S-2-hydroxyglutarate regulates CD8+ T-lymphocyte fate

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R-2-hydroxyglutarate accumulates to millimolar levels in cancer cells with gain-of-function isocitrate dehydrogenase 1/2 mutations. These levels of R-2-hydroxyglutarate affect 2-oxoglutarate-dependent dioxygenases. Both metabolite enantiomers, R- and S-2-hydroxyglutarate, are detectible in healthy individuals, yet their physiological function remains elusive. Here we show that 2-hydroxyglutarate accumulates in mouse CD8+ T cells in response to T-cell receptor triggering, and accumulates to millimolar levels in physiological oxygen conditions through a hypoxia-inducible factor 1-alpha (HIF-1α)-dependent mechanism. S-2-hydroxyglutarate predominates over R-2-hydroxyglutarate in activated T cells, and we demonstrate alterations in markers of CD8+ T-cell differentiation in response to this metabolite. Modulation of histone and DNA demethylation, as well as HIF-1α stability, mediate these effects. S-2-hydroxyglutarate treatment greatly enhances the in vivo proliferation, persistence and anti-tumour capacity of adoptively transferred CD8+ T cells. Thus, S-2-hydroxyglutarate acts as an immunometabolite that links environmental context, through a metabolic-epigenetic axis, to immune fate and function.

In response to T-cell receptor (TCR) triggering, quiescent CD8+ T lymphocytes transition to a proliferative effector state. During this response, memory CD8+ T lymphocytes form and can persist for the entire lifespan of the organism, mounting rapid recall responses, thereby providing long-term immunity. The metabolic programs of these different CD8+ T lymphocyte states are distinct and important for function1–4. Effector CD8+ T lymphocytes generate most ATP and biomass through glycolysis5; both naive and memory cells rely heavily on oxidative phosphorylation6,7. Various cytokines and transcription factors are important for the differentiation of CD8+ T lymphocytes, and it is evident that immunological memory is influenced by epigenetic mechanisms8–12.

CD8+ T-lymphocytes traffic into severely hypoxic areas within tumours and inflammatory tissue13. The response to oxygenation, mediated by the von Hippel–Lindau (VHL) and HIF-1α proteins, is an essential regulator of metabolism and CD8+ T-lymphocyte function14–16. Here we demonstrate that CD8+ T lymphocytes produce 2-hydroxyglutarate (2HG) in response to TCR triggering and environmental hypoxia. Using CD8+ T-lymphocytes isolated from Mus musculus with CD8-specific genetic deletions of Vhl, Hif-1α and Hif-2α, driven by cre expressed under the distal promoter of the lymphocyte protein tyrosine kinase (dLckcre), we highlight the dependency of this metabolic feature on the HIF pathway. S-2HG constitutes the majority of the 2HG pool, and we show that S-2HG alters the phenotypic and functional characteristics of CD8+ T lymphocytes, maintaining a state of increased proliferative, survival and anti-tumour capacity.

The VHL–HIF-1α axis regulates 2HG production

To elucidate the metabolic effects of HIF-1α activation, we profiled the metabolome of CD8+ T lymphocytes with low (Vhl+/–) or high (Vhl+/+dLckcre, denoted as Vhl+/-) HIF signalling, and knockout of both Hif-1α and Vhl (Hif1a–/–/Vhl/–/–dLckcre, denoted as Hif1a–/–/Vhl–/–) to control for a specific contribution of HIF-1α.14,15. Unsupervised clustering and principal component analysis (Fig. 1a, b) separate Vhl+/– from Vhl+/+ CD8+ T lymphocytes. Vhl+/–/Hif1a+/– cluster with Vhl+/+ indicating that Hif-1α mediates significant metabolic changes following Vhl deletion. Glycolysis is important for sustaining effector function5–7 and these data indicate that Vhl suppresses glycolysis via inhibition of Hif-1α, (Extended Data Fig. 1a–c).

Vhl loss suppresses late and increases early tricarboxylic acid (TCA) cycle intermediates (Extended Data Fig. 1a). Notably, 2HG is significantly enriched in Vhl+/– CD8+ T lymphocytes (Fig. 1c, Extended Data Fig. 1d). Furthermore, increases in 2HG are dependent on Hif-1α when Vhl is deleted (Extended Data Fig. 1a). This was validated using quantitative mass spectrometry in Vhl+/– and Vhl+/–/Hif1a+/– CD8+ T lymphocytes (Fig. 1d), as well as in VHL-null cell lines, that express either Hif-1α (RCC4) or HIF-2α (786-O), reconstituted with Vhl (Fig. 1e, Extended Data Fig. 1e). Deletion of Vhl in murine embryonic fibroblasts from Vhl+/fl mice increases 2HG levels (Fig. 1f, Extended Data Fig. 1f). Hence, the VHL–HIF signalling axis regulates 2HG levels, and constitutive Hif-1α signalling underlies this effect in Vhl-null CD8+ T lymphocytes.

HIF-1α regulates S-2HG production

R-2HG production is increased by isocitrate dehydrogenase 1 and/or isocitrate dehydrogenase 2 mutations in different cancers18,20, accumulation of the S-2HG enantiomer occurs in the context of hypoxia21,22 and mitochondrial dysfunction3,4,23,24. We thus sought to determine 2HG levels in CD8+ T lymphocytes following activation. 2HG before activation and at sea-level oxygen is undetectable, whereas levels at the same oxygenation are elevated 2–4 days after TCR stimulation (Fig. 2a). When activated CD8+ T lymphocytes are exposed to 1% oxygen, the intracellular concentration of 2HG reaches millimolar levels (Fig. 2b, Extended Data Fig. 2a) and is proportional to the degree of oxygenation (Fig. 2c). We confirmed this using 1H NMR spectroscopy (Extended Data Fig. 2b). Given such high levels of 2HG, we sequenced25
We carried out deletion of loxp-flanked Hif1a or Hif2a (also known as Epa1) genes in CD8+ T lymphocytes, using dLckcre (ref. 26; Extended Data Fig. 2c). 2HG accumulation is abolished in Hif1afl/fl dLckcre (denoted as Hif1a−/−), but not Hif2afl/fl dLckcre (denoted as Hif2a−/−) cells under hypoxia (Fig. 2g, h; Extended Data Fig. 2d, e), with no difference in viability (Extended Data Fig. 2f). We next examined 2HG levels in vivo, in the spleens of mice. There is more R-2HG than S-2HG (Fig. 2i); furthermore, the levels of S-2HG are significantly decreased in spleens of Hif1afl/fl dLckcre mice (Fig. 2i). There is also a slight decrease in the levels of R-2HG (Fig. 2i). 2HG is present in the urine of healthy individuals and is elevated in patients with 2HG acidaemia27. Hif1afl/fl dLckcre mice have lower levels of S-2HG in urine (Fig. 2j) indicating that Hif-1α in the T-lymphocyte (CD4+ and CD8+) compartment makes a contribution to S-2HG production in vivo. Activated Hif1a−/− CD8+ T lymphocytes in 21% oxygen have lower 2HG at extended time points (Extended Data Fig. 2g), indicating a contribution of Hif-1α in non-hypoxic conditions also.

