulate the cellular lifespan of primary mouse embryonic fibroblasts (35). Pgam1-overexpressing cells were isolated based on the expression of the reporter marker GFP and compared with control cells engineered to express GFP alone (Figure 3A). Enforced expression of Pgam1 induced CD8+ T cells to adopt a predominantly glycolytic phenotype, as revealed by augmented uptake of radiolabeled 2DG, increased ECAR levels, and lower OCR/ECAR ratio compared with controls (Figure 3, B–D).

To explore whether ectopic expression of Pgam1 influences CD8+ T cell differentiation in vivo, we adoptively transferred Pgam1-overexpressing pmel-1 CD8+ T cells into wild-type mice and monitored the expansion and long-term persistence of antigen-specific T cells after gp100-VV infection. At the peak of the immune response, the frequency of Pgam1-transduced T cells in the spleen was significantly reduced, but no major phenotypic differences were observed compared with controls (Supplemental Figure 6), which indicates that the high glycolytic activity mainly impaired T cell engraftment and expansion in vivo. Strikingly, at 1 month after transfer, these differences in frequency became more pronounced, as Pgam1-transduced T cells had decreased long-term survival in both lymphoid and peripheral tissues (Figure 3, E and F). To determine whether enforced glycolysis affects the ability of memory T cells to respond to antigen reexposure, at 30 days after primary infection with gp100-VV, we challenged the hosts with a heterologous adenovirus type 2 encoding gp100 antigen (referred to herein as ad-gp100) and evaluated the proliferative responses of transduced T cells at the peak of the immune response. We found that secondary progeny of Pgam1-transduced T cells were severely impaired, with a 100-fold reduction in frequency and absolute numbers (Figure 3, G and H). Taken together, these findings using enforced overexpression of a key glycolytic enzyme were consistent with the view that acquisition of a high glycolytic metabolism severely limits the formation of CD8+ T cell memory in vivo.

**Inhibition of glycolysis enhances memory CD8+ T cell formation.** Our experiments using adoptive transfer of CD8+ T cells with intrinsic differences in glucose metabolism and studies using a genetic approach to augment glycolytic function indicated that CD8+ T cells displaying higher glycolytic flux had a limited capacity to establish immunological memory. It remained to be addressed,
Inhibition of glycolysis triggers activation of energy stress signaling, which reinforces glycolysis shutdown. (A) ECAR and OCR/ECAR ratio of CD8+ T cells activated by antibodies specific to CD3 and CD28 in the presence of 2DG (2 mM) or DMSO vehicle. Data are mean ± SEM of 4 measurements. (B) Immunoblot analysis of p-AMPK and Hif1-α protein in cells as in A. β-actin was used as a loading control. (C) Quantitative RT-PCR analysis of the expression of glycolytic enzymes in CD8+ T cells. Results are presented relative to Actb. Tpi, triose phosphate isomerase; Pkm2, pyruvate kinase muscle; Slc16a3, solute carrier family 16, member 3; Ldha, lactate dehydrogenase A. Data are mean ± SEM of 3 measurements. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, 1-tailed (A, OCR/ECAR) or 2-tailed (A, ECAR, and C) Student’s t test. Data are representative of 3 (C) or 2 (A and B) independent experiments.

