CTLA-4 blockade drives loss of T_{reg} stability in glycolysis-low tumours

Limiting metabolic competition in the tumour microenvironment may increase the effectiveness of immunotherapy. Owing to its crucial role in the glucose metabolism of activated T cells, CD28 signalling has been proposed as a metabolic biosensor of T cells. By contrast, the engagement of CTLA-4 has been shown to downregulate T cell glycolysis. Here we investigate the effect of CTLA-4 blockade on the metabolic fitness of intra-tumour T cells in relation to the glycolytic capacity of tumour cells. We found that CTLA-4 blockade promotes metabolic fitness and the infiltration of immune cells, especially in glycolysis-low tumours. Accordingly, treatment with anti-CTLA-4 antibodies improved the therapeutic outcomes of mice bearing glycolysis-defective tumours. Notably, tumour-specific CD8$^{+}$ T cell responses correlated with phenotypic and functional destabilisation of tumour-infiltrating regulatory T (T_{reg}) cells towards IFN$\gamma$- and TNF-producing cells in glycolysis-defective tumours. By mimicking the highly and poorly glycolytic tumour microenvironments in vitro, we show that the effect of CTLA-4 blockade on the destabilisation of T_{reg} cells is dependent on T_{reg} cell glycolysis and CD28 signalling. These findings indicate that decreasing tumour competition for glucose may facilitate the therapeutic activity of CTLA-4 blockade, thus supporting its combination with inhibitors of tumour glycolysis. Moreover, these results reveal a mechanism by which anti-CTLA-4 treatment interferes with T_{reg} cell function in the presence of glucose.

Cellular energy metabolism reprogramming is a crucial hallmark of cancer. High glucose consumption and lactate production by tumour cells restrict nutrient availability in the tumour microenvironment for effector T cells, which also rely on glycolysis to proliferate and function. We corroborated the negative effect of tumour glucose metabolism on T cell function using the highly glycolytic mouse mammary carcinoma 4T1. We found that 4T1 cells, cultured either alone or with activated T cells, consume more glucose and produce more lactate than activated T cell monocultures. In the presence of 4T1 cells, or similar non-toxic concentrations of exogenous lactate, activated T cells were significantly less activated and viable (Extended Data Fig. 1b), which indicates the negative effect of tumour glycolysis on T cell function and potentially response to immunotherapy. To explore the role of tumour glycolysis in response to immunotherapy, we correlated tumour immune infiltration and glycolysis before and after CTLA-4 blockade using RNA sequencing (RNA-seq) data from patients with advanced melanoma receiving ipilimumab treatment.

At baseline, the expression of glucose catabolism genes was frequently negatively correlated with the infiltration of major immune cell subsets (Fig. 1a, left, Extended Data Fig. 1e), in agreement with previous observations, which suggests that tumour glycolysis may contribute to a non-inflamed tumour phenotype. After treatment with ipilimumab, these negative correlations were alleviated, with some glycolysis-related genes becoming positively correlated with most immune cell subsets (Fig. 1a, right, Extended Data Fig. 1f). However, key glycolytic genes such as Ldha, which encodes the crucial enzyme subunit for lactate production, and Scl6a1, which encodes the lactate transporter MCT1, remained inversely correlated with immune infiltrates after ipilimumab treatment (Fig. 1a, right)—which suggests that anti-CTLA-4 alone may not be sufficient to potentiate immune cell fitness in highly glycolytic tumours that overexpress these genes. We investigated these effects in a more controlled setting, by using a glycolysis-defective 4T1 variant...
in tumour cells (Extended Data Fig. 2a, e), we observed positive correlations between Ldha expression and the immune infiltrate, which were eventually amplified by CTLA-4 blockade, pointing to activation of glycolysis in immune cells (Fig. 1c, right). This suggested that glycolysis-low tumours may be more responsive to CTLA-4 blockade, whereas the treatment of glycolysis-high tumours may require dampening tumour glycolysis together with anti-CTLA-4.

**LDHA-KD tumours respond better to anti-CTLA-4**

We next investigated the effect of slowing tumour glycolysis on the efficacy of CTLA-4 blockade and the underlying mechanisms.
Plots of circulating anti-tumour AH1-specific CD8+ T cells before (pre) and one week after (post) 4T1-Sc (left) or 4T1-KD (right) re-implantation in survivor mice BALB/c mice implanted in the mammary fat pad (mfp) with 10^6 4T1-Sc or 4T1-KD = 2 out of 4 tumour-free after 4T1-KD re-implantation, n = 4T1-KD (opposite mfp) completely eradicated a second tumour implantation, especially with n = 4T1-KD compared to naive control mice (Fig. 2b). Despite the high number of tumour cells by flow cytometry plots of circulating anti-tumour AH1-specific CD8+ T cells before (pre) and one week after (post) 4T1-Sc (left) or 4T1-KD (right) re-implantation in survivor mice after neoadjuvant anti-CTLA-4 treatment, as in b, d. Frequency (left) and memory phenotype (based on CD44 and CD62L expression) (right) in circulating AH1-specific CD8+ T cells in survivor mice as in n = 4 per group) or in treatment-naive mice (n = 5) one week after injection with 4T1-Sc or 4T1-KD (n = 1 experiment with the IgG2b 9D9 anti-CTLA-4; similar results were obtained with the IgG2a 9D9 antibody). Quantification of tumour-infiltrating T cells by flow cytometry in the indicated treatment groups (n = 5 mice per group; representative of two independent experiments). aC, anti-CTLA-4; Teff, effector CD4+FOXP3− T cells. Data are mean ± s.e.m.* P < 0.05, ** P < 0.01, log-rank test (a), two-way ANOVA with Bonferroni’s correction (b) or two-sided unpaired t test (d,e).

To simulate the clinical management of breast cancer, we treated mice orthotopically implanted with 4T1-KD or 4T1-Sc cells with anti-CTLA-4 before surgical tumour resection. Again, we resected 4T1-KD and 4T1-Sc tumours three days apart to equalize tumour size on the day of surgery (Fig. 2a, Extended Data Fig. 2d). Notably, lactate dehydrogenase (LDH) activity remained potently downregulated in 4T1-KD tumours compared with control 4T1-Sc tumours up to the day of surgery (Extended Data Fig. 2e). Neoadjuvant CTLA-4 blockade significantly prolonged survival in 4T1-KD but not 4T1-Sc-bearing mice (Fig. 2a). To clarify the immunological nature of this effect, we re-implanted disease-free mice approximately 100 days after anti-CTLA-4 treatment and surgical resection of 4T1-Sc tumours with either 4T1-KD or the more aggressive 4T1-Sc model. The growth of both 4T1-Sc and 4T1-KD tumours was significantly delayed in the re-implanted mice compared to naive control mice (Fig. 2b). Despite the high number of tumour cells re-injected (n = 10^6), approximately 20–25% of these mice completely eradicated a second tumour implantation, especially with 4T1-KD (n = 2 out of 4 tumour-free after 4T1-KD re-implantation, n = 0 out of 4 tumour-free after 4T1-Sc re-implantation) (Fig. 2b). In mice re-implanted with 4T1-KD, this response was associated with greater expansion and maturation of anti-tumour CD8+ T cells recognizing the 4T1-associated antigen AH1 (Fig. 2c, d). By contrast, mice that survived after treatment with neoadjuvant anti-CTLA-4 and surgical resection of 4T1-Sc tumours were not able to control the growth of re-implanted 4T1-Sc tumours (Extended Data Fig. 2f). This underscored the importance of limiting tumour glycolysis for the development of long-lasting memory anti-tumour responses after anti-CTLA-4.

We next examined the immune changes that specifically occur in 4T1-KD-bearing mice responding to neoadjuvant anti-CTLA-4. We either resected or injected 4T1-Sc and 4T1-KD tumours three days apart, which produced similar-sized tumours and similar survival outcomes (Fig. 2a, Extended Data Fig. 3a, b) and used the latter schedule for simultaneous flow cytometry analyses of 4T1-Sc and 4T1-KD samples. As previously described^{13,15}, the downregulation of tumour LDHA resulted in increased T cell infiltration. Notably, this effect extended to Treg cells and occurred irrespective of treatment (Fig. 2e, Extended Data Fig. 3c), pointing to functional modulation or changes in other immune cells in tumour or periphery as potential mechanisms that underlie the enhanced activity of anti-CTLA-4 in LDHA-KD tumours. Peripheral Treg cells increased after anti-CTLA-4 treatment irrespective of tumour...
LDHA expression (Extended Data Fig. 3c), in line with previous studies. There were no specific changes in myeloid cells in 4T1-KD-bearing mice treated with anti-CTLA-4 that could explain the different therapeutic outcomes (Extended Data Fig. 3d). By contrast, tumour-infiltrating CD4+ T cells—especially Treg cells—the production of IFNγ and TNF was consistently upregulated in 4T1-KD-bearing mice after anti-CTLA-4 treatment in either schedule (Fig. 3a, b, Extended Data Fig. 4a–e).

The overexpression of CTLA-4 in intratumoral Treg cells may explain their preferential targeting by anti-CTLA-4 (Extended Data Fig. 4f). Of note, the production of IFNγ in Treg cells positively correlated with IFNγ expression in CD8+ T cells (Fig. 3a, b), which suggests that functional destabilization (that is, IFNγ production) of Treg cells could, in turn, favour the activation of CD8+ tumour-infiltrating lymphocytes (TILs) in vivo. The production of IFNγ by 4T1-KD-infiltrating Treg cells after anti-CTLA-4 treatment was not associated with substantial loss of FOXP3 expression (Fig. 3c) but was coupled to the downregulation of CD25 and/or CTLA-4 (Fig. 3d). Accordingly, CTLA-4lo Treg cells, which proportionally increased after CTLA-4 blockade in 4T1-KD tumours (Fig. 3d), preferentially expressed IFNγ (Fig. 3e).

We confirmed these results in three further experimental settings. First, we used the 4T1 A3-8KD model, generated using a different LDHA-targeting shRNA (A3) that was previously shown to have less LDHA downregulation than the 4T1 A2-10KD cell variant used here as 4T1-KD, and which corresponds to less severe impairments in glycolysis (Extended Data Fig. 6c). In this setting, CTLA-4 blockade was slightly less effective and prevented metastasis formation in about 50% of the mice (Extended Data Fig. 5a). Similar to 4T1-KD tumours, CTLA-4 blockade in 4T1 A3-8KD tumours led to phenotypic instability of Treg cells (CD25 and CTLA-4 downregulation), and increased production of IFNγ by Treg cells and granzyme B by CD8+ TILs (Extended Data Fig. 5b, c). Even in this setting, CTLA-4lo Treg cells preferentially expressed IFNγ and further upregulated IFNγ after anti-CTLA-4 treatment (Extended Data Fig. 5d). Accordingly, CTLA-4 expression and IFNγ production in Treg cells were inversely correlated, and IFNγ production by CTLA-4lo Treg cells positively correlated with granzyme B expression in CD8+ TILs (Extended Data Fig. 5e).

