Distinct metabolic programs established in the thymus control effector functions of γδ T cell subsets in tumor microenvironments

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Metabolic programming controls immune cell lineages and functions, but little is known about γδ T cell metabolism. Here, we found that γδ T cell subsets making either interferon-γ (IFN-γ) or interleukin (IL)-17 have intrinsically distinct metabolic requirements. Whereas IFN-γ+ γδ T cells were almost exclusively dependent on glycolysis, IL-17+ γδ T cells strongly engaged oxidative metabolism, with increased mitochondrial mass and activity. These distinct metabolic signatures were surprisingly imprinted early during thymic development and were stably maintained in the periphery and within tumors. Moreover, pro-tumoral IL-17+ γδ T cells selectively showed high lipid uptake and intracellular lipid storage and were expanded in obesity and in tumors of obese mice. Conversely, glucose supplementation enhanced the antitumor functions of IFN-γ+ γδ T cells and reduced tumor growth upon adoptive transfer. These findings have important implications for the differentiation of effector γδ T cells and their manipulation in cancer immunotherapy.

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Peripheral lymphoid organs and within tumors in various experimental models of cancer. We further show that the provision of glucose or lipids has a major impact on the relative expansion and function of the two γδ T cell subsets and that this can be used to enhance antitumor γδ T cell responses.

**Results**

**Intratumoral γδ T cell subsets display distinct metabolic profiles.** The analysis of metabolic profiles of tumor-infiltrating γδ lymphocytes (γδ TILs) presented a major challenge: the low numbers that can be retrieved from tumor lesions in mice are largely incompatible with techniques such as Seahorse metabolic flux analysis. To overcome this difficulty, we used a newly developed protocol, SCENITH (Single Cell Metabolism by Profiling Translation Inhibition), which is a flow-cytometry-based method for profiling energy metabolism with single-cell resolution. This method is based on metabolism-dependent translation rates and puromycin's incorporation into nascent proteins (Extended Data Fig. 1). The use of specific inhibitors allows the estimation of glucose dependence, mitochondrial dependence, glycolytic capacity and fatty acid and amino acid oxidation. We employed SCENITH to analyze the metabolic profiles of γδ TILs isolated from tumor lesions in well-established mouse models of breast (E0771) and colon (MC38) cancer. In both cancer models, and at both earlier time points (Fig. 1a,b) and earlier time points (Fig. 1c,d), we observed that γδ T cells had substantially higher glycolytic capacity, whereas γδ cells were strongly dependent on mitochondrial activity (Fig. 1). These data, obtained in cancer models, prompted us to investigate the metabolic phenotypes of γδ T cell subsets in multiple tissues at steady state.

Peripheral γδ T cell subsets show different mitochondrial and metabolic phenotypes. To explore the metabolic differences between γδ T cell subsets in peripheral tissues, we analyzed mitochondrial and metabolic phenotypes. The metabolic differences between γδ T cell subsets in peripheral tissues, we analyzed mitochondrial and metabolic phenotypes. To explore the metabolic differences between γδ T cells in peripheral tissues, we employed CD27 expression. CD27 - γδ T cells displayed increased MitoTracker and
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showed higher levels of basal OXPHOS in γδ-test.***

Pt 0.0003; TMRM n γδ (P = 0.0003, TMRE MFI of Pc7, data pooled cells ex vivo. Relative MitoTracker was calculated by dividing the MFI of MitoTracker by the MFI of FSC-A and multiplying by 100 (expanded in vitro) from LNs (γδ stained with MitoTracker (green) and Hoechst 33342 (blue). Scale bar, 5 γδ 17 cells, data were validated in independent experiments using SCENITH on splenic and LN γT cell subsets (Fig. 2g).

To assess whether this metabolic dichotomy had an underlying transcriptional basis, we measured the mRNA levels of key mitochondrial and glycolysis-associated genes in purified peripheral γδ 17 and γδ 17 cells. We found systematic biases in gene expression that matched the differential metabolic programs (Fig. 2h,i). Of particular note is the clear-cut segregation of two master transcriptional regulators: nuclear respiratory factor 1 (Nrf1), which orchestrates mitochondrial DNA transcription 36, found to be enriched in γδ 17 cells (Fig. 2h), and Mac proto-oncogene, BHLH transcription factor (Myc), which controls glycolysis 36, that was highly overexpressed in γδ 17 cells (Fig. 2i). Myc expression was further validated using a Myc-green fluorescent protein (GFP) reporter mouse (Fig. 2j). These data collectively demonstrated that γT cell subsets possess distinct mitochondrial and metabolic features in peripheral organs at steady state.

γδ T cell subsets are metabolically programmed in the thymus.

We next aimed to understand when, during their differentiation, the metabolic differences between the two effector γT cell subsets were established. Since most γδ T cells are functionally preprogrammed in the thymus, we examined γδ thymocyte subpopulations. Studies have identified sequential stages of thymic γδ T cell progenitor development marked by CD24, CD44 and CD45RB 3. Early CD24+ (γδ 17) precursors downregulate CD24 to become a CD24−CD44−CD45RB− (γδ 17) population that generates cells committed to either IL-17 or IFN-γ expression, which display, respectively, CD44+CD45RB− (γδ 17) and CD44−CD45RB+ (γδ 17) phenotypes (Extended Data Fig. 3). By using SCENITH, we found that, in both the adult (Fig. 3a) and newborn (Fig. 3b) thymi, these subsets showed the same metabolic dichotomy as in the periphery (Fig. 2g), although this was less distinct in γδ thymocytes, likely due to the dynamic subset segregation process 36.

To investigate any potential switching of metabolic programming during γδ thymocyte development, we first compared early thymic γδ progenitors with more mature subpopulations already committed to IL-17 or IFN-γ production. We found that γδ 17+ and γδ 17+ progenitors stained highly for TMRE, which was lost when ΔΨ 17 was dissipated by the ionophore carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (Fig. 3c,d). Although γδ 17 cells retained a high level of TMRE staining, γδ 17 cells showed a marked reduction in ΔΨ 17, suggesting a metabolic switch away from OXPHOS (Fig. 3d). Moreover, imagestream analysis of γδ 17 cells stained with either MitoTracker or TMRE revealed large and active mitochondria, in contrast with γδ 17 cells, which displayed negligible staining for either dye (Fig. 3e), in line with our previous observations in peripheral subsets (Fig. 2a–c). Furthermore, Seahorse extracellular flux analysis showed that γδ 17 thymocytes have both higher maximal respiration potential and spare respiratory capacity than those of their γδ 17 counterparts (Fig. 3f,g). Thus, γT cell subsets acquire distinct mitochondrial features during their acquisition of effector function in the thymus.