We next sought to determine the metabolic route by which Hif-1α promotes S-2HG production in CD8+ T lymphocytes. Transcriptionally, hypoxic CD8+ T lymphocytes show induction of glycolysis and suppression of the TCA cycle (Extended Data Fig. 2h). Moreover, TCA cycle intermediates are decreased (Extended Data Fig. 2i). Recent reports implicate lactate and malate dehydrogenases (Ldha and Mdh1/2) as enzymatic sources of 2HG in hypoxia22,23. In Hif1a−/− CD8+ T lymphocytes, the hypoxic expression of these enzymes suggests that Mdh1 and Mdh2 are unlikely to mediate the hypoxia-induced accumulation of S-2HG (Extended Data Fig. 2j). Confirming this, knockdown of Mdh1 or Mdh2 does not decrease S-2HG in hypoxia (Extended Data Fig. 2k–m); knockdown of Mdh1 leads to marginal increases in S-2HG (Extended Data Fig. 2l). Knockdown of Mdh1 or Mdh2 increases R-2HG levels (Extended Data Fig. 2m, n). Knockdown of Ldha (Extended Data Fig. 2k) decreases S-2HG (Fig. 2k), and also increases R-2HG levels in hypoxic CD8+ T lymphocytes (Extended Data Fig. 2n). Overexpression of Ldha in hypoxic Hif1a−/− CD8+ T lymphocytes (Extended Data Fig. 2o) rescues S-2HG production (Fig. 2l). Consistent with this, Ldha

Figure 1 | VHL-HIF signalling regulates 2-hydroxyglutarate levels.

a, Unsupervised hierarchical clustering and heat map of all detected metabolites. b, PCA of metabolomes. Percentage variance of each PC is in parentheses. c, Metabolites ranked in order of decreasing P value.

Figure 2 | Hypoxic induction of 2-hydroxyglutarate depends on Hif-1α in CD8+ T lymphocytes.

a, 2HG in naive and activated cells; n ≥ 4 mice per time point. b, 2HG in cells cultured at 21% or 1% oxygen for 48 h; n = 12 mice. c, 2HG in cells cultured at 21% (n = 15), 10% (n = 6), 5% (n = 10) or 1% (n = 6) oxygen for 48 h. d, Enantioselective mass spectrometry (MS) for S- and R-2HG. e, 2HG in human cells (n = 4 healthy donors). f, Enantioselective MS for S- and R-2HG from cells in e, g, H2G in Hif1afl/fl and Hif1afl/fl dLckcre (g) or Hif2afl/fl and Hif2afl/fl dLckcre (h) cells, cultured as in b (n = 4 mice per genotype). i, 2HG in splenic extracts from Hif1afl/fl (n = 20) and Hif1afl/fl dLckcre (n = 15) mice. j, 2HG in urine from Hif1afl/fl (n = 49) and Hif1afl/fl dLckcre (n = 25) mice. k, S-2HG in cells with shLdha (n = 4 individual transfections), l, Ldha–Flag (n = 6) or Pdk1–Flag (n = 4) overexpression in Hif1afl/fl dLckcre cells. The dotted line represents S-2HG levels in Hif1afl/fl cells (n = 4/6 individual transfections).

m, 13C-isotopologue profile of 2HG in cells cultured as in b (n = 7 mice per condition). Unpaired t-test (i, j). Paired t-test (b, l, m). One-way ANOVA (c, k) and two-way ANOVA (g, h). Error bars denote s.d.; each dot in a–e, i and j represents a mouse. *P < 0.05, **P < 0.01, ***P < 0.001; NS, not significant.
of restrained cell expansion (Extended Data Fig. 5g) and there is a clear decreased cytotoxicity (Extended Data Fig. 5f). Furthermore, α and Vegfa production (Extended Data Fig. 5d), indicating Hif-1 and hypoxic (Extended Data Fig. 2v). Hence, HIF-1 increases (Extended Data Fig. 2s), and re-expression of Pdk1 in this context (Extended Data Fig. 2s–u). Pdk1 expression is impaired in hypoxic (Fig. 2l, Extended Data Fig. 2o). Inhibition of pyruvate dehydrogenase (Pdh) by pyruvate dehydrogenase kinases (Pdk), promotes glomalin synthesis. We reasoned that Pdk supports S-2HG production by diverting glutamine-derived 2-oxoglutarate to 2HG. Furthermore, the glutamate pool increases in hypoxic lymphocytes (Extended Data Fig. 2p) and in Vhli/−/CD8 T lymphocytes (Extended Data Fig. 1a) and depends on Hif-1α (Extended Data Fig. 2q) but not Hif-2α (Extended Data Fig. 2r). Inhibition of pyruvate dehydrogenase (Pdh) by pyruvate dehydrogenase kinases (Pdk), promotes glomalin synthesis. We reasoned that Pdk supports S-2HG production by diverting glutamine-derived 2-oxoglutarate to Ldhα and, consistent with this, dichloroacetate (DCA) abrogates hypoxia-induced 2HG accumulation (Extended Data Fig. 2s–u). Pdk1 expression is impaired in hypoxic Hif1a−/−/CD8 T lymphocytes (Extended Data Fig. 2j), as is phosphorylation of Pdh (Extended Data Fig. 2s), and re-expression of Pdk1 in this context increases S-2HG in hypoxia (Fig. 2l, Extended Data Fig. 2o). Inhibition of Pdk activity also impedes hypoxia-induced increases in the glutamate pool (Extended Data Fig. 2v). Hence, Hif-1α drives S-2HG production in hypoxic CD8 T lymphocytes through the Pdk–Pdh signalling axis and Ldhα induction (Extended Data Fig. 4a).