As a second approach, we perturbed Treg cells more robustly by using an IgG2a version of the anti-CTLA-4 clone 9D9, which binds to the mouse FcγRI more efficiently than the standard 9D9 IgG2b antibody, crosslinks CTLA-4 better, and has been reported to deplete tumour-infiltrating Treg cells more efficiently in other tumour models. In our experimental setting, the neoadjuvant 9D9 IgG2a antibody maximized metastasis protection and overall survival in mice bearing 4T1-KD, downregulated CTLA-4lo GITR+ intratumoral Treg cells more extensively, and more strongly upregulated IFNγ in the remaining tumour-infiltrating Treg cells without affecting their expression of FOXP3 (Extended Data Fig. 5f–i). Again, we found that production of IFNγ by Treg cells inversely correlated with Treg cell stability (CTLA-4 and GITR expression) and positively correlated with IFNγ production in CD8+ TILs (Extended Data Fig. 5j). Lastly, we investigated these effects in a different tumour model and mouse genetic background (C57BL/6) by knocking down LDHA in the mouse melanoma B16F10 (B16-KD) (Extended Data Fig. 5k). B16-KD cells displayed lower LDH activity and glycolytic capacity than the scramble B16-Sc control (Extended Data Fig. 5l, m). Similar to the effects in the 4T1 model, CTLA-4 blockade significantly downregulated CTLA-4 and CD25 and increased IFNγ expression in B16-KD but not B16-Sc-infiltrating Treg cells (Extended Data Fig. 5n–p). Together, these findings suggested that inhibition of tumour glycolysis may promote the ability of CTLA-4 blockade to induce the loss of Treg cell stability associated with the development of anti-tumour immunity.

### Tumour glycolysis supports Treg cell stability

To clarify the link between tumour glycolysis and Treg cell stability in vivo, we implanted 4T1-KD in Matrigel plugs containing sodium lactate—a strategy that was shown to partially reverse the anti-tumour effect of LDHA-KD15,18—and assessed Treg cell phenotypic and functional changes associated with CD8+ TIL activation after anti-CTLA-4 treatment. In this condition, intratumoral Treg cell stability was better retained (increased CTLA-4 and decreased IFNγ), whereas the expression of FOXP3 and tumour burden were not substantially affected (Fig. 3f–j). Furthermore, the positive correlation between IFNγ-producing Treg cells and CD8+ TILs was lost after the addition of lactate (Fig. 3k). This effect appeared to be driven by IFNγ changes in Treg cells, as lactate did not directly affect IFNγ-producing CD8+ T cells (Fig. 3l). We corroborated these results by maximizing tumour glycolysis (as opposed to exogenously supplying lactate into the tumour microenvironment). For this, we generated hyper-glycolytic 4T1 Rho-0 cells (4T1-EtBr) by ethidium bromide treatment in vitro to reduce mitochondrial function and compared and contrasted Treg cells infiltrating these hyper-glycolytic tumours versus glycolysis-defective 4T1-KD tumours. 4T1-EtBr cells tended to further upregulate LDHA, displayed stronger LDH activity than 4T1-Sc or 4T1-KD cells, and produced ATP mostly by glycolysis, in sharp contrast to 4T1-KD cells, which produced ATP mainly through mitochondria respiration (Extended Data Fig. 6a–c). 4T1-EtBr cells also appeared slightly more aggressive than 4T1-KD in vivo (Extended Data Fig. 6d). We found that CD4+ T cells infiltrating 4T1-EtBr tumours in comparison to 4T1-KD tumours are enriched in FOXP3+ Treg cells that are phenotypically and functionally more stable, expressing CTLA-4 and CD25 more broadly and producing less IFNγ (Extended Data Fig. 6e–g). These results illustrated a direct relationship between tumour glycolysis and intratumoral Treg cell stability, and suggested that the local glucose: lactate ratio may alter the susceptibility of Treg cells to anti-CTLA-4-mediated re-programming. In glycolysis-defective tumours, glucose may be more largely available and CTLA-4 blockade can more efficiently induce Treg cells to metabolize glucose. Using a bilateral 4T1-Sc and 4T1-KD tumour system to control for nutrient input, we found that 4T1-KD cells take up glucose less efficiently, and Treg cells infiltrating these tumours increase glucose uptake after anti-CTLA-4 (Extended Data Fig. 6h–j). We confirmed these effects using a more precise system to detect intra-tumour Treg cells for surface glucose staining with FOXP3–GFP transgenic C57BL/6J mice implanted with syngeneic B16-KD and B16-Sc tumours (Extended Data Fig. 6k–m). Notably, the increased uptake of glucose in Treg cells infiltrating glycolysis-defective B16-KD tumours was associated with the tendency of these Treg cells to be less suppressive ex vivo (Extended Data Figs. 6n, 7a).

### Glucose and CD28 axis limit Treg cell stability

We next studied in vitro the conditions that lead to loss of Treg cell stability in glycolysis-defective tumours after anti-CTLA-4 treatment in vivo. 4T1-KD cells also use less glucose than 4T1-Scs cells in vitro (Fig. 4a, b) and Treg cells co-cultured with 4T1-KD versus 4T1-Scs cells modestly, but significantly, upregulated the expression of IFNγ and/or TNF after anti-CTLA-4 treatment (Extended Data Fig. 7c). Similarly, Treg cells cultured in medium conditioned by either 4T1-KD or the poorly glycolytic benign mammary gland cell line NMuMG produced more IFNγ and/or TNF than Treg cells exposed to 4T1-Scs-conditioned medium, and anti-CTLA-4 enhanced this effect (Extended Data Fig. 7d, e). We thus asked whether higher glucose availability in cultures with glycolysis-low tumours contributed to anti-CTLA-4-mediated Treg cell destabilization and whether this effect was linked to CD28 co-stimulation, which has been extensively shown to modulate the glucose metabolism of T cells. We found that CTLA-4 blockade directly enhances Treg cell glucose uptake and IFNγ production in the presence of glucose (11 mM) and physiological CD28 co-stimulation by irradiated B cells (Fig. 4c, d) independent of mitochondrial metabolism (Extended Data Fig. 8a). By contrast, blocking oxidative phosphorylation with oligomycin reduced FOXP3 expression and IL-10 production in Treg cells (Extended Data Fig. 8b), which indicates that mitochondrial respiration is
important for $T_{reg}$ cell integrity, as previously suggested\textsuperscript{23-25}, and that forcing glycolysis in $T_{reg}$ cells makes them more susceptible to losing their stability, in support of our new findings here. We then evaluated the effect of anti-CTLA-4-induced $T_{reg}$ cell glucose consumption on $T_{reg}$ cell suppression in relation to glucose loads and CD28 co-stimulation (Fig. 4e). We found that anti-CTLA-4 inhibits $T_{reg}$ cell suppression and...
increases CD86 expression as a function of glucose concentration (Fig. 4f, g, Extended Data Fig. 8c, d). Notably, this effect was lost with CD28-deficient Treg cells (Extended Data Fig. 8e), which suggests that anti-CTLA-4-mediated inhibition of Treg cell suppression in the presence of glucose is dependent on CD28 signalling in Treg cells. Direct CD28 co-stimulation using an agonist antibody fully overcame the need for glucose to counteract Treg cell suppression (Fig. 4h–j, Extended Data Fig. 9a, b). However, despite complete inhibition of Treg cell function, CD28 agonism delayed the proliferation of CD8+ Tcells, especially at low glucose concentrations (Extended Data Fig. 9a). By titrating CD28 stimulation, we found that above a certain concentration (approximately 0.05 μM), anti-CD28 slows the proliferation of T cells, especially in low glucose concentrations (Extended Data Fig. 9c, d). According to previous observations that in glucose-restricting conditions, CD28 co-stimulation triggers mitochondrial respiration in activated T cells, which less efficiently sustains proliferation. In contrast to CTLA-4 blockade, the inhibition of the other major immune checkpoint PD-1 did not affect Treg cell suppression, either when under high glucose concentrations (11 mM) or when PD-1 Treg cells were tested (Extended Data Fig. 9d, e), and did not promote Treg cell glucose uptake in vitro or loss of Treg cell stability in vivo (Extended Data Fig. 9f, g). Because anti-PD-1 acts downstream and requires CD28 signalling, these results may be explained by high CTLA-4 expression in Treg cells that prevents CD28 co-stimulation.

**Fitness opposes stability in glycolytic Treg cells**

To prove that anti-CTLA-4-mediated Treg cell destabilization in glycolysis-defective tumours depends on the glucose catabolism of Treg cells, we tested whether this effect is lost in mice with conditional deletion of LDHA or the glucose transporter GLUT1 (encoded by Slc2a1) in Treg cells. We used B16-KD as a glycolysis-defective tumour model compatible with the genetic background of these mutant mice (C57BL/6). In comparison to control Treg cells, GLUT1-deficient Treg cells infiltrating glycolysis-defective tumours after treatment with CTLA-4 blockade restored expression of CTLA-4 and CD25, did not extensively

**Fig. 4 | Glucose-dependent loss of Treg cell functional stability induced by CTLA-4 blockade**. a. Flow cytometry representative plot (left) and quantification (right) of 2-NBDG staining on 4T1-KD and 4T1-Sc cells in two independent experiments (n = 2). Rel. MFI denotes 2-NBDG MFI of stained samples relative to matched unstained control. b. Glucose consumption by 4T1-KD and 4T1-Sc cells cultured in hypoxic conditions (n = 3; representative of two independent experiments). c. Flow cytometry representative plot (left) and quantification (right) of glucoseCy3 staining on Treg cells treated with anti-CTLA-4 or IgG control in 11 mM glucose (n = 2; representative of two independent experiments). d. Quantification of IFNγ by Luminex-based bead immunoassay in supernatants from Treg cell cultures as in e.

e–g. In vitro suppression assays with increasing glucose concentrations and anti-CTLA-4 or IgG control. f. Percentage of inhibition of the proliferation of CD8+ Tcells co-cultured with Treg cells relative to CD8+ Tcells cultured alone in the same treatment conditions (n = 3; representative of three independent experiments). g. Percentage of CD86-expressing B cells from cultures as in f.

h–j. In vitro suppression assays with increasing glucose concentrations and anti-CD28 or IgG control. i. Percentage of inhibition of proliferation of CD8+ Tcells co-cultured with Treg cells relative to CD8+ Tcells cultured alone in the same treatment conditions (n = 3; representative of three independent experiments). j. Percentage of CD86-expressing B cells from cultures as in i.

k. Model for loss of functional stability of Treg cells according to glucose availability and CTLA-4 blockade. Left, under glucose restriction, such as in 4T1-Sc tumours, anti-CTLA-4 has limited activity against Treg cell-mediated immunosuppression of Teff cells. Right, when competition for glucose is diminished, T cells infiltrate the tumour better and anti-CTLA-4 promotes glucose metabolism of Treg cells via CD28 co-stimulation, leading to functional destabilization of Treg cells and increased Teff cell activation. APC, antigen-presenting cell; TCR, T cell receptor. Data are mean ± s.d. P values determined by two-sided unpaired t test.

