Impaired mitochondrial oxidative phosphorylation limits the self-renewal of T cells exposed to persistent antigen

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The majority of tumor-infiltrating T cells exhibit a terminally exhausted phenotype, marked by a loss of self-renewal capacity. How repetitive antigenic stimulation impairs T cell self-renewal remains poorly defined. Here, we show that persistent antigenic stimulation impaired ADP-coupled oxidative phosphorylation. The resultant bioenergetic compromise blocked proliferation by limiting nucleotide triphosphate synthesis. Inhibition of mitochondrial oxidative phosphorylation in activated T cells was sufficient to suppress proliferation and upregulate genes linked to T cell exhaustion. Conversely, prevention of mitochondrial oxidative stress during chronic T cell stimulation allowed sustained T cell proliferation and induced genes associated with stem-like progenitor T cells. As a result, antioxidant treatment enhanced the anti-tumor efficacy of chronically stimulated T cells. These data reveal that loss of ATP production through oxidative phosphorylation limits T cell proliferation and effector function during chronic antigenic stimulation. Furthermore, treatments that maintain redox balance promote T cell self-renewal and enhance anti-tumor immunity.

Suppression of endogenous anti-tumor immune responses is frequently required for tumor initiation and growth\textsuperscript{1}. Intratumoral T cell failure often occurs despite the ability to recognize cancer-specific antigens, due to a series of functional defects known collectively as T cell ‘exhaustion’. T cell exhaustion is defined by loss of proliferative capacity, decreased production of cytotoxic effector molecules and upregulation of inhibitory immunoreceptors such as programmed death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (ref. \textsuperscript{2}). Enhancing T cell function via immune checkpoint blockade or ectopic expression of chimeric antigen receptors (CARs) has shown promise for treating cancer;\textsuperscript{1,2,3} unfortunately, the majority of patients still fail to durably respond to immune-based therapy.

How immune checkpoint blockade reactivates endogenous immune responses remains the subject of some controversy. Intratumoral T cells from patients with durable response to immunotherapy express genes associated with memory and self-renewal\textsuperscript{4,5}, whereas T cells from patients who fail to respond to immunotherapy acquire a ‘terminally exhausted’ phenotype marked by impaired proliferation\textsuperscript{6,7}. Loss of proliferative capacity ultimately limits the clinical activity of checkpoint inhibitors and underscores a need to identify strategies to restore or maintain a self-renewal program in exhausted T cells.

Our laboratory previously demonstrated that T cell proliferation requires activation-induced glucose uptake as well as a rewiring of glucose metabolism to support macromolecular synthesis required for proliferation\textsuperscript{8}, and that inhibitory immunoreceptors such as PD-1 and CTLA-4 act in part by reducing activation-induced glucose uptake\textsuperscript{9}. T cells extracted from tumors show signs of metabolic dysfunction\textsuperscript{10–13}. However, whether altered metabolism is responsible for either the functional defects or the altered gene expression observed in exhausted T cells remains unknown.

Here, we show that impaired mitochondrial ATP production during chronic antigenic stimulation limits T cell self-renewal capacity and promotes terminal differentiation. Despite exhibiting a glycolytic phenotype, terminally exhausted T cells are unable to proliferate. Functional interrogation of T cells during chronic stimulation revealed impairment of ADP-coupled oxidative phosphorylation. The uncoupling of electron transport from mitochondrial reduction of NAD\textsuperscript{+} to NADH resulted in generation of reactive oxygen species (ROS) rather than maintenance of cellular nucleotide triphosphate levels. Direct inhibition of mitochondrial oxidative phosphorylation suppressed proliferation as well as expression of genes associated with T cell self-renewal while activating genes associated with terminal differentiation. Finally, preventing persistent antigen-driven accumulation of mitochondrial ROS with antioxidants restored T cell proliferation, effector function and memory cell-associated gene expression and enhanced anti-tumor T cell immunity in vitro and in vivo. These data establish that chronic stimulation-dependent mitochondrial dysfunction is a major factor in limiting anti-tumor immunity and highlight the importance of metabolic cues in directing T cell fate decisions within the tumor microenvironment.
Results

T cell exhaustion is driven by persistent antigen. To better understand how changes in cellular metabolism might contribute to the development of T cell exhaustion, we developed an in vitro system in which activated T cells were expanded in the absence (‘acute’) or presence (‘chronic’) of persistent antigenic stimulation in the form of either tumors with or without specific antigen (OVA) or anti-CD3-mediated stimulation of the T cell antigen receptor (TCR). Cells were passed every 2 d with or without persistent stimulation, and both acutely and chronically stimulated T cells were briefly re-stimulated for 6 h before collection. Chronically stimulated T cells generated using these protocols failed to produce effector cytokines and upregulated expression of inhibitory immunoreceptors associated with exhausted T cells (Fig. 1a, Extended Data Figs. 1a–c and 9). Moreover, chronic stimulation in vitro was sufficient to activate a transcriptional signature associated with T cell exhaustion. RNA sequencing (RNA-seq) revealed that T cells that were chronically stimulated for 8 d were highly enriched for genes upregulated in tumor-infiltrating exhausted T cells from both mouse models and patients as well as T cells isolated from mice with chronic lymphocytic choriomeningitis virus (LCMV) infections, but not significantly for genes upregulated in anergic T cells (Fig. 1b and Extended Data Fig. 1e)⁹⁻¹⁰,¹⁴. Accordingly, in sharp contrast to activated OT-I T cells expanded without persistent TCR stimulation, chronically stimulated T cells were unable to kill cognate antigen-expressing tumor cells in vitro, nor were they able to suppress tumor growth following adoptive transfer in vivo (Fig. 1c and Extended Data Fig. 1f). Collectively, these results establish that chronic in vitro T cell stimulation can recapitulate the hallmarks of in vivo T cell exhaustion and offer the opportunity to evaluate the contribution of altered metabolic behavior to the development of this process.

Enhanced glycolysis is a hallmark of terminally exhausted T cells. During conventional immune responses, activated T cells increase glucose uptake as well as the fraction of glucose excreted as lactate, a phenomenon known as aerobic glycolysis¹⁷,¹⁸. We found that T cells expanded in the presence of persistent antigen maintained higher rates of both glucose transporter expression and glucose uptake than activated OT-I T cells expanded in the absence of repetitive antigen exposure (Fig. 1d and Extended Data Fig. 1d). They also exhibited higher rates of lactate excretion that was almost entirely glucose-derived (Fig. 1e and Extended Data Fig. 1g,h). Extracellular flux analysis of the expanded T cells demonstrated that chronically stimulated T cells maintained increased rates of extracellular acidification that were dependent on available glucose and inhibited by 2-deoxy-D-glucose, which blocks the conversion of glucose to glucose-6-phosphate (Extended Data Fig. 2a). The extracellular acidification rate (ECAR) of chronically stimulated T cells could only be marginally increased upon re-stimulation or treatment with rotenone and antimycin A, suggesting that chronically stimulated T cells have minimal glycolytic reserve (Fig. 1f and Extended Data Fig. 2b). In proliferating cells, activation of aerobic glycolysis reflects the need to balance glucose uptake with the production of anabolic precursors as byproducts of glycolytic and tricarboxylic acid (TCA) cycle activity¹⁷. In T cells, costimulation of the TCR and CD28 receptors synergize to induce aerobic glycolysis in support of both proliferation and effector function¹⁷. It was therefore puzzling that, despite high rates of glycolysis, chronically stimulated T cells displayed a progressively reduced ability to proliferate as they were passaged (Fig. 1g and Extended Data Fig. 2c). The decreased accumulation of chronically stimulated T cells over time was primarily attributable to a loss in proliferative capacity rather than a decrease in cell viability, which was only slightly decreased after 8 d in culture (Extended Data Fig. 2d,e).

While loss of proliferative capacity is a hallmark of a subset of exhausted T cells with a terminally exhausted phenotype⁴,⁶, it has not been reported that such cells display enhanced glycolysis compared with proliferating T cells or effector cells. To examine this issue, we analyzed single-cell transcriptomes from intratumoral T cells isolated from both mice bearing B16 melanoma tumors, as well as patients who were treated with immune checkpoint inhibition in independent clinical studies⁶,⁸,¹⁹. We found that a glycolytic gene signature was increased in terminally exhausted T cell subsets as compared with either progenitor-like exhausted or conventional effector T cells isolated from human melanomas, basal cell carcinomas and squamous cell carcinomas (Fig. 1h and Extended Data Fig. 2f). Similarly, glycolytic genes were significantly enriched in terminally exhausted T cells from B16 melanoma-bearing mice as compared with progenitor exhausted T cells (Extended Data Fig. 2g). Consistent with these findings, we observed an inverse correlation between expression of glycolytic genes and expression of TCF7, which is essential for intratumoral T cell self-renewal⁶, but observed no such correlation between tricarboxylic citric acid cycle genes and TCF7 expression in basal and squamous cell carcinomas (Extended Data Fig. 2h).

Given that terminally exhausted T cells were enriched in glycolytic genes, we next asked whether chronically stimulated T cells in vitro exhibited a terminally exhausted phenotype. T cells expanded in the presence of persistent antigen were significantly enriched for genes that distinguished terminally exhausted T cells from stem-like progenitor exhausted T cells (Fig. 1i and Extended Data Fig. 2i) and upregulated the exhaustion-associated transcription factor TOX, while decreasing expression of the transcription factor TCF-1, which marks progenitor-like exhausted cells (Fig. 1j)⁹⁻¹⁰,²⁴,²⁵. Additionally, T cells expanded in the presence of persistent antigen were unable to produce effector cytokines, even when PD-1–PD-L1 interactions were blocked during priming (Extended Data Fig. 2j), and were unable to recover functionally after antigen withdrawal (Extended Data Fig. 2k). This functional incapacitation is similar to that of terminally exhausted T cells, which exhibit a phenotype that cannot be reversed by immune checkpoint inhibition or antigen withdrawal.