The adoption of divergent metabolic programs by thymic γδ T cell subsets suggested that they could thrive under distinct metabolic environments. To begin to address this, we placed wild-type (WT) embryonic day 15 (E15) thymic lobes in organ culture for 7 days (E15 + 7-d fetal thymic organ cultures (FTOCs)) with media containing either low or high amounts of glucose (Fig. 3h). γδ 17 cells were readily detected in lower glucose conditions but failed to develop to normal numbers when glucose concentrations were raised. By contrast, γδ 17 cells were relatively enriched in high glucose conditions, as demonstrated by a significant decrease in the γδ 17/γδ 17 cell ratio (Fig. 3h). We next established E15 + 7-d FTOCs in the presence of the glycolysis inhibitor 2-deoxy-d-glucose (2-DG), and found increased numbers of γδ 17 cells and increased γδ 17/γδ 17 cell ratios (Fig. 3i). A similar result was observed in E15 + 7-d FTOC in the presence of metformin, which reduces glucose uptake (Extended Data Fig. 4). By contrast, running E15 + 7-d FTOC in the presence of metformin, which reduces the efficiency of OXPHOS by inhibiting complex I of the electron transport chain, impaired γδ 17 cell generation and decreased the γδ 17/γδ 17 cell ratio (Fig. 3j). Collectively, these results suggest that the mitochondrial characteristics adopted by γδ 17 and γδ 17 cells during thymic development directly impact their ability to thrive in distinct metabolic environments.

**Distinct mitochondrial activities underlie effector fate of thymic γδ T cell precursors.** We next aimed to investigate the association of distinct metabolic programs with the developmental divergence
of γδT and γδTN cells in the thymus. Although the γδTCR population, that is the progenitor γδ cell subset that immediately precedes the surface upregulation of either CD44 or CD45RB (marking commitment to the IL-17 or IFN-γ pathways, respectively19) was predominantly TMREhi, we observed a fraction of cells with reduced TMRE staining that we reasoned might be transitioning to the TMRElo.

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state shown by γδTN cells (Fig. 3c). We further hypothesized that the metabolic status of γδTN progenitors may predict their developmental fate. To test this, we sorted TMREhi and TMRElo cells from the γδTN subset obtained from E15 + 7-d FTOC, and cultured them for 5 d on OP9-DL1 cells, which are known to support appropriate development of thymocytes9. As predicted, virtually all cells from the TMRElo cultures upregulated CD45RB and entered the IFN-γ pathway (Fig. 4a); however, we were surprised that almost all cells from the TMREhi cultures entered the CD44hi IL-17 pathway (Fig. 4a). This strongly suggests that γδTN cells have already committed to an effector fate, and that this commitment associates with distinct mitochondrial activities.

To pursue this hypothesis further, we tested γδ24+ progenitors that immediately precede the γδ17 subset; again, we observed a segregation of effector fate, with the majority of TMRElo γδ24+ cells entering the IFN-γ pathway, and the majority of TMREhi γδ24+ cells entering the IL-17 pathway (Fig. 4b). The observation that so few TMREhi γδ24+ cells adopt an IFN-γ secreting fate again suggests that most γδ24+ progenitors have already committed to subsequent effector function. Moreover, we found that differences in TMRE levels correlated with the known3,11,13 effector biases of Vγ1 (γδ22+)-biased and Vγ4 (γδ17+)-biased progenitors (Fig. 4c), and allowed TMRE-based segregation of effector fates using only Vγ4 progenitors (Extended Data Fig. 5). Furthermore, among γδ24+ thymocytes along the γδTN pathway, we observed a progressive downregulation of TMRE levels from γδ20 to CD44 hi CD45RB+ cells and finally γδ24+ cells (Fig. 4d).

Given that we and others11,13 have previously shown a key role for TCR signaling in γδTN thymocyte differentiation, we next asked if downregulation of TMRE levels associated with hallmarks of TCR signaling. Indeed, we found that low TMRE associated with high expression of CD37 (Fig. 4e), one of the best established markers of TCR signaling in γδ T cell development3,12,23. Moreover, in E15 thymic lobes, TMRE staining was reduced along with CD25 downregulation, which is another hallmark of (developmentally early) TCRγδ signaling12,14,23. Furthermore, at this E15 stage, the cells with the lowest TMRE staining were Vγ5 progenitors (Fig. 4f) that are known to engage a Skint1-associated TCR ligand in the thymus and to uniformly commit to the IFN-γ pathway18.

These lines of evidence suggested that γδ progenitors receiving agonist TCRγδ signals shift away from OXPHOS as indicated by their reduced ΔΨm. To strengthen this point, we manipulated TCR signals using agonist GL3 monoclonal antibody, which, as expected9,11,16, promoted γδTN cell development while inhibiting the γδ17 pathway in E17 + 6-d FTOC (Fig. 4g). Upon specifically sorting TMRElo γδ24+ cells from E17 thymi and stimulating them with GL3 for 5 h, we found a subpopulation that downregulated CD24 together with TMRE, in a monoclonal-antibody-dose-dependent manner (Fig. 4h). These results strongly suggest that TCR signaling leads to ΔΨm downregulation as γδ thymocytes differentiate into IFN-γ producers.

To gain further molecular resolution, we performed single-cell RNA-seq on TMREhi and TMRElo γδ24+ cells from E15 + 2-d FTOCs (Extended Data Fig. 6a). Dimensionality reduction using UMAP (uniform manifold approximation and projection) showed that TMREhi cells clustered clearly away from TMRElo γδ24+ cells (Fig. 4i), and the former were enriched in genes involved in the regulation of antigen receptor signaling (Fig. 4j). In support of the metabolic phenotypes observed ex vivo, genes associated with OXPHOS were enriched specifically in TMREhi γδ24+ cells while genes involved in glucose metabolism were unregulated in TMRElo γδ24+ cells (Extended Data Fig. 6b).

These data collectively demonstrate that metabolic status of thymic γδ progenitors marks their developmental fate from a very early stage. Progenitors entering the IL-17 pathway display sustained high mitochondrial activity, whereas those in the IFN-γ pathway undergo a TCR-induced metabolic shift towards aerobic glycolysis. We next questioned how these intrinsic metabolic differences impacted the physiology of effector γδ T cell subsets.

