

Active aldehydes accelerate CD8⁺ T cell exhaustion through metabolic alteration in tumor microenvironment

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Abstract

Glycolysis and mitochondrial fatty acid oxidation (FAO) regulate CD8⁺ T cell differentiation fate. However, how this metabolic balance regulates T-cell exhaustion remains unknown. PD1 signal inhibits glycolysis and enhances FAO. Here, we find that CD8⁺ T cells in the tumors adhere to glycolysis with attenuated FAO despite high PD1 expression. Active aldehydes, final products of lipid peroxidation, accumulate in CD8⁺ T cells in proportion to their exhaustion depth, defined by mitochondrial mass and potential. Aldehydes promote glycolysis and inhibit the FAO activity in T cells. Mice deficient of an FAO enzyme in T cells generate more acrolein, a representative aldehyde, enhancing T-cell exhaustion and attenuating anti-tumor immunity. Acrolein is partly generated from mitochondria and damages mitochondrial architecture. Inhibitors of lipid peroxidation or aldehydes enhanced the PD1-blockade therapy effect by rectifying metabolic imbalance. Therefore, active aldehydes resulting from FAO impairment cause a vicious cycle of metabolic imbalance that leads to T cell exhaustion.

Introduction

A major challenge of PD1 blockade cancer immunotherapy is that a substantial number of patients do not respond to such treatment. The therapeutic effects of cancer immunotherapy are largely influenced by the exhaustion of tumor infiltrating CD8⁺ T cells^{1, 2, 3, 4}. Although the exhaustion stage of CD8⁺ T cells is a spectrum, exhausted CD8⁺ T cells (Tex cells) are roughly divided into progenitor Tex cells and terminal Tex cells^{5, 6, 7}. Progenitor Tex cells are characterized by longevity and proliferative capacity and can give rise to effector T cells. On the other hand, terminally Tex cells have lost their proliferation capacity and the majority of them undergo cell death^{4, 5, 6, 7}. Recent studies reveal that the anti-tumor effect of PD1 blockade therapy is mediated not by the rejuvenation of terminal Tex cells but by the expansion of functional effector T cells from progenitor Tex cells^{4, 8, 9}. Therefore, mechanisms and methodologies by which progenitor Tex cells are generated or maintained in the tumor microenvironment (TME) have been studied⁷. The manipulation of T cell metabolism is a practical strategy for inducing the progenitor Tex cells in tumor-bearing host^{10, 11, 12, 13, 14, 15}.

The balance of glycolysis and mitochondrial metabolism in T cells is finely controlled to fulfill their energy demands according to their differentiation stages such as naïve, effector, and memory T cells^{16, 17}. Although researchers in general agree that mitochondria in Tex cells are dysfunctional^{13, 18, 19, 20}, there are different conclusions of the glycolysis usage in Tex cells. Some reports indicate that Tex cells decrease the expression of glucose transporter 1 (Glut1) and/or decrease the glucose uptake^{21, 22, 23, 24}, whereas others indicate that Tex cells favor glycolysis with upregulated HIF-1 α , glucose transporters and/or glucose uptake^{25, 26}. This unresolved discrepancy might be attributed to differences in the definition of T-cell exhaustion among researchers.

Although glycolysis is critical for the differentiation of naïve T cells to effector T cells, extreme reliance on glycolysis paradoxically impairs the effector function and boosts the exhaustion of T cells^{26, 27, 28, 29}. Indeed, glycolysis inhibitors such as 2-deoxy-D-glucose (2-DG) and Akt inhibitors delay T-cell exhaustion, increase the number of long-surviving T cells, and, consequently, enhance the antitumor immunity^{10, 30}. Upon the glycolysis inhibition, T cells prefer to utilize the mitochondrial metabolism and upregulate the fatty acid oxidation (FAO) activity, which are important for the longevity of T cells^{30, 31}. Consistently, FAO activators, such as bezafibrate, fenofibrate, metformin, and spermidine, reduce the generation of terminal Tex cells and increase the number of long-survival T cells^{12, 15, 32, 33}. This growing evidence suggests that the balance between glycolysis and FAO is crucial for determining antitumor immune responses.

Recent studies have indicated that terminal Tex cells accumulate lipid peroxides and dampen their mitochondrial activity, which further accelerates exhaustion^{34, 35, 36}. As lipid peroxidation leads to ferroptosis, terminal Tex cells could be subjected to oxidative stress-induced cell death^{35, 37}. The lipid accumulation is associated with a reduced expression of very-long-chain acyl-CoA

dehydrogenase, a FAO-related enzyme, in CD8⁺ Tex cells, suggesting an important role of FAO in the robust function of CD8⁺ T cells in TME ³⁴.

PD1 is a checkpoint for T cells to avoid overactivation-induced cell death by inhibiting T cell receptor and CD28 signaling ^{38,39}. PD1 signaling is known to inhibit glycolysis and enhance FAO, which are advantageous for T cell longevity ^{21,40}. Nevertheless, PD1⁺ Tex in TME are usually short-lived and carry impaired mitochondrial metabolism, suggesting the dysregulated balance of glycolysis and FAO. How the glycolysis and FAO balance are associated with the T cell exhaustion process is not fully understood.

In this paper, we report that the mitochondrial status of CD8⁺ T cells, characterized by the mitochondrial mass and potential, correlates with the exhaustion depth of these cells. This mitochondrial compartmentation distinguishes terminal Tex cells into living and early apoptotic populations. Despite the high expression of PD1, the living terminal Tex cells show features of high glycolytic and low FAO activity, which we define as “metabolic exhaustion”. Tex increased accumulation of active aldehydes, final products of lipid peroxidation in an exhaustion depth-dependent manner. The accumulation of active aldehydes is more prominent in an FAO enzyme (HADHA)-deficient T cells. These results indicate that FAO activity is critical to prevent active aldehyde accumulation during T-cell exhaustion. Acrolein, a representative active aldehyde generated from mitochondria, impairs FAO activity and accelerates the glycolysis signal transduction. Therefore, active aldehyde shapes the metabolic exhaustion of T cells in TME.

Results

Mitochondrial status is associated with metabolic exhaustion of T cells in TME

Naïve CD8⁺ T cells could be divided into four populations (D1-D4) according to their mitochondrial potential and mitochondrial mass (Fig. 1A, day 0). Using the *in vitro* CD8⁺ T cell exhaustion model of repetitive stimulation with anti-CD3 antibody and IL-2 ⁴¹, we found that these stimulations promoted the sequential differentiation of CD8⁺ T cells from D1 to D4 *in vitro* (Fig. 1A). To know the mitochondrial status of exhausted CD8⁺ T (Tex) cells in tumors, we examined mitochondrial potential and mass of PD1⁺ CD8⁺ T cells among the tumor-infiltrating lymphocytes (TILs) after gating on CD45⁺ Zombie⁻ population (Extended Data Fig. 1A). We confirmed that the T cells, which have depolarized mitochondria and are recognized as “terminal” Tex cells in several literatures, were located in D4 in our mitochondrial compartmentation definition (Extended Data Fig. 1A) ^{13,18,22}. D4 cells were mostly small (FSC-A^{low}) and Zombie⁻ Annexin V^{high}, suggesting that they were early-stage apoptotic T cells (Fig. 1B and Extended Data Fig. 1A) ⁴². To trace the T cell differentiation trajectory *in vivo*, we intravenously transferred CD45.2⁺ naïve OT-1 T cells into CD45.1 recipient mice with MC38-OVA tumor. Over time,

tumor-infiltrating donor PD1⁺ CD8⁺ T cells progressively differentiated from D1 to D3 (Extended Data Fig. 1B). Although D4 cells did not change drastically, they were presumably eliminated via rapid dead-cell clearance mechanisms such as efferocytosis in response to phosphatidylserine exposure on the cell surface (Fig. 1B)⁴³.

To avoid analytic limitations of the color combination in flow cytometry for further analysis, we sought to find surrogate markers of D1-D4. If the D4 population was excluded, the fractions determined by the mitochondrial potential intensity alone (D1' D2' and D3') precisely corresponded to D1, D2 and D3, respectively (Extended Data Fig. 1C). We therefore used D1'-D4' as a surrogate marker of D1-D4 hereafter. The capacity of cytokine production and cytotoxic function of T cells in MC38 sequentially increased from D1' to D3' (Extended Data Fig. 1D). In accordance with the increase in Annexin V positivity (Fig. 1B), exhaustion markers gradually increased from D1 to D3 (Fig. 1C and Extended Data Fig. 1E). Most of these exhaustion markers were reduced in D4, possibly due to the death process of these cells. Accordingly, the expression of Slamf6, a progenitor Tex marker known to be correlated with Tcf1⁴, decreased from D1 to D3 (Fig. 1D). Similar cytokine production capacity and surface exhaustion marker results were obtained from the in vitro exhaustion model on day 8 (Extended Data Fig. 1F-G).

To further analyze the role of mitochondrial status in the definition of Tex, we conducted single-cell RNA sequencing (scRNA-seq) on the isolated D1, D2 and D3 populations from PD1⁺ CD8⁺ T cells in the MC38 tumor. Cluster analysis demonstrated five distinctive populations, where cluster 0, 1 and 2 exhibited exhaustion signatures and cluster 3 showed high stem-like gene signatures (Extended Data Fig. 1H-I). The D1-D3 populations were broadly distributed with a degree of polarization: D2 and D3 populations were enriched for exhausted signatures whereas the D1 population showed higher stem-like signatures (Fig. 1E-F). GO Enrichment analysis revealed that pathways associated with cell division were significantly upregulated in D3 compared to D1, indicating the differentiated or proliferating state of D3 in the TME (Fig. 1G and Supplementary Table 1). The cell differentiation trajectory analysis revealed a clear gradient in the pseudotime score, reflecting the progressive differentiation from D1 to D3 (Fig. 1H). Results of analyzing the scRNA-seq dataset for the CDR3 region of TCR demonstrated directional clonal expansion from D1 to D3 (Fig. 1I). Although the cell cycle-related genes were transiently upregulated in D3 in the TME (Fig. 1G), their proliferation capacity was gradually declined from D1 to D3 upon ex vivo re-stimulation, indicating that D3 cells have already entered a state in which they can no longer effectively divide by further stimulation (Fig. 1J). Although D2 and D3 showed little or only marginal difference in their exhaustion markers reported (Fig. 1C and F), they are clearly different in their differentiation trajectories, clonal expansions, and proliferative capacities (Extended Data Fig. 1B and Fig. 1H-J). Therefore, mitochondria-based classification provides a higher resolution definition of exhaustion, which remains ambiguous due to their

heterogeneity^{44, 45}. The mitochondrial status of Tex cells in TME also demonstrates that terminal Tex cells existed in either a live state with high membrane potential or an apoptotic state with depolarized mitochondria, a previously undefined feature.

We next investigated metabolic flows of D1-D4 PD1⁺ CD8⁺ T cells in tumor tissues, focusing on glycolysis and FAO. Glucose transporter-1 (Glut1) expression and 2-NBDG, a fluorescent glucose analog, uptake increased from D1' to D3' but decreased in D4' (Fig. 2A). To measure the precise glycolysis activity, we incubated isolated D1-D3 with ¹³C-labeled glucose for three hours and measured the ¹³C-labeled lactic acid. D4 was excluded from this ¹³C tracing assay because this population was mostly trypan blue-positive (dead) after incubation. As shown in Fig. 2B, the glycolytic activity increased from D1 to D3 progressively. These data suggest that live "terminal" Tex cells (D3) had high glycolysis activity, whereas early apoptotic "terminal" Tex cells (D4) did not, which might explain the discrepancy in glycolysis usage of Tex cells between reports^{21, 22, 23, 24, 25, 26}. Regarding mitochondrial metabolism, the central fatty acid oxidation (FAO) enzyme HADHA gradually decreased in response to the exhaustion depth (Fig. 2C). A similar reduction in HADHA expression was also observed in human cancers, relating with exhaustion depth (Fig. 2D). The carnitine-acylcarnitine translocase SLC25A20 on mitochondrial inner membrane, which is critical for FAO in shuttling acylcarnitine into the mitochondrial matrix and exporting free carnitine into the cytosol^{46, 47}, also gradually decreased from D1' to D4' (Fig. 2E). Furthermore, because elaborated and densely folded cristae is associated with greater oxidative phosphorylation and FAO activity^{31, 48}, we analyzed the complexity of cristae architecture in mitochondria using Transmission Electron Microscope (TEM). This TEM analysis revealed a loosed cristae structure with progressively increased spacing between folds from D1 to D3 (Fig. 2F). These data suggest impaired mitochondrial FAO activity according to the exhaustion depth.

PD1 signal is reported to inhibit glycolysis and enhance FAO^{21, 40}. However, these data suggested that live "terminal" Tex cells (D3) adhered to glycolysis with an attenuated mitochondrial function, such as FAO, despite PD1 expression, which is partly supported by previous reports^{13, 18, 19, 20, 25, 26}. This unbalanced metabolism in Tex in TME was referred to as "metabolic exhaustion" in this work hereafter. We focused on live Tex cells (D1-D3) for the following mechanistic analysis.

Active aldehydes accumulate in proportion to the exhaustion depth

Lipid peroxidation induced by reactive oxygen species (ROS) is one of the exhaustion processes in T cells^{34, 35, 36}. Mitochondrial ROS (mROS) and lipid peroxides (lipid radical) accumulated in PD1⁺ CD8⁺ T cells in TME in proportion to the exhaustion depth from D1' to D3' (Fig. 2G and Extended Data Fig. 2A). Although active aldehydes are the final products of lipid peroxidation and are more than 10 times toxic than ROS^{49, 50}, the role of active aldehydes in the T cell

exhaustion has not been extensively addressed (Fig. 2H). We measured the level of acrolein, a representative active aldehyde, by AcroleinRED, which reacts specifically to the free acrolein in live cells. We also used an antibody against protein-conjugated acrolein (PC-Acro) to detect the reacted acrolein in the cells. Acrolein levels measured by both methods were elevated in correspondence with the exhaustion depth (Fig. 2I and Extended Data Fig. 2B). Like acrolein, levels of other active aldehydes, including malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE), were also elevated in highly Tex cells (Extended Data Fig. 2C). Elevated levels of lipid radical, acrolein and PC-Acro were similarly observed among D1' to D4' in the in vitro exhaustion model by repetitive stimulation (Extended Data Fig. 2D). The acrolein levels in CD8⁺ T cells were also increased by repetitive stimulation (Extended Data Fig. 2E). Moreover, in human carcinomas, a greater accumulation of acrolein was observed in accordance with the exhaustion depth in tumor-infiltrating CD8⁺ T cells (Fig. 2J). Therefore, the gradual accumulation of active aldehydes in accordance with the exhaustion depth is a general phenomenon.

Acrolein enhances glycolysis and attenuates the FAO activity

Although acrolein was known to be a detrimental metabolite, how acrolein contributed to metabolic exhaustion was unknown. The D3' population showed high glycolytic activity (Fig. 2A-B), which is reported as a hallmark of a metabolic feature of Tex cells^{25,26}. To investigate the effect of acrolein on glycolysis, we stimulated mouse total CD8⁺ T cells with CD3/CD28 beads with or without acrolein and examined the activity of metabolic pathways. Although free acrolein concentration was approximately 10 μ M in MC38 tumor tissues (Extended Data Fig. 3A), we optimized and used acrolein (ACR) at 2-5 μ M range in vitro, which was appropriate for the proliferative capacity assay for primary CD8⁺ T cells. We found that the Akt-mTOR pathway, which promotes glycolysis during T-cell differentiation, was quickly upregulated in CD8⁺ T cells within 3 hours of the acrolein stimulation (Fig. 3A-B)^{40, 51}. Seahorse analysis revealed that acrolein increased the level of glycolysis, indicated by extracellular acidification rate (ECAR) (Fig. 3C). Moreover, acrolein stimulation significantly enhanced the glucose uptake, PD1 expression, and proliferation of CD8⁺ T cells, indicating accelerated T cell differentiation (Fig. 3D-F). Indeed, acrolein boosted T-cell differentiation from D1 to D4 within 7 and 21 hours (Fig. 3G and Extended Data Fig. 3B). Increases in Akt-mTOR pathway and PD1⁺ Tim3⁺ Tex cells by ACR stimulation were more obvious in the in vitro exhaustion model on day 8 (Extended Data Fig. 3C-D). In terms of mitochondrial metabolism, we investigated the two main pathways FAO and glutaminolysis using CpT1a inhibitor (Etomoxir) and glutaminase inhibitor (BPTES), respectively. Acrolein stimulation reduced mitochondrial respirations within 3 hours in an etomoxir-dependent but BPTES-independent manner, demonstrating that acrolein selectively attenuated FAO activity over glutaminolysis (Fig. 3H).