Chronic T cell stimulation leads to loss of mitochondrial function. Even with high rates of glycolysis, most cells require intact mitochondrial function to proliferate²⁶. We therefore asked whether the highly glycolytic phenotype exhibited by chronically stimulated T cells might reflect decreased mitochondrial function. T cell activation increases mitochondrial oxygen consumption by stimulating inositol-1-phosphate-dependent electron transport chain (ETC) activity²⁷,²⁸. We found that although, similar to actively activated T cells, mitochondrial oxygen consumption increased early during chronic stimulation, it progressively declined as the cells were passaged, such that mitochondrial oxygen consumption was significantly decreased relative to acutely stimulated cells by day 8 and nearly absent by day 14 (Fig. 2a). In contrast to activated T cells expanded in the absence of persistent antigen, T cells that had been chronically stimulated for 8 d were unable to increase oxygen consumption in response to anti-CD3 (Fig. 2b). In addition, steady-state concentrations of TCA cycle metabolites were reduced in chronically stimulated T cells as compared with acutely stimulated T cells (Extended Data Fig. 3a). The inability to activate mitochondrial oxidative phosphorylation in response to persistent antigenic stimulation correlated with an increasing reliance on glycolysis for ATP production and markedly reduced glucose-dependent spare respiratory capacity, suggesting a defect in the ability of chronically stimulated T cells to oxidize glucose to maintain cellular bioenergetics (Fig. 2c and Extended Data Fig. 3b).

To confirm the above findings, we traced the fate of uniformly labeled glucose ([U-¹³C] glucose) in acutely and chronically stimulated T cells (Fig. 2d). Condensation of glucose-derived acetyl-CoA and oxaloacetate to generate citrate was largely unaffected,
Fig. 1 | Aerobic glycolysis is a hallmark of terminally exhausted T cells. All experimental analyses were conducted 8 d after initial stimulation unless otherwise specified. a, PD-1 expression and TNF production by acutely and chronically stimulated T cells upon re-stimulation with PMA and ionomycin. The experiment was repeated three times with similar results. b, Gene set enrichment plot showing that genes associated with chronically stimulated OT-I T cells in vitro are enriched for genes upregulated in exhausted CD8+ T cells as compared with Texh, exhausted T cell; Tmem, memory T cell. c, Growth of B16-OVA xenografts. Tumor-bearing mice received no T cells or 1 million acutely or chronically stimulated OT-I T cells by adoptive transfer 5 d after tumor implantation. Tumor size at 14 d post-implantation is shown. d, Median glucose consumed (d) and lactate excreted (e) in acutely and chronically stimulated T cells following initial stimulation. f, ECAR of acutely and chronically stimulated polyclonal T cells at baseline, in response to re-stimulation (anti-CD3), or in the presence of ATP synthase inhibition (Oligo) or uncoupling agents (FCCP). g, Population doublings of acutely and chronically stimulated OT-I T cells following initial stimulation. h, Normalized expression of glycolytic genes in CD8+ T cell clusters from patients with melanoma treated with immune checkpoint blockade. i, Gene set enrichment plot showing that chronically stimulated OT-I T cells in vitro are enriched for genes upregulated in terminal Texh as compared with progenitor Texh (ref. 8). j, Flow cytometry plots of acutely and chronically stimulated T cells in vitro demonstrating suppression of TCF-1 and upregulation of TOX in chronically stimulated T cells. P values were calculated by unpaired, two-sided Student’s t-test (c–g) relative to acutely stimulated T cells, based on 1,000 permutations by the GSEA algorithm and not adjusted for multiple comparisons (b, i), or by Wilcoxon two-sided rank sum test with Benjamini–Hochberg false discovery rate (FDR) correction (h). Data are presented as the mean ± s.d. of n = 5 (e), n = 4 (f) or n = 3 (d, g) biologically independent samples from a representative experiment. **P < 0.01, ****P < 0.0001. ES, enrichment score; LCMV, lymphocytic choriomeningitis virus; NS, not significant; Texh, exhausted T cell; Tmem, memory T cell.

suggesting that despite high rates of aerobic glycolysis, delivery of glucose-derived acetyl-CoA to the TCA cycle was unimpaired (Fig. 2e). However, chronic T cell stimulation significantly decreased the fraction of downstream TCA cycle metabolites derived from glucose (Fig. 2e). The defect in mitochondrial oxidation of carbon substrates was not unique to glucose, as isotope tracing of [U-13C] palmitate demonstrated markedly reduced contribution of palmitate to all TCA cycle metabolites, indicating an impairment in
Fig. 2 | Chronic antigen stimulation induces mitochondrial dysfunction and limits nucleotide biosynthesis. **a, b**, OCR of acutely and chronically stimulated T cells after the indicated days in culture (a) and after 8 d of culture (b) at baseline, in response to re-stimulation (anti-CD3) or in the presence of ATP synthase inhibition (Oligo), uncouplers (FCCP) or complex III/IV inhibition (Rot/AA). **c**, Ratio of glycolytic to mitochondrial ATP produced by acutely or chronically stimulated cells following re-stimulation with anti-CD3. **d**, Schematic depicting how oxidative metabolism of [U-13C] glucose generates metabolites associated with the TCA cycle. Colored circles represent 13C-labeled carbons. **e**, Fractional labeling with [U-13C] glucose of citrate, glutamate, fumarate, malate and aspartate in acutely and chronically stimulated T cells following re-stimulation. **f**, Labeling of lipids by [U-13C] after 24 h of culture in acutely and chronically stimulated T cells beginning 4 d after initial stimulation. **g**, Quantification of relative metabolite pool sizes in chronically stimulated T cells compared with acutely stimulated T cells. Dashed line indicates median ratio in acutely stimulated cells. P values were calculated by unpaired, two-sided Student's t-test (a–c,e,f) or unpaired, two-sided Student's t-test with Benjamini–Hochberg FDR correction (g–i). Data are presented as the mean ± s.d. of n = 5 (a), n = 8 (a,c), n = 4 (b) or n = 3 (e,f,h) biologically independent samples from a representative experiment. *P < 0.05, **P < 0.01, ***P < 0.0001. a.u., arbitrary units; CPM, counts per million; D, day; m, mass.

mitochondrial beta-oxidation (Extended Data Fig. 3c,d). Taken together, these findings suggest a generalized defect in the mitochondrial oxidative capacity of chronically stimulated T cells.

Persistent antigen limits T cell nucleotide triphosphate synthesis. We next asked how defective mitochondrial oxidation might contribute to terminal T cell exhaustion during persistent antigen stimulation. Cellular proliferation depends on the net production of proteins, lipids and nucleic acids\(^{13,12,20}\). Chronically stimulated T cells demonstrated both intact glucose-dependent citrate production as well as an increased contribution of glucose to lipid synthesis, suggesting that lipid synthesis is not limiting for T cell proliferation during chronic stimulation (Fig. 2e,f). Furthermore, supplementation of acetate, which offers an independent source of acetyl-CoA for lipid synthesis\(^{20}\), did not restore proliferation in chronically stimulated T cells (Extended Data Fig. 3e).
A second role for the TCA cycle in proliferating cells is to generate precursors that support nucleotide biosynthesis\textsuperscript{11,12}. An unbiased liquid chromatography–mass spectrometry (LC–MS)-based assessment of steady-state metabolites in acutely and chronically stimulated T cells found that both purine and pyrimidine precursors as well as monophosphorylated purines and pyrimidines were increased in T cells expanded in the presence of antigen (Extended Data Fig. 3f). As we were unable to demonstrate a reduced ability to produce lipid and nucleotide precursors, we next asked whether nucleotide triphosphate pools might be compromised as a result of the reduced oxidative phosphorylation exhibited by T cells expanded in the presence of persistent antigen. LC–MS analysis revealed a decrease in all nucleotide triphosphates and increases in nucleosides and/or nucleotide monophosphates, such that the ratios of nucleotide triphosphates to their corresponding nucleotide monophosphates or nucleosides were all substantially decreased (Fig. 2g,h). As the iterative phosphorylation of nucleotides by nucleotide diphosphate kinase requires ATP, we assessed whether the ATP/AMP ratio was decreased in chronically stimulated T cells and found that this ratio progressively decreased over the course of chronic T cell stimulation (Fig. 2i). The syntheses of DNA and protein are highly dependent on a continuous supply of ATP, and this reduction is consistent with the progressive loss of the ability to proliferate or produce cytokine in T cells exposed to persistent antigen.

Oxidative stress promotes terminal T cell differentiation. Our observation that mitochondrial insufficiency develops when activated T cells are expanded during persistent antigen exposure is consistent with reports suggesting that tumor-infiltrating lymphocytes exhibit mitochondrial dysfunction\textsuperscript{13,14}. The defective oxidative phosphorylation we observed during chronic antigenic stimulation was not due to impaired mitochondrial biogenesis or mitochondrial protein expression, which were in fact increased during chronic stimulation (Fig. 3a,b). Rather, we observed an increase in the NADH/NAD\textsuperscript+ ratio during chronic T cell stimulation (Fig. 3c). Given the observed decrease in ATP/AMP ratios, this indicated uncoupling of mitochondrial NAD\textsuperscript+ generation from oxidative phosphorylation and ATP synthesis.

To test the effect of lowering the redox load of chronically stimulated T cells, we overexpressed water-forming NADH oxidases from Lactobacillus brevis (LbNOX) in both the cytoplasm and mitochondrial of chronically stimulated T cells (Extended Data Fig. 4a). Complementation of ETC activity by overexpressing LbNOX has been shown to decrease the NADH/NAD\textsuperscript+ ratio and decrease redox stress in cells with impaired mitochondrial function\textsuperscript{15}. We found that LbNOX overexpression reduced redox stress in T cells expanded in the presence of persistent antigen (Extended Data Fig. 4b) and partially restored the proliferative capacity of chronically stimulated T cells, suggesting that the increased reductive stress that accompanies persistent antigenic exposure contributed to the impaired oxidative phosphorylation we had observed (Extended Data Fig. 4c).

One way that increased reductive stress can contribute to uncoupling of oxidative phosphorylation is through the generation of ROS. ROS can directly interfere with mitochondrial ATP synthesis by inactivating iron–sulfur cluster-containing proteins within the ETC. We therefore hypothesized that the decreased mitochondrial ATP synthesis in the face of an increased NADH/NAD\textsuperscript+ ratio observed in chronically stimulated T cells might be due to ROS-mediated inactivation of mitochondrial electron transport. To test this possibility, we examined ROS generation in T cells proliferating in the presence or absence of persistent antigen exposure. In comparison with activated T cells expanded in the absence of chronic TCR engagement, T cells exposed to chronic antigen accumulated high levels of both cellular and mitochondrial ROS, resulting in increased levels of lipid peroxidation (Fig. 3d and Extended Data Fig. 4d).