Figure 4

Inhibition of glycolysis sustains Foxo1 activity and enhances lymphoid homing. Recent studies have demonstrated the importance of Foxo1 in regulating memory versus effector lineage commitment in CD8+ T cells. We assessed the expression levels of key transcriptional regulators implicated in memory T cell and Teff formation, such as Tcf7, Left1, Bcl6, and Prdm1, as well as T cell differentiation markers, including CD44, CD62L, CD27, and KLRG-1. 2DG-treated T cells displayed increased mRNA levels of Bcl6, Tcf7, and Left1, while control cells were enriched in Prdm1 mRNA (Figure 5A). Additionally, 2DG prevented the induction of effector molecule–encoding genes, such as Prf1 and GzmB (Figure 5A). Thus, gene transcription analysis provided evidence that blockade of glycolysis by 2DG enforces a transcriptional program characteristic of memory cells. Moreover we observed that 2DG-treated T cells retained higher expression of CD62L, a molecule found in Tns and early memory T cell subsets, compared with control cells (Supplemental Figure 9). To test whether inhibition of glycolysis during T cell priming programs CD8+ T cells to adopt a diverse fate in vivo, we transferred 2DG-treated pmel-1 CD8+ T cells into wild-type mice infected with gp100-YY and evaluated the expansion, persistence, and phenotypic changes of transferred cells. 2DG-treated CD8+ T cells expanded robustly, as indicated by increased frequencies and numbers of CD8+Thy-1.1+ T cells in the spleen at the peak of the immune response compared with control cells (P < 0.01; Supplemental Figure 10, A and B). Despite increased expansion, a higher proportion of 2DG-treated cells retained a memory phenotype, whereas the majority of control CD8+ T cells underwent terminal differentiation, as revealed by the loss of CD62L and concomitant acquisition of the senescence marker KLRG-1 (Supplemental Figure 10, C and D). These quantitative and qualitative differences between 2DG-treated T cells and controls became more prominent during the memory phase of immune response. We observed 100-fold higher frequencies of pmel-1 CD8+ T cells in both lymphoid and nonlymphoid tissues of animals that received adoptive transfer of 2DG-treated versus control T cells (Figure 5, B and C). Notably, the persisting progeny of T cells primed in the presence of 2DG were relatively enriched in Tcms compared with control cells, which preferentially gave rise to senescent KLRG-1+ T cells (Figure 5D). Consistent with these findings, recall responses were significantly increased in mice that received adoptive transfer of 2DG-treated T cells (Figure 5, E and F). Together, these findings indicate that specific inhibition of glycolysis during priming enhances not only the quantity, but also the quality, of antigen-specific memory CD8+ T cells.

Inhibition of glycolysis sustains Foxo1 activity and enhances lymphoid homing. Recent studies have demonstrated the importance of Foxo1 in regulating memory versus effector lineage commitment in CD8+ T cells (20, 41, 42). To determine whether inhibition of glucose metabolism in CD8+ T cells regulated Foxo1 activity, we first evaluated Foxo1 phosphorylation in differentiating CD8+ T cells stimulated in the presence or absence of 2DG. Stimulation of CD8+ T cells induced phosphorylation of Foxo proteins at T24/T36 residues, a process that was prevented by 2DG treatment however, whether direct inhibition of glycolysis would conversely enhance T cell survival and the capacity to form long-lived memory cells. To directly target the glycolytic pathway in CD8+ T cells, we used the Hk2 inhibitor 2DG. Previous reports have demonstrated that cell proliferation was blocked by high concentrations of 2DG (50 mM) (36). However, low doses of 2DG have been used to address the role of glycolysis in directing the fate decision of CD4+ Th17 cells versus Foxp3+ Tregs (37, 38). Based on these findings and titration experiments (Supplemental Figure 7), we chose a 2-mM dose for the present study. Activation of CD8+ T cells in the presence of 2DG was sufficient to inhibit glycolytic flux and dramatically reduce lactate production, as revealed by measurement of ECAR and OCR/ECAR ratio of CD8+ T cells activated by antibodies specific to CD3 and CD28 in the presence of 2DG (2 mM) or DMSO vehicle. Data are mean ± SEM of 4 measurements. (B) Immunoblot analysis of p-AMPK and Hif1-α protein in cells as in A. β-actin was used as a loading control. (C) Quantitative RT-PCR analysis of the expression of glycolytic enzymes in CD8+ T cells. Results are presented relative to Actb. Tpi, triose phosphate isomerase; Pkm2, pyruvate kinase muscle; Slc16a3, solute carrier family 16, member 3; Ldha, lactate dehydrogenase A. Data are mean ± SEM of 3 measurements. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, 1-tailed (A, OCR/ECAR) or 2-tailed (A, ECAR, and C) Student’s t test. Data are representative of 3 (C) or 2 (A and B) independent experiments.