**Enrichment of lipid storage and lipid metabolism in γδ17 cells.** Having shown that, in stark contrast to γδTN cells, γδ17 cell generation was reduced under high glucose concentrations (Fig. 3h), and enhanced upon inhibition of glycolysis (Fig. 3i) or glucose uptake (Extended Data Fig. 4), we questioned whether other metabolic resources might be important for γδ17 cell physiology. To address this question, we took advantage of Zbtb1618 reporter mice to segregate γδ8+ and γδ17 cells (Zbtb16 encodes the transcription factor PLZF18,21). We performed RNA sequencing (RNA-seq) of lymphoid and tissue-resident γδ T cells sorted into PLZF+ (γδ17) and PLZF– (γδ8) cells (Extended Data Fig. 7a). As expected, γδ17 cells across tissues expressed Il17a, whereas Il17f was also expressed in tissue-resident γδ17 cells (Fig. 5a). Different metabolic pathways...
were associated with lymphoid versus tissue-resident γδ T cells. However, the genes common to γδT cells across all tissues were related to lipid and mitochondrial metabolism, including glutamate transporter (Slc1a1), glucose/fatty acid metabolism (Pdk4), mitochondrial protein transport (Abim3) and lipid metabolism (Fabp1, Abdh5, Atp10a). These data highlight genes associated with lipid metabolism as a common feature of γδT cells across tissues.

Consistent with this, LN γδ17 cells had a higher neutral lipid content (assessed by LipidTOX staining) than γδ17 cells (Fig. 5b). This differential lipid content was further increased upon activation with IL-1β+IL-23 (Extended Data Fig. 7b), was associated with expression of IL-17A, IL-17F and RORγt (Extended Data Fig. 7c), and was observed across γδ T cells from multiple tissues, with the notable exception of the skin (Fig. 5c), where γδ T cells have been shown
to display specific mechanisms of tissue adaptation. In particular, Vγ6+ γδ T cells in the dermis are transcriptionally distinct from those in pLN s and display a highly activated but less proliferative phenotype. This tissue adaptation may alter the metabolic requirements of skin-resident γδ T cells and γδ T cells may adapt to utilize specific metabolites present within the skin.

Imaging analysis revealed that the increased LipidTOX staining was due to the accumulation of intracellular lipid droplets in γδ T cells (Fig. 5d,e). Lipid droplets store neutral lipids including triglycerides (TAGs) and cholesterol esters. The two γδ T cell subsets had equivalent TAG content (Fig. 5f) but free cholesterol, as determined by filipin III staining, was higher in γδ T cells (Fig. 5g). We next questioned whether γδ T cells engaged in lipid uptake which could account for lipid storage. Using labeled palmitate (Bodipy-FL-C16), we found that γδ T cells selectively took up lipids (Fig. 5h), which was further enhanced following activation (Extended Data Fig. 7d). Analysis of γδ T cell cytokine production confirmed that the ability to take up palmitate was specific to IL-17 producers (Fig. 5i,j). Of note, Vγ4+ and Vγ6+ (Vv1–Vv4+) γδ T cells showed a higher palmitate uptake than Vγ1+ cells (Fig. 5k). While Vγ6+ γδ T cells primarily produce IL-17, Vγ4+ cells can produce either IFN-γ or IL-17 (Fig. 5k). However, palmitate uptake was specific to Vγ4+ cells that produced IL-17 (Fig. 5l). Furthermore, γδ T cells also displayed a higher uptake of fluorescently labeled cholesterol ester (Bodipy CholEsteryl FL-C16) (Fig. 5m), emphasizing their ability to take up multiple types of lipids, including fatty acids and cholesterol.

These data demonstrate that γδ T cells have an exquisite capacity to take up and accumulate intracellular lipids, and display transcriptional signatures of enhanced lipid metabolism compared with γδT cells.

**High-fat diet promotes γδT cell expansion and their accumulation in tumors.** We next tested the effect of a lipid-rich, high-fat diet (HFD), on γδ T cell subsets in vivo. Unlike mice fed a standard-fat diet (SFD), which alternate between using lipids or carbohydrates for fuel during light–dark cycles, respectively, mice fed an HFD had reduced respiratory exchange ratio (RER), illustrating a systemic metabolic switch to constantly burning lipids as the main fuel source. (Fig. 6a). We found that both the percentage and absolute number of LN γδ T cells were increased during HFD (Fig. 6b), which was due to a specific increase in γδT (but not γδT) cells (Fig. 6c,d).

Tumors are another site reported to be lipid-rich. To explore the effect of the lipid-rich tumor environment on γδ T cells, we employed the B16F10 melanoma model. In SFD mice, we found an enrichment of γδ T cells within the tumor compared with draining LN (dLN) or spleen (Fig. 6e). These γδ T cells were found enriched compared with γδT cells (Fig. 6f). Given that γδ T cells were enriched in obese mice and in tumors, we next asked if obesity combined with the tumor model would further increase γδ T cells. Mice fed HFD exhibited enhanced tumor growth (Fig. 6g) and further increased percentages and numbers of tumor-infiltrating γδ T cells compared with the SFD group (Fig. 6h–j). These data demonstrate that a lipid-rich environment selectively accumulates γδ T cells but not γδT cells in the tumor.

Given the preferential uptake of cholesterol by γδ T cells (Fig. 5i), we next investigated its effect on γδ T cell proliferation and function. We incubated purified γδ T cells with cholesterol-loaded cycloextrin (CLC), which we found to promote γδ T cell proliferation when compared with control culture conditions (Fig. 6k). To determine its impact on tumor growth in vivo, we injected CLC-pretreated (or control) γδ T cells twice (within 2 d) into subcutaneous E0771 tumors (as established in Fig. 1b), which allow local T cell delivery. Strikingly, γδ T cells pretreated with CLC substantially enhanced tumor growth (Fig. 6l–n).

Conversely, we also tested the effect of reducing lipids in vivo, by injected orlistat, which inhibits lipases and thus prevents uptake of dietary fat, into B16F10-tumor-bearing mice. Mice injected with orlistat exhibited reduced body weight and tumor growth compared to vehicle-treated mice (Extended Data Fig. 7a,b). Importantly, these mice showed decreased numbers of tumor-infiltrating γδ T cells, which had lower neutral lipid content (Extended Data Fig. 7c,d). Together, these data show that lipid-rich environments promote the selective expansion of γδ T cells that support tumor growth.

**Glucose supplementation enhances antitumor functions of γδT cells.** We next aimed to use the knowledge gathered in this study to boost antitumor γδ T cell responses, which are known to rely on γδT cells.** Given our data showing that glucose promotes the development of γδT cells over γδ T cells in the thymus (Fig. 3a,b), and the higher glycolytic capacity of γδT cells in peripheral organs (Fig. 2g) and also within tumors (Fig. 1b–e), we hypothesized that glucose supplementation would enhance γδT cell functions. Further supporting this hypothesis, we found that intratumoral γδ T cells preferentially took up fluorescently labeled glucose (2-NDBG) when compared with γδT TILs (Fig. 7a).