Mitochondrial uncoupling suppresses T cell self-renewal. Free radicals such as superoxides generated within the mitochondria are rapidly converted to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) via superoxide dismutase. H\textsubscript{2}O\textsubscript{2} reacts with metal ions such as iron present within multiple components of the ETC as well as with several enzymes within the TCA cycle, and can rapidly lead to their inactivation\textsuperscript{16}. To test whether disruption of iron–sulfur cluster-containing complexes involved in electron transport could reproduce the phenotype of exhausted T cells, we treated activated T cells with inhibitors of complexes I (rotenone) and V (oligomycin) of the ETC or cobalt chloride (CoCl\textsubscript{2}), which inactivates iron–sulfur cluster-containing proteins, in the absence of further antigenic stimulation\textsuperscript{17}. Cells were treated after 2 d of initial stimulation given the known requirement of mitochondrial oxidative phosphorylation for T cell activation\textsuperscript{18}. All treatments increased ROS, reduced ATP/AMP ratios and reduced subsequent T cell proliferation, suggesting that impairing oxidative phosphorylation alone is sufficient to cause bioenergetic compromise and impair growth (Fig. 3e,f and Extended Data Fig. 4e). In addition to reducing T cell proliferation, all three inhibitors reduced TCF-1 expression and activated TOX expression (Fig. 3g). We confirmed loss of expression of self-renewal genes by quantitative PCR (Extended Data Fig. 4f).

To determine whether ROS accumulation is also a hallmark of exhausted T cells in patients, we analyzed single-cell RNA-seq data from T cells isolated from patients with melanoma, basal cell carcinoma and squamous cell carcinoma from independent datasets\textsuperscript{19}. We found that terminally exhausted T cells were significantly enriched in genes associated with response to oxidative stress in both patient datasets (Fig. 4h and Extended Data Fig. 4g). Notably, in patients in whom both single-cell RNA-seq and TCR sequencing were performed, we observed a progressive increase in oxidative stress genes with increasing clone size only in exhausted T cells, but not in memory T cells (Extended Data Fig. 4h). Finally, we noted an inverse relationship between expression of oxidative stress response genes and TCF7 in exhausted T cells from patient tumors (Fig. 4i and Extended Data Fig. 4i). Collectively, these results demonstrate that oxidative stress is a consequence of chronic antigen-driven mitochondrial dysfunction and is sufficient to impair T cell proliferation and self-renewal.

Antigen-driven ROS is associated with sustained NFAT activity. We next asked how mitochondrial dysfunction might activate the exhaustion-associated gene expression program. Nuclear translocation of NFAT is known to require complex III-dependent mitochondrial ROS production\textsuperscript{20}; furthermore, NFAT is known to bind to the TOX promoter and activate TOX transcription\textsuperscript{21}, and constitutively active NFAT has been shown to activate many aspects of the exhaustion-associated transcriptional program\textsuperscript{22}. We confirmed strong enrichment of NFAT-binding motifs at sites with increased chromatin accessibility early during the development of intratumoral T cell dysfunction in vivo (Extended Data Fig. 5a)\textsuperscript{23}. Nuclear NFAT translocation requires calcium-dependent calcineurin phosphatase activity, suggesting that sustained intracellular calcium elevation might promote both mitochondrial ROS and downstream NFAT activation. Consistent with this hypothesis, we found that chronically stimulated T cells exhibited higher concentrations of intracellular calcium both at baseline and in response to monomeric T cell receptor stimulation, whereas activated T cells expanded in the presence of interleukin 2 (IL-2) increased intracellular calcium levels only in response to TCR crosslinking (Extended Data Fig. 5b). As a result, RNA-seq of activated T cells expanded in the presence of persistent antigen demonstrated increased expression of NFAT target genes as compared with activated T cells expanded in the presence of IL-2 alone (Extended Data Fig. 5c). To confirm that elevated NFAT activity was associated with oxidative stress and terminal T cell exhaustion in patient samples, we analyzed

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single-cell RNA-seq data from intratumoral T cells isolated from patients with melanoma. We found that expression of NFAT target genes correlated highly with expression of genes related to oxidative stress; as a result, an NFAT target gene signature was highly enriched within terminally exhausted T cells (Extended Data Fig. 5d,e). Taken together, these findings suggest that heightened NFAT activity in response to calcium-driven mitochondrial ROS contributes to development of T cell exhaustion.
N-acetylcysteine (N-AC) reverses antigen-driven T cell metabolic defects. Given that ROS accumulation restricts both proliferation and expression of genes required for self-renewal in chronically stimulated T cells, we asked whether neutralizing intracellular ROS could restore T cell self-renewal. Lymphocyte function in standard culture media can be improved by the addition of β-mercaptoethanol (β-ME)36, which prevents the oxidation of free sulfhydryl residues and acts as a buffer against the generation of free radicals. Supplementation of which prevents the oxidation of free sulfhydryl residues and acts as a buffer against the generation of free radicals. Endogenous glutathione synthesis is required to prevent ROS-dependent toxicity during T cell activation37. We therefore hypothesized that chronic stimulation-dependent ROS accumulation might increase the cellular dependence on glutathione-antioxidant defense. To investigate this hypothesis, we cultured T cells in medium containing either standard tissue culture concentrations (100 μM) or tenfold reductions (10 μM) in the concentrations of extracellular cysteine to limit endogenous glutathione synthesis. Consistent with a requirement for de novo glutathione for T cell activation and proliferation, acutely stimulated T cells were partially sensitive to reductions in available cysteine (31.6% reduction in proliferation) (Fig. 4c). However, T cells cultured in the presence of persistent antigen had an even greater reduction (71.1%) in their proliferation (Fig. 4c) and exhibited increased expression of PD-1 (Fig. 4d). To confirm the requirement of

Fig. 4 | Endogenous antioxidants are required for T cell proliferation. a. Effects of β-ME supplementation on T cell proliferation during acute (upper panel) and chronic (lower panel) stimulation. b. Effects of β-ME supplementation on intracellular accumulation of IFN-γ and TNF following re-stimulation of acutely and chronically stimulated T cells with PMA and ionomycin. c. Effects of extracellular cysteine availability on T cell proliferation during acute (left) and chronic (right) stimulation. d. Effects of extracellular cysteine availability on PD-1 expression as measured by flow cytometry following re-stimulation of acutely and chronically stimulated T cells with PMA and ionomycin. e. Quantification of clonogenic B16 cells following 24 h of coculture with acutely or chronically stimulated OT-I T cells in media containing nutrients at the indicated concentrations. f. Expression of TCF-1 and TOX in acutely stimulated T cells with or without the addition of BSO or diamide following 2 d of initial stimulation. *P < 0.05, **P < 0.01, ****P < 0.0001. Chr, chronic; Cys, cysteine; Glc, glucose.
glutathione for T cell self-renewal, we blocked endogenous glutathione synthesis using buthionine sulfoximine (BSO), an inhibitor of gamma-glutamylcysteine synthetase, or increased endogenous glutathione consumption using the oxidizing agent diamide. Acutely stimulated T cells exhibited diminished proliferation as well as decreased expression of TOX in the presence of TCF-1 and increased expression of gamma-glutamylcysteine synthetase, or increased endogenous glutathione synthesis using buthionine sulfoximine (BSO), an inhibitor of glutathione for T cell self-renewal, we blocked endogenous glutathione synthesis (Extended Data Fig. 5g). Chronically stimulated T cells were unable to survive in the absence of de novo glutathione synthesis (Extended Data Fig. 5h). Finally, using two-dimensional killing assays, we found that the ability of T cells to kill tumor cells was particularly sensitive to cysteine withdrawal (Fig. 4e). Thus, chronic stimulation-driven mitochondrial dysfunction and ROS accumulation increases sensitivity to glutathione depletion.

One mechanism to increase cellular uptake of cysteine is to add a cell-permeant form of cysteine to the culture medium. We therefore assessed whether supplementing T cells with N-AC (10 mM) could restore metabolic T cell function during chronic stimulation.

Fig. 5 | Antioxidants reverse metabolic T cell dysfunction. a, Quantification of reduced glutathione pool sizes in chronically stimulated T cells with or without N-AC supplementation as measured by LC–MS. b, Fluorescence intensity of acutely and chronically stimulated T cells cultured with or without N-AC during acute (left) or chronic (right) stimulation at baseline or in the presence of ATPO synthase inhibition (Oligo), uncoupling agents (FCCP) or complex III/IV inhibition (Rot/AA). d, Fractional labeling by [U-13C] glucose of citrate, aconitate, α-KG, glutamate, malate and aspartate in T cells cultured with or without N-AC during chronic stimulation. e, Quantification of relative metabolite pool sizes in chronically stimulated T cells with or without N-AC supplementation. Colored dots represent nucleotides, nucleosides and nucleoside catabolites, as indicated. Dashed lines represent cutoffs of $P < 0.01$ and log2 fold-change $< -0.5$. f, g, Quantification of ATP/AMP ratios (f) and relative nucleotide ratios (g) in chronically stimulated T cells with or without N-AC supplementation. Dashed line indicates median ratio in acutely stimulated cells. *$P < 0.05$, ****$P < 0.0001$. GSH, glutathione.
N-AC supplementation increased glutathione synthesis, suggesting that cysteine availability is limiting for the maintenance of glutathione pools in chronically stimulated T cells (Fig. 5a and Extended Data Fig. 6a). As a result, N-AC decreased intracellular ROS and increased the oxygen consumption rate (OCR) of chronically stimulated T cells, suggesting that increasing intracellular cysteine levels can help maintain ADP-coupled oxidative phosphorylation during chronic T cell stimulation (Fig. 5b,c). Moreover, N-AC increased the oxygen consumption rate (OCR) of chronically stimulated T cells (Fig. 5b,c). As a result, N-AC decreased intracellular ROS and significantly depleted of genes associated with terminal phenotype (right)8.

Fig. 6 | Antioxidants restore the proliferation and self-renewal of chronically stimulated T cells. a, Population doublings of acutely (above) and chronically (below) stimulated T cells in medium with or without the addition of N-AC, as indicated. b, Principal component analysis of RNA-seq of T cells acutely or chronically stimulated with or without N-AC during chronic stimulation. Bar graphs depict genes predominantly contributing to variance. c, Expression of TCF-1 and TOX in acutely or chronically stimulated T cells with or without N-AC during chronic stimulation, as indicated. d, Gene set enrichment plot showing that chronically stimulated T cells cultured in the presence of N-AC are significantly enriched for genes associated with progenitor phenotype (left) and significantly depleted of genes associated with terminal phenotype (right)8. P values were calculated by unpaired, two-sided Student’s t-test (a) or based on 1,000 permutations by the GSEA algorithm and not adjusted for multiple comparisons (d). Data are presented as the mean ± s.d. of n = 3 biologically independent samples from a representative experiment. ****P < 0.0001.