We next investigated the molecular mechanisms by which acrolein stimulation enhanced glycolysis and inhibited FAO. Phosphoproteomic analysis of CD8⁺ T cells stimulated by acrolein for 1 hour revealed the activation of TCR-driven Akt/mTOR-related signaling pathways (including Rho GTPase cycle and phospholipase C pathways) and suppression of mitochondrial biogenesis, as predicted by Ingenuity Pathway Analysis (IPA) software (Fig. 3I and Supplementary Table 2-3). TCR signal is known to activate pyruvate dehydrogenase kinase 1 (Pdk1), leading to phosphorylation and inactivation of pyruvate dehydrogenase E1 alpha 1 (Pdha1), thereby promoting anaerobic glycolysis⁵². Among ~20,000 phosphopeptides analyzed, Pdha1 was one of the most significantly increased by ACR, ranking third in upregulation (Fig. 3J and Supplementary Table 2). We further investigated the expression levels of glycolysis and FAO-related enzymes by western blotting. Among the enzymes examined, only the acylcarnitine transporter SLC25A20 was reduced in 3-hour stimulation (Fig. 3K and Extended Data Fig. 3E). TEM analysis demonstrated that three-hour of ACR stimulation loosened the density of mitochondrial cristae or degraded their architecture (Fig. 3L). These data underpin our findings that acrolein upregulates glycolysis and inhibits FAO.

Treating CD8⁺ T cells with another active aldehyde, 4-HNE, upregulated glycolysis and proliferation (Extended Data Fig. 3F-G). In contrast to active aldehydes, treating T cells *in vitro* with hydrogen peroxide (H₂O₂), a ROS, did not enhance glycolysis or differentiation (Extended Data Fig. 3H-J). Therefore, active aldehydes promoted the differentiation of CD8⁺ T cells toward an exhausted state with glycolysis upregulation and FAO downregulation despite the high expression of PD1 in these cells^{21, 40}.

Endogenous acrolein dictates the metabolic exhaustion

Acrolein is a primary active aldehyde showing the highest toxicity, it reacts immediately with neighboring cysteine and lysine and denatures those proteins^{53, 13, 18, 19, 20}. Therefore, we addressed the role of active aldehydes in forming metabolic exhaustion, focusing on acrolein. We stimulated CD8⁺ T cells with CD3/CD28 beads in the presence of an acrolein scavenger, N-benzylhydroxylamine (NBHA)⁵⁰. NBHA reduced glycolysis (ECAR), increased the D1 population, and reduced D2 and D3 populations, suggesting that endogenous acrolein enhanced the differentiation of T cells (Fig. 4A-B). This NBHA effect of inhibiting differentiation was more obvious in the *in vitro* exhaustion model after long culture, NBHA treatment significantly reduced the frequency of PD1⁺ Tim3⁺ Tex cells on day 8 (Extended Data Fig. 4A). We therefore examined the relation between cellular acrolein levels and metabolic features in tumor-infiltrating PD1⁺ CD8⁺ T cells. As expected, compared to the CD8⁺ T-cell population with a low amount of acrolein, that with a high amount of acrolein exhibited a stronger Akt-mTOR signal transduction, Glut1 expression, and 2-NBDG incorporation (Fig. 4C-D). Similarly, Akt-mTOR signal transduction,

Glut1 expression, and 2-NBDG incorporation were increased in Tex cells infiltrating human renal cell carcinoma (RCC) and lung cancer with high amount of acrolein (Fig. 4E).

Given that acrolein impacted T-cell metabolic regulation, we investigated the intracellular location of acrolein production. Super-resolution fluorescence microscopy analysis of CD8⁺ TILs demonstrated that signals of mitochondria (PhenoVue 551) and PC-Acro exhibited approximately 40 % spatial overlap (Fig. 4F). In addition, mitochondrial membrane peroxidation progressively increased in accordance with the hierarchical accumulation of acrolein from D1 to D3 (Fig. 2I-J and Fig. 4G), supporting the acrolein production being partly from mitochondria⁵⁴. CD8⁺ T cells might be inefficient in detoxifying acrolein due to low expression of aldehyde dehydrogenase 2 (Aldh2): we could not detect mRNA of Aldh2 in scRNA-seq analysis (Extended Data Fig. 4 B). These data suggested that the intrinsic acrolein contributed to the dysregulation of mitochondrial function and metabolic exhaustion.

FAO dysfunction accelerates metabolic exhaustion

To determine the role of FAO in metabolic exhaustion in T cells, we analyzed *Hadha*^{flox/flox} *Cd4*-Cre mice that were deficient of HADHA in T cells (HADHA-T KO)¹⁵. HADHA-T KO mice develop without any apparent defects and show a similar frequency of CD44⁺ population in CD8⁺ T cells compared with wild-type mice¹⁵. Further, naïve peripheral CD8⁺ T cells in HADHA-T KO mice did not increase the expression of FAO-related molecules or functions, including lipid radicals, acrolein, Akt-mTOR signal transduction, 2-NBDG incorporation, fatty acid transporter CD36, and fatty acid uptake, compared to those in wild-type mice (Extended Data Fig. 5A-C). However, tumor-infiltrating PD1⁺ CD8⁺ T cells in HADHA-T KO mice upregulated the expression of the fatty acid transporter CD36 and increased the fatty acid uptake compared with those cells from the wild-type mice (Fig. 5A). These HADHA-deficient T cells accumulated more lipid radicals and acrolein as well as presented stronger Akt-mTOR signal transduction and 2-NBDG incorporation than did wild-type cells (Fig. 5B-C). To investigate the metabolic balance of glycolysis and mitochondrial pathways in T cells from HADHA-T KO mice, we measured metabolic flow of naïve CD8⁺ T cells during the CD3/CD28 beads stimulation *in vitro*. The Real-Time ATP Rate Assay in Seahorse revealed that ATP demands during stimulation were fulfilled mostly by glycolysis in HADHA-deficient T cells, in contrast to mainly by mitochondrial pathways in control T cells (Fig. 5D). This dependence on glycolysis rather than mitochondria metabolism in CD8⁺ T cells from HADHA-T KO mice resembled metabolic exhaustion. Indeed, more tumor-infiltrating CD8⁺ T cells from HADHA-T KO mice expressed exhaustion markers Tim3 and TOX and the cell death marker Annexin V than did those from the wild-type mice (Fig. 5E-F). Functionally, tumor-infiltrating PD1⁺ CD8⁺ T cells from HADHA-T KO mice had less

capacity of proliferation than those from wild-type ctrl mice (Fig. 5G). Accordingly, tumor growth was faster and the frequency of tumor-infiltrating CD8⁺ T cells was lower in the HADHA-T KO mice than in the wild-type mice (Fig. 5H-I).

Fatty acids were highly incorporated in CD8⁺ TIL from HADHA-T KO mice compared to wild-type mice (Fig. 5A). Since terminal Tex cells exhibited FAO dysregulation in wild-type mice (Fig. 2C-F), we investigated the fatty acid uptake in the terminal Tex cells. PD1⁺ Tim-3⁺ Tex cells from wild-type mice increased the fatty acid incorporation compared with PD1⁺ Tim-3⁻ T cells (Extended Data Fig. 5D), which might be linked to further accumulation of lipid radical and acrolein in the terminal Tex cells (Fig. 2G and I). These data demonstrate that FAO dysfunction exacerbates the exhaustion state and increases oxidative stress by promoting acrolein accumulation in T cells through a feedback loop mechanism (Extended Data Fig. 5E).

A lipid radical or acrolein scavenger enhances anti-tumor immunity

We finally examined whether lipid radical or acrolein scavengers could reduce the exhaustion and enhance antitumor immunity. The lipid radical scavenger α -tocopherol (vitamin E: VtE) and the aldehyde-specific scavenger N-benzylhydroxylamine (NBHA) reversed the low antitumor activity of HADHA-T KO mice (Fig. 6A-B). While glycolytic activity was increased in effector CD8⁺ T cells from HADHA-T KO mice (Fig. 5C-D), NBHA administration suppressed the Akt-mTOR pathway, increased the D1 population, and decreased the D2 population in PD1⁺ CD8⁺ TIL in HADHA-T KO mice (Fig. 6C-D). These data validated that acrolein accumulation in T cells of HADHA-T KO mice was one of the primary reasons for the low antitumor immunity, as acrolein promotes their differentiation.

We also investigated whether acrolein undermines the response to the PD-L1 mAb therapy. Administrating either VtE or NBHA enhanced the antitumor activity of the PD-L1 mAb (Fig. 7A-B). Although the frequency of PD1⁺ CD8⁺ TILs among CD45⁺ cells was increased by PD-L1 mAb treatment with or without NBHA (Fig. 7C), their metabolic phenotypes were altered. Consistent with the previous report²¹, PD-L1 mAb treatment increased the tendency of glycolysis-associated signaling and significantly reduced FAO activity measured by tracing the metabolites (acylcarnitine) derived from ¹³C-labeled palmitate (Extended Data Fig. 6 and Fig. 7D). Importantly, adding NBHA to PD-L1 mAb treatment abolished effects of PD-L1 mAb treatment on glycolysis-related signals and FAO activity in PD1⁺ CD8⁺ TILs (Extended Data Fig. 6 and Fig. 7D). Because D1-D4 defined by mitochondrial states correlated with the exhaustion depth (Fig. 1), we examined D1-D4 population changes upon PD-L1 mAb treatment alone or in combination with NBHA. While PD-L1 mAb treatment alone reduced the D1 population and increased the D3 population among the PD1⁺ CD8⁺ TILs, adding NBHA to PD-L1 mAb treatment abolished these effects (Fig. 7E). Moreover, NBHA recovered the reduction of proliferative capacity induced by

PD-L1 mAb alone (Fig. 7F). These data indicate that acrolein scavenger enhances anti-tumor immunity by inhibiting metabolic alteration and differentiation of CD8⁺ T cells in TME.

Discussion

In this paper, we demonstrate two key points. First, the mitochondrial status of CD8⁺ T cells characterizes the exhaustion stages in TME, which the conventional exhaustion markers can not capture. Terminally differentiated Tex cells are divided into living and early apoptotic populations: living terminal Tex cells increase mitochondrial potential and glycolysis activity, and apoptotic terminal Tex cells reduce metabolic fitness characterized by depolarized mitochondria and low glycolysis activity. These results may explain the different conclusions on the utility of glycolysis in terminal Tex cells among literatures^{21, 22, 23, 24, 25, 26}. Second, T cells with attenuated FAO accelerate their lipid peroxidation and active aldehyde production, which are prominently observed in the terminal Tex cells. Active aldehydes, partly produced by mitochondria, enhance glycolysis and reduce FAO activity in Tex cells, leading to metabolic exhaustion, despite high PD1 expression (Extended Data Fig. 7A-C).

The metabolic balance of glycolysis and FAO regulates the differentiation fate of T cells toward short- or long-lived cells with low or high proliferation capacity, respectively^{10, 30, 31}. Effector T cells use both glycolysis and FAO to meet their anabolic and catabolic demands^{16, 17, 32}. However, chronic stimulation by tumor antigens in TME gradually attenuates the mitochondrial FAO activity, potentially via the mechanism that acrolein, generated from mitochondria as a final product of lipids-ROS reactions, reduces mitochondrial biogenesis and damages mitochondrial membrane structure directly. The HADHA level decreases progressively from D1 to D4, but its level does not decrease immediately after acrolein treatment, unlike SLC25A20. These results suggest that the reduction of HADHA may result from the acrolein-associated signal pathway rather than the direct damage. Although mitophagy is critical for the clearance of damaged mitochondria⁵⁵, active aldehydes might inhibit autophagy and mitophagy through the activation of the Akt-mTOR pathway⁵⁶. Active aldehydes would, therefore, reduce the ability to clear damaged mitochondria, resulting in the accumulation of more aldehydes. A robust FAO activity shunts this loop and renders T cell longevity.

Our data indicate that D1 and D2 T cells use both glycolysis and FAO in TME whereas the highly exhausted D3 T cells rely mostly on glycolysis. As the Slamf6 expression in D1 and D2 populations is higher than in the D3 population, the majority of Tcf1⁺ progenitor Tex cells should be located around D1-D2. These data, therefore, support that progenitor Tex cells utilize the balanced glycolysis and FAO metabolism to retain their self-renewal ability⁵⁷. Considering the report that Tcf1-knockdown T cells carry dysfunctional and depolarized mitochondria⁵⁸, the

transition from D1-D2 to D3-D4 partly reflects the differentiation from progenitor to terminal Tex cells⁴. Because the frequency of progenitor Tex cells correlates to the higher response to the PD1 blockade therapy^{4, 8, 9}, the frequency of D1-D2 in TME might be a good biomarker for better responsiveness. Further studies are warranted.

In the TME with low glucose and high fatty acid levels, T cells are known to rely on FAO to survive³². Tumor-infiltrating CD8⁺ T cells in HADHA-T KO mice incorporate fatty acids greater than those of wild-type mice. A possible reason is that FAO dysfunction reduces the ATP production of T cells, and the consequent alteration of AMP/ATP ratio upregulates the AMPK/CD36 pathway⁵⁹. Therefore, FAO plays an important role in not only consuming fatty acids but also preventing the uptake of unnecessary fatty acids, contributing to the retention of T cell function and their longevity.

Some groups have indicated that ROS accelerates the exhaustion of T cells. ROS scavengers or inhibitors such as N-acetylcysteine and mitochondrial anti-oxidants inhibit T cell exhaustion^{19, 20, 60}. As active aldehydes are the final products of lipids peroxidation, these ROS scavengers could also inhibit the production of active aldehydes. Given that the reactive toxicity of active aldehydes is higher than that of ROS^{49, 50}, active aldehydes are likely to be a major factor for exhaustion in addition to ROS. Since the reaction mode of active aldehydes is different from that of ROS, the subsequent signal cascades between ROS and reactive carbonyl species (RCS), such as active aldehydes, could be different⁶¹. As we did not find a quick upregulation of ECAR by ROS, ROS and RCS may differently contribute to the exhaustion steps. Further studies on the signal cascade of active aldehydes, especially on glycolysis and FAO, are needed.

Our in vitro analysis was conducted using extracellular acrolein (ACR) treatment, which may not fully replicate the intracellular signaling environment driven by endogenously generated acrolein from mitochondria. However, acrolein, a small molecule, can act as a diffusive agent through the cell membrane⁶². Mitochondrial acrolein, therefore, should diffuse into the cytoplasm, which is highly likely to activate Akt/mTOR signaling. Acrolein is highly reactive toward cysteine residues through the addition reaction⁶³. Acrolein may not only affect Akt/mTOR signaling-associated molecules in the way of post-translational regulation, but also covalently modify and inactivate PTEN, thereby activating the PI3K/Akt pathway and promoting phosphorylation of Akt substrates⁶⁴. For SLC25A20 reduction by acrolein, several cysteine residues have been reported to be critical for its functional regulation⁴⁷. Thus, the rapid reduction of SLC25A20 following acrolein exposure may result from direct cysteine modification, potentially leading to protein denaturation and functional impairment.

Although the active aldehyde-specific scavenger NBHA enhanced the anti-tumor immune activity in HADHA-TKO mice and during PD-L1 mAb therapy, NBHA alone rarely affected the anti-tumor activity. A few potential reasons may explain these observations: 1) The FAO

dysfunction-exhaustion feedback loop (vicious cycle) is more prominent in these two models, and NBHA may attenuate such a severe vicious cycle. 2) PD1 blockade therapy is known to promote the clonal replacement, where terminally exhausted T cells are replaced by newly recruited, less differentiated clones⁸. NBHA may exert a more pronounced effect for the effector T cell differentiation, but not the already exhausted T cells.

In conclusion, we have clarified the mechanism by which active aldehydes, the final products of lipid peroxidation, accelerate CD8⁺ T cell exhaustion through formatting metabolic exhaustion in TME. Mitochondrial FAO dysfunction leads to the production of active aldehydes from mitochondria, further impairs FAO activity and enhances glycolysis. Our findings shed light on the development of a strategy to improve cancer immunotherapy by preventing metabolic exhaustion.

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Author contributions

Y.H., K.K., and K.C. designed research; Y.H., K.K., K.Ic., J.W., K.S., A.M., S.H., Y.W., K.Y., M.K., and H.K. performed research; Y.H., K.K., K.Ic., T.Hi., J.W., S.H., Y.W., T.Koz., T.Y., and K.C. analyzed data; Y.H., K.K., K.Ic., J.W., K.It., T.M., H.D., T.Kob., K.O., and T.Y. harvested human samples; and Y.H., K.K., T.Ho., and K.C. wrote the paper. All authors reviewed the results and approved the manuscript.

Competing interests

T.Ho. and K.C. received research funding from Shimadzu Corporation. All other authors declare no competing interests.