We first tested the impact of glucose on γδ T and γδ T cell functions in vitro. We cultured purified γδT– (γδT) or γδT– (γδT) cells in standard culture conditions containing a low dose of glucose (5 mM) or in high glucose (50 mM). We found high glucose to be detrimental to γδT cells (Extended Data Fig. 8), in stark contrast to γδ T cells. Indeed, supplementation with high glucose augmented (whereas provision of 2-DG reduced) the percentage and numbers of γδT cells (Fig. 7b), with parallel effects on their proliferation.

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**Fig. 4** | Distinct mitochondrial activities underlie effector fate of thymic γδ T cell progenitors. a,b. Flow cytometry profiles and percentage of thymic γδT and γδT cell output from sorted TMRE+ and TMRE+ γδT cells (a) or γδT cells (b) after 5-d culture on OP9DL1 cells. Data are representative of 3 independent experiments (n = 4 mice pooled per group per experiment). c. Percentage of Vγ1+ and Vγ4+ cells in TMRE+ and TMRE+ γδT progenitors. Vγ1+ TMRE+ versus TMRE+ P < 0.0001 and Vγ4+ TMRE+ versus TMRE+ P < 0.0001. Data are representative of 3 independent experiments (cells sorted from n = 4 mice pooled per group per experiment). d. TMRE MFI of thymic γδT cells (CD44+CD45RB–, CD44+CD45RB– γδ T cells and γδ T cells (CD44+CD45RB–) from 6-d FTOCs of E17 B6 thymic lobes; γδT versus CD44+CD45RB– γδ T cells (P = 0.002), and CD44+CD45RB– γδ T cells versus γδT cells (P = 0.0301). e. TMRE staining in CD24+CD73+, CD24+CD73–, CD24+CD73+ and CD24+CD73– γδ T cells from 7-d FTOCs of E15 B6 thymic lobes. f. TMRE staining in CD25+CD24– (γδT cells), CD25– and CD25+ and Vγ5+ γδT progenitors from E15 thymus. g. Flow cytometry profiles of thymic γδT, γδT and γδT cells from 6-d FTOCs of E17 B6 thymic lobes stimulated or not with anti-TCRα monoclonal antibody (GL3; 1 μg ml−1). The graph shows the percentage of γδT cells (γδ–GL3 versus +GL3; P < 0.0001) and γδT cells (GL3 versus +GL3; P = 0.0002) in each condition. Data are representative of 2 independent experiments (n = 4 thymi pooled per point per group and per experiment). h. FACS-sorted γδT and γδT cells from E17 thymus were cultured (or not) for 5 h with different concentrations (as indicated) of anti-TCRα monoclonal antibody (GL3). TMRE levels were analyzed by flow cytometry in γδT and γδT cells. CTRL vs GL3 (1 μg ml−1, P = 0.0271; GL3 (1 μg ml−1) versus GL3 (5 μg ml−1), P = 0.0021 and GL3 (5 μg ml−1) versus GL3 (10 μg ml−1), P = 0.0475). Data are representative of 2 independent experiments (n = 3 mice pooled per group per experiment). i. Single-cell RNA-seq clustering of TMRE+ and TMRE+ γδT cells from E15+2-d FTOCs using UMAP. j. Gene Ontology term analysis of genes upregulated in TMRE+ versus TMRE+ γδT cells shown in i. Error bars show mean ± s.d., *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 using unpaired two-tailed Student’s t-test.
(Fig. 7c) and on the levels of expression of both IFN-γ (Fig. 7d) and its master transcriptional regulator, T-bet (Fig. 7c).

To specifically address the importance of aerobic glycolysis for γδTN cells, we cultured γδTN cells with galactose (compared with glucose), since cells grown in galactose enter the pentose phosphate pathway instead of using aerobic glycolysis. We observed a reduction in the percentage and absolute numbers of γδTN cells (Fig. 7f), as well as in their IFN-γ (Fig. 7g) and T-bet (Fig. 7h).
expression levels, thus establishing that aerobic glycolysis is required for optimal IFN-γ production by γδTIN cells.

Next we asked if the cytotoxic function of γδTIN cells was also enhanced by glucose supplementation. For this, we cocultured γδTIN cells that were previously supplemented (or not) with a high dose of glucose with E0771 breast cancer cells at different effector:target (E:T) ratios. ‘Glucose-enhanced’ γδTIN cells displayed substantially higher cytotoxic potency against the cancer cells than did the respective controls at each E:T ratio (Fig. 7i). These data reveal a new, metabolism-based means to enhance the antitumor functions of γδ T cells that could be explored for adoptive cell immunotherapy of cancer.

Discussion

Metabolism dysregulation is viewed as an immune evasion strategy in cancer. To overcome it, and thus enable antitumor immune responses, it is critical to understand immune cell metabolism and its interplay with tumor cells in the TME. Although our knowledge on αβ T cell metabolism has increased significantly3,37, little is known about γδ T cells. Here, we identified a metabolic dichotomy between the main effector γδ T cell subsets that play opposing roles in cancer immunity20,30. Whereas immunoregulatory γδTIN cells are almost exclusively glycolytic, protumoral γδTIN cells require mitochondrial metabolism; and their activities within tumors can be promoted by glucose or lipid metabolism, respectively.

Unexpectedly, the metabolic dichotomy of γδ T cell subsets is established early during thymic development, which contrasts with the peripheral metabolic (re)programming of effector αβ T cells. Naïve αβ T cells require activation to undergo rewiring of cellular metabolism, namely the transition from OXPHOS to aerobic glycolysis, through which glucose is fermented into lactate rather than oxidized in mitochondria. Furthermore, depending on metabolic cues in the tissue or during immune challenge, naïve T cells are pushed toward T_{H}1, T_{H}2, T_{H}17 or T_{reg} fates, dependent on intrinsic metabolic pathways engaged outside the thymus. By contrast, we show that an equivalent metabolic shift occurs in early thymic γδ progenitors as they commit to the IFN-γ pathway, seemingly as a result of strong TCRγδ signaling. Indeed, analysis of various hallmarks of TCR signaling suggest that γδ progenitors receiving agonist TCRγδ signals shifted away from OXPHOS as indicated by their reduced ΔΨm. Moreover, upon TCR (GL3 monoclonal antibody) stimulation, a small population of γδ progenitors downregulated CD24 together with ΔΨm (TMRE), thus associating strong TCRγδ signaling in the γδTIN developmental pathway with metabolic reprogramming. This draws a parallel with αβ T cell activation, during which early TCR signaling is required for induction of aerobic glycolysis20. This acts as a switch for Myc mRNA (and protein) expression, such that strength of TCR stimulus determines the frequency of T cells that transcribe Myc mRNA. The common denominator of the metabolic switches in effector γδ and αβ T cells may thus be upregulation of Myc, which is required for transcription of genes encoding glycolytic enzymes15–19. Indeed, our data show a striking enrichment of Myc (mRNA and protein) in γδTIN cells compared with γδTIN cells. On the other hand, the sustained dependence of γδTIN cells on mitochondrial OXPHOS is in line with that recently reported for their functional αβ T cell equivalents, T_{H}17 cells40. Of note, IL-17-producing type 3 innate lymphoid cells (ILC3) were recently shown to require both glycolysis and mitochondrial-derived reactive oxygen species for activation24, but a direct comparison with type 1 ILCs is still missing.