N-AC restores self-renewal in exhausted T cells. N-AC restored the proliferation of chronically stimulated T cells (Fig. 6a). This rescue was abrogated under hypoxic conditions (Extended Data Fig. 7a), suggesting that the ability of N-AC to restore T cell self-renewal depends on oxygen-dependent, ETC-mediated ATP synthesis. To determine whether the restoration of proliferation by N-AC was accompanied by a change in gene expression, we performed RNA-seq on acutely and chronically stimulated T cells in the presence or absence of N-AC. Principle component analysis demonstrated that T cells chronically stimulated in the presence of N-AC clustered separately from both acutely and chronically stimulated cells (Fig. 6b). Among the genes most strongly contributing to variance between N-AC-treated and untreated chronically stimulated T cells were genes encoding memory-associated transcription factors such as Tcf7, Pou2af1 and Pou2af1, as well as genes associated with terminal exhaustion, such as M1i, M1i2, Il10 and Il13 (Fig. 6b), suggesting that N-AC treatment might regulate the interconversion between progenitor and terminally differentiated T cells within the exhausted T cell pool. Indeed, flow cytometry analysis revealed that while N-AC treated, chronically stimulated T cells still expressed TOX, up to half of cells recovered expression of TCF-1, consistent with a transition from a terminally exhausted to a progenitor exhausted phenotype (Fig. 6c)15,38,39. More broadly, analysis of RNA-seq from N-AC-treated chronically stimulated T cells demonstrated a global shift in gene enrichment from genes upregulated in terminally exhausted T cells to genes upregulated in self-renewing progenitor exhausted T cells (Fig. 6d). Quantitative PCR analysis confirmed that N-AC supplementation during chronic stimulation restored expression of key genes required for self-renewal in exhausted T cells, including Tcf7 and Myb, while suppressing expression of genes associated with terminal T cell exhaustion, such as Prdm1 (Extended Data Fig. 7b)30–42. Notably, chronically
N-AC reverses T cell effector dysfunction. While N-AC treatment restored T cell proliferation during chronic stimulation, the requirement of mitochondrial free radical production for full T cell activation and cytokine production raised the possibility that N-AC-treated T cells might lack effector function. However, we found that N-AC treatment actually increased cytokine production by chronically stimulated T cells, whereas immune checkpoint blockade was unable to do so (Fig. 7a). This rescue was dependent on ETC function, as N-AC was able to rescue cytokine production during hypoxia (Extended Data Fig. 8a). Functionally, N-AC was able to improve the killing capacity of chronically stimulated T cells, and this capacity synergized with anti-PD-L1 therapy to fully restore T cell effector function (Fig. 7b). We also observed a synergistic effect of anti-PD-L1 therapy and N-AC on T cell metabolism, as anti-PD-L1 therapy, which is known to increase glucose uptake, increased mitochondrial oxygen consumption only in the

![Figure 7](image_url)

Fig. 7 | Antioxidants reverse chronic stimulation-driven loss of T cell effector function. a, Intracellular accumulation of IFN-γ and TNF following re-stimulation of T cells with PMA and ionomycin following acute or chronic stimulation in the presence or absence of N-AC or anti-PD-L1, as indicated. b, Quantification of clonogenic B16 cells following 24 h of coculture with acutely or chronically stimulated OT-I T cells that had been treated with N-AC or anti-PD-L1 throughout the coculture period. c, d, Viability (c) and intracellular production of IFN-γ and TNF (d) of CD8+ T cells isolated from EL4 tumors 3 d after re-stimulation in the presence or absence of N-AC. e, f, Viability (e) and production of IFN-γ and TNF (f) by CAR-T cells 3 d after re-stimulation in the presence or absence of N-AC. g, Kaplan–Meier curve showing survival of B16-OVA-bearing recipient mice following adoptive transfer of OT-I T cells that had been chronically stimulated in the presence or absence of N-AC. All mice received anti-PD-L1 therapy twice weekly. h, Immunohistochemistry showing enhanced granzyme B expression in tumor-infiltrating T cells treated with N-AC. Stainings of tumors extracted from two individual mice are shown. P values were calculated by one-way ANOVA with Sidak’s multiple comparisons post-test (b), unpaired, two-sided Student’s t-test (c,e) or log-rank (Mantel–Cox) test (g) relative to vehicle-treated cells. Data are presented as the mean ± s.d. of n = 3 biologically independent samples or n = 5 independent mice (g) from a representative experiment. *P < 0.05, ****P < 0.0001. IHC, immunohistochemistry; Veh, vehicle.
presence of N-AC (Extended Data Fig. 8b,c). The ability of antioxidants to maintain T cell functionality was not restricted to N-AC, as supplementation of T cells with either the mitochondrionally targeted antioxidant MitoTEMPO or the water-soluble vitamin E analog Trolox similarly restored cytokine production in chronically stimulated T cells (Extended Data Fig. 8d).

We next sought to confirm whether induction of oxidative stress is limiting for T cells undergoing exhaustion in vivo. Intratumoral T cells in vivo exhibit a terminally differentiated phenotype marked by an inability to proliferate or produce effector cytokines as well as higher rates of apoptosis in response to ex vivo re-stimulation. We found that both tumor-infiltrating cells from mice bearing EL4 T cell lymphoma tumors as well as CD19-directed CAR-T cells from mice bearing A20 B cell lymphoma tumors exhibited impaired cytokine production and high rates of apoptosis upon ex vivo re-stimulation (Fig. 7c–f). N-AC treatment improved the viability and cytokine production of both endogenous and CAR-transduced exhausted T cells (Fig. 7c–f). These results demonstrate that oxidative stress limits the proliferative and functional capacity of T cells undergoing exhaustion in vivo. Finally, we evaluated whether buffering oxidative stress with N-AC could enhance anti-tumor T cell responses in vivo. In a B16 melanoma model, which is largely refractory to anti-PD-L1 therapy, adoptive transfer of T cells cultured in the presence of N-AC improved the survival of tumor-bearing mice (Fig. 7g). Immunohistochemical analysis confirmed an improvement in the functional capacity of tumor-infiltrating T cells as reflected by an increase in granzyme B expression (Fig. 7h). Collectively, these results demonstrate that N-AC reverses in vivo T cell effector dysfunction and enhances anti-tumor immunity.

**Discussion**

While immune checkpoint inhibitors and CAR-modified T cell therapies have produced encouraging clinical responses in patients with cancer, persistent antigen exposure ultimately leads to terminal dysfunction of tumor-infiltrating T cells and limits immune-mediated tumor control. Recent findings demonstrating that successful response to anti-PD-1 therapy requires a subset of exhausted T cells that retain proliferative capacity strongly suggest that overcoming antigen-driven limitations on self-renewal is key to achieving durable anti-tumor immunity. Our laboratory previously established that during initial antigenic activation of T cells, both cytokine and CD28 stimulation promote nutrient uptake and catabolism to support cell growth and proliferation. It remained unclear, however, what the metabolic effects of persistent antigenic stimulation would be and how such metabolic alterations might affect T cell proliferation and differentiation.

We now establish that altered metabolism resulting from persistent antigenic exposure is essential to limiting intratumoral T cell proliferation and self-renewal. The hallmark of this metabolic phenotype is a rapid induction of mitochondrial oxidative stress that limits the ability of T cells to engage in oxidative phosphorylation, resulting in bioenergetic limitations that are sufficient to block T cell proliferation. Moreover, we show that free radical-driven impairment of mitochondrial oxidative phosphorylation is both necessary and sufficient to repress gene expression programs associated with self-renewal while inducing gene expression programs associated with terminal T cell exhaustion.

The metabolic alterations observed in chronically stimulated T cells may underlie the phenotypic changes observed in immunotherapy failures. Failure to respond to immune checkpoint blockade is often associated with a depletion of T cells within the tumor parenchyma. While this has been ascribed to mechanical barriers such as fibroblast-associated extracellular matrix, our findings suggest that intratumoral T cell depletion may actually reflect impaired survival under conditions known to promote redox dysfunction, including hypoxia and cysteine depletion. Another predictor of immunotherapy failure is a lack of TCF-1+ progenitor cells. Our data demonstrate that chronic stimulation-dependent redox dysfunction suppresses TCF-1 expression and promotes a terminally exhausted phenotype that underlies checkpoint blockade unresponsiveness.

Recently, the transcription factor TOX was found to be essential for activation of the exhaustion-associated gene expression program, and T cells lacking Tox were more prone to terminal differentiation and death within tumors. Our data demonstrating that oxidative stress can promote Tox expression suggest that negative regulatory programs driven by TOX may protect T cells from bioenergetic catastrophe during chronic antigen exposure, permitting survival at the expense of effector capacity. Initial Tox expression and phenotypic exhaustion have been shown to require Nfat, but sustained Tox expression subsequently becomes calcineurin-independent. Whether persistent calcium-driven mitochondrial ROS sustains Tox expression via Nfat-independent mechanisms remains to be determined.

Pharmacologic targeting of T cells to enhance immunotherapy has largely centered around blocking inhibitory immune checkpoints, which, while effective in many malignancies, is typically not durable. This may in part be because immune checkpoint blockade does not reprogram the chromatin landscape of exhausted T cells. The underlying cause of epigenetic reprogramming during the development of intratumoral T cell dysfunction remains to be elucidated. However, our finding that reversing redox stress can reactivate gene expression even at loci known to be inaccessible in exhausted T cells suggests that even epigenetic silencing can be suppressed if mitochondrial redox damage is prevented. While these data provide evidence that drugs such as N-AC can restore and/or maintain an anti-tumor immune response in vivo, whether other therapies that preserve immune cell bioenergetic integrity can also help to prevent T cell exhaustion remains to be explored.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41590-020-0725-2.

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Methods

**Articles**

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**Articles**

**Sigma**, 500 ng ml$^{-1}$ l-glutamine, 50 μM β-ME. All cells were tested routinely for mycoplasma and kept in culture for no more than 30 or 15 passages.

**Plasmids.** LbNOX and MitolbNOX were a gift from V. Mootha (Howard (Addgene plasmid nos. 75285, 74488) and were subcloned into pMIG-II vectors. All vectors were sequence-confirmed using Sanger sequencing.

**T cell isolation and activation.** For activation of polyclonal CD8$^+$ T cells, T cells were isolated from the spleen and inguinal lymph nodes of C57BL/6 mice (Jackson 000664) using the Dynabeads Untouched CD8$^+$ T cell kit. T cells were activated with plate-bound anti-CD3 (2C11, 3 μg ml$^{-1}$, 1:1000 dilution) and anti-CD28 (19.51, 3 μg ml$^{-1}$, 1:500 dilution) for 48 h in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 50 μM β-ME and 10 ng ml$^{-1}$ IL-2 (Peprotech). For activation of OT-I transgenic CD8$^+$ T cells, single-cell suspensions were generated from spleens and inguinal lymph nodes of OT-I mice (Jackson 003831). Cells were cultured at 10 × 10$^6$ cells per ml in the presence of 1 μM SiINFEK F peptide (Invivogen) for 48 h in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 50 μM β-ME and 10 ng ml$^{-1}$ IL-2 (Peprotech).