Figure Legends

Fig. 1. CD8⁺ T cells undergo differentiation accompanied by changes in mitochondrial status

(A) Schema of repetitive stimulation. Naïve (CD44^{low}) CD8⁺ T cells isolated from mouse spleens and LNs were stimulated by anti-CD3/CD28 coated plates. Cells were harvested and re-stimulated every 2 days. Representative flow cytometry profiles and frequencies of D1-D4 cells, based on mitochondrial mass and mitochondrial potential on day 0, 2, and 8. Technical replicates: day 0 and 2, n = 3; day 8, n = 5. (B-D) Frequencies of Annexin V⁺ (B), Tim-3⁺, LAG-3⁺, TIGIT⁺ (C) and Slamf6⁺ (D) cells in D1-D4 populations among PD1⁺ CD8⁺ T cells in MC38 tumors on day 14 (n = 8 mice). (E-I) Single-cell RNA-sequencing (scRNA-seq) analysis of D1, D2 and D3 populations sorted from PD1⁺ CD8⁺ T cells in MC38 tumors on day 14. (E) UMAP plot according to scRNA-seq profiles of each cell. Colors of dots indicate D1, D2, or D3 category. (F) Violin plots of exhausted or stem-like signature scores in D1-D3. (G) Gene Ontology (GO) enrichment analysis of genes that were significantly upregulated in D3 compared to D1 (adjusted p < 0.05 and log₂ fold change (D3/D1) > 1). (H) Pseudotime and trajectory analysis mapped onto the UMAP. Violin plots of pseudotime score in D1-D3. (I) Clonal distribution of TCR clonotypes in D1-D3. T cell clones were categorized as rare (range, 0 –≤ 0.003), small (range, 0.003 –≤ 0.01), middle (range, 0.01 –≤ 0.03) and large (range, 0.03 –≤ 0.1), Hyperexpanded (range, 0.1 –≤ 1) based on the proportion of each clonotype in D1-D3. (J) D1, D2 and D3 of PD1⁺ CD8⁺ T cells were sorted from MC38 tumors on day 25, labeled with CellTrace Violet and stimulated with anti-CD3 and anti-CD28 coated beads for 49 hours. An unstimulated sample from D1 group was included to assess baseline dye intensity. The figure shows a representative CellTrace histogram with CellTrace^{low} frequencies shown next to the sample annotations, along with their quantification (D1 and D2, n = 12; D3, n = 8 technical replicates). Data are means ± SEMs (J).

P values were determined using paired two-tailed Student's t tests (B to D), one-way ANOVA with Tukey's multiple comparisons tests (F, H and J), or a one-sided hypergeometric test and adjusted for multiple testing using the Benjamini-Hochberg (BH) method (G). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. All data are representative of three or more independent experiments with similar results, except (E) to (I).

Fig. 2. Acrolein accumulates in CD8⁺ T cells in TME in an exhaustion depth-dependent manner.

(A) MFI of Glut1 and 2-NBDG (a glucose analog) in D1'-D4' populations among PD1⁺ CD8⁺ T cells in tumors on day14 (Glut1, n = 6 mice; 2-NBDG, n = 8 mice). (B) Sorted D1, D2 and D3 of PD1⁺ CD8⁺ T cells from MC38 tumors on day 17 were incubated in medium containing 10

mM ^{13}C glucose for 3 hours. Data shows the levels (peak area) of ^{13}C -labeled lactic acid produced by each subset (D1, n = 4; D2, n = 5; D3, n = 6 technical replicates). (C) Representative flow cytometry profiles (including corresponding isotype control) and MFI of HADHA in PD1⁻ Tim-3⁻ and PD1⁺ Tim-3⁺ cells among CD8⁺ T cells in MC38 tumors on day 9 (n = 10 mice), and MFI of HADHA in D1'-D4' cells among PD1⁺ CD8⁺ T cells in MC38 tumors on day 14 (n = 8 mice). (D) MFI of HADHA in D1'-D4' populations among PD1⁺ CD8⁺ T cells in human tumors (4 RCC and 3 lung cancer). (E) Representative flow cytometry profiles (including corresponding isotype control) and MFI of SLC25A20 in D1'-D4' cells among PD1⁺ CD8⁺ T cells in MC38 tumors on day 15 (n = 5 mice). (F) Quantification of mitochondrial inter-cristae distances (nm) based on transmission electron microscopy (TEM) images of D1-D3 of PD1⁺ CD8⁺ T cells from MC38 tumors on day 20. For D1, D2, and D3, n = 12, 18, and 11 cristae distances were measured from n = 5, 9, and 4 mitochondria derived from n = 2, 4, and 2 cells, respectively. (G) MFI of mitochondrial superoxide (Mito SOX) in D1-D4 populations and lipid radical in D1'-D4' populations among PD1⁺ CD8⁺ T cells in tumors on day 14 (Mito SOX, n = 6 mice; lipid radical, n = 8 mice). (H) Schema of the lipid peroxidation pathway. Reactive oxygen species (ROS) create lipid radicals from cellular lipids and then lipid radicals produce active aldehydes, e.g., acrolein, in a chain reaction. Created with BioRender.com, elements adapted and assembled using PowerPoint. (I) MFI of acrolein in D1-D4 populations and protein-conjugated acrolein (PC-Acro) in D1'-D4' populations among PD1⁺ CD8⁺ T cells in tumors on day 14 (n = 6 mice). (J) MFI of lipid radical in D1'-D4', acrolein in D1-D4 and PC-Acro in D1'-D4' populations among PD1⁺ CD8⁺ T cells in human tumors (4 RCC and 3 lung cancer for lipid radical, 6 RCC and 4 lung cancer for acrolein, and 6 RCC and 4 lung cancer for PC-Acro). Data are means $\pm$ SEMs (B and F). P values were determined using paired two-tailed Student's t tests (A, C, E, G and I), one-way ANOVA with Tukey's multiple comparisons tests (B and F), or two-tailed Wilcoxon matched-pairs signed rank tests (D and J). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. All data are representative of three or more independent experiments with similar results, except (D) and (J).

Fig. 3. Acrolein increases glycolysis and reduces fatty acid oxidation *in vitro*.

(A) The Akt-mTOR and glycolysis boost the differentiation of CD8⁺ T cells from naïve T cells to exhausted T cells. (B) MFI of pAkt, pmTOR and pS6 in primary CD8⁺ T cells stimulated for 3 hours with anti-CD3 and anti-CD28 coated beads in the presence or absence of 2 μM acrolein (ACR) evaluated by flow cytometry (n = 5 technical replicates). (C) Seahorse measurements of real-time ECAR of CD8⁺ T cells 3 hours after stimulation with anti-CD3/CD28 beads in the presence or absence of 5 μM ACR (Ctrl, n = 6; ACR, n = 7 technical replicates). (D) MFI of 2-

NBDG in CD8⁺ T cells 3 hours after stimulation with anti-CD3/CD28 beads in the presence or absence of 2 μ M ACR measured by flow cytometry (n = 5 technical replicates). (E) Frequencies of PD1⁺ cells among CD8⁺ T cells 3 hours after stimulation with anti-CD3/CD28 beads in the presence or absence of 2 μ M ACR measured by flow cytometry (n = 5 technical replicates). (F) Thymidine incorporation of CD8⁺ T cells 7 hours after stimulation with anti-CD3/CD28 beads in the presence or absence of 5 μ M ACR (n = 4 technical replicates). (G) Frequencies of D1-D4 populations among CD8⁺ T cells 7 hours after stimulation with anti-CD3/CD28 beads in the presence or absence of 2.5 μ M ACR (n = 12 technical replicates). (H) Seahorse measurements of OCR of CD8⁺ T cells 3 hours after stimulation with anti-CD3/CD28 beads in the presence or absence of 5 μ M ACR. 5 μ M etomoxir or 3 μ M BPTES was added about 10 minutes before running the assay (All Ctrl groups, n = 3; All ACR groups, n = 4 technical replicates). To measure the oxidation activity of endogenous fatty acids or glutamine, low-nutrient medium (RPMI + 2.5 mM glucose) was used during the assay. (I)-(J) Phosphoproteomic analysis of total CD8⁺ T cells stimulated with anti-CD3/CD28 beads for 1 hour in the presence or absence of 5 μ M ACR (n = 5 technical replicates). (I) Volcano plot of canonical pathways analyzed by Ingenuity Pathway Analysis (IPA). For visualization purposes, pathways with p values equal to 1 or with undefined z-scores were excluded from the figure. 545 pathways are shown in the figure (upregulated: z-score > 0.8 and p value < 0.05; downregulated: z-score < -0.8 and p value < 0.05; the others: -0.8 $\leq$ z-score $\leq$ 0.8 or p value $\geq$ 0.05). (J) Bar graph showing the top 5 upregulated and downregulated gene names based on phosphorylation changes among approximately 20,000 phosphopeptides. (K) Western blotting images of SLC25A20 in CD8⁺ T cells stimulated with anti-CD3/CD28 beads for 3 hours in the presence or absence of 5 μ M ACR. (L) Quantification of mitochondrial inter-cristae distances (nm) based on TEM images of naïve CD8⁺ T cells stimulated with anti-CD3/CD28 beads for 3 hours in the presence or absence of 5 μ M ACR. For Ctrl and ACR, n = 125 and 136 cristae distances were measured from n = 33 and 41 mitochondria derived from n = 14 and 18 cells, respectively. Data are means $\pm$ SEMs. P values were determined using unpaired two-tailed Student's t tests (B to G and L) or one-way ANOVA with Tukey's multiple comparisons test (H) or right-tailed Fisher's Exact Test (I). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. All data are representative of three or more independent experiments with similar results, except (I) and (J).

Fig. 4. Endogenous acrolein shapes metabolic states of Tex in TME.

(A) Seahorse measurement of real-time ECAR of CD8⁺ T cells 3 hours after stimulation with anti-CD3/CD28 beads in the presence or absence of 10 μ M NBHA (n = 4 technical replicates). The control data are shared with those in Extended Data Fig. 3H to compare results under the same

conditions in the same experiment, as supportive data. (B) Frequencies of D1-D4 cells among CD8⁺ T cells in the same condition as (A) measured by flow cytometry (n = 6 technical replicates). (C) Representative flow cytometry profiles and MFI of pAkt, pmTOR and pS6 in PC-Acro low and PC-Acro high populations among PD1⁺ CD8⁺ T cells in MC38 on day 14 (n = 5 mice). A fluorescent antibody for PC-Acro was used instead of staining dye for acrolein due to the limitation of staining condition. (D) Representative flow cytometry profiles and MFI of Glut1 and 2-NBDG in PC-Acro and acrolein, respectively, low and high populations among PD1⁺ CD8⁺ T cells in MC38 on day 14 (Glut1, n = 6 mice; 2-NBDG, n = 5 mice). (E) MFI of pAkt, pmTOR, pS6, Glut1 and 2-NBDG between PC-Acro or acrolein low and high populations of PD1⁺ CD8⁺ T cells in human tumors (6 RCC and 4 lung cancer). (F) Representative fluorescence microscopy image and Pearson's correlation between mitochondria and PC-Acro in sorted PD1⁺ CD8⁺ T cells from MC38 on day 19 (n = 12 cells). An experimental control group was not included, as the experiment aimed to examine the colocalization of mitochondria and PC-Acro. (G) MFI of MitoPeDPP (mitochondrial membrane peroxidation) in D1'-D4' cells among PD1⁺ CD8⁺ T cells in MC38 on day 14 (n = 6 mice). Data are means $\pm$ SEMs (A and B). P values were determined using unpaired two-tailed Student's t tests (A and B), paired two-tailed Student's t tests (C, D, and G), or two-tailed Wilcoxon matched-pairs signed rank test (E). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. All data are representative of three or more independent experiments with similar results, except (E).

Fig. 5. FAO dysfunction in T cells accelerates the acrolein production and exhaustion.

(A) CD36 expression and BODIPY C₅, C₁₂, and C₁₆ (fatty acid uptake) in PD1⁺ CD8⁺ T cells from MC38 tumors grown in HADHA^{flx/flx} (Ctrl) and HADHA^{flx/flx} CD4-Cre (KO) mice on day 14 (CD36, Ctrl n = 8 mice, KO n = 7 mice; BODIPY C₅, n = 5 mice; BODIPY C₁₂, n = 5 mice; BODIPY C₁₆, Ctrl n = 8 mice, KO n = 7 mice). (B) MFI of lipid radical, acrolein and PC-Acro in PD1⁺ CD8⁺ T cells in MC38 tumors from Ctrl and KO mice on day 14 (lipid radical, Ctrl n = 8 mice, KO n = 6 mice; acrolein, n = 5 mice; PC-Acro, Ctrl n = 8 mice, KO n = 6 mice). (C) MFI of pAkt, pS6 and 2-NBDG in PD1⁺ CD8⁺ T cells in MC38 tumors from Ctrl and KO mice on day 14 (pAkt and pS6, n = 5 mice; 2-NBDG, Ctrl n = 8 mice, KO n = 6 mice). (D) ATP productions derived from glycolysis and mitochondrial OXPHOS in naïve CD8⁺ T cells from Ctrl and KO mice before and after 3-hour stimulation with anti-CD3/CD28 beads measured by Seahorse (n = 6 technical replicates). (E) Frequencies of PD1⁻ Tim-3⁻, PD1⁺ Tim-3⁺, and TOX⁺ cells among CD8⁺ T cells in MC38 tumors from Ctrl and KO mice on day 14 (n = 5 mice). (F) Frequencies of Annexin V⁺ cells among PD1⁺ CD8⁺ T cells in MC38 tumors from Ctrl and KO mice on day 14 (Ctrl, n = 8 mice; KO, n = 7 mice). (G) PD1⁺ CD8⁺ T cells sorted from MC38

tumors of Ctrl and KO mice on day 14 were labeled with CellTrace Violet and stimulated with anti-CD3/CD28 beads for 49 hours. An unstimulated sample from Ctrl group was included to assess baseline dye intensity. The figure shows a representative CellTrace histogram with CellTrace^{low} frequencies shown next to the sample annotations, along with their quantification (n = 10 technical replicates). (H) MC38 tumor growth in Ctrl and KO mice (Ctrl, n = 5 mice; KO, n = 7 mice). Statistical analysis on day 20. (I) Representative flow cytometry profiles on day 14 and frequencies of CD8⁺ T cells among tumor-infiltrating CD45⁺ cells from Ctrl and KO mice on day 10, 14 and 20 (day 10, Ctrl n = 6 mice, KO n = 8 mice; day 14, Ctrl n = 8 mice, KO n = 7 mice; day 20, Ctrl n = 5 mice, KO n = 6 mice). Data are means $\pm$ SEMs. P values were determined using unpaired two-tailed Student's t tests. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. All data are representative of two or more independent experiments with similar results.

Fig. 6. Lipid radical and acrolein scavengers recover the anti-tumor immunity attenuated by FAO dysfunction.

(A) MC38 tumor growth in littermate ctrl (HADHA^{flox/flox}) and KO (HADHA^{flox/flox} CD4-Cre) male mice intraperitoneally (i.p.) treated with α -tocopherol (vitamin E: VtE) (2 mg/kg) or DMSO (Ctrl) every 3 days from day 7. (n = 5 mice). Statistical analysis on day 28. (B) MC38 tumor growth in littermate ctrl and KO female mice i.p. treated with N-benzylhydroxylamine (NBHA) (100 mg/kg) or PBS (Ctrl) every 3 days from day 7 (n = 5 mice). Tumor growth in female mice is usually slower than that in male mice. Statistical analysis on day 16. (C-D) MFI of pAkt and pS6 (C) and frequencies of D1-D4 cells (D) on day 10 among PD1⁺ CD8⁺ T cells in TME from KO male mice treated with NBHA or PBS (Ctrl) (n = 5 mice). Data are means $\pm$ SEMs. P values were determined using unpaired two-tailed Student's t tests (A-B). *P < 0.05; **P < 0.01. All data are representative of two or more independent experiments with similar results.

Fig. 7. Acrolein scavengers restores anti-tumor immunity by reactivating fatty acid oxidation (FAO).

(A) MC38 tumor growth in wild-type (WT) female mice i.p. treated with anti-PD-L1 mAb every 6 days and α -tocopherol (vitamin E: VtE) (2 mg/kg) every 3 days from day 7 (Ctrl, n = 8 mice; α PD-L1, n = 7 mice; α PD-L1 + VtE, n = 7 mice). Statistical analysis on day 22. (B) MC38 tumor growth in WT female mice i.p. treated with anti-PD-L1 mAb every 6 days and N-benzylhydroxylamine (NBHA) (100 mg/kg) every 3 days from day 7 (Ctrl, n = 7 mice; α PD-L1, n = 6 mice; α PD-L1 + NBHA, n = 7 mice). Statistical analysis on day 22. (C)-(F) Analysis of tumor-infiltrating PD1⁺ CD8⁺ T cells from MC38 tumors on day 14 in WT mice treated with anti-

PD-L1 mAb and NBHA as indicated in (B). (C) Frequencies of PD1⁺ CD8⁺ T cells among tumor-infiltrating CD45⁺ cells (α PD-L1, n = 8 mice; the other groups, n = 9 mice). (D) Sorted PD1⁺ CD8⁺ T cells were incubated in medium containing 200 μ M BSA-conjugated palmitate-¹³C₁₆ for 3 hours. Data show the levels (fmol) of ¹³C-labeled acylcarnitines (C16: Palmitoylcarnitine, C14: Myristoylcarnitine, C12: Lauroylcarnitine, C10: Decanoylcarnitine, C8: Octanoylcarnitine, C6: Hexanoylcarnitine) (n = 6 technical replicates). (E) Frequencies of D1-D4 among PD1⁺ CD8⁺ T cells in tumors (α PD-L1, n = 8 mice; the other groups, n = 9 mice). (F) Sorted PD1⁺ CD8⁺ T cells were labeled with CellTrace Violet and stimulated with anti-CD3/CD28 beads for 45 hours. An unstimulated sample from Ctrl group was included to assess baseline dye intensity. The figure shows a representative CellTrace histogram with CellTrace^{low} frequencies shown next to the sample annotations, along with their quantification (Ctrl, n = 4; α PD-L1, n = 12, α PD-L1 + NBHA, n = 10; NBHA, n = 12 technical replicates). Data are means $\pm$ SEMs. P values were determined using unpaired two-tailed Student's t tests (A and B), one-way ANOVA with selected column comparisons between the untreated and α PD-L1 groups, or α PD-L1 and α PD-L1 + NBHA groups (C to F). *P < 0.05; **P < 0.01; ****P < 0.0001. All data are representative of two or more independent experiments with similar results.