**S–2HG alters CD8 T-cell differentiation**

S-2HG inhibits 2-oxoglutarate-dependent dioxygenases. Consistent with this, Hif-1α is stabilized in normoxic (Extended Data Fig. 5a) and hypoxic (Extended Data Fig. 5b) CD8 T lymphocytes by treatment with cell-permeable S-2HG, suggesting that S-2HG augments HIF signalling in normoxia and hypoxia. Additionally, there is increased phosphorylation of Pdh-E1α (Extended Data Fig. 5a, b), elevated glucose uptake, lactate secretion (Extended Data Fig. 5c) and Vegfa production (Extended Data Fig. 5d), indicating Hif-1α-dependent effects. As Hif-1α supports effector functions in CD8 T lymphocytes, we reasoned that S-2HG promotes effector differentiation through Hif-1α. However, unexpectedly, there is suppression of effector cytokine production (Extended Data Fig. 5e) and decreased cytotoxicity (Extended Data Fig. 5f). Furthermore, S-2HG restrains cell expansion (Extended Data Fig. 5g) and there is a clear increase in apoptosis at doses greater than 300 μM (Extended Data Fig. 5h, i). Further characterization revealed decreased secretion of IFN-γ (Extended Data Fig. 5j), yet elevated production of IL-2 (Fig. 3a), with increased viability in the absence of IL-2 supplementation (Extended Data Fig. 5k). This possibly reflects an autocrine pro-survival effect. These effects are robustly mediated at the transcriptional level after prolonged treatment with S-2HG (Fig. 3b, Extended Data Fig. 5l) and are independent of HIF-1α (Fig. 3a, Extended Data Fig. 5j, k).

We then characterized the phenotype of cells that had been treated with a prolonged course of S-2HG. There is increased expression of CD62L (Fig. 3c, Extended Data Fig. 5m) and this is reversible upon withdrawal of treatment (Extended Data Fig. 5n). The effect does not occur when treating cells cultured in vehicle for 7 days (Extended Data Fig. 5n), demonstrating that S-2HG treatment of newly activated cells maintains this phenotypic marker. Importantly, CD62L downregulation does not occur when Hif-1α is absent, which masks the effect of S-2HG on CD62L following Hif-1α deletion (Extended Data Fig. 5o). Hif-2α is dispensable for CD62L maintenance in response to S-2HG (Extended Data Fig. 5p). With S-2HG, CD62L maintenance depends on the level of antigenic stimulation (Fig. 3d). Furthermore, S-2HG-treated cells express more IL-2 (Fig. 3c), CD44, 41BB, Eomes and less PD-1 in a Hif-1α-dependent manner (Extended Data Fig. 5q).

To determine the role of endogenously produced S-2HG, overexpression of L2hgdh (Extended Data Fig. 5r), a dehydrogenase that oxidizes S-2HG, was performed. Overexpression of L2hgdh promotes the downregulation of CD62L in both 21% and 1% oxygen (Fig. 3e), indicating that endogenously produced S-2HG regulates CD62L expression. Furthermore, L2hgdh overexpression leads to an increase in the proportion of Klrg1hi cells, which are decreased in the presence of exogenous S-2HG (Fig. 3f). Conversely, successful short hairpin RNA (shRNA)-mediated knockdown of L2hgdh by hairpin 3 (Extended Data Fig. 5s) increases endogenous S-2HG levels (Fig. 3g), especially in 1% oxygen, promoting maintenance of CD62L (Fig. 3h). Knockdown of L2hgdh blocks loss of CD62L in response to low oxygen (Fig. 3h). The same effect is seen with CD127 in low oxygen (Extended Data Fig. 5i). These data demonstrate that L2hgdh activity regulates the expression of key phenotypic markers of CD8 T lymphocytes, by controlling endogenous S-2HG levels. Transcriptionally, S-2HG treatment increases expression of Eomes, Ccr6, Bcl6, Sell (Cd62l) and Tcf7,
with repression of Pdml, after 7 days (Extended Data Fig. 5u). This transcriptional program is similar to gene expression changes in memory CD8\(^+\) T lymphocytes, suggesting that S-2HG treatment of CD8\(^+\) T lymphocytes \textit{ex vivo} may enhance long-term persistence and survival in the context of adoptive cell transfer\(^3\).

We thus co-transferred CFSE-labelled vehicle and S-2HG treated CD45.1.1 or CD45.1.2 OT-I CD8\(^+\) T lymphocytes into lymphocyte-depleted mice (Extended Data Fig. 6a) to assess their capacity for homeostatic proliferation\(^34,35\). S-2HG-treated cells display greater homeostatic proliferation (Fig. 4a, b), with more cells dividing more than 5 times (Fig. 4c). We then assessed the capacity of S-2HG treated cells to persist in lymphoporeate mice. Adoptively transferred ovalbumin-specific CD45.1 OT-I CD8\(^+\) T lymphocytes, pre-treated with S-2HG, showed markedly enhanced persistence 30 days after transfer (Fig. 4d), expressing elevated CD44, CD127 and Bcl-2 levels relative to naive cells\(^36,37\) (Fig. 4e). In response to vaccination with ovalbumin-derived protein SIINFEKL-loaded dendritic cells, S-2HG-treated OT-I CD8\(^+\) T lymphocytes robustly recall (Fig. 4f and Extended Data Fig. 6b, c). Consistent with this, OT-I CD8\(^+\) T lymphocytes, pre-treated with S-2HG are more proficient at controlling tumour growth \textit{in vivo} in both lymphocyte-depleted (Fig. 4g) and lymphoporeate (Fig. 4h) mice. These data demonstrate that S-2HG treatment \textit{ex vivo} maintains cells in a state with increased proliferative and survival capacity, when transferred \textit{in vivo}, that is otherwise decreased by effector differentiation.