**Acute and chronic T cell stimulation.** For OT-I transgenic T cells, following 48 h of initial stimulation as described in "T cell isolation and activation," T cells were cocultured with B16-F10 melanoma cells that had been plated the day before at subconfluent density in the presence of 1 ng ml$^{-1}$ IFN-γ (Peprotech) to induce MHC-I expression. Cells were cocultured at 1 × 10$^6$ T cells per ml in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 5 μM β-PE and 10 ng ml$^{-1}$ IL-2 (Peprotech). For activation of OT-I transgenic T cells, T cells were passed into fresh cocultures every 48 h until 8 d following initial stimulation, at which point cells were used for experiments. For polyclonal CD8$^+$ T cells, following 48 h of initial stimulation as described in "T cell isolation and activation," T cells were cultured at 1 × 10$^6$ cells per ml in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 5 μM β-PE and 10 ng ml$^{-1}$ IL-2, and with (chronic) or without (acute) plate-bound anti-CD3 (3 μg ml$^{-1}$). Every 2 d for the duration of the experiment, cells were collected, and cell number was counted using a Beckmann Coulter Counter with a cell volume gate of 75–4,000 femtoliters. Then, 50% of the cells were re-plated in 1 ml of fresh T cell medium. For nutrient deprivation experiments, cells were cultured in RPMI-1640 medium without glucose, glutamine or cysteine, with 10% dialyzed FBS, 5 μM β-PE, 10 ng ml$^{-1}$ IL-2 and the indicated concentrations of glucose, glutamine and cysteine, with (chronic) or without (acute) plate-bound anti-CD3 (3 μg ml$^{-1}$). All experiments were performed at least two independent times.

**Quantification of gene expression.** RNA was isolated from T cells using Trizol (Invitrogen) according to manufacturer instructions and 200 ng of RNA was used for complementary DNA synthesis using iScript (Bio-Rad). Quantitative real-time PCR analysis was performed in technical triplicates using QuantiStudio 7 Flex (Applied Biosystems) with Power SYBR Green (Life Technologies). All data were generated using qDNA from triplicate wells for each condition. Actin was used as an endogenous control for all experiments. Primers used for quantitative PCR with reverse transcription are shown in Supplementary Table 1. For RNA-seq, RNA was isolated from T cells using Trizol and then prepared as instructed using the TruSeq RNA Sample Preparation Kit v.2 (Illumina) in accordance with the manufacturer's instructions. RNA-seq samples were sequenced using Illumina NexSeq 500, generating 150-base pair pair-end reads. Fastq files were aligned to genome mm9 using TopHat2 v.2.1.1 with default parameters. Aligned features were counted with hiseq-count v.0.11.1 and differential expression was determined using the edgeR package v.3.28.1 in Bioconductor v.3.10 as previously described.

**Gene set enrichment analysis (GSEA).** GSEA$^+$ was performed using preranked gene lists. GSEA Preranked v.6.0.12 was used with default parameters and data was exported and graphed in GraphPad Prism v.8.

**Single-cell RNA-seq analysis.** Gene expression counts by unique molecular identifier for single cells were used as generated previously$^{18–20}$ and further normalized by dividing by the total counts for that cell, multiplied by a scale factor of 10,000 and log10 normalized using the NormalizedData function in Seurat v2.3.4 (ref. 21). The glycolysis score for each cell was calculated as the average z-score over all genes in the HALLMARK_GLYCOLYSIS gene set$^{22,23}$. ROS score for each cell was calculated as the average z-score of all genes in the GO_RESPONSE_TO_OXIDATIVE_STRESS gene set$^{24}$. NFAF score for each cell was calculated as the average z-score over all genes in the PID_NFAF_TPATHWAY gene set$^{25}$. TCR annotation was performed as described previously$^{26}$. Single cells were grouped by TCR clonotype based on matching T cell receptor-β amino acid sequence. P values were calculated using a one-sided Student’s t-test.

**Nutrient consumption and secretion.** Cells were plated in 12-well plates at 5 × 10$^5$ cells per well and treated and/or stimulated as indicated in the figures, while keeping cell density at subconfluency. Medium was exchanged for the assay period of 5×24 h, then collected, centrifuged at 300g for 3 min to remove cellular components and analyzed using a 2990T Biochemistry Analyzer (YSI Life Sciences) to determine enzyme activity. Absolute rates of consumption/secrection of these metabolites were calculated by subtracting their concentrations in medium incubated for the same amount of time without the cells, then normalized to the cell number, medium volume and hours of incubation. These experiments were performed independently at least two times.
NADH/NAD+ levels. NADH and NAD+ levels were measured using a modified version of manufacturer instructions supplied with the NADH/NAD+ Glu Assay (Promega) as previously described. Briefly, T cells were acutely and chronically stimulated as described in “Acute and chronic T cell stimulation” before preparation of cell extracts. For extraction, cells were washed three times in ice-cold PBS, extracted in 100 μl of ice-cold lysis buffer (1% dodecylmaltoside/0.2 mM NaOH diluted 1:1 with PBS) and immediately frozen at −80 °C. To measure NADH, 20 μl of sample was incubated at 75 °C for 30 min to selectively degrade NAD+. To measure NAD+, 20 μl of the sample was combined with 20μl of lysis buffer and 20 μl of 0.4 N HCl and incubated at 60 °C for 15 min to degrade NADH. Following incubations, samples were allowed to equilibrate to room temperature and were then quenched by neutralizing with 20 μl of 0.25 M Tris in 0.2 N HCl (NADH) or 20 μl of 0.5 M Tris base (NAD+). Manufacturer instructions were followed thereafter to measure NADH/NAD+ using a luminometer.

Extracellular flux analysis. ECAR and OCR were measured using a Seahorse XF96 Extracellular Flux Analyzer (Agilent Technologies). Cells were plated on Cell-Tak (Fisher)-coated XF-96 well-plates at 2 × 10^5 cells per well in assay medium (MEM or CMEM supplemented with 10% FBS, 10^5 M 13C15N-labeled glucose and 1 mM sodium pyruvate), as previously described. Analyses of the ECAR and OCR were performed at basal level, and after subsequent injections of oligomycin (1 μM), carboxyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP; 1 μM) and rotenone/antimycin mix (0.5 μM). Where indicated, acute injection of 10 μM 2-deoxy-D-glucose was performed. 13C-labeled metabolites in some experiments, 2-deoxy-D-glucose was injected at a final concentration of 5 mM. Following measurements, cell number was determined and averaged per condition, and the ECARs and OCRs were normalized to these values. Mitochondrial ATP production was calculated by subtracting the minimum respiration rate following oligomycin injection from the final respiration rate before oligomycin injection.

Immunoblotting. Protein lysates were extracted in RIPa buffer (Cell Signaling), separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% milk in Tris (pH)-buffered saline with 0.1% Tween-20 (TBST) and incubated at 4 °C with primary antibodies overnight. After TBST washes the next day, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, GEN931 or GEN934, 1:5,000) for 1 h, incubated with enhanced chemiluminescence substrate (Pierce or GE Healthcare) and imaged using a ChemiDoc Touch Imaging System (Bio-Rad). Antibodies used (at 1:1,000 unless otherwise noted) were FLAG (Sigma-Aldrich, F1804), total OXPHOS (Abcam, ab110413), Glut1 (Cell Signalling, 129395) and Actin (Sigma, A3854 at 1:20,000).

Metabolomic analyses. Stable isotope labelling and metabolite extraction. T cells were acutely or chronically stimulated for 8 d as described in “Acute and chronic T cell stimulation.” On day 8 following stimulation, cells were washed with PBS and re-plated on plates coated with anti-CD3 (3μg/ml) and 3,10,000,000,000 NADH/NAD+ in fresh RPMI-1640 containing 10% FBS (for metabolite profiling studies) or 10% dialyzed FBS (for isotope profiling studies), 1% penicillin-streptomycin, 2 mM l-glutamine, 5 μM BME and 10 mM sodium pyruvate, as previously described. For profiling studies, RPMI-1640 without glucose or glutamine was used, supplemented with either 16C-glucose (Sigma) and 16C-glutamine (Gibco) or 16C-glutamine and 16C-glucose (Cambridge Isotope Laboratories), to a final concentration of 10 mM (glucose) and 245 μM (l-glutamine). For [16C]palmitate (Sigma) isotopologue tracing studies, 14C or [16C]labeled palmitate was fed to cells for 24 h, without glucose containing 10% dialyzed FBS, 2 mM l-glutamine, 5 μM BME, 10 μg/ml IL-2, 10 mM glucose and 0.5 μg/ml [1-13C] glucose (Perkin Elmer), and with (chronic) or without (acute) plate-bound anti-CD3 (3 μg/ml). After 24 h cultures were washed twice with PBS, and lipids were collected with 50% methanol containing 0.1 M HCl on dry ice, extracted with chloroform and transferred to scintillation vials. A Perkin Elmer Tri-Carb 2910 TR scintillation counter was used to measure 13C counts per minute for 1 min per sample. Counts per minute were normalized to cell number measured from duplicate samples.

Animal models. All animal experiments were performed according to Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee guidelines (Protocol Number 11–03–007, Animal Welfare Assurance Number FW00009998).

In vivo tumor models. C57BL/6-scid (Jackson 001913) mice were injected subcutaneously with 2 × 10^6 BLE-OVA cells in a 1:1 mix of PBS and Matrigel (Corning). At 5 d later, 2 × 10^6 OT-I T cells that had been acutely or chronically stimulated in the presence or absence of N-AC, as described in “Acute and chronic T cell stimulation,” were adoptively transferred to mice via retro-orbital injection. All mice were treated with blocking antibodies against PD-1-L (1 μg 1D.11 (BioXCell, 200 μg/g body weight, 2 × ) or anti-CD40L (BioXCell, 200 μg/g body weight, 2 × ). Mice were monitored daily and all deaths were killed for signs of morbidity. At the time of killing, tumors were fixed in 4% paraformaldehyde and sequentially dehydrated in ethanol. Paraffin embedding and immunohistochemistry for granzyme B were performed by HistoWiz. Kaplan–Meier analysis of survival was performed using GraphPad Prism 8 software.