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Methods

Mice

C57BL/6N (5 to 11 weeks old) mice were obtained from Charles River Laboratories Japan or Japan SLC, Inc. OT-1 TCR-transgenic mice (CD45.2) were obtained from The Jackson Laboratory (originally developed by M. B. Bevan at the University of Washington, Seattle, WA). C57BL/6N-background *Hadha*^{flox} mice were obtained from Cyagen Biosciences. *Hadha*^{flox/flox} *Cd4*-Cre mice¹⁵ were generated by crossing *Hadha*^{flox} mice with *Cd4*-Cre mice⁶⁵. C57BL/6-background CD45.1 congenic mice have been maintained at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. Littermate or age/sex-matched mice were used for analysis. Female mice were used except in the experiments in which the usage of male mice was described explicitly. All mice were used under protocols approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (protocol approval number: 25086). They were maintained under specific pathogen-free (SPF) conditions. They were fed with F-2 from Funabashi Farm Co., Ltd. They were kept in the dark from 20:00 to 8:00, in the light from 8:00 to 20:00, at 25 °C, and at 45% humidity.

Cell lines

Details of the MC38 murine colon adenocarcinoma cell line have been described previously⁶⁶. The identity of the cells was confirmed based on morphology, functional characteristics and gene expression profiles. MC38 cells were cultured in RPMI medium (Gibco) with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich) and 1% (v/v) penicillin-streptomycin mixed solution (Nacalai Tesque). MC38-OVA cells were generated by transfection of ovalbumin (OVA) cDNA into parental MC38 cells and maintained under Geneticin selection (0.5 mg/ml G418; InvivoGen) in the same culture medium as the parental cells.

Chemical reagents

The following chemical reagents were used for the mouse therapies and the in vitro assays. Acrolein (100 µg/mL in water) (AccuStandard), 4-Hydroxynonenal (MCE) prepared in DMSO (Nacalai Tesque), Etomoxir (Sigma-Aldrich) prepared in MilliQ, BPTES (AdipoGen) prepared in DMSO, N-Benzylhydroxylamine hydrochloride (TCI), α-tocopherol (Wako) prepared in DMSO, IL-2 (PeproTech) prepared in MilliQ, and ³H thymidine (PerkinElmer). Other chemical reagents used were equipped with the kits. The concentration of the reagents in vitro was determined following experimental optimization.

Mouse therapy model

MC38 cells (5×10^5) were injected intradermally (i.d.) into the right flank of C57BL/6N mice on day 0. For anti-PD-L1 combination treatment, the mice were injected intraperitoneally (i.p.) with 25-30 µg of an anti-PD-L1 mAb (clone 1-111A, made in-house) every 6 days⁶⁷, i.p. with 2 mg of N-benzylhydroxylamine hydrochloride (NBHA) every 3 days, or i.p. with 40 µg of α-tocopherol (Vitamin E) every 3 days from day 7. Tumor measurement was performed on every 2 or 3 days, and tumor volume was calculated using the formula for a typical ellipsoid: $3.14 \times (\text{length} \times \text{breadth} \times \text{height}) / 6$. Accidentally dead mice on the way of the therapies were excluded from the data. All mice experiments were performed under protocols approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (protocol approval number: 25086).

Cell preparation and culture condition for in vitro analysis of primary CD8⁺ T cells

For in vitro analysis, axillary, brachial and inguinal LNs on both sides of mice without tumors were harvested, homogenized and pooled. Spleens were harvested, homogenized and then treated with ACK lysing buffer for 2 minutes. After pooling the cells of LNs and spleens, total or naïve (CD44^{low}) CD8⁺ T cells were purified using MojoSort system (BioLegend) according to the manufacturer's instructions. Isolated mouse CD8⁺ T cells were directly analyzed, or cultured in RPMI with 10% (v/v) heat-inactivated FCS, 1% (v/v) penicillin-streptomycin, 25-50 µM 2-mercaptoethanol (Nacalai Tesque), and other reagents or materials as necessary.

Tumor collection

Tumor-bearing mice were euthanized on the indicated days. Tumor samples were harvested and minced into 1 to 2 mm pieces with scissors and digested with collagenase type IV (Thermo Fisher Scientific) using a gentleMACS Dissociator (Miltenyi Biotec).

PBMC collection

Blood was sampled from a retro-orbital sinus of mice. It was treated with ammonium-chloride-potassium (ACK) lysing buffer for 2 minutes twice to lyse red blood cells.

Human samples

Patients admitted to Kyoto University Hospital for cancer surgeries consented to the collection of a part of resected tumors. Obtained samples were minced into 1 to 2 mm pieces with scissors and digested with Dri Tumor & Tissue Dissociation Reagent (BD Biosciences) using a gentleMACS Dissociator. For CD45⁺ cell enrichment, tumor digestions were processed by Manual MACS Magnetic Separators (Miltenyi Biotec) according to the manufacturer's instructions. All human samples were obtained from subjects who provided informed consent in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of the Kyoto University (G1012).

Adoptive transfer model using mouse OT-1 naïve CD8⁺ T cells

MC38-OVA cells (2×10^6) were injected i.d. into the right flank of CD45.1 congenic male and female mice on day 0. Male and female mice were equally distributed across groups. On day 9, naïve (CD44^{low}) OT-1 CD8⁺ T cells (CD45.2⁺) were isolated from spleens and lymph nodes using MojoSort system. Isolated cells (1.25×10^6 cells per mouse) were intravenously transferred into the tumor-bearing mice. The isolated cells were also analyzed by flow cytometry prior to transfer. Tumors were collected on days 13, 16, and 20 after tumor inoculation (i.e., days 4, 7, and 11 after transfer) for flow cytometric analysis.

In vitro exhaustion experiment by repetitive stimulation

Naïve CD8⁺ T cells were resuspended at a concentration of 1×10^6 cells/mL in RPMI with 10% FCS, 1% penicillin-streptomycin, 50 μ M 2-mercaptoethanol, and 10 ng/mL IL-2. They were seeded on a flat plate coated with 1 μ g/mL anti-CD3 antibody (eBioscience) and 1 μ g/mL anti-CD28 antibody (eBioscience). The plate was kept in a 37 °C CO₂ incubator. Cells were passaged at 1×10^6 cells/mL and re-stimulated every two days. From day 2, plates were coated with only anti-CD3, in the absence of anti-CD28. The cells were analyzed on day 2 and 8 or 10. As necessary, acrolein or N-benzylhydroxylamine hydrochloride was added at a concentration of 2 μ M (until day 4) or 10 μ M (from day 4).

Thymidine incorporation assay

2×10^5 total CD8⁺ cells were stimulated with anti-CD3 and anti-CD28 coated Dynabeads (Gibco) in 200 μ L of RPMI with 10% FCS, 1% penicillin-streptomycin, 25 μ M 2-

mercaptoethanol and 12.3 kBq of ³H thymidine, supplied with 5 μM acrolein or 1 μM 4-hydroxynonenal, in a 37 °C CO₂ incubator for 7 hours. These cells were collected by Filtermate 196 (Packard) and irradiation of ³H thymidine was measured by MicroBeta2 (PerkinElmer). Thymidine incorporation assay was performed at Radioisotope Research Center, Agency for Health, Safety and Environment, Kyoto University.

Flow cytometry analysis

The following antibodies recognizing murine surface antigens were used: CD8a (53-6.7), CD45 (30-F11), CD45.2 (104), CD11b (M1/70), PD-1 (29F.1A12), PD-1 (RMP1-30), Tim-3 (RMT3-23), LAG-3 (C9B7W), TIGIT (1G9), CD38 (90), CD73 (TY/11.8), CD44 (IM7), and CD36 (HM36) from BioLegend; CD8a (53-6.7), CD45 (30-F11), CD45.2 (104), Tim-3 (5D12/TIM-3), TCR β Chain (H57-597), PD-1 (29F.1A12), and Slamf6 (13G3) from BD Biosciences; CD45.1 (A20), PD-1 (RMP1-30), CD39 (24DMS1), CD62L (MEL-14), CD44 (IM7), and CD8a (53-6.7) from eBioscience. The following antibodies recognizing murine intracellular targets, corresponding isotype controls, and secondary antibodies for the non-fluorescent primary antibodies were used: IFN-γ (XMG1.2), TNF-α (MP6-XT22), and Granzyme B (GB11) from BioLegend; Phospho-Akt (SDRNR), Phospho-mTOR (MRRBY), Phospho-S6 (cupk43k), and Perforin (eBioOMAK-D) from eBioscience; TOX (REA473) from Miltenyi Biotec; Acrolein [10A10, used for protein-conjugated acrolein (PC-Acro)] and Malondialdehyde [6H6, used for protein-conjugated malondialdehyde (PC-MDA)] from Novus Biologicals; HADHA (Polyclonal), SLC25A20 (Polyclonal) from Proteintech; Glut1 (EPR3915), HADHA (EPR17940), Anti-Rabbit IgG H&L (Polyclonal), and Rabbit IgG Isotype Control (EPR25A) from Abcam; 4-Hydroxynonenal [Polyclonal, used for protein-conjugated 4-hydroxynonenal (PC-4-HNE)] from Bioss; Phospho-Akt (Polyclonal) from Cell Signaling Technology; Anti-Rabbit IgG H+L (Polyclonal) from Invitrogen. The following antibodies recognizing human surface antigens were used: CD45 (HI30), CD11b (ICRF44), and CD8 (SK1) from BioLegend; CD8 (RPA-T8), CD45 (HI30), PD-1 (MIH4), and PD-1 (EH12.1) from BD Biosciences. The following antibodies recognizing human intracellular targets and a secondary antibody for the non-fluorescent primary antibody were used: Phospho-Akt (SDRNR), Phospho-mTOR (MRRBY), and Phospho-S6 (cupk43k) from eBioscience; Acrolein [10A10, used for protein-conjugated acrolein (PC-Acro)] from Novus Biologicals; HADHA (Polyclonal) from Proteintech; Glut1 (EPR3915) and Anti-Rabbit IgG H&L (Polyclonal) from Abcam. Mitochondrial potential, mitochondrial mass and mitochondrial superoxide were determined by 1.2 nM MitoTracker Deep Red, 15.6 nM MitoTracker Green and 5 μM MitoSOX Red, respectively (all Invitrogen). Lipid radical and acrolein were determined by 1 μM LipiRADICAL Green and 0.5 μM AcroleinRED, respectively (both Funakoshi). Glucose uptake was determined by 100 μg/ml 2-NBDG (Abcam).

Mitochondrial membrane peroxidation was determined by 0.5 μ M MitoPeDPP (Dojindo). Fatty acid uptake of C5, C12, C16 was determined by 1 μ M BODIPY FL C₅, 1 μ M BODIPY FL C₁₂ and 1 μ M BODIPY FL C₁₆, respectively (all Invitrogen). Dead cells were excluded by Zombie NIR, Zombie Violet or Zombie Aqua (All Biolegend). 2×10^5 to 1×10^6 cells were used for staining. For MitoTracker Deep Red, MitoTracker Green, or MitoSOX Red staining, cells were incubated with appropriately diluted dyes in 2% FCS in PBS for 30 minutes in a 37 °C CO₂ incubator. After incubation, cells were washed twice with 2% FCS in PBS. For staining with LipiRADICAL Green, AcroleinRED, 2-NBDG, BODIPY, or MitoPeDPP, cells were washed twice with 1% BSA in PBS, then stained with appropriately diluted dyes in 1% BSA in PBS for 30 minutes in a 37 °C CO₂ incubator. After incubation, cells were washed twice with 1% BSA in PBS. Subsequently, cells were stained with appropriately diluted Zombie dye and Fc-blocked by anti-CD16/32 antibody (BioLegend or BD Biosciences) for mice or by Normal Mouse Serum (Invitrogen) for humans, and then stained with appropriately diluted antibodies for 12 minutes at 4°C and washed with 2% FCS in PBS. To measure apoptosis, cells were stained with PE-Cy7 conjugated Annexin V (BioLegend, Cat# 640950) diluted in Annexin V Binding Buffer (BioLegend) for 15 minutes at 4°C, and then washed with Annexin V Binding Buffer. To measure cytokine production, cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Selleck) and 500 ng/ml ionomycin calcium (Cosmo Bio) in the presence of Brefeldin A Solution (eBioscience) for 4 hours in a 37 °C CO₂ incubator before starting staining. For intracellular staining, cells were processed with Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions, and subsequently stained with antibodies appropriately diluted with Permeabilization Buffer (eBioscience) for 30 minutes at 4°C, and then washed with 2% FCS in PBS. For further detection of unconjugated intracellular primary antibodies, cells were stained with a fluorophore-conjugated secondary antibody diluted in Permeabilization Buffer for 15 minutes at 4°C and then washed with 2% FCS in PBS. All flow cytometry experiments were performed on a LSRFortessa X-20 or a FACSymphony A5 (both BD Biosciences) and analyzed using FlowJo software (v10.7.2 or v10.10.0). Please refer to the reporting summary for detailed information about the antibodies.

Cell sorting of CD8⁺ TIL subset by Flow cytometry

Murine tumor samples were digested as described above, and CD8⁺ TILs were enriched by Manual MACS Magnetic Separators. Enriched cells were stained with Zombie dye, Fc-blocked by anti-CD16/32 antibody, and stained with antibodies. The following antibodies were used: CD8a (53-6.7), CD45.2 (104), PD-1 (29F.1A12) from BioLegend; CD45.2 (104) from BD Biosciences; PD-1 (RMP1-30) from eBioscience. If D1, D2 and D3 CD8⁺ TIL subsets were needed, cells were stained with MitoTracker Deep Red and MitoTracker Green for 30 minutes in

a 37 °C CO₂ incubator before Zombie staining. Among these stained cells, target CD8⁺ T cell subsets were sorted by FACSMelody (BD Biosciences). Please refer to the reporting summary for detailed information about the antibodies.

Flow cytometric analysis of CD8⁺ TIL proliferation using CellTrace Violet

To assess the proliferative capacity of mouse CD8⁺ TIL subsets sorted by flow cytometry, they were labeled with 5 µM CellTrace Violet (Invitrogen) in PBS following the manufacturer's instructions. Labeled cells were stimulated with anti-CD3/CD28 coated Dynabeads for 2 days in RPMI with 10% FCS, 1% penicillin-streptomycin, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate (Gibco) and 1% Non-Essential Amino Acids (Gibco). Cells were harvested, stained with Zombie NIR, Fc-blocked by anti-CD16/32 antibody, and stained with surface antibodies, which were identical to those used for the previous sorting. The fluorescence of stained cells was measured by LSRFortessa X-20. Highly proliferative cells were defined as CellTrace^{low} cells among live and singlet CD8⁺ TILs. An unstimulated sample was also measured to assess baseline CellTrace dye intensity.

Immunofluorescence imaging

For immunostaining, isolated cells were incubated with 0.3 µM PhenoVue 551 (PerkinElmer) and 5.5 µg/mL Hoechst 33342 (Invitrogen) in 2% FCS in PBS for 30 minutes in a 37 °C CO₂ incubator. After washing twice with 2% FCS in PBS, cells were processed with Foxp3/Transcription Factor Staining Buffer Set, and subsequently incubated with APC conjugated Acrolein antibody (Novus Biologicals) appropriately diluted with Permeabilization Buffer (eBioscience) at 4°C for 30 minutes. Following incubation, cells were washed with 2% FCS in PBS. Immunofluorescence images were acquired and analyzed using a Nikon N-SIMS super-resolution fluorescence microscope system. Please refer to the reporting summary for detailed information about the antibody.