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upregulate IFNγ and TNF, and retained stronger suppression function ex vivo (Extended Data Fig. 10a–e). Notably, GLUT-1 or LDHA-deficient tumour-infiltrating Treg cells displayed poor expansion and proliferation potential after treatment (Extended Data Fig. 10f–i). Similarly, LDHA-deficient Treg cells remained less fit in culture despite high concentrations of glucose (10 mM), and increasing CD28 co-stimulation further reduced their fitness to levels comparable to control Treg cells activated in acute glucose restriction (0.5 mM) (Extended Data Fig. 10m, n). In addition, the prevalent Ki67-negative fraction in LDHA-deficient Treg cells better retained suppressive markers after activation (Extended Data Fig. 10m, o). These observations reveal a dual implication for glycolysis in Treg cells: it is needed to sustain Treg cell expansion, but it makes Treg cells more susceptible to re-programmability, especially when CD28 can be engaged, such as after CTLA-4 blockade. This also suggests that glucose is dispensable for active Treg cell suppression and that alternative sources of fuel can support this function.

Discussion

Previous studies have shown that glucose metabolism is dispensable for Treg cell differentiation and that Treg cells use fatty acids as preferential metabolic substrates. Our results indicate that Treg cell avoidance of glucose metabolism has functional importance and may be mediated by CTLA-4 overexpression, which by blocking CD28 signalling towards glucose utilization, can ensure the functional stability of Treg cells. We found that removal of this control with anti-CTLA-4 promotes CD28 co-stimulation and counteracts Treg cells suppression as a function of glucose availability both in vitro and in vivo (Fig. 4k). A major outcome of CTLA-4 inactivation is CD28 ligand upregulation on antigen-presenting cells, which is associated with loss of Treg cell suppression. However, whether this effect could result in enhanced Treg cell glucose metabolism was not known. Our findings point to glucose metabolism as a functional vulnerability in Treg cells that is generally prevented by CTLA-4 overexpression. We demonstrate that CTLA-4 blockade achieves superior efficacy linked to anti-tumour immunological memory in mice bearing tumours that can spare glucose for infiltrating immune cells, and that this effect is associated with functional destabilization of Treg cells towards IFNγ-producing cells (Fig. 4k). Aerobic glycolysis can enhance IFNγ production in CD4+ T cells by three main mechanisms: by alleviating GAPDH-mediated inhibition of IFNγ translation; by increasing acetyl-coenzyme A for epigenetic modification of histones; and by sustaining Ca2+-NFAT signalling through phosphoenolpyruvate. If forced to engage in glycolysis after CTLA-4 blockade in the presence of glucose, Treg cells may undergo similar mechanisms that lead to IFNγ upregulation. Overall, these results suggest that CTLA-4 blockade can be best exploited to treat glycolysis-low tumours or in combination with inhibitors of tumour glycolysis that can increase glucose availability in the tumour microenvironment, thus maximizing Treg cell destabilization and anti-tumour immunity.

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/s41586-021-03326-4.

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Methods

No statistical methods were used to predetermine sample size. Unless stated otherwise, the experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

Tumour cell lines

The mouse mammary carcinoma 4T1 cell line was provided by F. Miller and the benign mammary gland NMuMG cell line by J. Koutcher. Mouse breast tissue cell lines were cultured in DMEM supplemented with 10% heat-inactivated FBS, 25 mM glucose, 6 mM L-glutamine, 1 mM penicillin-streptomycin, and 4 mg/l puromycin. The B16F10 mouse melanoma cell line was originally obtained from I. Fidler and cultured in RPMI1640 supplemented with 10% heat-inactivated FBS, 1% nonessential amino acids and 2 mM L-glutamine. Cell lines were authenticated by STR profiling or morphology and expression of specific antigens and were routinely tested for mycoplasma contamination. Tumour cells were transfected with SureSilencing LDHA-targeting shRNA plasmids (KD: A2 = GTACGTCCATGATGCATAC; A3 = GGAATCTCATTCGATGCATAC) or scramble control plasmids (Sc = GGAATCTCATTCGATGCATAC) (QIAGEN). Stable LDHA-KD (4T1 A2-10KD = 4T1-KD, 4T1 A3-8KD and B16-KD) and scramble control (4T1-Sc and B16-Sc) cell lines were generated as previously described. Hyper-glycolytic/poorly oxidative Rho-0 cells were generated by in vitro treatment with ethidium bromide as previously described. LDHA modulation in these cell variants was confirmed at protein level by western blot, using a rabbit anti-LDHA antibody (1:1,000; Cell Signaling Technology, 2012s) coupled with an HRP-conjugated anti-rabbit IgG (1:3,000; Cell Signaling Technology, 7074S) as secondary antibody, with vinculin (1:1,000, Santa Cruz, sc-73614; revealed by an HRP-conjugated anti-mouse IgG, 1:5,000, Cell Signaling Technology, 7067S) or β-actin (1:5,000, Sigma, A21203; revealed by Molecular Devices Evaluation Kit R8202) as protein loading control, and at enzymatic activity level by using the Cytotoxicity Detection Kit PLUS (LDH) (Roche Diagnostics), as previously reported. Altered glycolytic and mitochondrial metabolism capacity of these tumour cells was also confirmed by Glycolytic Proton Flux Rate and ATP rate assays using a Seahorse XF 96 Analyzer according to the manufacturer’s instructions (Seahorse XF Glycolytic Rate Assay and Real-Time ATP Rate Assay, Agilent Technologies).

Mice

Female BALB/cAnN mice were from Charles River Laboratory, RAG2-knockout BALB/c were from Taconic, and wild-type, CD28-knockout and CD45.1 congenic C57BL/6J mice were from Jackson Laboratory. FOXP3–GFP transgenic mice were provided by A. Rudensky and backcrossed to C57BL/6J at MSK. Foxp3GFP-cre-ERT2, Foxp3FRGFP or B10F10, Slc2a1ΔRho, Foxp3FRGFP or Foxp3ΔRho or Slc2a1ΔRho, C57BL/6J mice were provided by G. Delgoffe. Foxp3ΔRho or LDHaΔRho mice were provided by P.-C. Ho and bred at MSK. Same-sex, same-aged mice were used in each experiment. All mice were bred and maintained under specific pathogen-free conditions (with a 12 h light-dark cycle at temperature of 21–23 °C and humidity of 35–55%) and used at the ages of 5–10 weeks. The maximal tumour size of 20 mm in any direction was not exceeded in any experiment. All animal experiments were conducted according to protocols approved by the MSK and University of Pittsburgh Institutional Animal Care and Use Committee.

In vivo experiments

Five-to-six-week-old female BALB/c mice were injected orthotopically with 10⁴ 4T1 cells in the mammary fat pad. Two days later, the tumour burden was quantified by BLI and mice were randomized in the different treatment groups to receive three intraperitoneal injections with 100 μg anti-CTLA-4 (clone 9D9 IgG2b, BioXcell; clone 9D9 IgG2a was provided by Bristol Myers Squibb) or isotype control (clone MPC-11, BioXcell) 3 days apart. 4T1-Sc and 4T1-KD tumours were injected or resected 3 days apart to equalize tumour size before surgery. Disease-free mice, approximately 100 days after surgery, were re-inoculated with 10⁴ 4T1-Sc or 4T1-KD cells in the opposite mammary fat pad and monitored for tumour growth to test development of anti-tumour immunological memory. In some experiments, 4T1-KD cells (0.2 × 10⁴–1 × 10⁴ per mouse) were implanted in the mammary fat pad in Matrigel (Matrigel Matrix Growth Factor Reduced, Becton Dickinson) with or without 30–50 mM sodium lactate as previously reported. Metastasis development was monitored every week by BLI after intraperitoneal injection of 50 μl of d-luciferin (30 mg ml⁻¹, Gold Biotechnology). At the time of euthanization, lungs were collected to quantify lung metastases either by ex vivo BLI or by haematoxylin and eosin staining of formalin-fixed and paraffin-embedded tissue sections. B16-Sc and B16-KD cells (250,000 cells per injection) were implanted intradermally in 5–8-week-old wild-type or FOXP3–GFP, Foxp3GFP-creB10F10, Foxp3ΔRho or Slc2a1ΔRho, Foxp3FRGFP or Slc2a1ΔRho or Foxp3ΔRho or LDHaΔRho C57BL/6J mice, which were then treated with 100 μg anti-CTLA-4 or the isotype control for four administrations, three days apart. Primary tumour growth was measured twice a week by caliper. Survival was defined as time to death or to euthanization for those mice that had to be euthanized because they were sick and/or their tumours reached the size limits. All mouse procedures were performed in accordance with the Institutional Animal Care and Use Committee of MSK and the University of Pittsburgh.