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Inhibition of glycolysis triggers activation of energy stress signaling, which reinforces glycolysis shutdown. (A) ECAR and OCR/ECAR ratio of CD8+ T cells activated by antibodies specific to CD3 and CD28 in the presence of 2DG (2 mM) or DMSO vehicle. Data are mean ± SEM of 4 measurements. (B) Immunoblot analysis of p-AMPK and Hif1-α protein in cells as in A. β-actin was used as a loading control. (C) Quantitative RT-PCR analysis of the expression of glycolytic enzymes in CD8+ T cells. Results are presented relative to Actb. Tpi, triose phosphate isomerase; Pkm2, pyruvate kinase muscle; Slc16a3, solute carrier family 16, member 3; Ldha, lactate dehydrogenase A. Data are mean ± SEM of 3 measurements. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, 1-tailed (A, OCR/ECAR) or 2-tailed (A, ECAR, and C) Student’s t test. Data are representative of 3 (C) or 2 (A and B) independent experiments.

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Phosphorylation of Foxo1 facilitates its binding to cytosolic 14-3-3 scaffold proteins, which prevents translocation of Foxo1 into the nucleus, thus resulting in a loss of Foxo1 transcriptional activity (42). Consistently, we found that the majority of Foxo1 protein was sequestered into the cytoplasm after CD8+ T cell stimulation, while treatment with 2DG promoted Foxo1 nuclear localization (Figure 6B).

To confirm that changes in Foxo1 compartmentalization resulted in differences in Foxo1 transcriptional activity, we measured the expression of Kruppel-like factor 2 (Klf2), a well-characterized Foxo1 target gene (43, 44). Quantitative RT-PCR analysis revealed that while control cells displayed low levels of Klf2 expression, 2DG treatment resulted in a dose-dependent increase in Klf2 and its target genes, such as selectin L (SelL; which encodes CD62L), chemokine (C-C motif) receptor 7 (Ccr7), and sphingosine-1-phosphate receptor 1 (S1p1r), which control the ability of T cells to home to secondary lymphoid structures (Figure 6C and refs. 44, 45). Finally, to determine whether modulation of glucose metabolism could regulate such lymphoid homing behavior, we adoptively transferred congenically marked control (Thy-1.1+) or 2DG-treated (Ly5.1+Thy-1.1+) cells in the same hosts and evaluated the presence of transferred cells in both lymphoid and peripheral tissues 24 hours later. Strikingly, CD8+ T cells treated with 2DG exhibited 10-fold greater migration to lymph nodes compared with control cells, whereas both groups were found at similar frequency in lungs (Figure 6, D and E). Our results indicate that sustained glycolytic activity is required to maintain cell migratory properties characteristic of cytotoxic effector T lymphocytes and that direct blockade of glycolysis reprograms T cells to preferentially migrate to lymphoid tissues.
Diverse metabolic changes accompany CD8+ T cell activation (9, 11). For instance, after antigen stimulation, T cells switch to glycolytic metabolism to sustain effector function (18). Metabolic reprogramming to glycolysis, however, has been found to be dispensable for proliferation or survival of T cells in the short term in vitro studies in the presence of alternative fuel (18). Whether specific changes in glucose metabolism can influence long-term survival of activated T cells and their capacity to enter into the memory pool in vivo remains unknown. In this study, we found that graded levels of glycolysis can act as a metabolic rheostat determining the decision between memory and terminal effector differentiation in CD8+ T cells. This conclusion was supported by 3 main lines of evidence. First, activated CD8+ T cells sorted on the basis of intrinsic differences in glucose metabolism experienced a divergent fate in vivo: CD8+ T cells displaying high glycolytic activity tended to be short-lived, whereas cells with lower glycolytic metabolism established memory.