Western blotting

Cultured CD8⁺ T cells were lysed in RIPA buffer [150 mM NaCl, 5 mM EDTA, 30 mM Tris-HCl (pH 7.4), 0.05% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 10% glycerol in distilled H₂O] supplemented with a phosphatase inhibitor cocktail (Nacalai Tesque) and protease inhibitor cocktail (Roche) for 30 minutes at 4°C. They were subsequently centrifuged and the supernatants containing the soluble protein fractions were collected. The protein amount was determined by DC protein assay (Bio Rad) and the cell lysates were diluted to appropriate protein concentration. The lysates were denatured with sample buffer with reducing reagent (Nacalai Tesque) and incubated at 95°C for 5 minutes. The proteins were resolved by a

10% polyacrylamide gel and subjected to immunoblotting assay. The transblotted membranes were blocked with a Blocking One (Nacalai Tesque) and incubated with a primary antibody followed by a secondary antibody as below: antibody to Hexokinase II (C64G5), β -Actin (13E5), HRP-linked anti-rabbit IgG secondary antibody (polyclonal) and HRP-linked anti-mouse IgG secondary antibody (polyclonal) from Cell Signaling Technology; antibody to HADHA (polyclonal), SLC25A20 (polyclonal), and HRP-linked GAPDH antibody (1E6D9) from Proteintech; antibody to CPT1A (8F6AE9) and ACADVL/VLCAD (polyclonal) from Abcam. The HRP-linked antibodies were diluted with 5% skim milk in Tris Buffered Saline with Tween 20 (TBS-T), and the others were diluted with 5% BSA in TBS-T supplemented with 0.02% sodium azide. The indicated antibody-incubated membranes were reacted with a Chemi-Lumi One Super (Nacalai Tesque) and the images were acquired with Amersham ImageQuant 800 (Cytiva). Please refer to the reporting summary for detailed information about the antibodies.

Transmission electron microscopy

For transmission electron microscopy (TEM) observation, cells were embedded in iPGell (GenoStaff) following the manufacturer's protocol. The resulting cell blocks were fixed with 4% formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) overnight at 4°C, then post-fixed with 1% osmium tetroxide in 0.1M PB for 2 hours. After dehydration using a graded ethanol series, the samples were embedded in epoxy resin (Luveak-812; Nacalai Tesque). Ultrathin sections (70-nm thickness) were prepared using an ultramicrotome (EM UC7; Leica). The sections were stained with uranyl acetate and lead citrate, and examined using a transmission electron microscope (H-7650; Hitachi). TEM was performed at the Division of Electron Microscopic Study, Center for Anatomical Studies, Graduate School of Medicine, Kyoto University. Cristae distance was calculated by using open-source software ImageJ (v1.53m).

Enrichment of phosphopeptides and mass spectrometry

Cultured CD8⁺ T cells (n=5 technical replicates) were lysed in 150 μ L of a buffer containing 6 M guanidine-HCl, 100 mM HEPES-NaOH (pH 7.5), 10 mM tris (2-carboxyethyl) phosphine hydrochloride, and 40 mM chloroacetamide. The lysates were solubilized by heating and sonication, followed by centrifugation at 20,000 g for 15 minutes at 4 °C. The supernatants were collected, and 100 μ g of total protein from each sample were purified by methanol–chloroform precipitation and resuspended in 100 μ L of 0.1% RapiGest (Waters) in 50 mM triethylammonium bicarbonate solution. Following sonication, the protein solutions were digested with 2 μ g of trypsin/Lys-C mix (Promega) at 37 °C overnight. The resulting peptide solutions were acidified with trifluoroacetic acid, centrifuged, and subjected to the High-Select Fe-NTA Phosphopeptide

Enrichment Kit (Thermo Fisher Scientific). Eluates were acidified, desalted using GL-Tip SDB (GL Sciences), evaporated in a SpeedVac concentrator (Thermo Fisher Scientific), and reconstituted in 0.1% trifluoroacetic acid and 3% acetonitrile. LC-MS/MS analysis of the resultant peptides was conducted using a nanoElute 2 coupled with a timsTOF HT mass spectrometer (Bruker). Peptides were separated on a C18 reversed-phase column (75- μ m inner diameter $\times$ 150 mm, Nikkyo Technos). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Samples were loaded onto the column at a flow rate of 0.2 μ L/min starting at 3% B, which was linearly ramped to 32% B over 90 minutes, then raised to 95% B at 91 minutes, and maintained at that level until 101 minutes. The mass spectrometer was set to operate in data-dependent acquisition mode using parallel accumulation–serial fragmentation (DDA-PASEF). The m/z range for both MS1 and MS2 spectra was 100–1700, and the ion mobility range was 0.6–1.6 V $\cdot$ s/cm³. The ramp time was 100 ms, with a duty cycle of 100%. Each acquisition cycle consisted of 10 PASEF MS2 scans. A polygon filter was applied in the m/z and ion mobility space to exclude low m/z , singly charged ions from precursor selection. The raw data were processed using the FragPipe (v22.0). Database searches were performed using the MSFragger (v4.1), using the default parameters of the LFQ-phospho workflow against the UniProt mouse database. Carbamidomethylation of cysteine (+57.0215 Da) was set as a fixed modification. The following variable modifications were included: acetylation of protein N-terminus (+42.0106 Da); oxidation of methionine (+15.9949 Da); phosphorylation (+79.9663 Da) of serine, threonine, or tyrosine. The resulting identifications were filtered using Philosopher with default parameters (MS Booster was disabled), and IonQuant (v1.10.27) was employed for quantification with default software settings.

Phosphopeptide Data analysis

Raw data files were analyzed using FragPipe-Analyst⁶⁸. In this analysis, firstly contaminants were filtered out. In addition, peptides that were not identified/quantified consistently in same condition have been removed. The quantification values of intensity were converted to log2 scale and biological replicates were grouped by experimental condition. Peptide-wise linear models combined with empirical Bayes statistics were used for the differential expression analyses. The limma package from R Bioconductor was used to generate a list of differentially expressed proteins for each pair-wise comparison. To maintain high data quality and specifically assess phosphorylation changes, phosphopeptides with N-terminal acetylation (+42.0106 Da) were excluded from the analysis. For Ingenuity Pathway Analysis (IPA) software (v01-22-01), identified molecules with log2 fold change (ACR/Ctrl) values greater than 0.5 or less than -0.5 were analyzed for canonical pathway analysis.

In vitro metabolic trace analysis of ¹³C-labeled glucose and palmitate

To assess glycolysis activity, isolated CD8⁺ T cell subsets (2×10³ cells per well) were incubated in glucose-free RPMI medium (Gibco) supplemented with 10mM D-Glucose-¹³C₆ (Isotec) for 3 hours in a 37 °C CO₂ incubator. After incubation, cells were lysed by 80% methanol and mixed by vortex mixer. The lysed solution was then dried by centrifugal evaporation. The dried sample was reconstituted with 50% methanol and subjected to LC/MS analysis. To assess fatty acid oxidation (FAO) activity, isolated cell subsets (3×10³ cells per well) were incubated in glucose-free RPMI medium supplemented with 2.5mM glucose, 0.75mM carnitine (Sigma-Aldrich) and 200 μM BSA-conjugated palmitate-¹³C₁₆ [palmitic acid-¹³C₁₆ (Isotec) bound to BSA (molar ratio of fatty acid to BSA, 5:1)] for 3 hours in a 37 °C CO₂ incubator. After incubation, cells were lysed with methanol containing stable isotope-labeled acylcarnitine standards (palmitoylcarnitine-d₃, myristoylcarnitine-d₉, lauroylcarnitine-d₃ and octanoylcarnitine-d₃, Cambridge Isotope Laboratories). The lysed solution was then dried by centrifugal evaporation. The dried sample was reconstituted with 50% methanol and subjected to LC/MS analysis. The levels of each metabolite were either presented as peak area, or assessed by comparing the peak area ratio, calculated by dividing the peak area of each metabolite by that of the internal standard, and then converted to the absolute amount (fmol) .

Acrolein quantification in tumor tissue

For free acrolein detection in tissue, 2,4-dinitrophenylhydrazine (DNPH) (Tokyo Chemical Industry) derivatization was applied. 450 μL solution of 80% acetonitrile containing 1% formic acid and 1mg/mL of DNPH was added to 50 mg of tissue. The tissue was homogenized three times at 4,000 rpm for 30 seconds using a bead homogenizer (Micro Smash 100R, TOMY) with 8 to 10 zirconia beads (1mm diameter). For acrolein standard solution, Acrolein (AccuStandard) was prepared at the concentration of 0.4, 1, 2, 4, 10 and 20 μg/mL, and 50 μL of them were mixed with 450 μL of the same reaction mixture to make acrolein standard solutions. The homogenized tissues or acrolein standard solutions were then incubated for 2 hours at 37 °C to complete DNPH derivatization. After centrifugation at 16,000 g for 30 minutes at 4 °C, 50 μL of supernatant was transferred to a glass vial and subjected to LC/MS analysis. Acrolein concentration was quantified by the external standard method. The correlation coefficient of the standard curve was higher than 0.99. Tumor acrolein concentrations were calculated assuming a tumor density to be 1 g/mL, based on reference data from murine solid tumor models ⁶⁹.

LC/MS analysis

All data acquisition was performed on the LCMS system consisting of a Nexera UHPLC and LCMS-8060 (Shimadzu) with the electrospray ionization and selected reaction monitoring mode.

LC separation was conducted on a Shim-pack GIST C18-AQ column (3 μ m, 150 mm $\times$ 2.1 mm id, Shimadzu). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). For palmitate metabolite analysis, LC gradient was modified as follows: 0 to 3 min, 35% B; 3 to 15 min, linear gradient to 95% B; 15 to 20 min, 95% B; 20.1 min, 95% B; hold for 4 min. For DNPH-derivatized acrolein, LC gradient was changed as follows: 0 min, 20% B; 0 to 15 min, linear gradient to 95% B; 15 to 16 min, 95% B; 16.1 min, 20% B; hold for 4 min.

A Shim-pack Mix-HILIC column (5 μ m, 150 mm $\times$ 2.1 mm id, Shimadzu) was adapted, and the mobile phase consisted of 40 mM ammonium bicarbonate in water (pH 9.7) (A) and acetonitrile (B) for glucose metabolite analysis. The gradient program was as follows: 0 to 0.5 min, 95% B; 0.5 to 15.5 min, linear gradient to 40% B; 15.5 to 16.5 min, linear gradient to 0% B; 16.5 to 26.5 min, 0% B; 26.5 to 27.5 min, linear gradient to 95% B; hold for 7.5 min; flow rate, 0.4 mL/min. The ion transitions (precursor-product ion) for DNPH-derivatized acrolein was m/z (mass/charge ratio) 235.05 to 158.30. The targeted metabolite for glycolysis and FAO activity assay are summarized in the Supplementary Table 4.

Single-cell RNA sequencing experiment

Tumor sample from MC38 tumor-bearing mice (n = 20 mice) on day 14 were pooled and digested. CD8⁺ TILs were enriched by Manual MACS Magnetic Separators. They were subsequently stained with appropriately diluted MitoTracker Deep Red and MitoTracker Green for 30 minutes in a 37 °C CO₂ incubator. After washing with 2% FCS in PBS twice, cells were stained with Zombie Violet, Fc-blocked by anti-CD16/32 antibody and stained for 12 minutes at 4 °C with appropriately diluted fluorescent-labeled and oligo-conjugated antibodies (including oligo-conjugated isotype controls) targeting cell surface antigens as follows: PE CD8a (clone: 53-6.7, BioLegend), PE-Cy7 PD-1 (clone: 29F.1A12, BioLegend), and BV786 CD45.2 (clone: 104, BD Biosciences), along with a panel of oligo-conjugated antibodies (BioLegend), including CD279 (PD-1, clone: RMP1-30), Ly108 (Slamf6, clone: 330-AJ), CD226 (clone: 10E5), KLRG1 (clone: 2F1/KLRG1), CD122 (clone: 5H4), CD366 (Tim-3, clone: RMT3-23), CX3CR1 (clone: SA011F11), TIGIT (clone: 1G9), CD39 (clone: Duha59), CD49d (clone: R1-2), CD183 (clone: CXCR3-173), CD185 (clone: L138D7), CD103 (clone: 2E7), CD107a (clone: 1D4B), CD95 (clone: SA367H8), CD127 (clone: A7R34), CD44 (clone: IM7), CD62L (clone: MEL-14), CD278 (clone: 7E.17G9), and isotype controls (Rat IgG2a κ , clone: RTK2758; Rat IgG2b κ , clone: RTK4530; Mouse IgG1 κ , clone: MOPC-21; Mouse IgG2a κ , clone: MOPC-173; Armenian Hamster IgG, clone: HTK888). The oligo-conjugated antibodies were included during sample preparation; however, Antibody Capture-derived signals from these antibodies were not used for analysis in this study. Corresponding oligo sequences and feature types are available in the GEO submission. Cells were washed with 2% FCS in PBS twice, filtered through a 40- μ m cell strainer

before sorting. Viable single PD1⁻ CD45.2⁺ CD8a⁺ cells and D1, D2, D3 subsets of PD1⁺ CD45.2⁺ CD8a⁺ cells were sorted using FACSMelody. To identify each subset in scRNA-seq, the sorted cells' Fc receptor was blocked by anti-CD16/32 antibody (BioLegend) and stained with oligonucleotide-labelled Hashtag 1 antibody for PD1⁻ population, Hashtag 4 antibody for D1, Hashtag 5 antibody for D2 and Hashtag 6 antibody for D3 (all Hashtag antibodies clone: M1/42; 30-F11, BioLegend). After incubation for 30 minutes at 4 °C, each cell subsets were washed three times with 2% FCS in PBS and pooled. A total of 10,000 pooled cells were loaded into a Chromium controller (10x Genomics). cDNA and library preparation were performed according to the manufacturer's indications using Chromium Next GEM Single Cell 5' Reagent Kits v2 (Dual Index) (10x Genomics), and the library was sequenced by a NextSeq 2000 sequencer (Illumina). Please refer to the reporting summary for detailed information about the antibodies.

ScRNA-seq data analysis

The sequencing reads were aligned to the GRCm39-2024-A reference genome for 5' gene expression sequencing data and to vdj_GRCm38_alts_ensembl-7.0.0 for V(D)J data, and assembled using the 'cellranger multi' function in CellRanger (v9.0.1, 10x Genomics)⁷⁰. The resulting raw barcodes, features, and matrix files were loaded into R (v4.4.0) and processed using Seurat (v5.2.1)⁷¹, along with hashtag oligo (HTO) count data and V(D)J data. Low-quality cells were filtered out based on the following criteria: < 200 detected genes, > 4,000 detected genes, and > 5% mitochondrial genes. Samples were demultiplexed based on HTO counts using the 'HTODemux' function. Cells identified as doublets (containing two hashtags) or without a hashtag were removed, and only cells from D1–D3 were retained for further analysis. The D1-D3 singlet data were normalized and log-transformed to mitigate the effects of varying sequencing depths. Dimensional reduction and clustering were performed using principal component analysis and visualized with UMAP via Seurat functions. Gene signature scoring was conducted using the 'AddModuleScore_UCell' function in the R package UCell (v2.8.0)⁷². Gene signatures for exhausted and stem-like CD8⁺ T cells were cited from Deak et al⁷³. Pseudotime was calculated, and trajectory visualization was performed using Monocle 3 (v1.3.7)⁷⁴ after transferring the Seurat object via SeuratWrappers (v0.3.5). T cell clonotypes were analyzed using scRepertoire (v2.0.7)⁷⁵ based on V(D)J gene sequences. Differentially expressed genes in D1 vs D3 were identified using the 'FindMarkers' function in Seurat. Genes upregulated in D3 were selected based on the criteria: p.adjust < 0.05 and log2 fold change (D3/D1) > 1. Gene ontology (GO, Biological Process) analysis was performed using clusterProfiler (v4.12.6)^{76,77}, with annotation provided by org.Mm.eg.db (v3.19.1). To reduce redundancy in GO terms, the 'simplify' function was applied.

ECAR, OCR and ATP production rate measured by Seahorse

Cultured cells were seeded in an XFe96 plate (2 to 4×10^5 cells per well) and their ECAR and OCR were measured by the XFe96 Extracellular Flux Analyzer (Seahorse Bioscience) as previously described¹¹. The Seahorse XF Glycolysis Stress Test Kit, the Seahorse XF Cell Mito Stress Test Kit, and the Seahorse XF Real-Time ATP Rate Assay Kit (all Seahorse Bioscience) were used for measurement of ECAR, OCR, and ATP production, respectively. For ECAR assays, XF DMEM Base Medium (Agilent Technologies) was used, supplemented with 2 mM glutamine (Agilent Technologies). To measure OCR derived from FAO or glutaminolysis activity, XF RPMI Base Medium (Agilent Technologies) was used, supplemented with 2.5mM glucose (Agilent Technologies) on the day of the assay. Etomoxir (5 μ M) or BPTES (3 μ M) was added about 10 minutes before running the assay. For Real-Time ATP Rate Assay, XF DMEM Base Medium was used, supplemented with 1 mM pyruvate (Agilent Technologies), 2 mM glutamine and 10 mM glucose. Mitochondrial and glycolytic ATP production rates were automatically calculated by the Seahorse machine operating software Wave Controller in accordance with the manufacturer's instructions.