**Figure 4** S-2HG treatment promotes \textit{in vivo} homeostatic renewal, persistence and anti-tumour capacity of transferred cells. a, Recovery of co-transferred CD45.1\(^+\) OT-I cells, from spleens (n = 6). b, \textit{In vivo} CFSE levels in cells from a, c. Percentage of cells in a that divided 0–9 times \textit{in vivo}. d, Representative flow cytometry plots and associated statistics of recovered cells from spleens (n = 6). e, Representative analysis of cells in d relative to naive cells (n = 6). f, Recovery of co-transferred cells, from spleens, lymph nodes and livers of vaccinated mice (n = 6). g, Lymphocyte-depleted mice bearing EG7-OVA tumours treated with no T cells (n = 7) or OT-I cells treated with or without (n = 6) S-2HG. Error bars denote s.e.m. h, Lymphoporeate mice bearing EG7-OVA tumours treated with no T cells or OT-I cells treated with or without S-2HG (n = 6). Error bars denote s.e.m. Paired t-test (a, b, f), unpaired t-test (d), one-way ANOVA (g, h). Error bars denote s.d. (not in g and h). Each dot in a, b, d and f represents a mouse. *P < 0.05, **P < 0.01, ***P < 0.001; NS, not significant.

\section*{S-2HG alters methylation in CD8\(^+\) T cells}

Mechanistic target of rapamycin (mTOR) is a modifier of CD8\(^+\) T-lymphocyte differentiation; however, we do not observe mTOR inhibition\(^36,38\) at doses of S-2HG needed for this to occur (Extended Data Fig. 7). S-2HG treatment \textit{ex vivo} may be selecting cells that express higher levels of anti-apoptotic genes. Two critical anti-apoptotic genes implicated in CD8\(^+\) T-lymphocyte survival are Bcl-2 and Bcl-XL\(^39,40\). These genes are not induced by S-2HG treatment (Extended Data Fig. 8a–d). Moreover, overexpression of Bcl-2 or Bcl-XL (Extended Data Fig. 8e) does not influence the expression of CD62L, CD44 or CD127 in the presence or absence of S-2HG (Extended Data Fig. 8f–i). Indicating that S-2HG is exerting these phenotypic changes independently of Bcl-2 or Bcl-XL.

Inhibition of 2-oxoglutarate-dependent dioxygenases that demethylate histones (Jumonji C containing proteins) or oxidise 5-methylcytosine in DNA (Ten-eleven translocation (Tet) proteins) may mediate the effect of S-2HG\(^32,41–43\). S-2HG alters global levels of various histone methylation marks (Extended Data Fig. 9a); in particular, di- and tri-methylation on H3K27 are reciprocally altered, indicating inhibition of H3K27me3 demethylation (Fig. 5a,b, Extended Data Fig. 9b). The H3K27me3/2/3 demethylase Utx (also known as Kdm6a) is an important regulator of thymocyte differentiation\(^44\); changes in H3K27me3 levels correlate with genes associated with regulation of CD8\(^+\) T lymphocyte differentiation\(^45\). Global levels of H3K27me3 in CD8\(^+\) T lymphocytes are reduced following activation, but remain high with S-2HG treatment in a HIF-1\(\alpha\)-independent manner (Fig. 5c, Extended Data Fig. 9c). Utx is induced following TCR stimulation (Extended Data Fig. 9d) and inhibition of Utx reproduces the effect of S-2HG treatment on CD62L expression (Fig. 5d, Extended Data Fig. 9e, f). In \textit{ vivo}, levels...
of H3K27me3 are highest in central memory (CD62L<sup>hi</sup>CD44<sup>hi</sup>) and naive (CD62L<sup>lo</sup>CD44<sup>hi</sup>) CD8<sup>+</sup> T lymphocytes, relative to effectors (CD62L<sup>lo</sup>CD44<sup>hi</sup>) (Fig. 5e). To determine whether histone methylation changes occur at the transcription start site (TSS) of CD62L with S-HG treatment, we performed chromatin immunoprecipitation (ChIP)–PCR for H3K27me3, H3K4me3 and RNA polymerase II (RNA Pol II), on naive and activated CD8<sup>+</sup> T lymphocytes cultured with or without S-2HG (Fig. 5f, Extended Data Fig. 9g). We could find no enrichment for H3K27me3 at or around the TSS. However, naive and S-2HG-treated CD8<sup>+</sup> T lymphocytes have higher enrichment for H3K4me3 at the TSS that is reduced in vehicle-treated cells. Additionally, S-2HG treated CD8<sup>+</sup> T lymphocytes have markedly higher RNA Pol II binding than both naive and vehicle-treated cells. Thus, S-2HG promotes CD62L transcription directly through enrichment of H3K4me3 at the TSS and indirectly through preservation of H3K27me3 elsewhere in the genome.

Total levels of 5-methylcytosine (5mC) in genomic DNA are largely unchanged by TCR triggering (Fig. 6a). However, total levels of 5-hydroxymethylcytosine (5hmC) decrease following TCR triggering (Fig. 6a). 5mC removal in genomic DNA can occur through Tet-mediated oxidation (active) as well as DNA replication (passive).<ref>25, 46</ref> S-2HG treatment induces small changes in total 5hmC and 5mC in a time-dependent manner (Fig. 6b, c); at day 3, there are marginally higher levels of 5hmC (statistically non-significant) with no changes in 5mC (Fig. 6b, c). This is probably due to decreased proliferation in the presence of S-2HG (Extended Data Fig. 5g). Following sustained treatment, at days 7 and 9, cells have less 5hmC and more 5mC (Fig. 6b, c). 5mC changes did not reach statistical significance at any time point; changes in 5hmC were statistically significant at day 7 only. Nonetheless, the changes at days 7 and 9 are consistent with inhibition of Tet proteins in the presence of sustained S-2HG treatment.