RNA sequencing analyses

We interrogated RNA-seq data for 22 human melanoma samples (n = 7, before ipilimumab; n = 15, after ipilimumab) from previously published analyses and from 4T1-Sc and 4T1-KD tumours treated with anti-CTLA-4 (n = 5 per tumour type) or an IgG control (n = 4, 4T1-Sc and n = 5, 4T1-KD). For mouse samples, frozen tissue was homogenized in TRIzol Reagent (Thermo Fisher 15596018) using the QIAGEN TissueLyser at 15Hz for 2–3 min with a Stainless Steel Bead (QIAGEN 69989). Phase separation was induced with chloroform. RNA was precipitated with isopropanol and linear acrylamide and washed with 75% ethanol. RNA samples were resuspended in RNase-free water. After Ribogreen quantification and quality control by Agilent BioAnalyzer, 50 ng of total RNA underwent polyA selection and TruSeq library preparation according to instructions provided by Illumina (TruSeq Stranded mRNA LT Kit, RS-122-2102), with 8 cycles of PCR. Samples were barcoded and run on a HiSeq 4000 in a PE50 run, using the HiSeq 3000/4000 SBS Kit (Illumina). An average of 51 million paired reads was generated per sample and the percent of mRNA bases averaged 69%. Heat maps of expressed genes were generated using log₂-transformed and standardized counts. The composition of immune cells was estimated from bulk RNA-seq data using the mean z-score approach with CIBERSORT LM22 signatures. In this approach, values for each gene are first z-transformed across all samples. Resulting z-scores are then averaged across genes to arrive at a single signature score for each sample. Pearson correlation test was used to analyse dependency between variables. All analyses after gene count generation were conducted in the R statistical environment (R development Core Team, 2008; ISBN 3-900051-07-0) (version 3.6.3).

In vitro T cell assays

Mouse T cells were cultured in RPMI1640 supplemented with 10% heat inactivated FBS, 1% nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol (complete RPMI1640).

Total T cells were immunomagnetically sorted from spleens of naive BALB/c mice using CD5 microbeads (Milteny) according to the manufacturer’s instructions. T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) and activated using phytohemagglutinin (PHA, 5 μg ml⁻¹, Sigma) or anti-CD3/anti-CD28 microbeads.
Glucose consumption = (glucose in base medium – glucose in conditioned medium)/no. of cells.

YSI-based measurements of glucose consumption and lactate production were calculated as follows: Glucose consumption = (glucose in conditioned medium – glucose in base medium)/(no. of cells/10^6 × hours). Lactate production = (lactate in conditioned medium – lactate in base medium)/(no. of cells/10^6 × hours)

**Multiplex cytokine analysis**

Cytokine concentrations in culture supernatants were quantified by using Luminex-based bead multiplex high sensitivity immunoassays (Millipore) and the DropArray system (Curiox BioSystems) according to the manufacturer's instructions.

**Quantitative PCR**

Total RNA was extracted by using TRIzol reagent (Invitrogen) and reverse-transcribed into complementary DNA (cDNA) using the High Capacity cDNA Transcription kit (Applied Biosystems). Expression of the indicated transcripts was quantified with the Fluidigm Biomark system by using the appropriate 6-fluorescein amide (6-FAM)-minor groove binder (MGB)-conjugated TaqMan primer probes (Applied Biosystem) on target gene pre-amplified according to the manufacturer's protocol. Gene expression was normalized relative to Actb. Data were analysed by applying the 2-ΔΔC_t calculation method.

**Flow cytometry analyses**

Tumours were dissociated after 30 min incubation with Liberase TL and DNase I (Roche) to obtain single-cell suspensions. When tumour mass exceeded 0.1 g, immune cell infiltrates were enriched by Percol (GE Healthcare) gradient centrifugation. Surface staining was performed after 10 min incubation on ice with an anti-mouse CD69/CD32 antibody (clone 2G2, BD Biosciences) to block Fcγ receptors, by using panels of appropriately diluted fluorochrome-conjugated antibodies (from BD Biosciences, eBioscience, Invitrogen or Biolegend) against the following the mouse proteins in different combinations: CD45 (clone 30-F11; 1:250), CD45.1 (clone A20; 1:200), CD3 (clone 145-2C11; 1:200), CD4 (clone RM4-5; 1:200), CD8a (clone 5H10; 1:200), CD11b (clone M1/70.15; 1:200), F4/80 (clone BM8; 1:200), MHC-II (clone M5/114.15.2; 1:200), Gr1 (clone RB6-8C5; 1:200) and an eFluor506 fixable viability dye. For intracellular staining, mouse cells were fixed and permeabilized (FOXP3 fixation/permeabilization buffer, eBioscience) and incubated with appropriately diluted FITC- or AF488-labelled anti-mouse FOXP3 (clone FJK-16s; eBioscience; 1:200), PE-labelled anti-CTLA-4 antibody (clone UC10-4F10-11; 1:200), and PE- or PECy7-labelled anti-Ki67 (clone M1/69.15.2; 1:200) antibodies (from BD Biosciences, eBioscience, Invitrogen or Biolegend) against the following mouse proteins in different combinations: CD45 (clone 30-F11; 1:250), CD45.1 (clone A20; 1:200), CD3 (clone 145-2C11; 1:200), CD4 (clone RM4-5; 1:200), CD8a (clone 5H10; 1:200), CD11b (clone M1/70.15; 1:200), F4/80 (clone BM8; 1:200), MHC-II (clone M5/114.15.2; 1:200), Gr1 (clone RB6-8C5; 1:200) and an eFluor506 fixable viability dye. For intracellular staining, mouse cells were fixed and permeabilized (FOX3 fixation/permeabilization buffer, eBioscience) and incubated with appropriately diluted FITC- or AF488-labelled anti-mouse FOXP3 (clone FJK-16s; eBioscience; 1:200), PE-labelled anti-CTLA-4 antibody (clone UC10-4F10-11; 1:200), and PECy7 or PE-labelled anti-Ki67 (clone B56, BD Biosciences; 1:50; clone 16A8, Biolegend, 1:250) antibodies for 30 min on ice. CD206 was revealed following cellular permeabilization (F4/80 staining) and the addition of 1 μg ml−1 plate-bound anti-CD3, 2,000 U ml−1 IL-2 and 50 μg ml−1 anti-CTLA-4. Alternatively, Treg cells were cultured for 48 h in 4 h tumour-conditioned medium (complete RPMI1640, 11 mM glucose) and CD86 expression in CD19 B cells (B cell co-stimulation). Treg cell suppression was calculated with the following formula:

\[
T_{reg} \text{cell suppression} = \left(1 - \frac{CTV_{low}(CD8^{+} T \text{cells} + T_{reg} \text{cells})}{CTV_{low}(CD8^{+} T \text{cells alone})}\right) \times 100
\]

For Treg cell:4T cell conditioned medium assays, CD25+ Treg cells were immunomagnetically purified from naive BALB/c splenocytes (Miltenyi Biotec). Treg cells were isolated with established 4T1-Sc or 4T1-KD cell cultures in 5 mM glucose complete RPMI1640 for 24 h in the presence of 1 μg ml−1 soluble anti-CD3, 2,000 U ml−1 IL-2 and 50 μg ml−1 anti-CTLA-4. Alternatively, Treg cells were cultured for 48 h in 4 h tumour-conditioned medium (complete RPMI1640, 11 mM glucose) with 1 μg ml−1 plate-bound anti-CD3, 2,000 U ml−1 IL-2 and 50 μg ml−1 anti-CTLA-4 or an isotype control. Monensin (BD GolgiStop, BD Biosciences) and brefeldin A (BD GolgiPlug, BD Biosciences) were added for the last 5 h of culture before performing intracellular cytokine staining.

Activation of Treg cell monocultures was performed by incubating FACs-sorted FOXP3−GFP Treg cells with 30 Gy irradiated immunomagnetically purified CD45.1+CD19− T cells (1:1 ratio) in the presence of 0.5 μg ml−1 soluble anti-CD3 and 50 μg ml−1 anti-CTLA-4, anti-CD10 and anti-Ig control for 48 h in a humidified chamber with 5% CO2 at 37 °C. To block mitochondrial metabolism in this assay, 2–5 nM rotenone + antimycin A or 4 nM oligomycin were added. IL-2 (1000.0 U ml−1) was added for the last 24 h incubation.

**Glucose and lactate measurements**

Glucose and lactate were quantified in culture supernatants by either 1H-NMR, luminescent assays (Glucose-Glo, Promega), or by YSI meter (MSK Metabolism Core Facility).

Quantification of glucose consumption and lactate production by 1H-NMR was conducted by integration of spectral line-shapes, as previously reported. Glucose and lactate concentrations were calculated with the following formula: 

\[
Cu = Ck \times (Iu/Ik) \times (Nk/Nu) \times (C6-glucose = 1 proton, C3-lactate = 3 protons)
\]

The Glucose-Glo Assay (Promega) was used to quantify glucose consumption in supernatants from 4T1-Sc, 4T1-KD, B16-Sc and B16-KD cells. Glucose consumption was calculated with the following formula:

\[
(T_{reg} \text{cell suppression} = \left(1 - \frac{CTV_{low}(CD8^{+} T \text{cells} + T_{reg} \text{cells})}{CTV_{low}(CD8^{+} T \text{cells alone})}\right) \times 100
\]
For intracellular cytokine staining, mouse tumour immune infiltrates were re-stimulated with 0.1 μg ml⁻¹ PMA and 0.5–1 μg ml⁻¹ ionomycin in complete RPMI1640 in a humidified chamber with 5% CO₂ at 37 °C. After 1 h, 1× GolgiStop and 1× GolgiPlug (BD Biosciences) were added to the cultures and incubated for 4–5 h at 37 °C. Surface staining was performed after blocking Fcγ receptors by incubating cells with PerCP-Cy5.5-labelled anti-CD4, BV650-labelled anti-CD8, and APC-Cy7-labelled anti-CD45 antibodies and an eFluor506-labelled fixable viability dye for 30 min on ice. Cells were then washed, fixed and permeabilized with the Foxp3 fixation/permeabilization buffer (eBioscience) and stained for 45 min with FITC- or AF488-labelled anti-FOXP3, BV450- or BV510-labelled anti-IFNγ (clone XMG1.2, BD Biosciences or Biolegend, 1:200–250) and APC-labelled or PE-Cy7-labelled anti-TNF (clone MP6-XT22, BD Biosciences or Biolegend, 1:200–250) antibodies. Samples were acquired on an LSRII or Symphony X50 flow cytometer (BD Biosciences) and a human melanoma RNA-seq datasets investigated in this study were analysed with Flowjo 10.6.1 software (Tree Star).

Mouse Tregs cells were sorted from FOXP3–GFP, Foxp3GFP-cebpeR2 and Foxp3GFP-cebpeR1, Slc2a1GFP transgenic mice by using CD4 pre-enriched splenocytes (CD4 Microbeads, Miltenyi Biotec). In brief, following Fcγ receptor blockade with anti-mouse CD16/CD32, samples were stained with a PE-Cy7- or APC-labelled anti-CD4 antibody for 30 min on ice. Cells were then washed and DAPI was added immediately before acquisition. FACS sorting was conducted on a FACS Aria II cell sorter (BD Biosciences).