The concept of TCR signaling playing a key role in the metabolic programming of γδ T cell subsets builds upon, but provides a novel perspective to, previous models of their thymic development. Thus, the unequivocal dependence on strong TCR signals for γδTIN cell differentiation15–19 may be linked to a required metabolic shift to aerobic glycolysis. Moreover, the detrimental impact of agonist TCR signals on γδTIN cell development may be due to metabolic conflict with their OXPHOS requirements, documented by our FTOC experiments using specific inhibitors. Importantly, these distinct metabolic phenotypes are maintained in peripheral γδ T cell subsets, which is consistent with and expands our previous epigenetic and transcriptional analyses15–19.

We were particularly interested in investigating the metabolic properties of peripheral γδ T cell subsets once they infiltrated tumor lesions, for which we employed three experimental models of cancer (melanoma, breast and colon). Critically, we found that the dichotomy between γδTIN and γδTIN subsets was preserved in the TME, which enabled metabolic interventions that may have therapeutic potential. In fact, while γδ T cell infiltration is largely perceived to associate with favorable prognosis in cancer patients41, recent clinical data have suggested that, in agreement with mouse experimental systems28, human γδTIN versus γδTIN cell subsets have antagonistic prognostic values4. Thus, improvement in the thera-

Fig. 5 | γδ Takes higher lipid uptake and lipid droplet content than γδTIN cells. a, Quadrant plot of genes upregulated in bulk RNA-seq of tissue-resident PLZF− γδ T cells (lower right), lymphoid PLZF+ γδ T cells (upper left), PLZF− γδ T cells from all tissues (upper right) or PLZF− γδ T cells from all tissues (lower left). Cells were isolated from PLZF−GFP (Zbtb16^{GFP}) mice. FC, fold change. b, Representative histogram of neutral lipid staining (LipidTOX) in γδTIN (CD27−) and γδTIN (CD27+) cells from LNs ex vivo. c, LipidTOX MFI in γδTIN and γδTIM cells from spleen (P = 0.0021), LNs (P < 0.0001), lungs (P = 0.0043), adipose (P = 0.0018), liver (P = 0.031) and skin (P = 0.9442) (n = 5–8, data pooled from 2 independent experiments). d, Confocal imaging of γδTIN and γδTIM cells expanded in vitro and stained with LipidTOX (red) and Hoechst 33342 (blue). Scale bar, 5 μM (data representative of a minimum 10 images from 2 independent experiments). e, Quantification of confocal imaging as shown in d (each data point represents the average per cell per image; LipidTOX P = 0.0018; lipid droplet no. P < 0.0001). f, Quantification of triglyceride (TAG) levels from γδTIN and γδTIM cells expanded in vitro (n = 7, each symbol represents one biological replicate). g, Filipin III staining of γδTIN and γδTIM cells ex vivo from LNs (n = 6, data pooled from 2 independent experiments; P = 0.0276). h, Bodipy-FL-C12 uptake in γδTIN and γδTIM cells from LNs ex vivo (n = 8, data pooled from 2 independent experiment). i, Representative plots of Bodipy-FL-C12 uptake and IL-17 on IFN-γ production by γδTIM and γδTIM cells from LNs stimulated with PMA/ionomycin. J, Bodipy-FL-C12 MFI in IFN-γ and IL-17 γδ T cells (n = 4, data representative of 3 independent experiments). k, Representative plot of Vγ1 and Vγ4 expression in total γδ T cells and percentage Bodipy-FL-C12 uptake by LN γδ T cell subsets (Vγ1, Vγ4, Vγ1γ4) (n = 6, data pooled from 2 independent experiments; Vγ1 versus Vγ4/Vγ6 P < 0.0001; Vγ4 versus Vγ6 P = 0.0143). l, Representative IFN-γ and IL-17 production by Vγ4 γδ T cells from LNs and percentage Bodipy-FL-C12 uptake by Vγ4^{IFN-γ} and Vγ4^{IL-17} γδ T cells (n = 6, data pooled from 2 independent experiments). m, Percentage Bodipy CholEsterolyt FL-C12 uptake by γδTIN (CD27−) and γδTIM (CD27+) cells from LNs ex vivo (n = 6, data pooled from 2 independent experiments). Error bars show mean ± s.d., *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 using unpaired two-tailed Student’s t-test.
The therapeutic performance of γδ T cells in the clinic is likely to require a better understanding of the factors that control the balance between γδ<sup>17</sup> and γδ<sup>IFN</sup> cell subsets in the TME.