Statistical analysis

For phosphoproteomics data, FragPipe-Analyst (v1.13) and Ingenuity Pathway Analysis (IPA) software (v01-22-01) were used for statistical analysis. Results of IPA were visualized using Graphpad Prism (v10.3.1 or v10.4.1). Statistical analyses and visualization of scRNA-seq data were performed using R (v4.4.0). To perform multiple-group analysis between D1-D3 or six distinct clusters of UMAP, one-way analysis of variance (ANOVA) was used. Statistical analyses of the other datasets were performed using Prism. For mice, to compare two groups, the two-tailed paired or unpaired Student's t test was used, and to perform multiple-group analysis, ANOVA was used. A p-value of < 0.05 was considered significant. The variations of data were evaluated as the means or standard errors of the mean (SEMs). Data distribution was assumed to be normal but this was not formally tested. Three or more samples were thought to be appropriate for the sample size estimate for in vitro experiments, and five or more samples for in vivo experiments. No statistical methods were used to pre-determine sample sizes. In some cases, cells were pooled from multiple mice in one experiment. Samples and animals were randomly chosen from the pool and treated. No blinding method was used for the treatment of samples and animals. To compare two groups for humans, the two-tailed Wilcoxon matched-pairs signed rank test was used, and a p-value of < 0.05 was considered significant. Seven or more samples were thought to be appropriate for the sample size estimate.

Data availability

ScRNA-seq data generated in this study have been deposited in the Gene Expression Omnibus (GEO) repository under accession number [GSE307051](#). The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Custom scripts supporting the data analysis were developed using standard computational tools and are available from the corresponding author upon reasonable request.

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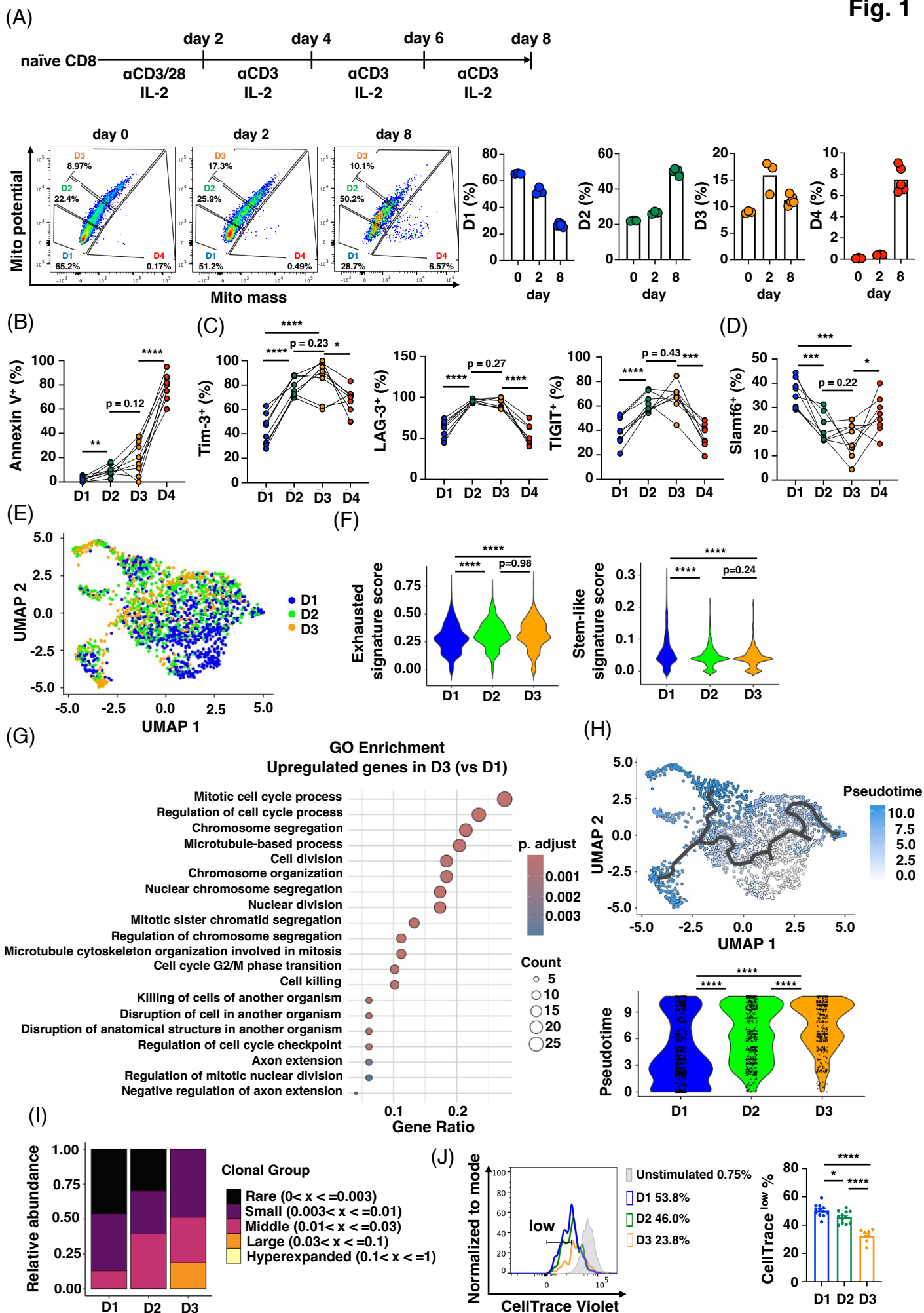


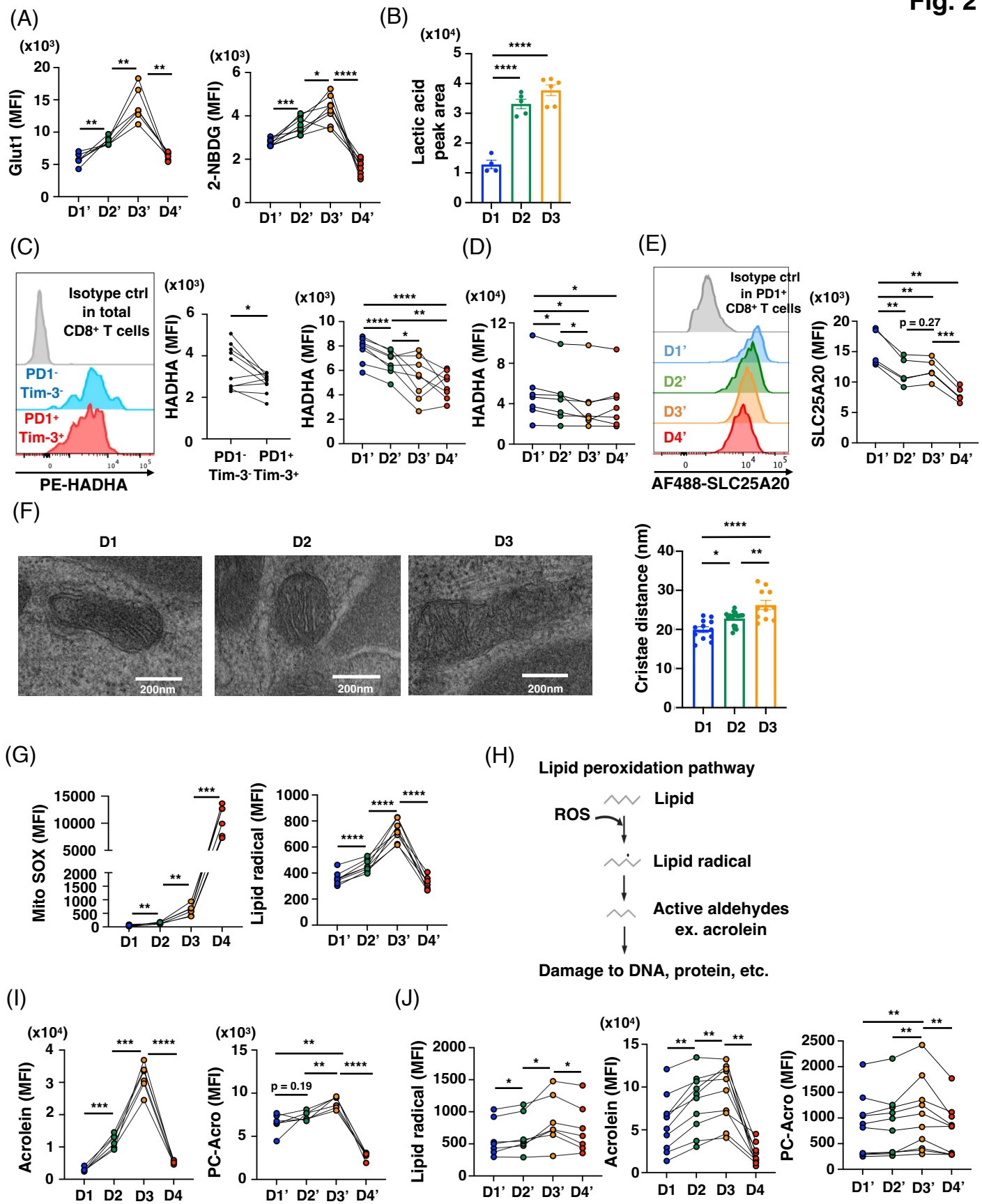
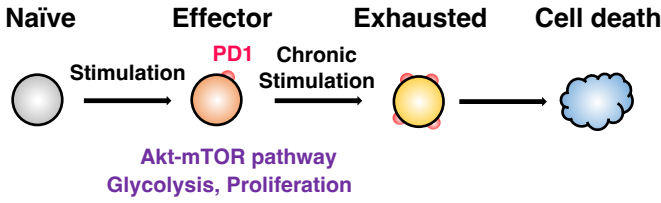
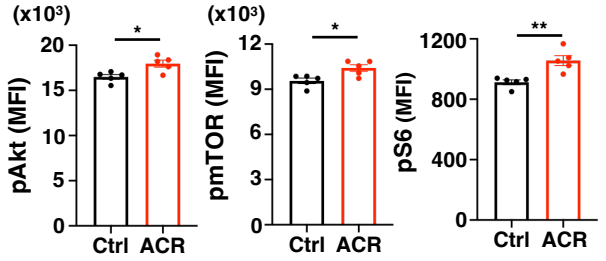
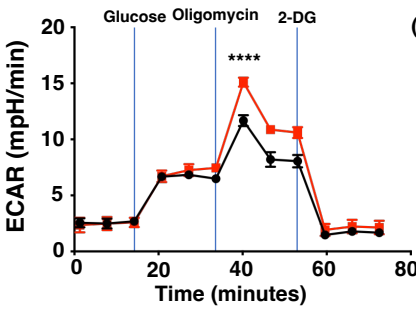
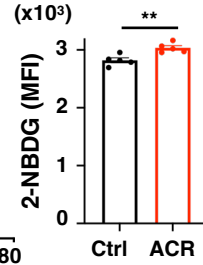
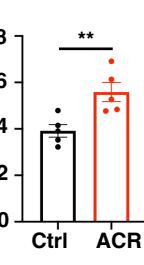
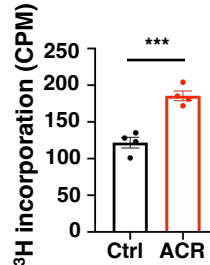
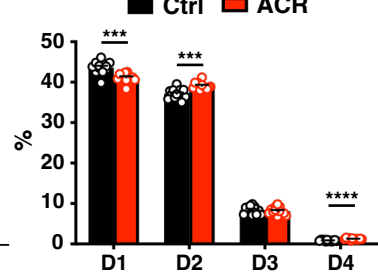
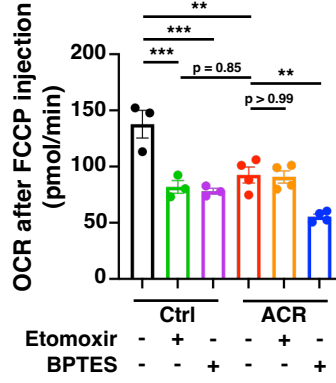
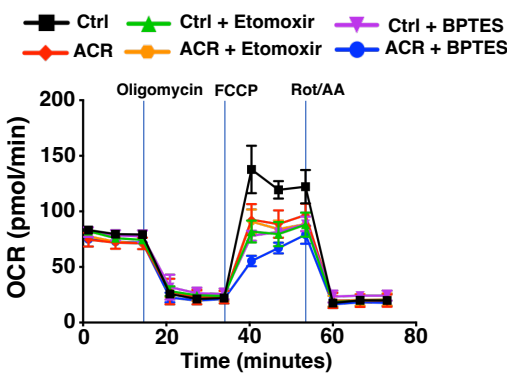
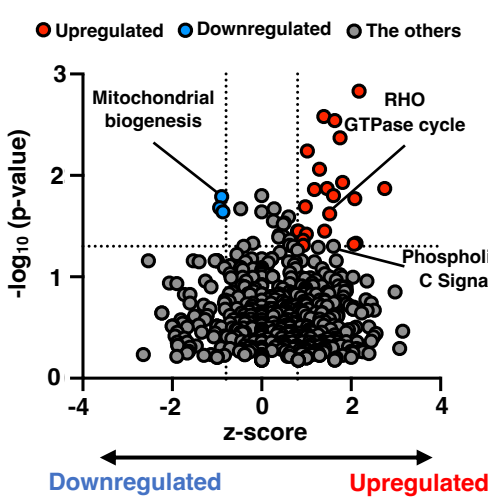
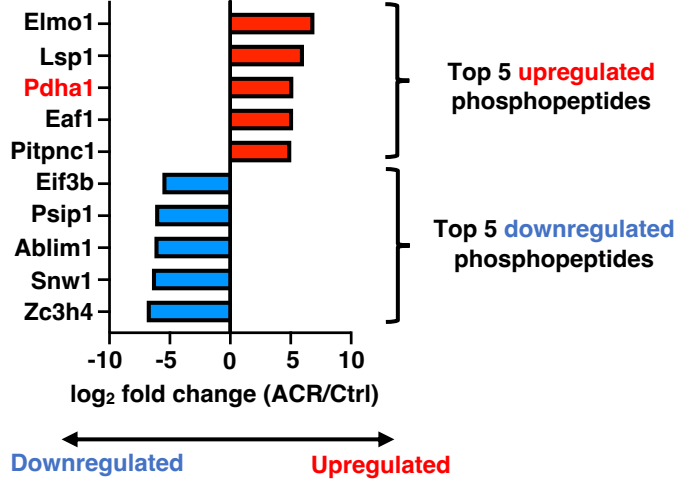
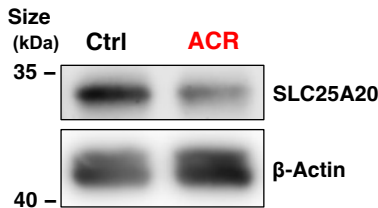
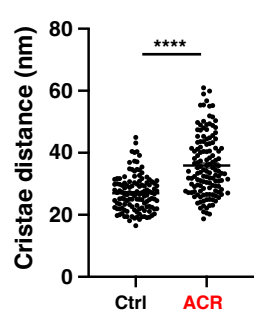
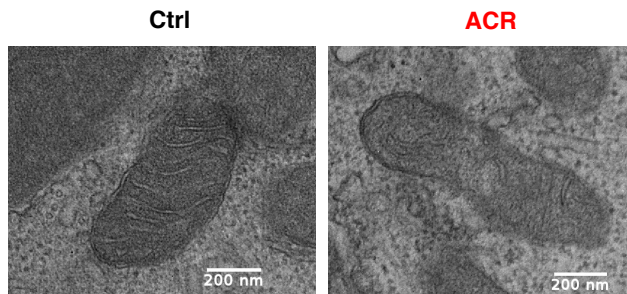
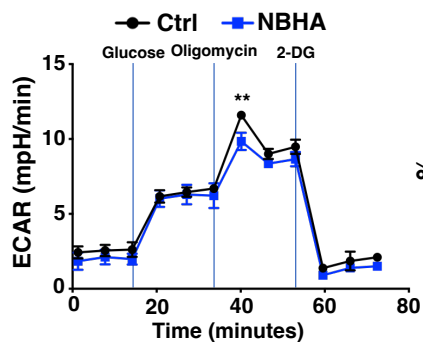
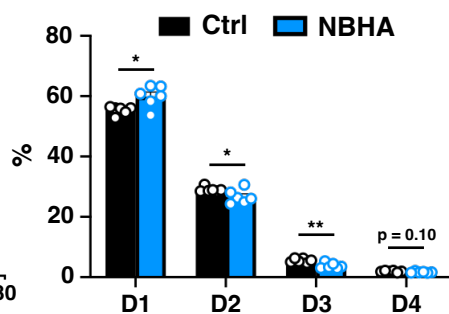
Fig. 2

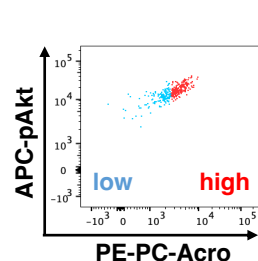
Fig. 3**(A)****(B)****(C)****(D)****(E)****(F)****(G)****(H)****(I)****(J)****(K)****(L)**



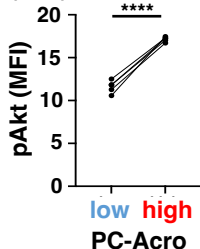
(B)



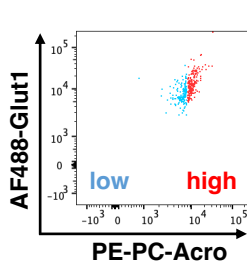
(C)



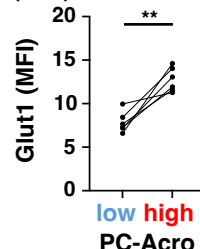
(x10³)



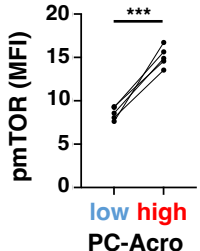
(D)



(x10³)

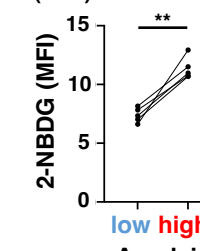
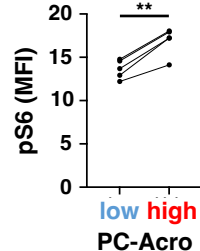


(x10³)

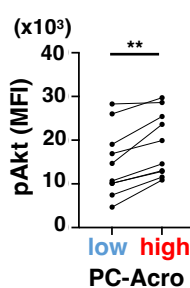


A scatter plot showing the relationship between 2-NBDG fluorescence (y-axis, log scale from 10^{-3} to 10^5) and $\log_{10}(\Delta F/F_0)$ (x-axis, log scale from 10^{-1} to 10^5). Data points are colored blue for 'low' responders and red for 'high' responders. The 'low' group is clustered at lower values for both axes, while the 'high' group is clustered at higher values.