Tet2 regulates CD4<sup>+</sup> T-lymphocyte function and is inhibited by 2HG<ref>47, 48</ref>. Knockdown of Tet2 recapitulates the effect of S-2HG on CD62L, indicating that Tet2 also contributes to CD8<sup>+</sup> T-lymphocyte effector differentiation (Fig. 6d, Extended Data Fig. 10a), implicating DNA demethylation as an added epigenetic modifier of CD8<sup>+</sup> T-cell differentiation<ref>12</ref>. We performed both 5hmC and 5mC DNA immunoprecipitation (DIP)–PCR around the TSS of CD62L (Fig. 6e, Extended Data Fig. 10b). Naïve cells have the highest and lowest enrichment for 5hmC and 5mC respectively, whereas S-2HG-treated cells display the opposite pattern. Vehicle-treated cells have an intermediate level of these two marks.

**Discussion**

Our data support a model in which both Utx and Tet2 contribute to effector differentiation of CD8<sup>+</sup> T lymphocytes in vitro. The activity of these epigenetic modifiers is altered by S-2HG in a fashion that inhibits effector differentiation.

TCR-triggering induced loss of 5hmC in genomic DNA, whereas 5mC levels were relatively stable (Fig. 6a). 5mC levels stabilized by four days after activation (Fig. 6a) and S-2HG treatment produced reciprocal changes in 5mC and 5hmC levels at later time points (Fig. 6b, c). We investigated 5mC and 5hmC presence at and around the TSS of CD62L (Fig. 6e). S-2HG treatment causes reciprocal 5mC increases and 5hmC decreases in this region consistent with inhibition of Tet proteins and transcriptional repression<ref>17</ref>, yet there is robust expression of CD62L with S-2HG treatment (Fig. 3c–e, h, Extended Data Fig. 5m–p). Inhibition of Tet2 maintains CD62L expression (Fig. 6d, Extended Data Fig. 10a), indicating that S-2HG-induced DNA methylation changes elsewhere in the genome may indirectly promote CD62L expression.

We also investigated H3K4me3 and H3K27me3 patterns at the TSS of CD62L (Fig. 5f). S-2HG-treated and naive CD8<sup>+</sup> T lymphocytes have high enrichment for the active H3K4me3 mark<ref>30</ref> at the TSS that is lost in vehicle-treated cells. In S-2HG-treated cells, this is accompanied by binding of RNA Pol II. Despite global increases in H3K27me3 with S-2HG (Fig. 5a–c), the lack of H3K27me3 at the TSS of CD62L is not surprising, as this mark is associated with repression<ref>49</ref>. Nevertheless, inhibition of Utx promotes CD62L maintenance (Fig. 5d, Extended Data Fig. 9e, f), indicating that H3K27me3 deposition at other genomic sites can indirectly promote CD62L expression. Owing to the relationship between histone methylation and other marks such as histone acetylation, modulation of the latter may have similar effects to those seen with S-2HG treatment<ref>30</ref>

Adaptively transferred cells treated with S-2HG ex vivo have an increased capacity to proliferate and persist in vivo, with enhanced anti-tumour efficacy (Fig. 4), demonstrating a new strategy to improve persistence of adoptive cell therapies for cancer. The data presented uncover a metabolic–epigenetic axis that controls aspects of T-cell fate. Factors regulating endogenous S-2HG levels, such as HIF signalling, TCR triggering, L2hdgh activity and potentially others, can alter the differentiation of CD8<sup>+</sup> T lymphocytes and thus shape the immune response.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

**Data Availability** The data that support the findings are available from the corresponding author upon reasonable request.

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Supplementary Information is available in the online version of the paper.

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Author Contributions P.A.T. and A.P. designed experiments and carried out ex vivo and in vivo experiments, analysed the data, and wrote the manuscript. A.T.P., A.D., A.W.G. and R.S.J. carried out the initial metabolome survey. A.W.G. was funded by the NIH (A1096852, A1072117). A.P. was funded by Marie-Curie IEF. A.T.P. was funded by UCSD NIH Grant (ST32GM07240-36). A.W.G. was funded by the NIH (A1096852, A1072117). Leukemia and Lymphoma Society, and Pew Scholars Fund. K.L.L., J.Y., G.S.C. and L.P. were funded by the Singapore National Research Foundation and Singapore Ministry of Education, the NRMC Clinician Scientist (NRMC/CIRG/1389/2014) and the Swedish Research Council. R.S.J. and co-workers are funded by the Wellcome Trust (grant WT092738MA), the Swedish Cancer Foundation (Cancerfonden), and the Swedish Research Council (Vetenskapsrådet). This work is dedicated to the memory of Lorenz Poellinger.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.S.J. (rsj33@cam.ac.uk).
Extended Data Figure 1 | VHL–HIF-1α regulate central carbon metabolism and 2HG levels in CD8+ T lymphocytes. a, Illustration of central carbon metabolism in CD8+ lymphocytes, including glycolysis and the tricarboxylic acid cycle, depicting relative levels of detected metabolites between Vhlfl/fl (n = 5), Vhlfl/flLckcre (n = 5) and Hif-1αfl/flVhlfl/flLckcre (n = 3) CD8+ T-lymphocyte groups. b, Glucose consumption and lactate production in Vhlfl/fl and Vhlfl/flLckcre CD8+ T lymphocytes 7 days after activation with anti-CD3 and anti-CD28 antibodies (n = 4 mice per genotype). c, Immunoblot analysis for Hif-1α and LaminB1, using nuclear extracts prepared from Vhlfl/fl and Vhl−/− CD8+ T lymphocytes cultured in 21% oxygen. d, Rank of metabolite loadings in PC1 from PCA. e, Immunoblot analysis for HIF-1α, HIF-2α and β-tubulin, on whole-cell extracts prepared from RCC4 and 786-O renal cancer cell lines, with and without expression of functional VHL. f, Deletion efficiency of Vhl in Vhlfl/fl MEFs following infection with adeno-Cre virus, n = 3 individual preparations. Accompanying immunoblot analysis for HIF-1α and β-tubulin, on whole-cell extracts. Two-tailed t-test (b), one-way ANOVA for multiple comparisons (a). Error bars denote s.d. and each dot represents an individual mouse in a and b. *P < 0.05, **P < 0.01, ***P < 0.001. For immunoblot source images, see Supplementary Fig. 1.
Extended Data Figure 2. For Intracellular 2HG Concentration