The finding that graded levels of glycolytic flux can act as a metabolic rheostat to regulate CD8+ T cell fate determination has parallels in CD4+ T cell biology. High levels of glycolytic activity were found to be dispensable for proliferation or survival of T cells in the short term in vitro studies in the presence of alternative fuel (18). Whether specific changes in glucose metabolism can influence long-term survival of activated T cells and their capacity to enter into the memory pool in vivo remains unknown. In this study, we found that graded levels of glycolysis can act as a metabolic rheostat determining the decision between memory and terminal effector differentiation in CD8+ T cells. This conclusion was supported by 3 main lines of evidence. First, activated CD8+ T cells sorted on the basis of intrinsic differences in glucose metabolism experienced a divergent fate in vivo: CD8+ T cells displaying high glycolytic activity tended to be short-lived, whereas cells with lower glycolytic metabolism established memory. Second, enforcing glycolysis by overexpression of Pgam1 severely impaired the ability of CD8+ T cells to persist in the long term and form memory in vivo. Finally, specific inhibition of glycolytic flux using 2DG during T cell priming enhanced the ability of CD8+ T cells to become long-lived memory cells.

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glycolytic shutdown, but were likely dependent on enforcement of cellular quiescence secondary to activation of glucose starvation signaling. Indeed, in all experimental settings used in the present studies, activated CD8+ T cells that were prone to form memory exhibited a lower overall bioenergetic profile, characterized not only by reduced levels of ECAR, but also by basal levels of OCR not exceeding those found in highly glycolytic cells.

Consistent with this view, we found that inhibition of glycolysis using 2DG was associated with increased activity of AMPK, an evolutionarily conserved protein kinase that enforces quiescence to limit energy demands in response to energy stress (9). Recently, AMPK has emerged as a key regulator of memory CD8+ T cell formation. For example, AMPKα1-deficient T cells were found to have profound defects in their ability to generate memory CD8+ T cell responses during Listeria monocytogenes infection (39), whereas pharmacologic activation of AMPK with metformin resulted in sublethally irradiated B16-tumor bearing mice after gp100-VV vaccination and exogenous IL-2. Numbers represent percent CD8+ Thy-1.1+ cells (B) Quantitative RT-PCR analysis of Slc2a1 and Pkm2 expression in pmel-1 CD8+ T cells isolated from tumors 5 days after adoptive transfer as in A. Results are presented relative to Actb. Data are mean ± SEM of 3 measurements. (C) Percent IFN-γ– and TNF-α–producing pmel-1 CD8+ Thy-1.1+ cells isolated from spleens 5 days after adoptive transfer as in A. (D and E) Tumor size (D) and survival (E) of sublethally irradiated B16 tumor-bearing mice as in A. Data are mean ± SEM of 5 samples. *P < 0.05, **P < 0.01, 2-tailed Student’s t test (B and C) or log-rank (Mantel-Cox) test (E). Data are representative of 2 independent experiments.

Figure 7
Inhibition of glycolysis during ex vivo expansion enhances the antitumor function of CD8+ T cells. (A) Flow cytometry analysis of CD8+ Thy-1.1+ T cells in the tumor at the indicated times after adoptive transfer of 10^6 pmel-1 CD8+ Thy-1.1+ T cells generated in vitro in the presence of 2DG or vehicle (culture media) into sublethally irradiated B16-tumor bearing mice after gp100-VV vaccination and exogenous IL-2. Numbers represent percent CD8+ Thy-1.1+ cells. (B) Representative expression of Actb. Data are mean ± SEM of 3 measurements. (C) Percent IFN-γ– and TNF-α–producing pmel-1 CD8+ Thy-1.1+ cells isolated from spleens 5 days after adoptive transfer as in A. (D and E) Tumor size (D) and survival (E) of sublethally irradiated B16 tumor-bearing mice as in A. Data are mean ± SEM of 5 samples. *P < 0.05, **P < 0.01, 2-tailed Student’s t test (B and C) or log-rank (Mantel-Cox) test (E). Data are representative of 2 independent experiments.

The levels of glycolytic flux appeared to be tightly linked to transcriptional programs regulating CD8+ T cell differentiation. Low levels of glycolysis were associated with high expression of transcription factors that promote CD8+ T cell memory, such as Tcf7, Lef1, and Bcl6, as well as increased activity of Foxo1, a key transcription factor that regulates memory T cell differentiation and the migratory pattern characteristic of Tcms. Conversely, high glycolytic flux was accompanied by upregulation of transcription factors that regulate terminal effector differentiation, such as Blimp-1, and increased expression of components of the effector machinery, including Prf1 and Gzmb. Our results are consistent with a model whereby elevated glucose metabolism is used by the cell to sustain a global program of effector differentiation that is antagonistic to the molecular circuit regulating memory differentiation.