Here, we also identified lipids as key γδ<sup>17</sup>-promoting factors, which is particularly relevant because tumors are known to be lipid-rich microenvironments. Palmitate and cholesterol ester...
Fig. 6 | HFD promotes the expansion of pro-tumoral γδT cells in lymph nodes and within tumors. a, RER of mice fed SFD or HFD for 8 weeks (n=3, data from 1 experiment). b, Bar graphs showing the percentage and absolute numbers of CD3+ γδ T cells from LNs of SFD and HFD mice (n=9, data pooled from 3 independent experiments). c, Proportion of γδT (CD27−) and γδTN (CD27+) cells in LNs of SFD and HFD fed mice (n=9, data pooled from 3 independent experiments). d, Percentage and absolute numbers of CD27+IFN-γ and CD27−IL-17+ γδ T cells from LNs of SFD and HFD mice (n=9, data pooled from 3 independent experiments). e, Proportion of infiltrating γδT cells in spleen, draining LN and tumor in the B16 tumor model (dLN and tumor n=30, data pooled from 4 independent experiments; spleen n=7; naive LN n=5). f, Bar graph showing the percentage of γδT and γδTN cells infiltrating tumors (n=9, data pooled from 2 experiments). g, Bar graph showing the size of tumors (mm³) in SFD and HFD fed mice. (n=7, representative of 3 independent experiments). h, Bar graph showing proportion of infiltrating γδT (CD27−) and γδTN (CD27+) cells in tumors of SFD- and HFD-fed mice (SFD n=10, HFD n=12, data pooled from 2 independent experiments). i, Representative plots of IL-17 and IFN-γ expression in γδ T cells infiltrating tumors of SFD- and HFD-fed mice. Bar graphs represent the percentage of γδT and γδTN cells infiltrating tumors (SFD n=17, HFD n=20, data pooled from 3 independent experiments). j, Bar graph showing the number per mm³ of γδT and γδTN cells in tumors of mice on SFD or HFD (SFD n=7, HFD n=8, data pooled from 2 independent experiments). k, Plots of proliferating Ki67+ γδ T cells cultured for 5 h with or without cholesterol-loaded cyclodextrin (CLC). Graph represents the percentage of Ki67+ γδ T cells (data are representative of two independent experiments; pool of 3–5 mice per experiment). l, yδT cells cultured (or not) with CLC for 5 h were injected subcutaneously into E0771 tumors at day 7 and day 9 after tumor cell injection. Representative picture of tumors observed at day 11 post-E0771 cell inoculation. m, Graph showing tumor weight at day 11 following E0771 inoculation. CTRL versus γδ − CLC (P=0.0361); γδ − CLC versus γδ + CLC (P=0.0003). n, E0771 tumor growth was monitored every 2 d after inoculation. Data in l–n are representative of 3 independent experiments (n=3 mice per experiment); P<0.0001. Error bars show mean ± s.d., *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 using unpaired two-tailed Student’s t-test or one-way ANOVA test with Šidák’s post-hoc analysis.
Fig. 7 | Glucose supplementation enhances the antitumor effector functions of γδT cells. 

a. Glucose uptake assessed upon intravenous injection of fluorescent 2-NBDG in tumor-bearing mice. Tumors were collected 15 min later for analysis. Histogram represents 2-NBDG uptake in γδT and γδFt cells (P = 0.047). Data are representative of 2 independent experiments (n = 4 mice per group and per experiment). b–d. Purified splenic and peripheral lymph node γδT cells (CD3−TCRγδ−CD27+) were cultured in the presence of IL-7 with medium containing low glucose (5 mM), 2-DG, high glucose (50 mM) or galactose (20 mM) for 78 h. b. Plots of peripheral γδT cells cultured with IL-7 and medium containing low glucose, 2-DG or high glucose. Histogram represents the fold change in number of γδT cells cultured with 2-DG or high glucose versus low glucose (P < 0.0001). c. Fold change in number of proliferating Ki67+ γδT cells cultured with 2-DG or high glucose versus low glucose (P < 0.0001). d. IFN-γ expression was analyzed by flow cytometry in γδT cells incubated with medium containing low glucose, 2-DG or high glucose. Histograms show the MFI of IFN-γ. Low glucose vs. 2-DG (P < 0.0001); 2-DG versus high glucose (P < 0.0001); low glucose versus high glucose (P = 0.015). e. T-bet expression was analyzed by flow cytometry in γδT cells incubated with medium containing low glucose, 2-DG or high glucose. Histograms show the MFI of T-bet. Low glucose versus 2-DG (P < 0.0001); 2-DG versus high glucose (P < 0.0001). f. Flow cytometry profiles of peripheral γδT cells cultured with IL-7 and medium containing glucose (50 mM) or galactose (20 mM). Histogram represents the numbers of γδT cells (P < 0.0001). g, h. IFN-γ (g) and T-bet (h) expression was analyzed by flow cytometry in γδT cells incubated with medium containing glucose or galactose (P = 0.0085 for IFN-γ expression and P = 0.0034 for T-bet expression). Histograms show the MFI of IFN-γ and T-bet. i. Summary of in vitro killing assay of E0771 tumor cells by γδT cells previously supplemented (or not) with glucose (5 h preincubation); P < 0.0001. Data are representative of 2 independent experiments (n = 3 mice per group and per experiment). j. Representative picture of tumors observed at day 11 following E0771 inoculation. γδT cells supplemented (or not) with glucose for 5 h were injected into the tumor at day 7 and day 9 after tumor cell injection. k. The E0771 tumor growth was monitored every 2 d for 11 d after E0771 inoculation. CTRL versus γδT− glucose (P = 0.0148); γδT− glucose versus γδT+ glucose (P < 0.0001).

b–e. Data are representative of 4 independent experiments (n = 3 mice per group and per experiment); f–h, data are representative of 2 independent experiments (n = 4 mice per group and per experiment); i, j. data are representative of 2 independent experiments (n = 5 mice per group and per experiment). Error bars show mean ± s.e.m. * P < 0.05, ** P < 0.01, *** P < 0.0001 using unpaired two-tailed Student’s t-test or ANOVA test.
uptake were higher in γδT than in γδFN cells. Thus, we propose that the increase in intracellular lipids is due to enhanced uptake, although endogenous lipid synthesis cannot be ruled out. Our findings that γδFN cell proliferation is boosted by cholesterol treatment, and that these cells expand substantially in obese mice, provide additional evidence that HFD causes a systemic increase in the γδT subset, consistent with previous findings in the skin and lungs, and may provide a mechanistic understanding for this expansion. Obesity is a known risk factor for cancer, and we have previously demonstrated the link between obesity and suppression of natural killer (NK) cell antitumor function. Given that γδT cells have strong protumoral effects and we find this population to be expanded in tumors of obese mice, this may represent an additional mechanism linking cancer and obesity, whereby abundant lipids favor γδT over γδFN cells to support tumor growth.

Conversely, we found γδFN cells, from their thymic development to intratumoral functions, to be boosted by glucose metabolism. Naturally, the large consumption of glucose by tumor cells creates a major metabolic constraint on γδFN TILs. Glucose restriction can impair T cell cytokine production, while production of lactate by tumor cells performing aerobic glycolysis can inhibit T cell proliferation and cytokotoxic functions. Therefore, we do not conceive glucose supplementation as an appropriate strategy to enhance endogenous T cell (including γδT) responses in vivo. Instead, we suggest that it should be considered in protocols used to expand/differentiate γδ T cells ex vivo for adoptive cell therapy. Such an ‘in vitro glucose boost’ may enable stronger antitumor activities (namely, IFN-γ production and cytotoxicity) upon T cell transfer, as suggested by our data using CD27+ γδFN cells in the breast cancer model, although evaluation of the duration and long-term impact of this ‘boost’ requires further investigation in slower growing tumor models.