Example Calculation For Intracellular 2HG Concentration

Total amount of 2HG measured (nmol): 2.646

Amount per cell: 2.646 (nmol) / 2.18x10^13 (mol/cell)

Intracellular concentration: 2.646x10^-13 / 5.53656x10^-10 = 2.18825 (mM)

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Extended Data Figure 2 | See next page for caption.
Extended Data Figure 2 | Hif-1α-dependent metabolic alterations underlie S-2HG production in CD8⁺ T lymphocytes. a, Example calculation of intracellular 2HG concentration. b, ¹H-NMR analysis for 2HG from CD8⁺ T lymphocytes cultured as in Fig. 2b. c, Deletion efficiency of Hif1a or Hif2a in CD8⁺ T lymphocytes, isolated from Hif1afl/fl dLckcre or Hif2afl/fl dLckcre mice (n = 4 mice). d, Total 2HG levels, normalized to viable cell count or protein content, in Hif1afl/fl and Hif1afl/fl dLckcre (d) and Hif2afl/fl and Hif2afl/fl dLckcre (e) CD8⁺ T lymphocytes cultured as in Fig. 2b (n = 4 mice per genotype). e, Illustration outlining the workflow for metabolite extraction, deletion efficiency and viability experiments in Hif1afl/fl, Hif1afl/fl dLckcre, Hif2afl/fl and Hif2afl/fl dLckcre CD8⁺ T lymphocytes. Also shown are viability measurements at day 4 (n = 4 mice per genotype). g, Total amount of 2HG in Hif1afl/fl (n = 6) and Hif1afl/fl dLckcre (n = 7) CD8⁺ T lymphocytes, at indicated times following activation (n ≥ 4 mice per time point). h, Heat map indicating qPCR measurement of expression of enzymes involved in central carbon metabolism in CD8⁺ T lymphocytes cultured as in Fig. 2b (n = 4 mice per condition). i, Liquid chromatography–tandem mass spectrometry (LC–MS/MS) quantification of total intracellular succinate, fumarate and malate levels in CD8⁺ T lymphocytes isolated from C57BL/6J mice and cultured as in Fig. 2b (n = 7 mice). j, Heat map indicating qPCR measurement, in Hif1afl/fl (n = 4) and Hif1afl/fl dLckcre (n = 3) CD8⁺ T lymphocytes growing in 1% oxygen, of expression of enzymes implicated in the hypoxic production of S-2HG. k, qPCR validation of shRNA-knockdowns in CD8⁺ T lymphocytes isolated from C57BL/6J mice. l–n, LC–MS/MS quantification of S- and R-2HG in CD8⁺ T lymphocytes isolated from C57BL/6J mice, with shRNA-mediated knockdown of Mdh1 (l), Mdh2 (m) and Ldh (n) (n = 4 pools of 4 mice per pool). o, Validation of Pdk1–Flag and Ldha–Flag expression in Hif1afl/fl dLckcre CD8⁺ T lymphocytes by immunoblot analysis for Flag. p, LC–MS/MS quantification of total intracellular glutamate levels in Hif1afl/fl, Hif1afl/fl dLckcre, Hif2afl/fl and Hif2afl/fl dLckcre CD8⁺ T lymphocytes cultured as in Fig. 2b; n = 7 mice. q, r, LC–MS/MS quantification of total intracellular glutamate levels in Hif1afl/fl, Hif1afl/fl dLckcre, Hif2afl/fl and Hif2afl/fl dLckcre CD8⁺ T lymphocytes cultured as in Fig. 2b; n = 4 mice per genotype. s, Immunoblot of cytosolic fractions for phospho-Pdh-E1α (S232) and total Pdh-E1α in CD8⁺ T lymphocytes cultured in 1% oxygen in the presence of the indicated concentration of DCA for 48h. t–v, Total intracellular concentration of 2HG (t), 2HG normalized to viable cell count or protein content (u) and glutamate (v) in CD8⁺ T lymphocytes from C57BL/6J mice cultured as in Fig. 2b and treated with 5mM DCA for the latter 48 h of culture (n = 4 mice). Two-way ANOVA for grouped data (d–f, q, r, t–v). Paired t-test for matched comparisons (i, p), one-way ANOVA for multiple matched comparisons (g, l–n). Error bars denote s.d. and each dot represents an individual mouse in g, i and p. NS, non-significant, *P < 0.05, **P < 0.01, ***P < 0.0001. Experiments were performed with indicated numbers of mice from multiple occasions. For immunoblot source images, see Supplementary Fig. 1.
Extended Data Figure 3 | Naive and expanding primary CD8+ T lymphocytes do not possess mutations in \( \text{Idh1} \) or \( \text{Idh2} \) that can explain the presence of high levels of 2HG. a, Illustration outlining the workflow for mutational analysis of \( \text{Idh1} \) and \( \text{Idh2} \). b, Sanger sequencing chromatograms validating the presence of wild-type \( \text{Idh1} \) as compared to the C57BL/6J NCBI reference sequence. c, Alignment of mouse and human IDH1 protein indicating conservation of active site arginine residues. d, Sanger sequencing chromatograms validating the presence of wild-type \( \text{Idh2} \) as compared to the C57BL/6J NCBI reference sequence. e, Alignment of mouse and human IDH2 protein indicating conservation of active site arginine residues.
Extended Data Figure 4 | Kinetics of 2HG labelling in 21% and 1% oxygen, by U-13C-glucose and U-13C-glutamine. a, Proposed mechanism by which Hif-1α controls S-2HG production in CD8+ T lymphocytes and 13C-labelling strategy using U-13C-glucose (m+6) and U-13C-glutamine (m+5) to label endogenous 2HG. Red and green represent pathways promoted and inhibited respectively by HIF-1α in hypoxia. b, Isotopologue distribution of 2HG (as a percentage of the total pool) in CD8+ T lymphocytes, after labelling with U-13C-glucose for 1, 5 and 24 h in both 21% and 1% oxygen conditions (n = 3 mice per time point). c, Isotopologue distribution of 2HG (as a percentage of the total pool) in CD8+ T lymphocytes, after labelling with U-13C-glutamine for 1, 5 and 24 h in both 21% and 1% oxygen conditions (n = 3 mice per time point). Error bars, s.d.