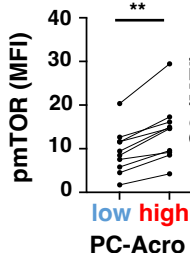
(x10

(x10³)

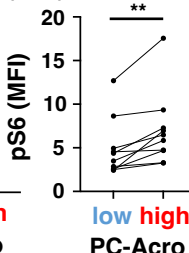
(E)



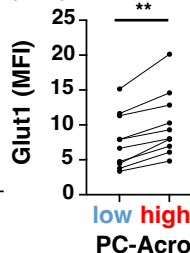
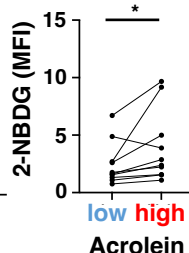
(x10³)



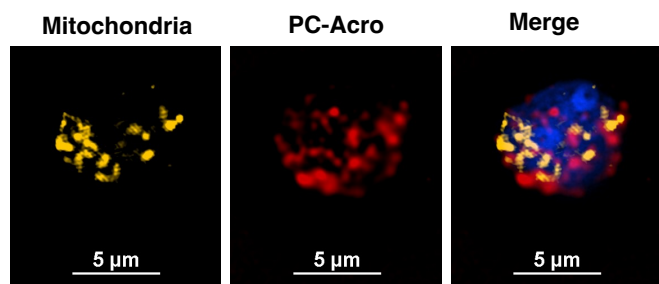
(x10³)



(x10³)

(x10³)

(F)



Yellow: mitochondria (PhenoVue551)
Red: PC-Acro (APC)
Blue: nucleus (Hoechst 33342)

Colocalization

Pearson's correlation

Pearson's correlation
0.15
0.30
0.32
0.35
0.38
0.40
0.42
0.45
0.48
0.60

(G)

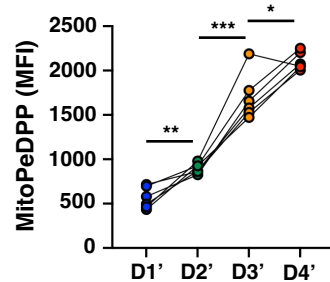


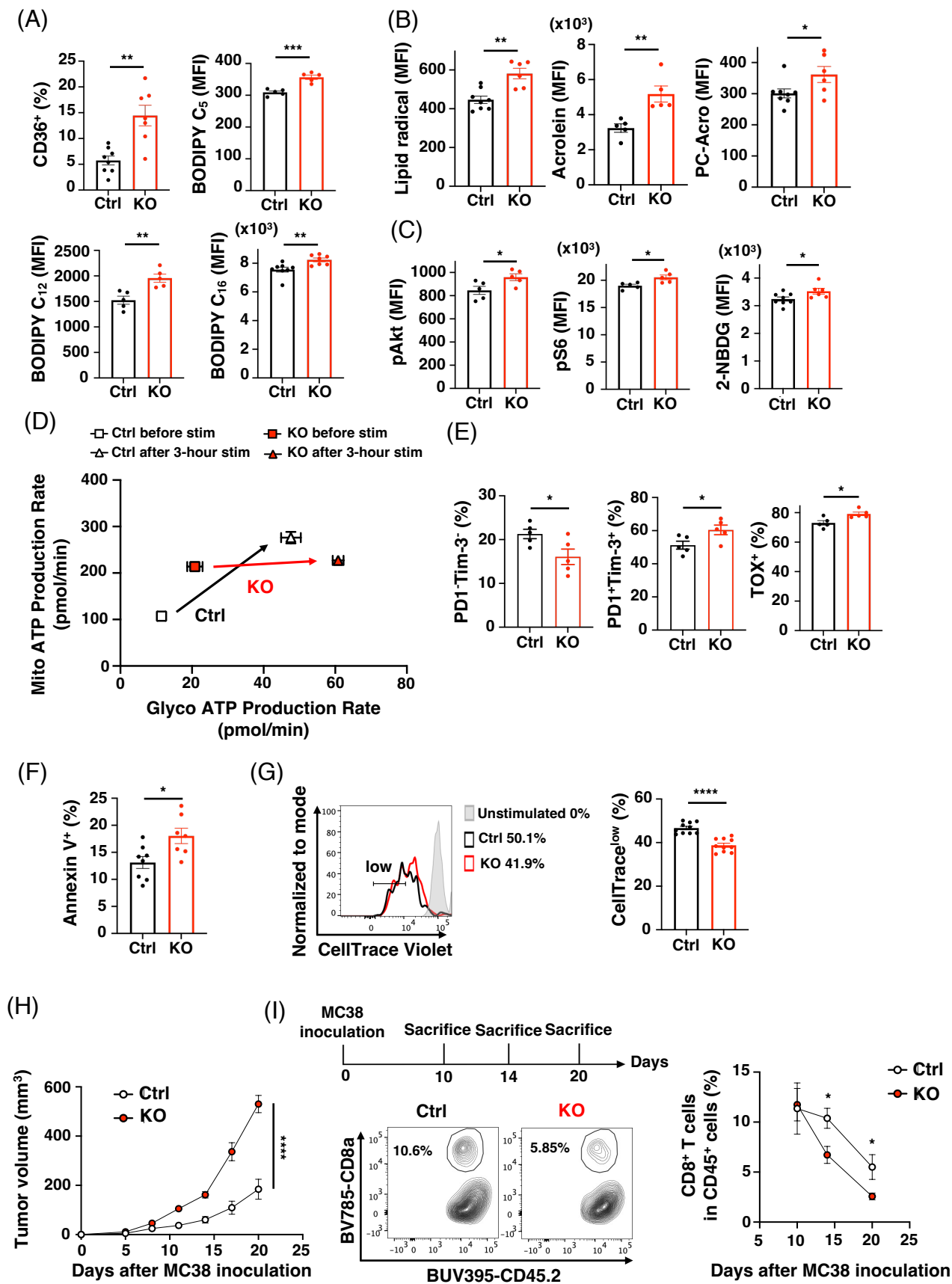
Fig. 5

Fig. 6

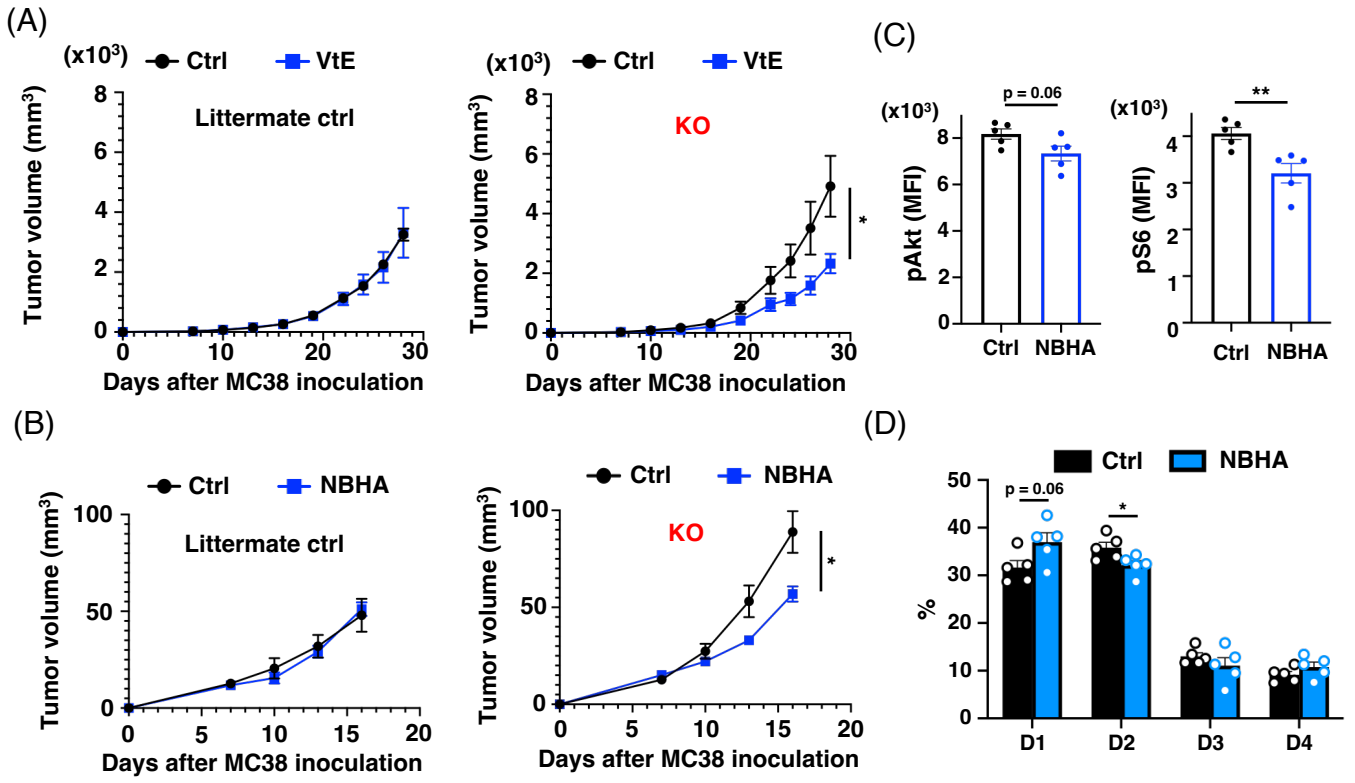
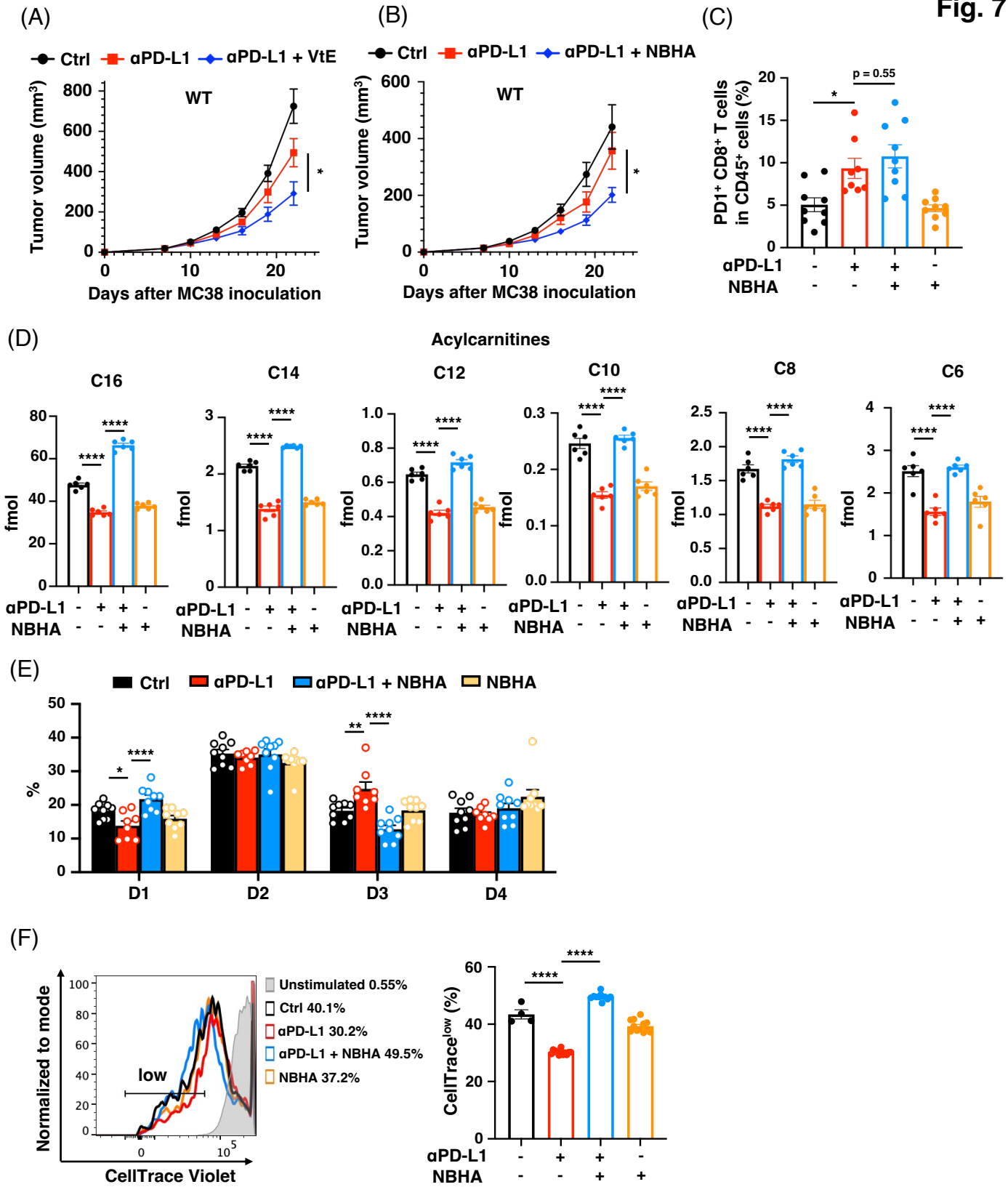
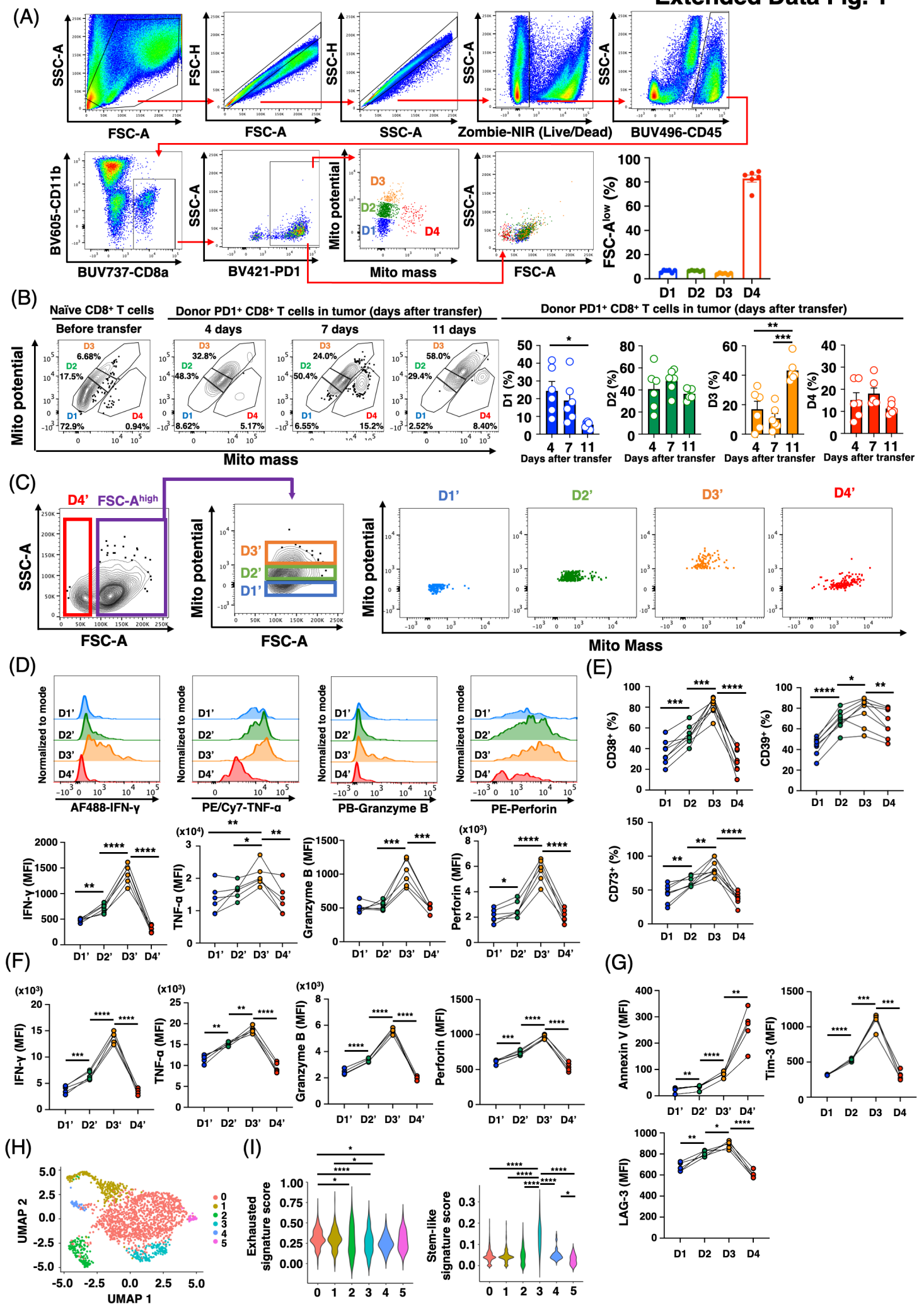
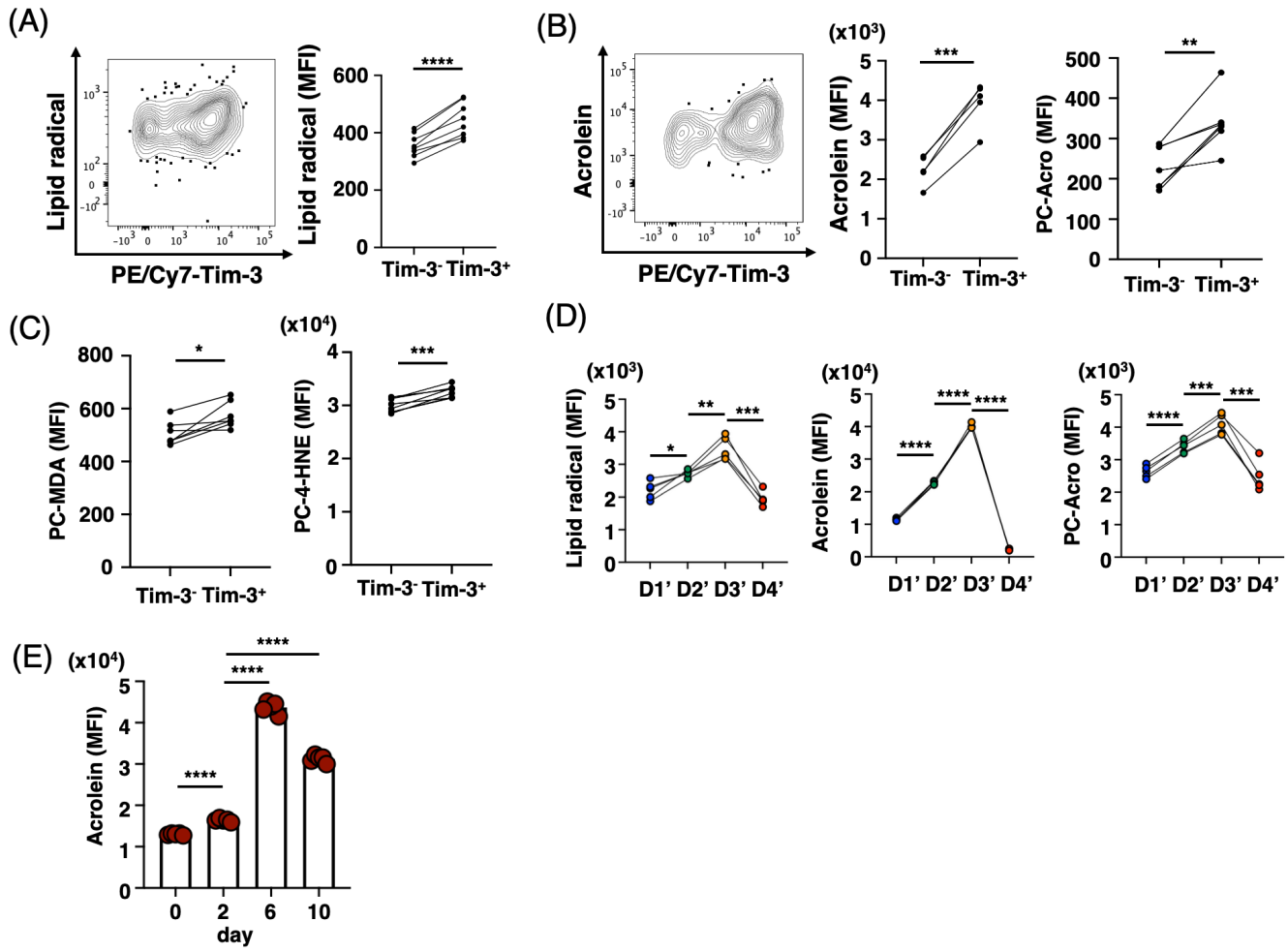