While we did not dissect the mechanistic link between aerobic glycolysis and IFN-γ production by CD27+ γδFN cells, previous studies on αβ T cells have shown that glycolysis controls (via the enzyme GAPDH) the translation of IFN-γ mRNA. Moreover, glycolysis was shown to be essential for the cytotoxic activity of NK cells, namely their degranulation and Fas ligand expression, upon engagement of NK cell receptors (NKRs). This is particularly interesting when considering the potential of a human γδ T cell product that we developed for adoptive cell therapy of cancer, Delta One T (DOT) cells. These human V81+ T cells are induced in vitro to express high levels of NKRs that enhance their cytotoxicity and IFN-γ production. We therefore propose high-dose glucose to be added to the DOT protocol as to further increase their antitumor potential.

In sum, this study demonstrates that thymic differentiation of effector γδ T cell subsets, besides well-established epigenetic and transcriptional regulation, includes divergent metabolic programming that is sustained in the periphery and, in particular, in the TME. It further identifies distinct metabolic resources that control the intratumoral activities of γδ T cell subsets, with lipids favoring γδT cells and glucose enhancing γδFN cells, which provides a new metabolism-based angle for therapeutic intervention in cancer and possibly other diseases.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of data and code availability are available at https://doi.org/10.1038/s41590-020-00848-3.

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Tumor transplantation in vivo. Mice were injected with 1 x 10^6 E0771 tumor cells s.c. in the flank, 1 x 10^5 E0771 tumor cells or 2 x 10^5 B16 tumor cells subcutaneously into the right shaved flank. Tumor growth was measured every 2–3 d using calipers, and animals were euthanized when tumors reached a diameter (D) of 15 mm; when tumors became ulcerous; or 1 or 2 weeks after tumor injection. Tumor size was calculated using the following formula: D^2 x D/2, D being the smaller value of the tumor diameter. In some experiments, mice were fed a HFD (60% calories from fat) for 10 weeks prior to tumor injection, and the HFD was continued throughout the experiment.

Comprehensive lab-animal monitoring system. Indirect calorimetry data were recorded using a Metabolic Cage System (Sable Systems) essentially as described previously. Mice were housed individually in metabolic chambers under a 12-h light–dark cycle at room temperature (22 °C) with free access to food and water. Mice were acclimated for 24 h in metabolic cages before recording calorimetric variables. Mice were fed either a standard diet or a high fat (58% energy from fat) diet for 12 weeks before the start of the experiments. Procedures for measuring metabolic activity were performed in accordance with the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Instituto de Medicina Molecular Orbea institutional ethical committee. Euthanasia was performed by CO2 inhalation. Anesthesia was performed by isoflurane inhalation.

In vitro killing assays. Purified CD27^γδ^ T cells based on death of E0771 cells (Annexin V staining) were sorted by FACS. CD27^γδ^ T cells were cocultured with 5 x 10^5 E0771 breast cancer cells in complete RPMI Medium (minus d-glucose). The killing capacities of CD27^γδ^ T cells based on death of E0771 cells (Annexin V staining) were assessed by flow cytometry after 24 h.

Flow cytometry. γδ T cells were analyzed by flow cytometry using standard procedures. For surface staining, cells were Fc-blocked with anti-Cd16/32 (clone 93; eBioscience) and incubated for 15 min at 4 °C with antibodies and LIVE/DEAD Fixable Near-IR (Thermo Fisher Scientific) or viability dye Zombie NIR Fixable Stain (Biolegend). For lipid depletion, cells were incubated in BFB (50 µM) for 24 h. For intracellular cytokine staining, cells were stimulated with 50 µg ml⁻¹ phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 2 µM brefeldin-A (Sigma-Aldrich) for 5 h at 37 °C, 5% CO2. Then, cells were fixed and permeabilized with Fixable Viability Stain (eBioscience) and stained with anti-IL-17A (TC11-18H10.1; 1:100), anti-IFN-γ (XMG1.2; 1:100), Ki67 (SolA01; 1:800), T-bet (4B10; 1:150) and RORγt (B2D; 1:100). Anti-IL-17F (9D3.1C8; 1:100), anti-Vγ1(2.11; 1:100), anti-Vγ4 (UC3-10A6; 1:200), anti-Vγ5 (536; 1:200) and anti-CDF45R (C363-16A; 1:400) were purchased from BioLegend and anti-IDC4 (IM4; 1:400) from BD Pharmingen. Cells were washed with FACS buffer. For intracellular cytokine staining, cells were stimulated with 50 µg ml⁻¹ phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 1 µg ml⁻¹ ionomycin (Sigma-Aldrich) for 3–4 h at 37 °C, 5% CO2, in the presence of 10 µg ml⁻¹ brefeldin-A (Sigma-Aldrich) and 2 µM monensin (eBioscience). Cells were fixed and permeabilized with Fixx staining kit (eBioscience)/Thermo Fisher Scientific, according to the manufacturer’s instructions. Cells were incubated for 30 min at 4 °C, with the following antibodies from eBioscience: anti-IFN-γ (XMG1.2; 1:100), Ki67 (SolA01; 1:800), T-bet (4B10; 1:150) and RORγt (B2D; 1:100). Anti-IL-17F (9D3.1C8; 1:100) and IL-17A (TC11-18H10.1; 1:100) were purchased from BioLegend. For Annexin V staining, the Annexin V Kit (eBioscience) was used following the manufacturer’s instructions.

The following dyes were purchased from Invitrogen and stained according to manufacturer’s instructions: Mitotracker Green FM, tetramethylrhodamine methyl ester perchlorate (TMRM), HCS LipidTOX Red Neutral Lipid Stain, Palmitate uptake was measured with 1 µM Bodipy FL-C16 (Invitrogen) incubated for 10 min at 37 °C. Cholesterol ester uptake was measured with 2 µM Bodipy Cholesterol, 50 µg ml⁻¹ of oil red O (Sigma-Aldrich) and 2 µM filipin III (Sigma-Aldrich) incubated for 1 h at 37 °C. Cholesterol content was measured using 50 µg ml⁻¹ Filipin III (Sigma-Aldrich) incubated for 1 h at room temperature.

Flow cytometry analysis was performed with a FACS Fortessa, LSRII or Canto II using FACS Diva Software (BD Biosciences) and data were analyzed using Flowjo software (BD Biosciences).
Seahorse metabolic flux analysis. Real-time analysis of OCRs and ECARs of IFN-γ- and IL-17-committed γδ T cells sorted from 5- to 6-d-old B6 pups and CD27+/−γδ T cells from spleen/lymph nodes expanded in vitro were assessed using the XFP Extracellular Flux or Seahorse XFe-96 analyzers, respectively (Seahorse Bioscience). Cells were added to a Seahorse XF96 Cell Culture Microplate (Agilent) and coated with Cell-Tak (Corning) to ensure adherence, and sequential measurements of OCR and ECAR were performed in XF RPMI Seahorse medium supplemented with glucose (10 mM), glutamine (2 mM) and sodium pyruvate (1 mM) following the addition of oligomycin A (2 μM), FCCP (2 μM), rotenone (1 μM) plus antimycin A (1–4 μM). Basal glycolysis, glycolytic capacity, basal mitochondrial respiration and maximal mitochondrial respiration were calculated. OCR and ECAR values were normalized to cell number.