Whether glucose metabolism directly or indirectly regulates distinct transcriptional factors of Teff versus memory T cell lineages needs further investigation. A recent study from Pearce’s group, however, has provided new insights on how metabolic enzymes can directly influence transcription (18). The authors found that the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase can bind to the adenylate uridylate-rich elements in the 3’-UTRs of IFN-γ, limiting its translation.

In the current study, we also addressed the metabolic qualities associated with increased effectiveness of adoptively transferred antitumor T cells. Current methods used to produce T cells for adoptive immunotherapy have the pitfall of driving cells toward terminal differentiation and senescence (52, 53). We found that inhibition of glycolysis limited the detrimental influence of ex vivo expansion on T cell differentiation, resulting in the generation of antitumor cells with improved fitness. T cells primed in the presence of 2DG accumulated at higher numbers in tumor deposits and displayed increased proliferative potential and effector function, as measured by enhanced tumor destruction. These findings have important implications for the design of both T cell–based therapies and vaccines for the prevention and treatment of cancer and infectious diseases, since they indicate that pharmacological targeting of metabolic pathways during T cell priming can promote the generation of long-lived CD8+ T cell immunity. To this end, reagents that are currently under evaluation in clinical trials because of their direct negative impact on glycolytic tumor cells, including 2DG, might be repurposed to enhance the formation of CD8+ T cell memory (54). Thus, modulation of the metabolism of the antitumor T cell response could be an important addition to the immunotherapist’s armamentarium.

Methods
Mice and tumor lines. pmel-1 Thy-1.1 (B6.Cg-Thy-1a/Cy Tg [TcraTcrb] B6.129S7-Tcrb/J) and C57BL/6 mice were obtained from the Jackson Laboratory. We crossed...
pmel-1 with Ly5.1 mice (B6.SJL-Ptpcr<sup>+</sup> Pep<sub>y</sub>/BoyJ) to obtain pmel-1 Ly5.1 mice. B16 (H-2<sup>D</sup>), a gp100<sup>+</sup> murine melanoma, was obtained from the National Cancer Institute Tumor Repository and maintained in culture media as previously described (4).

In vitro activation of T cells. CD8<sup>+</sup> T cells from pmel-1 mice were maintained in vitro with 1 μM hgp100<sub>25–33</sub> peptide or plate-bound anti-CD3 (2 μg/ml; 145-2C11; BD Biosciences) and soluble anti-CD28 (1 μg/ml; 37.5 I; BD Biosciences) and expanded in culture medium containing 10 ng·ml<sup>−1</sup> IL-2 (Chiron) for 4 days. T cells were incubated with 2DG (Sigma-Aldrich) or vehicle (culture media) at indicated doses in the main text and figure legends. CD8<sup>+</sup> T cells from C57BL/6 mice were stimulated using plate bound anti-CD3 and soluble anti-CD28 and expanded in culture medium containing 10 ng·ml<sup>−1</sup> IL-2 (Chiron) for 4 days.

Adaptive cell transfer, infection, recall response, and tumor experiments. Adoptive transfer of purified CD8<sup>+</sup> T cells and infection with gp100-VV were performed as previously described (4). Recall response experiments were performed 30 days after primary infection with gp100-VV by rechallenging mice with 10<sup>5</sup> pfu ad-gp100. Tumor experiments were performed using 10<sup>6</sup> activated pmel-1 Thy-1.1 CD8<sup>+</sup> T cells as previously described (4).

Cell proliferation assay. We labeled pmel-1 splenocytes with 5 μM Cell proliferation dye eFluor670 (eBioscience), stimulated in vitro with 1 μM hgp100<sub>25–33</sub> peptide, and expanded in culture medium containing 10 ng·ml<sup>−1</sup> IL-2 for 4 days. Dilution of Cell proliferation dye was then evaluated by flow cytometry.