Statistical analyses

Two-sided Student’s t-test and two-way ANOVA (with Bonferroni’s multiple comparisons test) were used to detect statistically significant differences between groups. P values for survival analyses were calculated with log-rank (Mantel–Cox) test. Statistical analyses were performed on the Prism 9 software (GraphPad Software) version for Macintosh Pro personal computer. Detailed information of the statistical test and number of observations or replicates used in each experiment, and the definition of centre and dispersion is appropriately reported in the legend of each figure. Significance was defined as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Reporting summary

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

Data availability

Human melanoma RNA-seq datasets investigated in this study were previously reported and have been deposited to the Gene Expression Omnibus (GEO) repository with accession number GSE165278. The 4T1 RNA-seq datasets generated for this study have been submitted to the GEO repository with accession number GSE164051. Source data are provided with this paper.

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Author contributions

R.Z., J.D.W. and T.M. developed the concept and discussed experiments; R.Z. wrote the manuscript, performed and analysed flow cytometry experiments and designed and performed in vitro T cell assays; R.Z. and I.S. designed and performed in vivo experiments; I.S. and I.J.C. developed and characterized LDHA-KD cell lines; M.M. and M.S. performed surgical tumour resections in mice; Y.S. performed bioinformatic analyses; M.J.W. performed in vivo experiments with Slc2a1 transgenic mice; R.M., A.L. and E.A. performed measurements of tumour metabolites; R.M. and S.J. assisted with western blot analyses and in vivo experiments; M.L., M.K. and M.M.M. provided assistance for in vivo experiments, Seahorse and western blot analyses; H.Z. maintained mouse colonies; C.L. processed human tumour tissue samples for RNA-seq analyses; A.G. and M.A. provided assistance with in vitro assays; J.A.K. and P.-C.H. discussed experiments for measurements of metabolites; P.-C.H. provided Treg cell-specific Ldhα knockout mice; G.M.D. provided glucose tracers, Foxp3-conditional Slc2a1 mutant mice, and scientific input; T.M., J.D.W. and R.B. supervised the research.

Competing interests

R.Z. is inventor on patent applications related to work on GITR, PD-1 and CTLA-4. R.Z. is consultant for Leap Therapeutics and ITEDOS Belgium SA. Y.S. is currently employed by Genentech and holds equity in Roche. P.-C.H. received research support from Roche-pRED and honorarium from Chungai and Pfizer. P.-C.H. is also a scientific advisory board member of Eliion Immunotherapeutics and Acepodia. G.M.D. consults for and/or is on the scientific advisory board of BlueSphere Bio, Century Therapeutics, BluePearls Pharmaceuticals, and Western Oncology/Kalivir; has grants from bluebird bio, Novasenta, Pfizer, Pieris Pharmaceuticals, TCR2, and Kalivir; G.M.D. owns equity in BlueSphere Bio and Novasenta. T.M. is a cofounder and holds an equity in IMVAQ Therapeutics. T.M. is a consultant of Imunos Therapeutics, Pfizer and Immunogenesis. T.M. has research support from Bristol-Myers Squibb; Surface Oncology; Kyn Therapeutics; Infinity Pharmaceuticals, Inc.; Peregrine Pharmaceuticals, Inc.; Adaptive Biotechnologies; Leap Therapeutics, Inc.; and Apea. T.M. has patents on applications related to work on oncolytic viral therapy, alpha virus-based vaccines, neo-antigen modelling, CD40, GITR, OX40, PD-1 and CTLA-4. J.D.W. is consultant for Adaptive Biotech; Amgen; Apicent; Ascentage Pharma; Astellas; AstraZeneca; Bayer; Beigene; Boehringer Ingelheim; Bristol Myers Squibb; Celgene; Chugai; Eli Lilly; Elucida; F Star; Geomunome; Imvaq; Kyowa Hakko Kirin; Linneaus; Merck; Neon Therapeutics; Polyomina; Piaoxiu; Recepta; Takara Bio; Triex; Truvax; Sellas; Sareratex, Surface Oncology; Syndax; Syntaligic, Werewolf Therapeutics. J.D.W. reports grants from Bristol Myers Squibb and Sephora. J.D.W. has equity in Tizona Pharmaceuticals, Adaptive Biotechnologies, Imvaq, Beigene, Linneaus; Apricity, Arsenal IO, Geomunome. J.D.W. is inventor on patent applications related to work on DNA vaccines in companion animals with cancer, assays for suppressive myeloid cells in blood, oncolytic viral therapy, alpahavirus-based vaccines, neo-antigen modelling, CD40, GITR, OX40, PD-1 and CTLA-4. The other authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to R.Z., J.D.W. or T.M.

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Extended Data Fig. 1 | Tumour glycolysis and immune cell function.

a, Quantification of glucose and lactate by \textsuperscript{1}H-NMR in supernatants from 72 h cultures of activated T cells (100,000 cells), 4T1 cells (3,000 cells) and the two cell types together. Plots show combined results from two independent experiments (n = 2 per experiment).

b, Percentage of proliferating (CFSE low) (left) and dead (right) CD4\(^+\) or CD8\(^+\) T cells assessed by flow cytometry after 48 h activation in the presence of the indicated concentrations of lactate acid to define the workable lactate dose range (n = 3 per condition, except for 0 μM lactic acid, n = 2).

c, d, Flow cytometry analysis of the indicated parameters in CD8\(^+\) and CD4\(^+\) T cells activated for 48 h in the presence or absence of 4T1 cells (c) or of 10 mM lactate (d) from two independent experiments (n = 3).

e, f, Expression of immune cell signatures by CIBERSORT (top) and glycolysis-related genes (bottom) in RNA-seq datasets from human melanoma samples at baseline (e, n = 7) and after ipilimumab treatment (f, n = 15). Each column in the heat maps represents an independent tumour sample. Data are mean ± s.d. Pvalues determined by two-sided unpaired t test.
Extended Data Fig. 2 | LDHA-deficient tumour model for neoadjuvant CTLA-4 blockade treatment. a, Expression of LDHA in 4T1-KD and 4T1-Sc whole-cell protein extracts by western blot analysis. Vinculin was used as a loading control. Representative of three independent experiments. b, Glycolytic proton efflux rate (glycoPER) in 4T1-KD and 4T1-Sc cultures (n = 20). 2-DG, 2-deoxy-d-glucose; Rot/AA, rotenone plus antimycin A. c, In vivo growth of 4T1-KD and 4T1-Sc tumours orthotopically implanted in the mammary fat pad of immunocompetent wild-type (WT) and immunodeficient RAG2 knockout (KO) BALB/c mice (n = 10 mice per group; representative of two independent experiments). d, Growth of primary 4T1-Sc and 4T1-KD tumours in mice treated as in Fig. 2a (left) and average tumour diameter on the day of tumour resection (right) (IgG, n = 9; anti-CTLA-4, n = 12). e, LDH activity in 4T1-Sc (n = 4) and 4T1-KD (n = 5) tumour extracts on the day of tumour resection after treatment as in d. f, Tumour growth after a second injection with 4T1-Sc in 4T1-KD- and 4T1-Sc-bearing mice that survived neoadjuvant treatment with CTLA-4 blockade as in Fig. 2a (n = 4 per group, except for naive, n = 5). Data are mean ± s.d. (b) or mean ± s.e.m. (c–f). P-values determined by two-way ANOVA with Bonferroni correction (b, c, f) or two-sided unpaired t-test (e).
Extended Data Fig. 3 | Neoadjuvant anti-CTLA-4 treatment schedule for same-day 4T1-Sc and 4T1-KD tumour resection. a, Top, additional treatment schedule modified to obtain 4T1-Sc and 4T1-KD tumours for flow cytometry analysis on the same day. Separate groups of BALB/c mice were injected with $10^6$ 4T1-KD and 4T1-Sc cells 3 days apart and then treated with three cycles of anti-CTLA-4 or the matched isotype control (IgG) every 3 days before surgery and flow cytometry analysis of tumour and tumour draining lymph node (DLN) samples. a, Bottom, primary tumour growth (left) and tumour weight (right) on the day of surgery, showing similar tumour size across groups during treatment and on the day of surgery ($n = 5$ mice per group). b, Overall survival of mice treated as in a ($n = 5$ mice per group). c, Frequency of the indicated T cell subsets among total CD45+ leukocytes in tumours and DLNs from the indicated treatment groups ($n = 5$ mice per group except for 4T1-Sc IgG, $n = 4$). d, Frequency of CD11b+ myeloid cell subsets among total CD45+ leukocytes, M1 and M2 macrophages according to MHC-II and CD206 staining among total CD11b+ myeloid cells in 4T1-Sc and 4T1-KD tumours as well as DLNs from mice treated as indicated ($n = 5$ mice per group except for 4T1-Sc IgG, $n = 4$). Representative plots (left) showing the flow cytometry gating strategy for M1 and M2 macrophages and granulocytes (granulo) are reported. Data are representative of at least two independent experiments. Data are mean ± s.e.m. *$P < 0.05$. P-values determined by log-rank test (b) or two-sided unpaired t-test (c, d).
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Selective loss of T<sub>reg</sub> cell functional stability in LDHA-deficient tumours treated with CTLA-4 blockade.

a, Representative gating strategy for tumour-infiltrating CD8<sup>+</sup>, CD4<sup>+</sup>FOXP3<sup>−</sup> Teff cells and CD4<sup>+</sup>FOXP3<sup>+</sup> T<sub>reg</sub> cells, in which expression of IFNγ and TNF was assessed. b, Representative flow cytometry plots showing IFNγ and TNF expression in T<sub>reg</sub> and Teff cells, CD8<sup>+</sup> TILs gated as in a from 4T1-Sc- and 4T1-KD-bearing BALB/c mice treated as in Fig. 3a. c, d, Quantification of TNF and IFNγ expression in CD4<sup>+</sup>FOXP3<sup>−</sup> Teff cells and CD8<sup>+</sup> TILs from 4T1-Sc- and 4T1-KD-bearing BALB/c mice treated as in Fig. 3a (c; n = 5 mice per group) and Fig. 3b (d; n = 5 mice per group except for 4T1-Sc IgG, n = 4). e, Quantification of IFNγ (top) and TNF (bottom) expression in CD8<sup>+</sup> T cells, CD4<sup>+</sup>FOXP3<sup>−</sup> Teff and T<sub>reg</sub> cells from DLNs of 4T1-Sc- and 4T1-KD-bearing BALB/c mice treated as in Fig. 3b (n = 5 mice per group except for 4T1-Sc IgG, n = 4). f, Quantification (left) and representative plots (right) of CTLA-4 expression by flow cytometry in CD8<sup>+</sup> T cells, CD4<sup>+</sup>FOXP3<sup>−</sup> Teff and T<sub>reg</sub> cells from tumour and DLN samples of 4T1-Sc and 4T1-KD tumour-bearing mice (n = 5 mice per group). Data are mean ± s.e.m. and representative of at least two independent experiments. P values determined by two-sided unpaired (c–e) or paired (f) t-test.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Treg cell destabilization and CD8\(^+\) TIL activation in additional LDHA-deficient tumour models treated with CTLA-4 blockade.