Ex vivo analysis of tumor-infiltrating T cells. C57BL/6-scid mice were injected subcutaneously with 2 × 10^6 EL4 cells in a 1:1 mix of PBS and Matrigel (Corning). After 14 d, tumors were harvested and single-cell suspensions were generated by manual dissociation. Cells were then re-stimulated on six-well plates coated with anti-CD3 (3 μg/ml) and 3,10,000,000,000 NADH/NAD+ in RPMI-1640 medium containing 10% FBS and 2 mM l-glutamine, or the 1:1,000 dilution of the following ions: aspartate, m/z 334–346; citrate, m/z 465; fumarate, m/z 245; glutamate, m/z 363; malate, m/z 335; and succinate, m/z 247. All peaks were manually inspected and verified relative to known spectra for each metabolite. Enrichment of 13C was assessed by quantifying the abundance of each metabolite. Enrichment of 13C was assessed by quantifying the abundance of 13C15N-labeled amino acid internal standards were analyzed to confirm <10% of inter-sample variability. Ion pair LC–MS analysis was performed with LC separation on a Zorbax RRHD Extend-C18 column (150 × 2.1 mm, 1.8-μm particle size, Agilent Technologies), and using a gradient of solvent A (10 mM tributylamine and 15 mM acetic acid in 97:3 water/methanol) and solvent B (100 mM tributylamine and 15 mM acetic acid in methanol) according to the manufacturer’s instructions (MassHunter Metabolomics dMRM Database and Method, Agilent Technologies).

Labeling of lipids by 13C-glucose. T cells were acutely or chronically stimulated as described in “Acute and chronic T cell stimulation.” At 4 d after initial stimulation, cells were plated in 24-well plates at 5 × 10^5 cells in 1 ml of RPMI-1640 medium without glucose containing 10% dialyzed FBS, 2 mM l-glutamine, 5 μM BME, 10 ng/ml IL-2, 10 mM glucose and 0.5 μg/ml [1-13C] glucose (Perkin Elmer), and with (chronic) or without (acute) plate-bound anti-CD3 (3 μg/ml). After 24 h cultures were washed twice with PBS, and lipids were collected with 50% methanol containing 0.1 M HCl on dry ice, extracted with chloroform and transferred to scintillation vials. A Perkin Elmer Tri-Carb 2910 TR scintillation counter was used to measure 13C counts per minute for 1 min per sample. Counts per minute were normalized to cell number measured from duplicate samples.

CAR-T cell models. CD19-specific CAR-T cells were generated as previously described. The CAR constructs included an scFv, composed of a mouse CD8 signal peptide, IgH rearrangement, a glycosine–serine linker and IgL rearrangement. The scFv was fused to the mouse CD8 hinge, the transmembrane region and mouse stimulatory domains, including mouse CD28 and/or mouse CD3z. The m1928z construct was cloned into pENTR1A in-frame followed by a sequence encoding the E2A self-cleaving peptide. For CAR-T cell generation, activated splenic T cells were spinfected with virus containing the m1928z-encoding construct. For CAR-T cell experiments, we used an HSCT-based allogeneic strategy with an MHC-disparate model (B6 → BALB/c), as previously described. Irradiated BALB/c recipients received B6 bone marrow, 1 × 10^6 A20 lymphoma cells and 1 × 10^6 CAR-T cells intravenously via tail vein injection. After 14 d, spleens...
were harvested, and CAR-T cells were isolated based on GFP expression using a Sony SH1000 sorter. Following sorting, cells were re-stimulated on six-well plates coated with anti-CD3 (3 μg ml⁻¹, 3:1,000 dilution) in 2 ml of RPMI-1640 medium containing 10% FBS, 2 mM l-glutamine, 5 μM β-ME and 10 ng/ml IL-2, and with or without N-AC (10 mM). At 3 d later, cells were re-stimulated with PMA and ionomycin and assessed for expression of CD8, PD-1, TNF and IFN-γ as described in “Measurement of viability, receptor expression and cytokine production.”

Statistical analysis. GraphPad Prism 8 software was used for statistical analyses. Error bars, P values and statistical tests are reported in figure legends.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Datasets are deposited in the NCBI Gene Expression Omnibus using the following accession code: RNA-seq, GSE138459. Additional information can be found in the Nature Research Reporting Summary. Further information and requests for reagents may be directed to, and will be fulfilled by, the corresponding author. Source data are provided with this paper.

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Author contributions
S.A.V. and C.B.T. conceived the study. S.A.V. performed all experiments with assistance from M.A.H. M.B. and J.R.C. assisted with LC–MS, extracellular flux and nutrient consumption experiments. D.K.W., B.K., A.T.S., H.Y.C. and K.E.Y. assisted with analysis of RNA-seq data. M.S., P.S.H. and M.R.M.v.d.B. assisted with CAR-T cell experiments. C.B.T. provided additional work in conception and study guidance. S.A.V. and C.B.T. wrote the manuscript.

Competing interests
C.B.T. is a founder of Agios Pharmaceuticals and a member of its scientific advisory board. He is also a former member of the Board of Directors and a stockholder of Merck and Charles River Laboratories. S.A.V. has received honoraria from Agios Pharmaceuticals and Rheos Pharmaceuticals, is an advisor for Immunai and has consulted for ADC Therapeutics. A.T.S. and D.K.W. are scientific founders and equity holders of, and receive consulting fees from, Immunai. A.T.S. received funding support from 10x Genomics and Arsenal Biosciences. K.E.Y. is an advisor for Immunai. H.Y.C. is a co-founder of Accent Therapeutics and Boundless Bio and is a consultant for 10x Genomics, Arsenal Biosciences and Spring Discovery.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41590-020-0725-2.
Supplementary information is available for this paper at https://doi.org/10.1038/s41590-020-0725-2.
Correspondence and requests for materials should be addressed to C.B.T.
Peer review information Peer reviewer reports are available. L. A. Dempsey was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team. Reprints and permissions information is available at www.nature.com/reprints.
Extended Data Fig. 1 | Chronic T cell stimulation induces T cell exhaustion. All experimental analyses were conducted eight days after initial stimulation unless otherwise specified. 

- **a–c**, Expression of inhibitory immunoreceptors (PD-1, LAG-3, PD-L1) and intracellular cytokine production (IFN-γ and TNF) in acutely and chronically stimulated T cells following re-stimulation with PMA and ionomycin. 

- **d**, Expression of Glut1 in acutely or chronically stimulated OT-I T cells with or without restimulation using bead-bound anti-CD3. Actin is used as a loading control. Experiment was repeated three times with similar results. Uncropped blot can be found within Source Data. 

- **e**, Gene set enrichment plot showing that genes associated with chronically stimulated polyclonal T cells in vitro are enriched for genes upregulated in exhausted CD8+ T cells (T exh) but not anergic T cells15. 

- **f**, Killing of peptide-pulsed B16 cells. Luciferase-expressing B16 cells pulsed with Ova peptide at the indicated doses for 4 h were co-cultured with acutely or chronically stimulated T cells for 24 h. The following day, cells were lysed and luciferase expression was assessed using a luminometer. 

- **g**, Normalized isotopologue abundance of intracellular lactate in acutely and chronically stimulated T cells following initial stimulation. P values were calculated by unpaired, two-sided Student's t-test (**–h**), relative to acutely stimulated T cells or based on 1,000 permutations by the GSEA algorithm and not adjusted for multiple comparisons (e). Data are presented as the mean ± s.d. of n=3 biologically independent samples from a representative experiment. **P < 0.01. 

NATuRE IMMuNologY | www.nature.com/natureimmunology
Extended Data Fig. 2 | Aerobic glycolysis is a hallmark of chronic stimulation-dependent terminal T cell dysfunction. a, Extracellular acidification rate of acutely and chronically stimulated polyclonal T cells in media containing or lacking glucose as indicated. b, Extracellular acidification rate of acutely and chronically stimulated polyclonal T cells at baseline and in response to electron transport chain inhibition. c, Population doublings of acutely and chronically stimulated polyclonal CD8+ T cells following initial stimulation. d, Viability of acutely and chronically stimulated T cells as determined by forward scatter and DAPI exclusion. e, Intracellular TOX expression and proliferation as measured by dilution of Cell Trace Violet fluorescence of acutely or chronically stimulated T cells. f, Normalized expression of glycolytic genes in CD8+ T cell clusters from patients with basal and squamous cell carcinoma treated with immune checkpoint blockade. g, Gene set enrichment plot showing that genes associated with terminally exhausted T cells isolated from murine B16 melanoma tumors are enriched for glycolytic genes. h, Correlation of glycolysis score (left) and TCA cycle score (right) with TCF7 expression in exhausted CD8+ T cell clusters from basal and squamous cell carcinoma patients treated with immune checkpoint inhibitors. i, Gene set enrichment plot showing that chronically stimulated OT-I T cells in vitro significantly downregulate genes upregulated in progenitor T_{exh} as compared to terminal T_{exh}. j, k, Intracellular cytokine production in acutely and chronically stimulated polyclonal T cells following re-stimulation. In (j), cells were cultured in the presence or absence of anti-PD-L1 (10 F.9G2) from D2-D8. In (k), "Chronic + 24 h rest" cells were rested in the absence of plate-bound anti-CD3 for 24 h prior to re-stimulation. Experiment was repeated three times with similar results. P values were calculated by unpaired, two-sided Student’s t-test relative to acutely stimulated T cells or based on 1,000 permutations by the GSEA algorithm and not adjusted for multiple comparisons. Data are presented as the mean ± s.d. of n = 3 biologically independent samples from a representative experiment. ****P < 0.0001.
Extended Data Fig. 3 | Chronic antigen stimulation impairs mitochondrial oxidation and ATP production. **a**, Quantification of relative tricarboxylic acid cycle metabolite pool sizes in acutely and chronically stimulated T cells. Columns represent biological replicates for each condition. **b**, Oxygen consumption rate (OCR) of acutely or chronically stimulated T cells at baseline or in the presence of ATP synthase inhibition (Oligo), uncoupling agents (FCCP), inhibition of glucose uptake (2-DG), and complex III/IV inhibition (Rot/AA). **c**, Schematic depicting how oxidative metabolism of uniformly-labeled palmitate ([U-\(^{13}\)C] palmitate) generates metabolites associated with the TCA cycle. Colored circles represent \(^{13}\)C-labeled carbons. **d**, Fractional labeling by [U-\(^{13}\)C] palmitate of citrate, glutamate, fumarate, malate and aspartate in acutely and chronically stimulated T cells following re-stimulation. **e**, Proliferation of T cells acutely or chronically stimulated in the presence or absence of supplemental sodium acetate (5 mM), as measured by dilution of Cell Trace Violet fluorescence. **f**, Quantification of pool sizes of metabolite intermediates in nucleotide synthesis in acutely and chronically stimulated T cells. Heatmap depicts pool size relative to row median. Columns represent biological replicates for each condition. Experiment was repeated two times with similar results. \(P\) values were calculated by unpaired, two-sided Student’s \(t\)-test (\(a,b,d\)). Data are presented as the mean \(\pm\) s.d. of \(n=3\) biologically independent samples from a representative experiment. **\(P\) < 0.01. ****\(P\) < 0.0001.
Extended Data Fig. 4 | Oxidative stress limits T cell proliferative capacity. a, Western blot depicting overexpression of FLAG-tagged recombinant NADH oxidase enzymes LbNOX and MitoLbNOX in T cells50. Experiment was repeated three times with similar results. Uncropped blot can be found within Source Data. b, Fluorescence intensity of acutely and chronically stimulated T cells expressing vector control, LbNOX, or MitoLbNOX following eight days in culture after loading with CM-H2DCFDA. c, Population doublings of acutely and chronically stimulated T cells expressing vector control, LbNOX, or MitoLbNOX. d, Fluorescence intensity of acutely and chronically stimulated T cells after loading with BODIPY-C11 to measure lipid peroxidation. Light-grey-shaded peak represents negative control. e, Fluorescence intensity of acutely or chronically stimulated T cells cultured with or without pharmacologic agents that impair ETC function following 2 days of initial stimulation. Cells were loaded with CM-H2DCFDA to measure ROS on D8 following initial stimulation. f, qRT-PCR of Myb and Tcf7 in acutely or chronically stimulated T cells with or without the addition of the indicated agents for 2 days following 2 days of primary stimulation. g–i, Expression of oxidative stress-related metabolic genes (“ROS score”) in tumor-infiltrating CD8+ T cells from basal and squamous cell carcinoma patients treated with immune checkpoint inhibitors. In (g), ROS score in independent CD8+ T cell clusters is shown. In (h), ROS score in exhausted and memory T cell populations is shown according to clone size as measured by TCR sequencing; box center line=median, box limits=upper and lower quartiles, box whiskers=1.58 x interquartile range. In (i), correlation of ROS score with TCF7 expression in exhausted CD8+ T cells is shown. Only cells with non-zero TCF7 expression were included. P values were calculated by one-way ANOVA with Sidak’s multiple comparisons post-test (g, i), or one-sided Student’s t-test relative to base mean (g, h). Data are presented as the mean ± s.d. of n=3 biologically independent samples from a representative experiment. **P < 0.01. ***P < 0.001. ****P < 0.0001.
Extended Data Fig. 5 | Endogenous anti-oxidant production is limiting for T cell proliferation. 