Antibodies, flow cytometry, and cell sorting. Mouse antibodies specific for anti-CD8<sub>x</sub> (53-6.7), anti-Thy-1.1 (OX-7), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD25 (PC61), anti-CD27 (LG.3A10), anti-IFN-γ (554143), anti-TNF-α (MP6-XT22), and anti-KLRG-1 (2F1) were from BD Biosciences. Mouse antibody specific for anti-CD69 (H1.2F3) was from eBiosciences. FACS Canto I or FACS Canto II (BD Biosciences) was used for flow cytometry acquisition. Samples were analyzed with FlowJo software (TreeStar). Cells were sorted on the basis of 2-NBDG levels with a FACS Aria instrument (BD Biosciences).

OCR and ECAR. OCR and ECAR were measured at 37°C using an XF24 extracellular analyzer (Seahorse Bioscience) as previously described (22). Briefly, cells were initially plated to unbuffered DMEM (DMEM with 10% FCS) and allowed to attach. Cells were washed with PBS, and 10 μM 2-NBDG (Invitrogen) was added to the media at indicated doses. Cells were incubated for 20 minutes. ECAR and OCR were calculated using Seahorse XF-24 proprietary software. In experiments evaluating the acute response to glucose supplementation, we used media with 0 mM glucose and added 25 mM glucose at time point through the injection port.

Real-time RT-PCR. We isolated RNA with the RNeasy mini kit (Qiagen) and generated cDNA by reverse transcription (Applied Biosystems). Real-time RT-PCR was performed for all genes with primers from Applied Biosystems by Prism 7900HT (Applied Biosystems). Gene expression was calculated relative to Actb.

Retroviral vector construction and virus production. Pgam1 cDNA sequence was cloned into retroviral MSCV vector that has GFP as a reporter gene (Addgene 9044). Platinum Eco cell lines (Cell Biolabs) were used for gamma retroviral production by transfection with DNA plasmids through the use of Lipofectamine 2000 (Invitrogen), and virus was collected 48 hours after transfection.

In vitro transduction of CD8<sup>+</sup> T cells. Mouse CD8<sup>+</sup> T cells were separated from non-CD8<sup>+</sup> T cells with a MACS negative selection kit (Miltenyi Biotec) and activated on plates coated with anti-CD3 (2 μg/ml; 145-2C11; BD Biosciences) and soluble anti-CD28 (1 μg/ml; 37.5 I; BD Biosciences) in cell culture media containing IL-2 (10 ng/ml; Chiron). Virus was “spin-inoculated” at 2,000 g for 2 hours at 32°C onto plates coated with retronectin (Takara). CD8<sup>+</sup> T cells were activated for 24 hours and transduced as previously described (33). 72 hours after retroviral transduction, GFP<sup>+</sup> cells were sorted with a flow cytometer and used for adoptive transfer experiments.

Immunoblot analysis. Western blot analysis was performed using standard protocols. Proteins were separated by 4%–12% SDS-PAGE, followed by standard immunoblot analysis using anti-Hif1α (R&D Biosciences), Hk2, p-AMPK, p-Foxo1/3α, total Foxo1, tubulin, and anti-β-actin (Cell Signalling). A 1:1,000 dilution was used for all antibodies.

Cell fractionation and protein analysis. Cytosolic and nuclear fractionation analysis was performed (Supplemental Methods).

GFP uptake assay. 2 x 10<sup>6</sup> CD8<sup>+</sup> T cells were incubated for 10 minutes in 500 μl glucose-free media. 500 μl glucose-free media containing 1 μCi/ml [3H]2DG (Perkin Elmer) was then added, and cells were incubated for a further 20 minutes. Cells were pelleted, washed in PBS, and then lysed in water. Lysate 3H content was then measured via liquid scintillation counting. For flow cytometry–based glucose uptake assays, we incubated CD8<sup>+</sup> T cells with 100 μM 2-NBDG (Invitrogen) for 2 hours before measuring fluorescence by flow cytometry.

Statistics. Data sets were compared using 1- or 2-tailed unpaired Student’s t test. A log-rank test was used for analysis of survival curves. For all analyses, a P value less than 0.05 was considered statistically significant.

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