\(a\)–\(e\), Primary tumour growth and overall survival, reporting the number of tumour-free mice at the end of the experiment, in BALB/c mice implanted in the mammary fat pads with the LDHA-KD 4T1A3-8KD cell line (10\(^6\) cells per mouse) treated with neoadjuvant anti-CTLA-4 \((n = 9)\) or IgG control \((n = 10)\), as indicated. CTLA-4 and CD25 in tumour-infiltrating T\(_{reg}\) cells (b), quantification of T\(_{reg}\) cells and CD8\(^+\) TILs as well as expression of the indicated markers by flow cytometry (c), and flow cytometry analysis of IFN\(\gamma\) expression in CTLA-4\(^+\) and CTLA-4\(^-\) tumour-infiltrating T\(_{reg}\) cells (d) from mice treated as in a (CTLA-4\(^-\) versus CTLA-4\(^+\) T\(_{reg}\) cells, two-sided paired \(t\)-test; IgG versus anti-CTLA-4 CTLA-4\(^-\) T\(_{reg}\) cells, two-sided unpaired \(t\)-test). e, Pearson correlation analyses of the indicated parameters in T\(_{reg}\) cells and CD8\(^+\) TILs from mice treated as in a (black, IgG; red, anti-CTLA-4). \(n = 9\)–\(10\) mice per group; one independent experiment.

\(f\)–\(j\), 4T1-KD-bearing BALB/c mice were treated with the standard IgG2b 9D9 anti-CTLA-4 antibody \((n = 10)\) or its IgG2a variant \((n = 9)\) or IgG control \((n = 10)\) \((f)\), and overall survival \((g)\), quantification of CTLA-4 and GITR expression in T\(_{reg}\) cells \((h)\), and tumour-infiltrating T\(_{reg}\) cells and their expression of FOXP3 and IFN\(\gamma\) by flow cytometry \((i)\) are shown. j, Pearson correlation analyses between the indicated parameters in T\(_{reg}\) cells and CD8\(^+\) TILs from mice treated as in f. \(n = 1\) experiment with 9D9 IgG2a. k–m, LDHA protein expression by western blot \((k)\), LDH activity \((l)\), and glycolytic proton efflux rate (GlycoPER) \((m)\) by Seahorse analysis in B16-KD and B16-Sc cells \((n = 3,\) representative of 2–3 independent experiments). \(n\)–\(p\), C57BL/6j mice were implanted with B16-KD and B16-Sc tumours and treated with anti-CTLA-4 or IgG control as indicated in n. Quantification of CTLA-4 and CD25 \((o;\) \(n = 5\) per group except for B16-KD IgG, \(n = 4\)) and IFN\(\gamma\) expression \((p)\) in tumour-infiltrating T\(_{reg}\) cells by flow cytometry (B16-Sc IgG, \(n = 4\); B16-Sc anti-CTLA-4, \(n = 6\); B16-KD IgG, \(n = 4\); B16-KD anti-CTLA-4, \(n = 3\), representative of two experiments). GzmB, granzyme B; i.d., intradermal; TM, tumour. Data are mean ± s.e.m. \((a–d, h–i, o–p)\) or mean ± s.d. \((l–m)\). Unless stated otherwise, \(P\) values were determined by two-sided unpaired \(t\)-test \((b, c, h, i, m, o, p)\). Pearson correlation coefficient \((e, j)\) or log rank test \((g)\).
Extended Data Fig. 6 | In vivo Treg cell response to tumour glucose metabolism and CTLA-4 blockade. a, b, LDHA protein expression by western blot (representative of three independent experiments) (a) and LDH activity (b) in 4T1-KD and 4T1-EtBr cells in comparison to control 4T1-Sc cells (n = 3). 

Extended Data Fig. 6 | In vivo Treg cell response to tumour glucose metabolism and CTLA-4 blockade. a, b, LDHA protein expression by western blot (representative of three independent experiments) (a) and LDH activity (b) in 4T1-KD and 4T1-EtBr cells in comparison to control 4T1-Sc cells (n = 3). c, Complete cell energetic map with mitochondrial and glycolytic production rates in the indicated 4T1 cell variants using a real-time ATP rate assay by Seahorse (Sc and EtBr, n = 22; KD and A3-8KD, n = 24) (representative of two independent experiments). d–g, BALB/c mice (n = 5 per group) were orthotopically implanted with 10^6 4T1-KD or 4T1-EtBr cells and tumours were surgically resected 13 days later (d). d, Overall survival and number of surviving mice out of total. Frequency of FOXP3 + Treg cells among tumour-infiltrating CD4+ T cells (e), CD25 and CTLA-4 (f) and IFNγ (g) expression in intra-tumour Treg cells by flow cytometry; representative of two independent experiments. 

Extended Data Fig. 6 | In vivo Treg cell response to tumour glucose metabolism and CTLA-4 blockade. h–j, Schematic representation of anti-CTLA-4 or control IgG treatment in BALB/c mice implanted with 4T1-Sc and 4T1-KD in opposite mammary fat pads, and tumour weight on day 13 for samples analysed in i and j. i, GlucoseCy3 staining by flow cytometry in CD45 + tumour cells gated as indicated to enrich in live CD45 + tumour cells by comparing CD45 and DAPI staining between tumour and spleen samples from mice treated as in h. j, GlucoseCy3 staining by flow cytometry in Treg cells gated based on surface staining of CD4, CD25 and GITR in tumour samples as in h. k, In vitro glucose consumption by B16-Sc and B16-KD cells. l, m, Ex vivo glucose uptake potential by flow cytometry analysis of glucoseCy3 staining in CD45 + tumour cells (l) and intra-tumour FOXP3-GFP + Treg cells (m) from B16-Sc- and B16-KD-bearing FOXP3–GFP transgenic C57BL/6J mice treated with anti-CTLA-4 (n = 3; representative of two independent experiments). Data are mean ± s.d. (b–k, m) or mean ± s.e.m. (e–g, h–j). P values determined by two-sided unpaired t test.
Extended Data Fig. 7 | Ex vivo and in vitro Treg cell response to tumour glucose metabolism and CTLA-4 blockade. a, b, FOXP3–GFP transgenic (Tg) mice were implanted with B16-Sc or B16-KD cells and treated with anti-CTLA-4 as indicated in a, and tumour-infiltrating FOXP3–GFP + Treg cells were FACS-sorted and tested in ex vivo suppression assays with CellTrace Violet (CTV)-labelled CD8^+ T cells activated with anti-CD3 in the presence of 0.5 or 10 mM glucose (b). b, Flow cytometry of CD44 and CD25 expression in CD8^+ T cells cultured with B16-Sc- and B16-KD-derived Treg cells (top) and quantification of proliferation (CTV dilution by CTV MFI) of dividing CTV^lo CD8^+ T cells and Treg cell suppression of CD8^+ T cell proliferation in the same culture conditions (bottom) (n = 3, representative of two independent experiments).

c, Quantification by flow cytometry of IFNγ and TNF expression in Treg cells co-cultured with 4T1-Sc or 4T1-KD cells in 5 mM glucose RPMI1640 for 24 h in the presence of soluble anti-CD3, IL-2 and anti-CTLA-4 (n = 3, n = 1 experiment).

d, Glucose consumption and lactate production by NMuMg benign mammary gland cell line in comparison with 4T1 cells (n = 6, n = 1 experiment with NMuMg).

e, Quantification by flow cytometry of IFNγ and TNF expression in Treg cells cultured for 48 h with 4T1-Sc, 4T1-KD- or NMuMg-conditioned medium (11 mM glucose complete RPMI1640) in the presence of plate-bound anti-CD3, IL-2 and anti-CTLA-4 or an IgG control (n = 3, n = 1 experiment). Data are mean ± s.d. *P < 0.05; **P < 0.01; ***P < 0.001. P values determined by two-sided unpaired t-test.
Extended Data Fig. 8 | Loss of functional stability of T<sub>reg</sub> cells induced by anti-CTLA-4 depends on T<sub>reg</sub> cell glycolysis and CD28 signalling.

**a**, Quantification and representative plots of GlucoseCy3 staining by flow cytometry of T<sub>reg</sub> cells activated as in Fig. 4c in the presence of 10 mM glucose ± rotenone/antimycin A or oligomycin and treated with anti-CTLA-4 or IgG control (average of two biological replicates per condition; representative of two independent experiments).

**b**, FOXP3 expression by flow cytometry and IL-10 production by Luminex-based bead immunoassay in T<sub>reg</sub> cells activated in the presence of 10 mM glucose ± rotenone/antimycin A or oligomycin (n = 3, representative of three independent experiments).