**a** Motif analysis of sites with increased accessibility in tumor-infiltrating CD8+ T cells (L7) as compared to T cells from Listeria-infected mice (E7) showing NFATc1 as among the motifs whose accessibility was most preferentially increased in L7 cells15. 

**b** Intracellular calcium flux as measured by ratio of bound to unbound Indo-1-AM in acutely and chronically stimulated T cells, at baseline, in response to monomeric anti-CD3, and in response to receptor clustering (streptavidin). 

**c** Gene set enrichment plot showing that chronically stimulated OT-I T cells are enriched for NFAT target genes. 

**d** Expression of NFAT target genes (“nfat score”) in independent CD8+ T cell clusters. 

**e** Correlation of expression of NFAT target genes (“nfat score”) with expression of oxidative stress-related metabolic genes (“ROS score”) in tumor-infiltrating CD8+ T cells from melanoma patients treated with immune checkpoint inhibitors. 

**f** Fluorescence intensity of acutely and chronically stimulated T cells cultured with or without BME supplementation after loading with CM-H2DCFDA to measure ROS. Light-grey-shaded peak represents negative control. 

**g** Proliferation of T cells acutely stimulated in the presence or absence of BSO or diamide as measured by dilution of Cell Trace Violet fluorescence. 

**h** Expression of TCF-1 and TOX in chronically stimulated T cells cultured in the presence or absence of BSO. P values were calculated by one-sided Student’s t-test relative to base mean (d, e). ****P < 0.0001.
Extended Data Fig. 6 | N-acetylcysteine reverses oxidative stress in chronically stimulated T cells. a, Quantification of relative metabolite pool sizes as measured by LC-MS in chronically stimulated T cells cultured with or without N-AC. Colored dots represent intermediates in glutathione synthesis as indicated. Dashed lines represent cutoffs of $p < 0.01$ and log$_2$ fold change $> 0.5$. b, ATP production by acutely or chronically stimulated T cells cultured with or without N-AC. P values were calculated by one-way ANOVA with Sidak’s multiple comparisons post-test compared to acutely stimulated T cells (b). Data are presented as the mean ± s.d. of $n = 4$ biologically independent samples from a representative experiment (b). *P < 0.05.
Extended Data Fig. 7 | Antioxidants restore T cell self-renewal during chronic stimulation. a, Population doublings of chronically stimulated T cells with or without N-AC supplementation under normoxic (left) or hypoxic (right) conditions. Experiment was repeated two times with similar results. b, qRT-PCR of Tcf, Myb, and Prdm1 in acutely or chronically stimulated T cells with or without the addition of N-AC as indicated. c, Intracellular calcium flux as measured by ratio of bound to unbound Indo-1-AM in acutely and chronically stimulated T cells cultured with or without N-AC. d, Gene set enrichment plot showing that the addition of N-AC during chronic stimulation reduces expression of NFAT target genes. P values were calculated by unpaired, two-sided Student’s t-test relative to cells cultured without N-AC (a), one-way ANOVA with Sidak’s multiple comparisons post-test (b) or based on 1,000 permutations by the GSEA algorithm and not adjusted for multiple comparisons (d). Data are presented as the mean ± s.d. of n = 3 biologically independent samples from a representative experiment. *P < 0.05. **P < 0.01. ***P < 0.0001.
Extended Data Fig. 8 | Antioxidants reverse endogenous tumor-associated T cell dysfunction. a, Production of IFN-γ and TNF following re-stimulation with PMA and ionomycin in chronically stimulated T cells with or without N-AC supplementation under normoxic or hypoxic conditions. Experiment was repeated two times with similar results. b, c, Oxygen consumption rate (OCR) of OT-I T cells chronically co-cultured with B16 melanoma cells with or without anti-PD-L1 antibodies and with or without N-AC supplementation at baseline or in the presence of ATP synthase inhibition (Oligo), uncoupling agents (FCCP), or complex III/IV inhibition (Rot/AA). d, Production of IFN-γ and TNF following re-stimulation with PMA and ionomycin in chronically stimulated T cells with or without MitoTEMPO or Trolox supplementation as indicated. Experiment was repeated two times with similar results. P values were calculated by unpaired, two-sided Student’s t-test (c). Data are presented as the mean ± s.d. of n = 4 biologically independent samples from a representative experiment (b, c). *P < 0.05.
Extended Data Fig. 9 | Gating strategy for fluorescence activated cell sorting analysis. For both polyclonal and OT-I transgenic T cells, gating was performed as shown. First, doublet exclusion was performed on cells gated by FSC-H versus FSC-W. Then, doublet exclusion was performed on cells gated by SSC-H versus SSC-W. Viable cells were identified by FSC-A and Live/Dead Blue exclusion. Finally, CD8 positivity was assessed by fluorescence in the BV-786 channel.
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- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever possible.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
Flow cytometry data was collected using FacsDiva (v8.0). Cell counts were collected using a Beckman Multisizer 4e. GC-MS data was collected using an Agilent 7890A GC coupled to an Agilent 5977C mass selective detector. LC-MS data was collected using a Zorbax RRHD Extend-C18 column coupled to a 6470 quadrupole mass spectrometer or a hydrophilic interaction chromatography column coupled to a 6545 Q-TOF mass spectrometer. Quantitative real-time PCR data was obtained using QuantStudio 7 Flex. RNA sequencing samples were sequenced using an Illumina NextSeq 500. Fasta files were aligned to genome build mm9 using TopHat2 v2.1.1 with default parameters. Aligned features were counted with htseq-count v0.11.1. Nutrient consumption was measured using a 23950D Biochemistry Analyzer. Extracellular flux analysis was performed using a Seahorse XF96 Extracellular Flux Analyzer. 14C-glucose labeling of lipids was measured using a Perkin Elmer Tri-Carb 2910 TR scintillation counter.

Data analysis
Flow cytometry analysis was performed using FlowJo (v10.0). GC-MS and LC-MS Metabolite data was extracted and integrated using MassHunter software (v8.09) and MassHunter Profiler Software v6.0. Correction for natural isotope abundance was performed using IsoCor (v6.0) software. Differential RNA-seq expression was determined using the edgeR package v3.28.1 in Bioconductor v3.10. Single-cell RNA-seq data was normalized using the NormalizeData function in Seurat v2.3.4. Statistical tests were performed using GraphPad Prism v8.0.

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Datasets are deposited in the NCBI Gene Expression Omnibus using the following accession numbers: RNA-seq, GSE138459. All data supporting the findings of this study are available from the corresponding author on reasonable request.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistics were used to pre-determine sample size. Cell biology experiments were performed in triplicate, which extensive experience has shown to be sufficient to determine reproducible metabolic results from cultured cells and immune cells (Frawirth et al, Immunity 2002, Carey et al, Nature 2015, Finley et al, Nat Cell Biol 2018, Vardhana et al, Nat Metab 2019). 4-8 replicates were used for extracellular flux experiments as has been described extensively in the literature (von der Windt et al, Curr Protoc Immunol 2016). |
| Data exclusions | No data were excluded. |
| Replication | All experiments were reliably reproduced. Each experiment was performed independently at least two times, but usually many more times. |
| Randomization | Samples were randomly distributed into groups. |
| Blinding | For assessment of tumor growth in vivo, T-cells were provided to the animal core facility and adoptively transferred in a blinded fashion. For cell culture experiments, blinding was not necessary as analyses were objective as is the standard for cell biology and immunology experiments in vitro (Frawirth et al, Immunity 2002, Carey et al, Nature 2015, Finley et al, Nat Cell Biol 2018, Vardhana et al, Nat Metab 2019). |

Reporting for specific materials, systems and methods

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### Materials & experimental systems

| n/a | Involved in the study |
| --- | --- |
| □ | X Antibodies |
| □ | X Eukaryotic cell lines |
| □ | X Palaeontology and archaeology |
| □ | X Animals and other organisms |
| □ | X Human research participants |
| □ | X Clinical data |
| □ | X Dual use research of concern |

### Methods

| n/a | Involved in the study |
| --- | --- |
| □ | X ChIP-seq |
| □ | X Flow cytometry |
| □ | X MRI-based neuroimaging |