Extended Data Figure 5 | See next page for caption.
Extended Data Figure 5 | S-2HG treatment promotes Hif-1α stability and alters the phenotypic and functional properties of CD8+ T lymphocytes in a Hif-1α-independent manner. a, b, Immunoblot analysis of nuclear and cytosolic fractions, prepared from CD8+ T lymphocytes cultured in 21% (a) and 1% (b) oxygen, for Hif-1α, Hdc1, phospho-Pdh-E1α (S232) and total Pdh-E1α. Cells were activated for 48 h with anti-CD3 and anti-CD28 antibodies and then expanded for a further 4 days in the presence of IL-2, followed by treatment with the indicated concentration of S-2HG for 16 h. The arrow indicates Hif-1α protein. c, Glucose consumption and lactate production of C57BL/6J, Hif1afl/fl (n = 12) and Hif1afl/fl dLckcre (n = 4) CD8+ T lymphocytes treated with or without 500 μM S-2HG-octyl ester. d, Vegfa production of wild-type C57BL/6J, Hif1afl/fl (n = 16) and Hif1afl/fl dLckcre (n = 4) CD8+ T lymphocytes treated with or without 500 μM S-2HG-octyl ester. e, Representative flow cytometry plots of IFN-γ versus TNF-α in SIINFEKL re-stimulated OT-I CD8+ T lymphocytes, as a function of increasing doses of S-2HG-octyl ester for 7 days. Associated quantification and statistics are shown in the graphs below. f, Specific killing of EG7-OVA cells by OT-I CD8+ T lymphocytes (n = 3 mice per condition). g, CFSE dilution assay (n = 4 mice per condition) at day 3 of CD8+ T lymphocytes activated with anti-CD3 and anti-CD28 antibodies and cultured with or without 500 μM S-2HG-octyl ester from day 0. Associated quantification and statistics are shown in the graph on the right. h, Viability and annexin V assay of CD8+ T lymphocytes treated with increasing S-2HG doses for 4 days (n = 4 mice). i, Viability of CD8+ T lymphocytes cultured with 300 μM S-2HG-octyl ester for the indicated number of days (n = 4 mice). j, Amount of IFN-γ protein in the media of wild-type C57BL/6J, Hif1afl/fl (n = 8) and Hif1afl/fl dLckcre (n = 4) CD8+ T lymphocytes treated for 24 h with or without 500 μM S-2HG-octyl ester. k, Viability of Hif1afl/fl (n = 8) and Hif1afl/fl dLckcre (n = 4) OT-I CD8+ T lymphocytes activated with 1,000 nM SIINFEKL peptide and cultured for 7 days with or without 500 μM S-2HG-octyl ester in the absence of IL-2 supplementation from day 0. l, Expression of Ifng mRNA in CD8+ T lymphocytes treated for either 24 h or 7 days with or without 500 μM S-2HG-octyl ester. n = 4 mice per group. m, CD44 and CD62L surface expression on OT-I CD8+ T lymphocytes treated with increasing doses of S-2HG for 7 days. Cells were activated with 1,000 nM SIINFEKL peptide; n = 3 mice. Gated on live, CD8+ cells. n, Illustration outlining the workflow for the experiment. Percentage of CD62L+ CD8+ T lymphocytes, treated for 7 days with 500 μM S-2HG-octyl ester (left) or vehicle (right), followed by washout or maintenance of the compound and follow up every three days, for 9 more days (n = 4 mice). Gated on live, CD8+ cells. o, p, CD44 and CD62L surface expression on Hif1afl/fl and Hif1afl/fl dLckcre (o) or Hif2afl/fl and Hif2afl/fl dLckcre (p) CD8+ T lymphocytes treated with or without 500 μM S-2HG-octyl ester for 1, 7 and 10 days following treatment. Data are representative of 3 (o) or 2 (p) mice per genotype. Gated on live, CD8+ cells. q, Flow cytometric characterization of indicated phenotypic markers on Hif1afl/fl and Hif1afl/fl dLckcre (n = 4) CD8+ T lymphocytes treated for 7 days with 500 μM S-2HG-octyl ester. Gated on live, CD8+ cells. r, Validation of L2hgdh–Flag expression in CD8+ T lymphocytes from C57BL/6J mice by immunoblot analysis for Flag. The arrow indicates L2hgdh–Flag protein. s, qPCR validation of L2hgdh knockdown in CD8+ T lymphocytes isolated from C57BL/6J mice. t, CD127 surface expression in response to L2hgdh knockdown (n = 4). Representative flow cytometry histogram of CD127 surface levels on transduced (GFP+) CD8+ T lymphocytes in response to shScramble or shL2hgdh 3 in 21% or 1% oxygen is shown on the right. u, qPCR quantification of Prdm1, Sell, Ieomes, Tcf7, Bcl6 and Ccr6 expression in CD8+ T lymphocytes treated for 1 or 7 days with or without 500 μM S-2HG-octyl ester. Paired t-test for matched comparisons (g) and two-way ANOVA for grouped data (c, d, j, k, l, q). One-way ANOVA for multiple comparisons (i, n, t). Error bars denote s.d. and each dot in c, d, j, k, l, q, t represents an individual mouse. NS, non-significant, *P < 0.05, **P < 0.01, ***P < 0.001. gMFI, geometric mean fluorescence intensity. Experiments were performed with indicated numbers of mice from multiple occasions. For immunoblot source images, see Supplementary Fig. 1.
Extended Data Figure 6 | *Ex vivo* treatment of CD8<sup>+</sup> T lymphocytes with S-2HG promotes *in vivo* homeostatic proliferation and recall of adoptively transferred cells. a, Diagram outlining the homeostatic proliferation experiments in Fig. 4a–c. Representative flow cytometry plots are shown for each pool before and after adoptive transfer. Flow cytometry plots show viable CD8<sup>+</sup> cells. b, Diagram outlining the recall experiments in Fig. 4f. c, Representative flow cytometry plots of recalling CD45.1<sup>+</sup>CD8<sup>+</sup> T lymphocytes in indicated organs on day 7 after vaccination (day 37 after transfer).