**c, d**, Representative plots of in vitro assays reported in Fig. 4f, g. Representative proliferation (CellTraceViolet dilution) by flow cytometry of activated CD8<sup>+</sup> T cells cultured alone or in the presence of T<sub>reg</sub> cells at the indicated glucose concentrations and treated with anti-CTLA-4 or an IgG control (e). **d**, Representative CD86 staining by flow cytometry on B cells from co-cultures with CD8<sup>+</sup> T cells and T<sub>reg</sub> cells treated as in c, d. **e**, In vitro suppression assay with CD25<sup>hi</sup> T<sub>reg</sub> cells immunomagnetically purified from spleens of naive wild-type or CD28-knockout mice cultured for 48 h with CellTraceViolet-labelled CD8<sup>+</sup> T cells and B cells and activated with anti-CD3 in the presence of anti-CTLA-4 or IgG control and the indicated glucose concentrations (n = 3 per conditions except for +CD28 KO Tregs at 1–10 mM glucose, n = 2; representative of two independent experiments). Data are mean ± s.d. P values determined by two-sided unpaired t-test. Oligo, oligomycin.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | CD28 agonism and CTLA-4 blockade, but not PD-1 blockade, drive loss of Treg cell functional stability. 

a, Representative flow cytometry plots of in vitro assays reported in Fig. 4i, j. 

b, Representative proliferation (CellTraceViolet dilution) by flow cytometry of activated CD8+ T cells cultured alone or in the presence of Treg cells at the indicated glucose concentrations and treated with anti-CD28 (2 μg ml⁻¹) or IgG control.

b, Representative CD86 staining by flow cytometry on B cells from co-cultures with CD8+ T cells and Treg cells treated as in a. 

c, Proliferation of CD8+ T cells cultured alone or with Treg cells in 0.5 mM (grey) or 10 mM glucose (black) and activated with increasing concentrations of anti-CD28 (0–0.2 μg ml⁻¹) (n = 3, representative of two independent experiments). 

d, Quantification and representative plots showing suppression of CD4+ T cell proliferation (left) and CD86 expression on B cells (right) by flow cytometry in culture with Treg cells treated with anti-CTLA-4, anti-PD-1 or an IgG control in complete RPMI1640 containing 11 mM glucose. Percentage suppression was calculated relative to proliferation of CD4+ T cells cultured alone in the same treatment conditions (n = 3; n = 1 experiment with anti-PD-1). 

e, Suppression of proliferation of CD8+ T cells cultured at the indicated ratios with FOXP3-GFP+PD-1+ Treg cells (top) or FOXP3-GFP+PD-1− Treg cells (bottom) FACS-sorted from spleens of naive FOXP3-GFP mice and incubated with anti-PD-1 or IgG control for 48 h (representative results from one experiment conducted with CD8+ and CD4+ as target T cells with similar results). 

f, Quantification and representative plots of GlucoseCy3 staining by flow cytometry in Treg cells activated as in Fig. 4c and treated with anti-PD-1 or IgG control (n = 3, representative of two independent experiments). 

g, Flow cytometry quantification and phenotypic analysis of Treg cells from 4T1-KD tumours treated with anti-CTLA-4, anti-PD-1 or IgG control as indicated (n = 10 mice per group, representative of two independent experiments). Data are mean ± s.d. (c, d, f) or mean ± s.e.m. (g). **P < 0.01; ***P < 0.001. P values determined by two-sided unpaired t-test.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Limiting Treg cell glucose metabolism prevents anti-CTLA-4-mediated Treg cell destabilization in glycolysis-defective tumours. a, Foxp3GFP-creERT2;Slc2a1fl/fl (Glut1 cKO) and Foxp3GFP-creERT2 control mice (ctrl) were implanted with B16-KD cells and treated with anti-CTLA-4 or IgG after induction of Slc2a1 deletion with tamoxifen as indicated. Tamoxifen treatment was continued throughout the treatment duration. b, Quantification of Slc2a1 mRNA relative to Actb in FOXP3–GFP– Teff cells and FOXP3–GFP+ Treg cells from the spleens of control and Glut1 cKO mice at the end of treatment as in a (n = 3). c, d, Flow cytometry analysis of CD25 and CTLA-4 (c; n = 3 except for ctrl IgG, n = 1), and IFNγ and TNF expression (d; n = 2) in tumour-infiltrating Treg cells from mice treated as in a (representative of two independent experiments). e, Ex vivo suppression assay with Treg cells sorted from the spleens of control and Glut1 cKO mice treated with anti-CTLA-4 as in a. Treg cell suppression of CD8+ T cell expansion after 48 h co-culture in 10 mM glucose and representative flow cytometry plot showing CTV dilution and generation (G) overlay of CD8+ T cells cultured alone (grey) or in the presence of control (black) or Glut1 cKO (red) Treg cell (n = 3; representative of two independent experiments). f–l, Foxp3GFP-cre:Slc2a1flo (Glut1 HET) or Foxp3GFP-cre:Ldhaflo (Ldha cKO) and Foxp3GFP-cre mice (ctrl) were implanted with B16-KD cells and treated with anti-CTLA-4 (f). g, Quantification of Slc2a1 mRNA relative to Actb in FOXP3–GFP+ Treg cells from spleens of control and Glut1 HET mice (n = 2).

h, i, Quantification by flow cytometry of intra-tumour Treg cells (h) and their expression of Ki67 (i) in control and Glut1 HET mice treated as in f (control, n = 4; HET, n = 2; representative of two independent experiments with mice carrying Glut1 HET or cKO Treg cells). j, Quantification of Ldha mRNA relative to Actb in FOXP3–GFP+ Treg cells from spleens of control and Ldha cKO mice (n = 3).

k, l, Quantification by flow cytometry of intra-tumour Treg cells (k) and their expression of Ki67 (l) in control or Ldha cKO mice treated as in f (ctrl, n = 3; Ldha cKO, n = 2; representative of two independent experiments). m, Schematic representation of the culture conditions used in n, o. CD5+ T cells from Ldha cKO or control mice were co-cultured for 48 h with CD45.1+ congenic antigen-presenting cells (either B cells or T-cell depleted splenocytes) as scaffold for soluble anti-CD3 crosslinking in low (0.5 mM) or higher (10 mM) glucose concentrations as indicated. n, Quantification by flow cytometry of Ldha cKO or control FOXP3–CD4+ Treg cells and their expression of Ki67 after activation as in m. o, FOXP3 and CTLA-4 expression by flow cytometry (MFI) in Ki67-negative Ldha cKO or control Treg cells from cultures as in m. Ctrl 0.5 mM glucose, n = 3 except for anti-CD28, n = 2; ctrl 10 mM glucose, n = 3; cKO, n = 4; representative of two independent experiments. aCTLA-4, anti-CTLA-4; aCD28, anti-CD28. Data are mean ± s.d. (b, e, g, j, n, o) or mean ± s.e.m. (c, d, h, i, k, l). **P < 0.01; ***P < 0.001. P values determined by two-sided unpaired t-test.
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**Statistics**

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- [x] n/a Confirmed
- [x] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- [ ] For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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- [x] 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.*

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**Software and code**

Policy information about availability of computer code

| Data collection | Flow cytometry data were collected using BD FACSDiva software. |
|-----------------|---------------------------------------------------------------|
| Data analysis   | Data were analyzed using the following software: Prism v9 (GraphPad Software); Flowjo v10; R Studio 1.0.143 versions for for Macintosh Pro personal computer. |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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**Data**

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Human melanoma RNAseq data sets investigated in this study were previously reported and have been deposited to the Gene Expression Omnibus (GEO) repository (GSE169278). The 4T1 RNAseq data sets generated for this study have been submitted to the GEO repository (GSE164051). All other relevant data supporting the findings of this study are available in the source data files.
Field-specific reporting

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- Life sciences
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Life sciences study design

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

**Sample size**
Mouse study: for the experiments requiring longitudinal measurements of tumor growth, we have determined that experimental groups of 8-10 animals allow to demonstrate a >50% improvement in tumor-free survival between treatment and control groups without overlapping confidence intervals. This is due to variability in the response to immunotherapy as well as variability in tumor growth following tumor challenge. For experiments that require harvesting of lymphocytes from various tissues (spleens or lymph nodes and tumors) we have estimated based on previous experience that 4-5 mice/group allow assessing statistically significant differences between groups.

**Data exclusions**
No data was excluded, except when samples did not pass the QC (i.e. poor cell viability and/or cell recovery; lymph node contamination in tumors for immune infiltration analysis). These criteria were pre-established. Specifically, samples in the following experiments did not pass the QC for poor cell viability and/or cell recovery: Extended Data Figure 1b, n=1 replicate at 0 μM lactate, poor viability in the absence of any treatment; Extended Data Figure 10h, n=1 tumor sample from Glut1 HET mice with too few Treg events for downstream analyses. Tumor samples in the following experiments did not pass the QC due to draining lymph node (DLN) contamination: Figure 3b, Extended Data Figure 3c,d, Extended Data Figure 4d, n=1 4T1.5c IgG tumor; Figure 3c,d, n=2 4T1.5c IgG and n=1 4T1.5c anti-CTLA-4 tumors; Figure 3g,h,i, n=1 Na Lac treated tumors; Extended Data Figure 4h,j, n=1 4T1.5c IgG and n=1 4T1.5c anti-CTLA-4 tumors.

**Replication**
Results have been replicated in at least 2 independent experiments or in similar experimental conditions as indicated in each figure legend.

**Randomization**
For in vivo experiments in mice, animals were randomized before treatment to make sure the average tumor size was homogeneous across groups.

**Blinding**
Blinding was not relevant to these studies, because the researchers needed to know the treatment groups to conduct the studies, and the data analyses were based on objectively measurable data.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a                              | n/a     |
| □ Antibodies                     | □ ChIP seq |
| □ Eukaryotic cell lines          | □ Flow cytometry |
| □ Palaeontology                  | □ MRI-based neuroimaging |
| □ Animals and other organisms    |         |
| □ Human research participants    |         |
| □ Clinical data                  |         |

Antibodies

**Antibodies used**

- Functional anti-mouse antibodies for in vitro studies:
  - anti-C03 (clone 145-2C11, BD Pharmingen; cat# 553706; https://www.bdbiosciences.com/us/applications/research/t-cell-immunology/th-1-cells/surface-markers/mouse/purified-hamster-anti-mouse-cd3e-145-2c11/p/553706; 0.5-1 μg/ml)
  - anti-CTLA-4 (clone 9H6, BioXcell; https://bxcell.com/product/Invivoplus-anti-m-ctla4/; 50 μg/ml)
  - anti-PD-1 (clone RMP1-14, BioXcell; https://bxcell.com/product/Invivoplus-anti-m-pd1/; 10-50 μg/ml)
  - anti-CD28 (clone 37.53, BD Pharmingen; cat# 553294; https://www.bdbiosciences.com/us/applications/research/t-cell-immunology/regulatory-t-cells/surface-markers/mouse/purified-nale-hamster-anti-mouse-cd28-37.53/p/553294; 0.0125-2 μg/ml)

- Functional anti-mouse antibodies for in vivo studies:
anti-CTLA-4 [clone 9D19 IgG2b, BioXcell; https://bxcell.com/product/invivoplus-anti-m-ctla4/; 100 μg/injection] anti-CTLA-4 [clone 9D19 IgG2a was kindly provided by Bristol Myers Squibb; 100 μg/injection]

Detection anti-mouse antibodies for flow cytometry analyses:

CD45 (clone 30-F11; BD Pharmingen; cat#557659 or 553079; https://www.bdbiosciences.com/ru/applications/research/stem-cell-research/cancer-research/mouse/apc-cy7-rat-anti-mouse-cd45-30-f11/p/557659; https://www.bdbiosciences.com/ru/applications/research/stem-cell-research/cancer-research/mouse/fitc-rat-anti-mouse-cd45-30-f1/p/561088; 1:200)
CD45.1 (clone A20; ebioscience; cat#47-0453-82; https://www.thermosphere.com/antibody/product/CD45-1-Antibody-clone-A20-Monoclonal/47-0453-82; 1:200)
CD3 (clone 145-2C11; BD Pharmingen; cat#564378; https://www.bdbiosciences.com/us/reagents/research/antibodies-buffers/immunology-reagents/anti-mouse-antibodies/cell-surface-antigens/bv650-hamster-anti-mouse-cd3e-145-2c11/p/564378; 1:200)
CD4 (clone RM4-5; ebioscience; cat#56-0042-82 or 17-0042-82; https://www.thermosphere.com/antibody/product/CD4-Antibody-clone-RM4-5-Monoclonal/56-0042-82; https://www.thermosphere.com/antibody/product/CD4-Antibody-clone-RM4-5-Monoclonal/17-0042-82; 1:200)
CD8a (clone 5H10; BD Pharmingen; cat#563234; https://www.bdbiosciences.com/us/reagents/research/antibodies-buffers/immunology-reagents/anti-mouse-antibodies/cell-surface-antigens/bv650-rat-anti-mouse-cd8a-5h10/p/563234; 1:200)
CD25 (clone PC61.5; BD Pharmingen; cat# 551071; https://www.bdbiosciences.com/us/applications/research/t-cell-immunology/regulatory-t-cells/surface-markers/mouse/percpcy55-rat-anti-mouse-cd25 pc61.5/p/551071; 1:200)
CD44 (clone IM7; BD Pharmingen; cat#560567; https://www.bdbiosciences.com/us/applications/research/t-cell-immunology/t-follicular-helper-t-th-cells/surface-markers/mouse/alexa-fluor-700-rat-anti-mouse-cd44 im7/p/560567; 1:200)
C6D2 (clone MEL-14; BD Pharmingen; cat# 553150; https://www.bdbiosciences.com/us/applications/research/t-cell-immunology/regulatory-t-cells/surface-markers/mouse/fitc-rat-anti-mouse-cd62jmel14/p/553150; 1:200)
GTR (clone DTA-1; BD Pharmingen; cat# 558140; https://www.bdbiosciences.com/us/applications/research/b-cell-research/surface-markers/mouse/pe-cy7-rat-anti-mouse-gtr-dta-1/p/558140; 1:200)
PD-1 (clone RMPI-130; ebioscience; cat# 17-9981-82; https://www.thermosphere.com/antibody/product/CD279-PD-1-Antibodyclone-RMPI130-Monoclonal/17-9981-82; 1:200)
CD86 (clone GL1; BD Pharmingen; cat# 558703 or 561963; https://www.bdbiosciences.com/us/applications/research/t-cell-immunology/regulatory-t-cells/surface-markers/mouse/apc-rat-anti-mouse-cd86 gl1/p/558703; https://www.bdbiosciences.com/us/applications/research/t-cell-immunology/regulatory-t-cells/surface-markers/mouse/pe-rat-anti-mouse-cd86 gl1/p/561963; 1:200)
CD11b (clone M1/70.15; Invitrogen; cat#RM2817; https://www.thermosphere.com/antibody/product/CD11b-Antibody-clone-M170-15-Monoclonal/RM2817; 1:200)
F4/80 (clone BM8; ebioscience; cat#11-480; https://www.thermosphere.com/antibody/product/F480-Antibody-clone-BM8-Monoclonal/11-4801-82; 1:200)
MHC-II (clone M5/114.15.2; ebioscience; https://www.thermosphere.com/antibody/product/MHC-Class-II-A4-E-Antibody-clone-M5114152-Monoclonal/485321; 1:200)
Gr1 (clone RB6-8C5; ebioscience; cat#45-5931-80; https://www.thermosphere.com/antibody/product/Ly6G-Ly6C-Antibodyclone-RB68C5-Monoclonal/45593180; 1:200)
Foxp3 (clone FJK-16s, ebioscience cat#11-5773-82, https://www.thermosphere.com/antibody/product/FOXP3-Antibody-clone-FJK16s-Monoclonal/11577382; 1:200, or cat#53-5773-82 https://www.thermosphere.com/antibody/product/FOXP3-Antibodyclone-FJK16s-Monoclonal/53577382; 1:200)
CTLA-4 (clone UC10.4F10-11; cat#553720; https://www.bdbiosciences.com/us/applications/research/t-cell-immunology/regulatory-t-cells/surface-markers/mouse/pe-hamster-anti-mouse-cd152 uc10-4f10-11/p/553720; 1:200)
K67 (clone 856, BD Biosciences cat#652883; https://www.bdbiosciences.com/us/applications/research/intracellular-flow/intracellular-antibodies-and-isotype-controls/anti-human-antibodies/pe-cy7-mouse-anti-ki-67-b66/p/561283, 1:50; or clone 16A8, Biolegend, cat#652403, https://www.biolegend.com/en-us/products/pe-anti-mouse-ki-67-antibody-8134; 1:250)
CD206 (clone CD68B2; Biolegend; cat#141712; https://www.biolegend.com/ja/jp/search-results/alexa-fluor-647-anti-mouse-cd206-mmra-antibody-7477; 1:200)
IFN-gamma (clone XM6G12, BD Biosciences cat#560661, https://www.bdbiosciences.com/us/applications/research/t-cell-immunology/th-1-cells/intracellular-markers/cytokines-and-chemokines/mouse/v450-rat-anti-mouse-ifn-igm12/p/560661; 1:200, or Biolegend, cat#505847, https://www.biolegend.com/en-us/products/brilliant-violet-510-anti-mouse-ifn-gammaintibody-8610; 1:250)
TNF-alpha (clone MP6-XT22, BD Biosciences cat#557644 or 554420, https://www.bdbiosciences.com/us/applications/research/t-cell-immunology/th-1-cells/intracellular-markers/cytokines-and-chemokines/mouse/percpcy55-rat-anti-mouse-tnf-mp6-xt22/ p/557644; https://www.bdbiosciences.com/us/applications/research/t-cell-immunology/th-1-cells/intracellular-markers/cytokines-and-chemokines/mouse/apc-rat-anti-mouse-tnf-mp6-xt22/561062, 1:200; or Biolegend cat#506322, https://www.biolegend.com/en-us/products/percp-cyanine55-anti-mouse-tnf-alpha-antibody-4438, 1:500)

Detection antibodies for western blot analyses:

LOHA rabbit antibody (1:1,000; Cell Signaling Technology; cat# 20125; https://www.cellsignal.com/products/primary-antibodies/lhda-antibody/20125)
Vinulcin mouse monoclonal antibody (1:1,000; Santacruz; cat#sc-73614; https://www.scbt.com/p/vinulcin-antibody-719) Anti-rabbit IgG HRP conjugated (1:5,000; Cell Signaling Technology; cat#70745; https://www.cellsignal.com/products/secondary-antibodies/anti-rabbit-igg-hrp-linked-antibody/7074)
Anti-mouse IgG HRP conjugated (1:5,000; Cell Signaling Technology; cat#70765; https://www.cellsignal.com/products/secondary-antibodies/anti-mouse-igg-hrp-linked-antibody/7076)
beta actin rabbit antibody (1:5,000, Sigma, cat#A2065; https://www.sigmaaldrich.com/catalog/product/sigma/a2065?lang=en&region=US)
Eukaryotic cell lines

Policy information about: cell lines

Cell line source(s)  The murine mammary carcinoma 4T1 cell line was kindly provided by Dr. Fred Miller (Karnans Cancer Institute) and the benign mammary gland NMuMG cell line by Dr. Jason Koutcher (MSK). The B16F10 mouse melanoma cell line was originally obtained from I. Fidler (M. D. Anderson Cancer Center, Houston, TX).

Authentication  Cell lines were authenticated by STR profiling (4T1) or morphology and expression of specific antigens (4T1 and B16). We routinely confirmed LDHA knock down in 4T1 and B16 LDHA-kO cell lines by western blot and LDH assay.

Mycoplasma contamination  Cell lines were routinely screened to avoid mycoplasma contamination.

Commonly misidentified lines (see iCLAR register)  No commonly misidentified lines were used in the study.

Animals and other organisms

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

Laboratory animals  Female BALB/cAnN mice were from Charles River Laboratory, RAG2 KO BALB/c from Taconic, and WT; CD28 KO and CD45.1 congenic C57BL/6j mice from Jackson Laboratory. Foxp3-GFP transgenic mice were generously provided by Dr. Alexander Rudensky and backcrossed to C57BL/6j at MSK. Foxp3-GFP-CreERT2, Foxp3-GFP-CreERT2;Slc2a1(Null1);/+, C57BL/6j mice were kindly provided by Dr. Greg Delgoffe (University of Pittsburgh). Foxp3-FLP-Cre;Ldhaf1f1 mice were kindly provided by Dr. Ping-Chih Ho and bred at MSK. Same sex, same age mice were used in each experiment. All mice were bred and maintained under specific pathogen-free conditions (with a 12 h light-dark cycle at temperature of 21–23°C and humidity of 35–55%), and used at the ages of 5–10 weeks. The maximal tumor size of 20 mm in any direction was not exceeded in any experiment. All animal experiments were conducted according to protocols approved by the MSK and University of Pittsburgh Institutional Animal Care and Use Committee.

Wild animals  The study did not involve wild animals.

Field-collected samples  The study did not involve samples collected from the field.

Ethics oversight  All mouse procedures were performed in accordance with the Institutional Animal Care and Use Committee of MSK and the University of Pittsburgh.

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

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  Sample preparation is described in details in the Methods section.

Instrument  Samples were collected on an LSRII or Symphony XSO flow cytometer (BD Biosciences).

Software  Flow cytometry data were collected using BD FACSDiva software (BD Biosciences) and analyzed with FlowJo software v. 10 (Tree Star Inc.).

Cell population abundance  The abundance of the cell populations of interest after FACS-based or immunomagnetic purification was >90%.

Gating strategy  The gating strategy is accurately described in the figure legend of each figure reporting flow cytometry data and also fully displayed in Extended Data Figure 4a.

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