### Antibodies

Antibodies used for T-cell activation were as follows:
- anti-CD3 (2C11, Thermo Fisher, 16-0031-86)
- anti-CD28 (19.5.1, BioLegend, 100304)

Antibodies used for flow cytometry were as follows:
- anti-CD3-biotin (145-2C11, BioLegend, 100304)
- anti-PD-L1 (10F.9G2, BioXCell, BE0101)
TNF-PE (BioLegend, MP6-XT22, 506306)
PD-1-PECy7 (BioLegend, RMPI-1, 109110)
IFN-γ-FITC (BioLegend, XM1G.1, 505806)
CD4-BV711 (BioLegend, RM4-5, 100550)
CD8a-BV786 (BioLegend, 53-6-7, 100750)
Tox-PE (Milenyi, REA473, 130-120-716)
Tcf1-Alexa647 (Cell Signaling, C6309, 6709)
PD-L1-APC (BioLegend, 10F.9G2, 124312)
LAG-3-PerCP-Cy5.5 (BioLegend, C987W, 125211)
TIM-3-PE Dazzle594 (BioLegend, B8.2C12, 134013)

Antibodies used for Western blotting were as follows:
FLAG (Sigma-Aldrich, F1804)
Total OXPHOS (Abcam, ab110413)
Glut1 (Cell Signaling, 129959)
Actin (Sigma, A3854)
anti-rabbit-HRP (GENA034, GE Healthcare, 1:5000)
anti-mouse-HRP (GENA4931, GE Healthcare, 1:5000)

Validation
Antibody validation was according to the manufacturer’s website and was as follows:
anti-CD3 (2C11, Thermo Fisher, 16-0031-86), validated in mouse splenocytes (Bachchi et al., Oncolimmunology 2019)
anti-CD28 (19.5, E Bioscience, 16-0281-85), validated in mouse splenocytes (Compeere et al., Nat Commun 2018)
anti-CD3-biotin (145-2C11, BioLegend, 100304), validated in mouse splenocytes (Bachchi et al., Oncolimmunology 2019)
anti-PD-1 (10F.9G2, BioXCell, BE0101), validated in mice in vivo (Stathopoulos et al., Immunity 2018)
TNFα-PE (BioLegend, MP6-XT22, 506306), validated in mice (Infante-Duarte et al., J Immunol 2000)
PD-1-PECy7 (BioLegend, RMPI-1, 109110), validated in mice (Matsumoto et al., J Immunol 2004)
IFN-γ-FITC (BioLegend, XM1G.1, 505806), validated in mice (Ferrick et al., Nature 1995)
CD4-BV711 (BioLegend, RM4-5, 100550), validated in mice (Bourdeau et al., Blood 2007)
CD8a-BV786 (BioLegend, 53-6-7, 100750), validated in mouse thymocytes (Takahashi et al., PNAS 1992)
Tox-PE (Milenyi, REA473, 130-120-716), validated in mouse T-cells (Scott et al., Nature 2019)
Tcf1-Alexa647 (Cell Signaling, C6309, 6709), validated in mouse T-cells (Scott et al., Nature 2019)
PD-L1-APC (BioLegend, 10F.9G2, 124312), validated in mice (Maer et al., J Immunol 2007)
LAG-3-PerCP-Cy5.5 (Biologend, C987W, 125211), validated in mice (Workman et al., J Immunol 2005)
TIM-3-PE Dazzle594 (BioLegend, B8.2C12, 134013), validated in mice (Mizukami et al., PNAS 2015)
FLAG (Sigma-Aldrich, F1804), validated with recombinant FLAG-tagged ZBTB48 (Jahn et al., EMBO Rep 2017)
Total OXPHOS (Abcam, ab110413), validated in mice (Svensson et al., Am J Physiol Endocrinol Metab 2020)
Glut1 (Cell Signaling, 129959), validated in mouse T-cells (Macintyre et al., Cell Metab 2014)
Actin (Sigma, A3854), validated in 3T3 cells (Vanderkerkhove and Weber, Eur J Biochem 1978)

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
B16-F10, B16-OVA, EL4, 293T, and A20 cell lines were all purchased from ATCC.

Authentication
Cell lines were not externally authenticated.

Mycoplasma contamination
Cell lines routinely tested negative for mycoplasma.

Commonly misidentified lines
No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBT Biosample.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals
For activation of polyclonal CD8+ T-cells, T-cells were isolated from the spleen and inguinal lymph nodes of C57/Bl6 mice (Jackson 000664). For activation of OT-I transgenic CD8+ T-cells, single cell suspensions were generated from spleens and inguinal lymph nodes of OT-I mice (Jackson 003831). Equal numbers of male and female mice were used and all mice were aged 8-12 weeks.

For in vivo tumor models, equal numbers of male and female C57Bl/6 scid (Jackson 001913) mice aged 8-12 weeks were injected subcutaneously with 200,000 B16-OVA cells in a 1:1 mix of PBS and Matrigel (Corning). OTI T-cells that had been acutely or chronically stimulated in the presence or absence of N-Ac as described were adoptively transferred to mice via retro-orbital injection. Mice were monitored daily and were sacrificed for signs of morbidity.

For ex vivo analysis of tumor-infiltrating T-cells, equal numbers of male and female C57Bl/6 mice aged 8-12 weeks were injected subcutaneously with 200,000 EL4 cells in a 1:1 mix of PBS and Matrigel (Corning). 14 days later, tumors were harvested mice were sacrificed. For CAR-T cell models, we utilized an HSCT-based allogeneic strategy with an MHC-disparate model (B6→BALB/c) as previously described. Equal numbers of male and female BALB/c recipients aged 8-12 weeks were irradiated and subsequently received B6 bone marrow, 1 million A20 lymphoma cells, and 1 million CAR-T cells intravenously via tail vein injection. 14 days later, mice were sacrificed and spleens were harvested.

The animals used for this study were housed in the Animal Care Facility maintained by Memorial Sloan Kettering Cancer Center. The animals were cared for by a full-time veterinary staff, in accordance with USPHS, USDA, and AAALAC requirements and the Guide for the Care and Use of Laboratory Animals. Animal studies were performed according to Memorial Sloan Kettering Cancer
Mice were maintained in individually ventilated polysulfone cages with a stainless-steel wire bar lid and filter top (no. 19, Thoren Caging Systems, Inc., Hazleton, PA). They were housed in autoclaved aspen chip bedding (PWI Industries, Quebec, Canada) with each cage provided 2 compressed cellulose squares (Nestlets®, Ancare, Bellmore, NY) for enrichment. Mice were fed a closed-formula, natural-ingredient, γ-irradiated diet (SO53 - PicoLab® Rodent Diet 20, Purina LabDiet, St Louis, MO) which was surface decontaminated using "flash" sterilization (100°C for 1 minute). Mice were provided reverse-osmosis acidified (pH 2.5 to 2.8, with hydrochloric acid) water in polyphenylsulfone bottles with stainless steel caps and slipper tubes (Outbreak 1; Techniplast, West Chester, PA) or drilled polysulfone bottles with neoprene stoppers (Outbreak 2; Thoren Caging Systems, Inc.). Cage bottoms were changed weekly, whereas the wire bar lid, filter top and water bottle were changed biweekly. The rooms were maintained on a 12:12-h light:dark cycle, relative humidity of 30% to 70%, and room temperature of 72 ± 2°F (22.2 ± 1.1°C). The animal care and use program at Memorial Sloan Kettering Cancer Center (MSKCC) is accredited by AAALAC, and all animals are maintained in accordance to the recommendations provided in the Guide for the Use and Care of Laboratory Animals 8th Edition. All animal use described in this investigation was approved by MSK’s Institutional Animal Care and Use Committee.

Wild animals
No wild animals were used in the study.

Field-collected samples
No field collected samples were used in the study.

Ethics oversight
All experiments were performed according to Memorial Sloan Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee (IACUC) guidelines. The animals used for this study were housed in the Animal Care Facility maintained by Memorial Sloan Kettering Cancer Center. The animals were cared for by a full-time veterinary staff, in accordance with USPHS, USDA, state, and AAALAC requirements and the Guide for the Care and Use of Laboratory Animals. Animal studies were performed according to Memorial Sloan Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee (IACUC) guidelines.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants
Policy information about studies involving human research participants

Population characteristics
N/A

Recruitment
N/A

Ethics oversight
N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data
Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration
N/A

Study protocol
N/A

Data collection
N/A

Outcomes
N/A

Flow Cytometry

Plots
Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a `group’ is an analysis of identical markers).
☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology
Sample preparation
For measurement of oxidative stress, lipid peroxidation, and mitochondrial mass, cells were loaded with CM-H2DCFDA (1 μM), MitoSOX Red (5 μM), BODIPY-C11 (5 μM), or MitoTracker Green (1 μM) for 30 minutes at 37°C in PBS. They were then quenched with RPMI containing 10% FBS, washed 2 times with FACS buffer, and analysed immediately using an LSR Fortessa machine (Beckman Dickinson). For measurement of intracellular calcium levels, T-cells were loaded with Indo-1 AM (Invitrogen) as per manufacturer’s instructions in serum-free media for 30 minutes at 37°C, quenched with media containing
serum, and used immediately for flow cytometry experiments. Indo-1-bound and unbound fluorescence was assessed per manufacturer’s instructions on an LSR Fortessa machine. Analysis of mean fluorescence intensity was performed using FlowJo v9.0. All experiments were performed at least two independent times.

For measurement of viability, receptor expression and cytokine production, T-cells were restimulated with phorbol myristate acetate (Sigma, 50 ng/mL) and ionomycin (Sigma, 500 ng/mL). 90 minutes later, cells were treated with Brefeldin A to block cytokine secretion. Three h later, cells were stained for surface markers and simultaneously labelled with Live/Dead Blue Viability Dye (Thermo Fisher) for 20 minutes at 4C. Cells were washed twice and fixed overnight using a FoxP3 Fixation/Permeabilization kit (Thermo Fisher). The following day, cells were washed and stained for intracellular cytokines at room temperature for one hour. They were then washed three times and analysed using an LSR Fortessa machine.

| Instrument          | BD LSR Fortessa (for acquisition) |
|---------------------|-----------------------------------|
| Software            | FACSDiva (v8.0) was used for data acquisition. FlowJo 10.0 was used for data analysis. |
| Cell population abundance | CD8+ T-cells were identified in all cases by CD8-BV786 staining. |
| Gating strategy     | The gating strategy was initially doublet exclusion using FSC-H and SSC-H followed by viable cell identification via LIV/DIEAD Blue exclusion using FSC-A by BUV396-A. CD8+ T-cells were then identified via CD8 expression using CD8-BV786 by CD4-BV711. |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.