Extended Data Figure 7 | S-2HG does not inhibit mTOR signalling at the doses necessary for the formation of memory-like CD8⁺ T lymphocytes. Immunoblot analysis on cytosolic extracts for mTOR signalling in CD8⁺ T lymphocytes treated with the indicated doses of S-2HG for 24 h. For immunoblot source images, see Supplementary Fig. 1.
Extended Data Figure 8 | S-2HG does not induce Bcl-2 or Bcl-XL that can explain the in vivo persistence of adoptively transferred CD8+ T lymphocytes. a, qPCR quantification of Bcl2 and Bcl2l1 (Bcl-XL) mRNA levels in response to 500μM S-2HG-octyl ester treatment for either 1 or 7 days (n = 4 mice). b, Immunoblot analysis for Bcl-2 and Bcl-XL protein in response to increasing doses of S-2HG-octyl ester for 9 days. c, qPCR quantification of Bcl2 and Bcl-XL mRNA levels in response to 300μM S-2HG-octyl ester treatment for either 1, 7 or 9 days (n = 4 mice). d, Representative flow cytometry histograms of Bcl-2 and Bcl-XL abundance in CD8+ T lymphocytes treated with 300μM S-2HG-octyl ester for 9 days. Quantification and associated statistics are shown in the graph on the right (n = 3 mice). e, Immunoblot analysis confirming the expression of Bcl-XL-Flag and Bcl-2-Flag in OT-1 in CD8+ T lymphocytes. f–h, CD62L (f), CD127 (g) and CD44 (h) surface expression in OT-1 CD8+ T lymphocytes transduced with retrovirus expressing either Bcl-2–Flag or Bcl-XL–Flag and treated with the indicated concentration of S-2HG-octyl ester for 7 days (n = 2 mice). i, Representative flow cytometry histograms of CD62L, CD127 and CD44 surface expression in OT-1 CD8+ T lymphocytes transduced with retrovirus expressing either Bcl-2–Flag or Bcl-XL–Flag and treated with the indicated concentration of S-2HG-octyl ester for 7 days. The associated statistics of these flow cytometry data are shown in f, g and h. **P < 0.01, NS, non-significant. Paired t-test for matched comparisons (d) and two-way ANOVA for grouped data (a). One-way ANOVA of matched samples for multiple comparisons (c, f, g, h). Error bars denote s.d. and each dot in a and c represents an individual mouse. Experiments were performed with indicated numbers of mice from at least two occasions. For immunoblot source images, see Supplementary Fig. 1.
Extended Data Figure 9 | S-2HG induces global histone H3 methylation changes in CD8⁺ T lymphocytes. a, Immunoblot analysis on nuclear extracts for histone H3 methylation marks in activated CD8⁺ T lymphocytes treated with the indicated doses of S-2HG for 7 days. b, Representative flow cytometry histograms of H3K27me3 staining as a function of increasing S-2HG-octyl ester concentration. c, H3K27me3 staining in CD8⁺ T lymphocytes treated with or without 500 μM S-2HG-octyl ester and stained with or without fluorophore-conjugated C36B11 antibody. d, qPCR measurement for expression of Utx in unstimulated and stimulated CD8⁺ T lymphocytes; n = 4 mice. Expression for Utx is displayed for each mouse individually. e, Representative flow cytometry plots of CD44 versus CD62L expression, with associated statistics, on activated CD8⁺ T lymphocytes after 4 days of treatment with 500 μM S-2HG-octyl ester or 1 μM GSKJ4. Gated on live, CD8⁺ cells. n = 3 mice. f, Representative flow cytometry plots of CD44 versus CD62L expression on CD8⁺ T lymphocytes with shRNA-mediated knockdown of Utx, 7 days after transduction. Gated on live CD8⁺ GFP⁺ cells. Graph on right shows knockdown hairpin fidelity for Utx. g, IgG control ChIP–qPCR for H3K4me3, H3K27me and RNA Pol II at and around the TSS for CD62L, in freshly isolated naive or activated CD8⁺ T lymphocytes treated with or without 500 μM S-2HG-octyl ester for 7 days. Each profile shows the fold change over the non-binding control primer. Each dot represents an individual primer pair. A pool of n = 6 mice was used for each condition and error bars denote s.e.m. One-way ANOVA for multiple matched comparisons (e). Each dot in e represents an individual mouse. Error bars (e, f) denote s.d. **P < 0.01. Experiments were performed with indicated numbers of mice from at least two occasions. For immunoblot source images, see Supplementary Fig. 1.
Extended Data Figure 10 | S-2HG induces global changes in the content of 5hmC and 5mC in genomic DNA of CD8⁺ T-lymphocyte genomic DNA. a, Representative flow cytometry plots of CD44 versus CD62L expression on CD8⁺ T lymphocytes with shRNA-mediated knockdown of Tet2, 7 days after transduction. Gated on live CD8⁺ GFP⁺ cells. Graph on right shows knockdown hairpin fidelity for Tet2 and error bars denote s.d.

b, IgG control DIP-qPCR for 5mC and 5hmC at and around the TSS for CD62L, in freshly isolated naive or activated CD8⁺ T lymphocytes treated with or without 500 μM S-2HG for 7 days. Each profile shows the fold change over the non-binding control primer. Each dot represents an individual primer pair. A pool of n = 6 mice was used for each condition and error bars denote s.e.m.