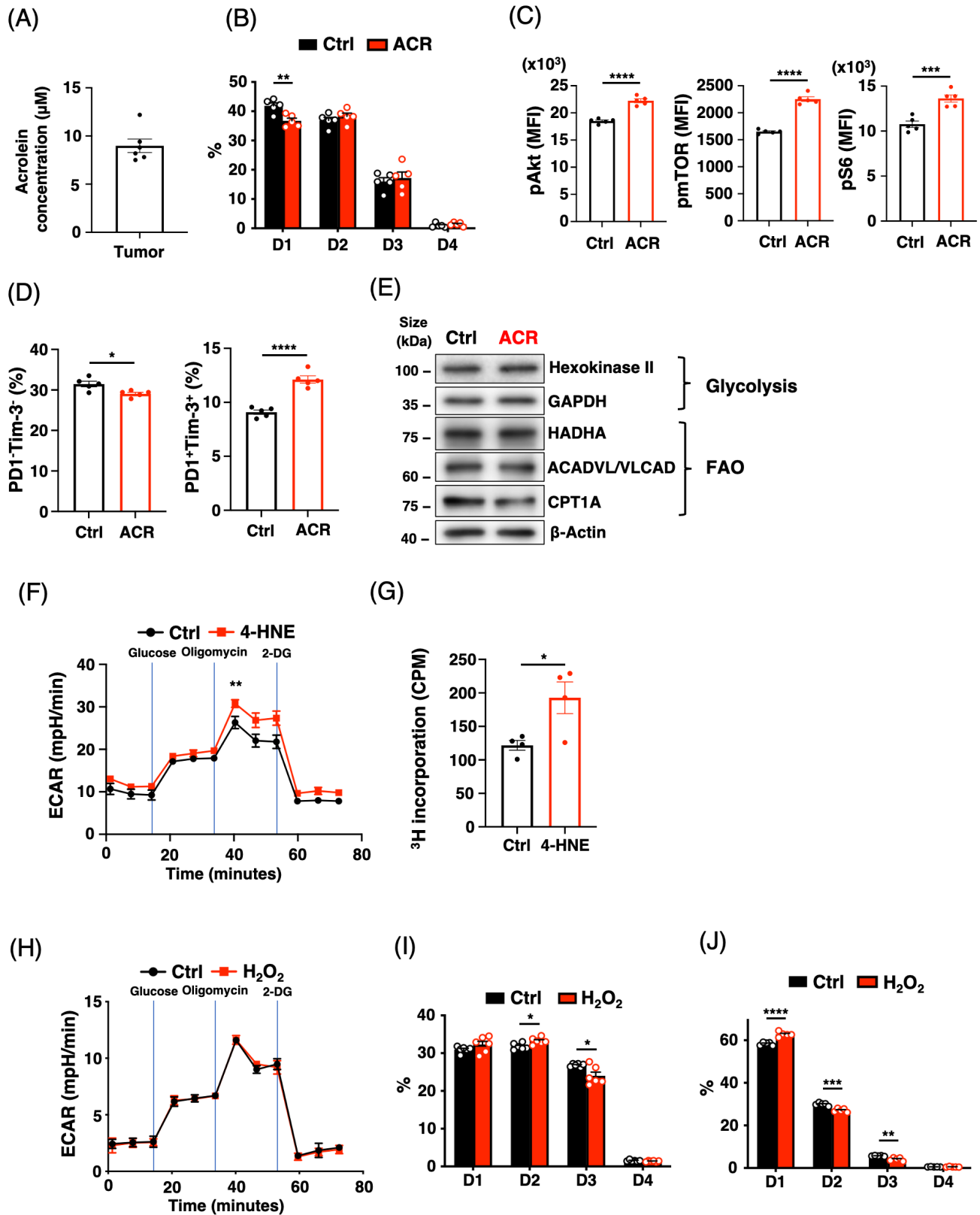
Fig. 7



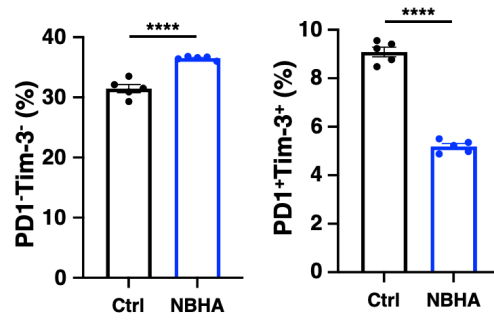
Extended Data Fig. 1



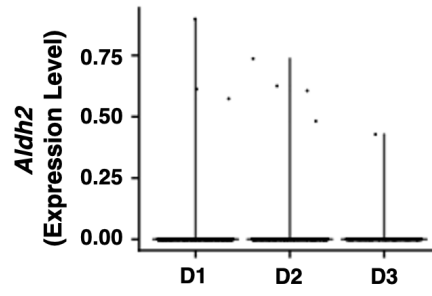


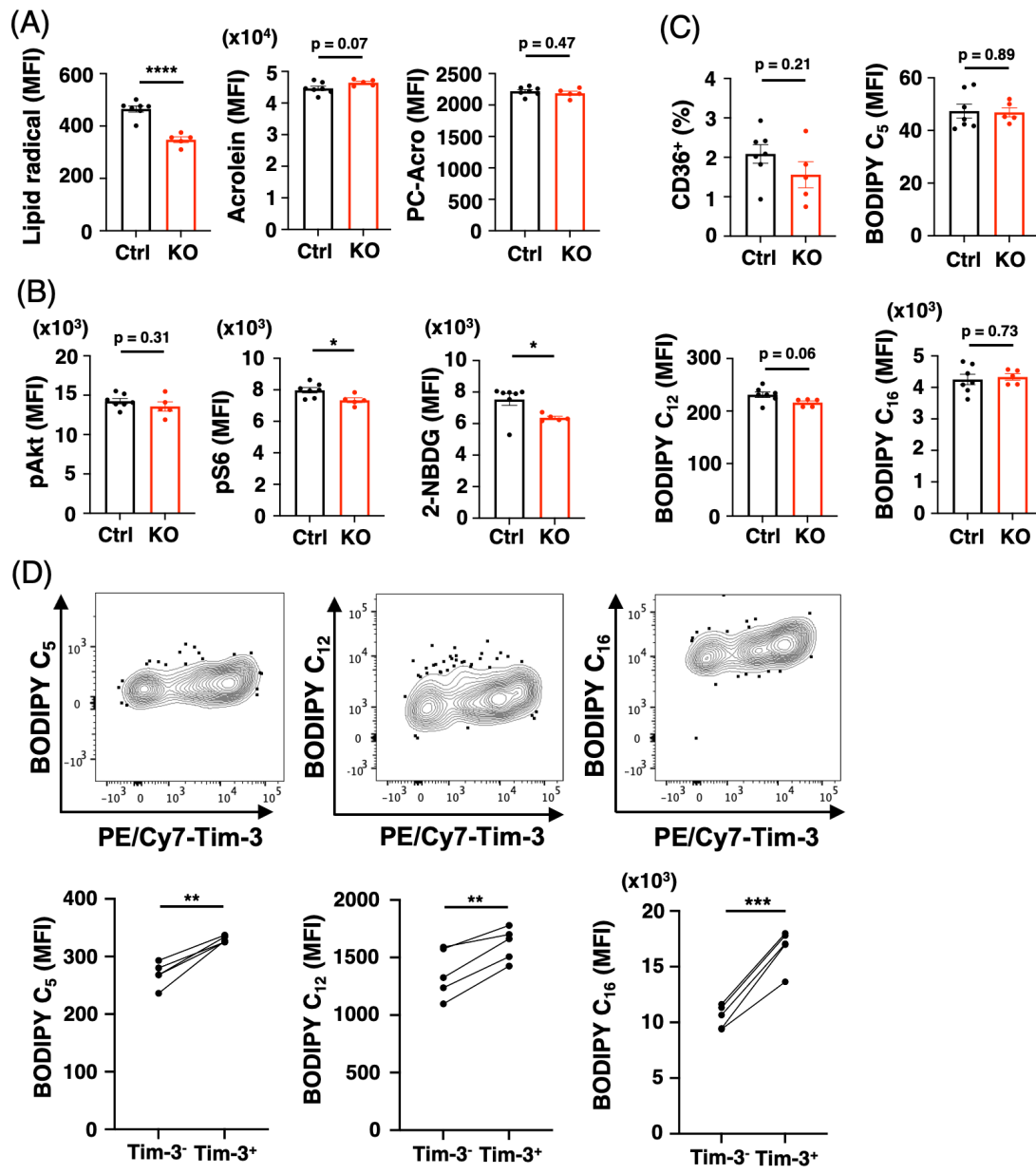


(A)



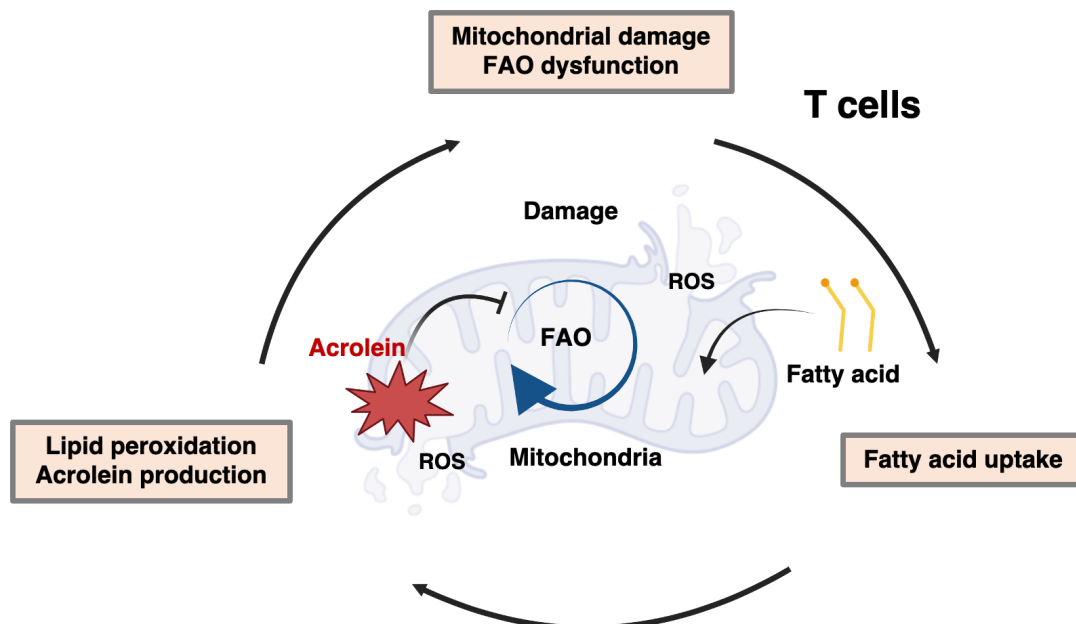
(B)

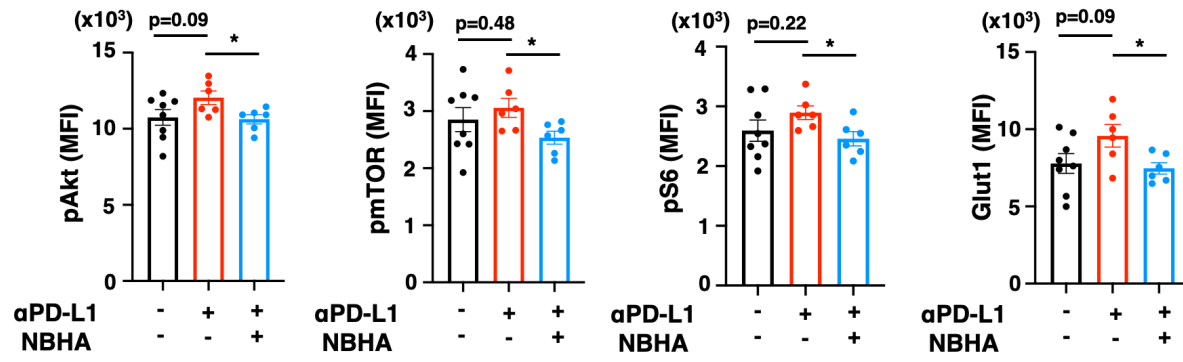




(E)

Loop for severe oxidative stress by FAO dysfunction in TME





Active aldehydes shapes metabolic exhaustion in T cells