SCENITH. Cells were plated at 20 x 10⁶ cells ml⁻¹ in 96-well plates. After activation of γδ T cells, cells were treated for 30 min at 37 °C. 5% CO₂ with control, 2-DG (100 mM; Sigma-Aldrich), oligomycin (1 μM; Sigma-Aldrich) or a combination of both drugs. Puromycin (10 μg ml⁻¹; Sigma-Aldrich) is added for 15 min at 37 °C. The SCENITH kit (http://www.scenith.com) containing different surface markers (as described above) for 15 min at 4 °C in FACS buffer washed in cold PBS and stained with primary conjugated antibodies against different surface markers (as described above) for 15 min at 4 °C in FACS buffer (PBS 1× 5% FCS, 2 mM EDTA). After washing with FACS buffers, cells were fixed and permeabilized using Cytofix/Cytoperm (BD) following the manufacturer’s instructions. Intracellular staining of puromycin using the anti-puro monoclonal antibody (clone R4/743L-EB) was performed by incubating cells during 30 min at 4 °C diluted in PermWash. Experimental duplicates were performed in all conditions.

In vivo glucose uptake. 2-NBDG (300 μg diluted in PBS 1×; Cayman Chemical) was injected intravenously in C57BL/6j mice; 15 min later, cells from tumors were collected.

Assessment of mitochondrial morphology. Mitochondrial membrane potential was measured using tetramethylrhodamin, ethyl ester (TMRE; 100 nM; Abcam) according to manufacturer protocols. Following TMRE staining, carbonyl cyanide 4-[(trifluoromethoxy)phenyl]hydrazone (FCCP; 25 μM; Abcam) was used as a positive control for mitochondrial membrane depolarization. Total mitochondrial mass was assessed using MitoTracker Green (Invitrogen) according to manufacturer’s instructions. All cells were subsequently analyzed by flow cytometry.

Triglyceride quantification. Triglycerides (TAGs) were quantified from expanded γδ T cells in vitro using Picroprobe Triglyceride Quantification Assay Kit, Fluorometric (Abcam) and absorbance measured using FLUOstar OPTIMA (BMG Labtech).

RNA isolation and real-time PCR. mRNA was prepared from FACs-sorted CD27+/− and CD27−γδ T cells from WT splenocytes and draining lymph nodes using High Pure RNA Isolation kit (Roche). Reverse transcription was performed with random oligonucleotides (Invitrogen). Results were normalized to actin mRNA. qPCR was performed with SYBR Premix Ex Taq master mix (Takara) on an ABI OPTIMA (BMG Labtech).

Assessment of mitochondrial morphology. Mitochondrial membrane potential was measured using tetramethylrhodamin, ethyl ester (TMRE; 100 nM; Abcam) according to manufacturer protocols. Following TMRE staining, carbonyl cyanide 4-[(trifluoromethoxy)phenyl]hydrazone (FCCP; 25 μM; Abcam) was used as a positive control for mitochondrial membrane depolarization. Total mitochondrial mass was assessed using MitoTracker Green (Invitrogen) according to manufacturer’s instructions. All cells were subsequently analyzed by flow cytometry.

RNA-seq and data processing. Single-cell sequencing libraries were generated using the Chromium Single Cell 5’ Library and Gel Bead Kit (10X Genomics) according to the manufacturer’s instructions. Data was analyzed using the R package Seurat v2.3 (refs. 54,55). UMI counts were normalized using regularized negative binomial regression with the scrantransform package56. For downstream analysis of normalized data, principal component analysis (PCA) was performed using n=50 dimensions, and PCA variability was determined using an Elbow plot. Differential gene expression analysis and GSEA was performed using the MAST and fgsea packages56. Pathways and gene lists for gene set enrichment analysis were obtained using the misegbld package from Molecular Signatures database (MSigDB)54,56. Adaptively thresholded Low Rank Approximation (ALRA) from the Seurat wrappers package was performed to correct for drop-out values for visualization of leading-edge and differentially expressed genes identified by MAST54. All downstream analysis was performed using R v3.6.3 and RStudio Desktop 1.2.5001 on an Ubuntu 19.10 linux (64-bit) system using the following R packages and libraries: dplyr v0.8.5, igsea v1.12.0, ggplot2 v3.3.0, MAST v1.12.0, scrantransform v0.2.1, Seurat v3.3.4, SeuratWrappers v0.1.0, uwot v0.1.8 and viridis v0.5.1.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software using non-parametric two-sided Mann–Whitney test or, if both groups followed a normal distribution (tested by D’Agostino and Pearson normality test), using two-tailed unpaired Student’s t-test or one-way ANOVA. All data are presented as means±standard error of mean (s.e.m. or standard deviation s.d. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

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

Data availability
The GEO public accession codes are GSE158585 for single-cell RNA sequencing; and GSE156782 for bulk RNA sequencing. The data that support the findings of this study are available from the corresponding author upon request.

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Author contributions
N.L., C.M., S.M. and M.R. performed most of the experiments and analyzed the data. G.J.F. designed and performed some experiments. N.S., A.C.K., L.D., H.K., A.D., S.C.,
H.P., R.L. and C.C. provided technical assistance in some experiments. M.K. and L.Z.A. performed bioinformatic analysis, and M.B. provided reagents, materials and support. P.P. and R.J.A. provided key assistance with the SCENITH™ methodology. B.S.-S., D.J.P. and L.L. conceived and supervised the study. N.L., C.M., B.S.-S., D.J.P. and L.L. wrote the manuscript.

**Competing interests**

B.S.-S. is an inventor of the patented ‘Delta One T cell’ technology, which has been acquired by GammaDelta Therapeutics (London, UK).

**Additional information**

Extended data is available for this paper at [https://doi.org/10.1038/s41590-020-00848-3](https://doi.org/10.1038/s41590-020-00848-3).

Supplementary information is available for this paper at [https://doi.org/10.1038/s41590-020-00848-3](https://doi.org/10.1038/s41590-020-00848-3).

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**Extended Data Fig. 1 | SCENITH™ methodology for analysis of cell metabolism.** **a.** Experimental design: E0771 breast or MC38 colon cancer cell lines were injected in WT mice; 6 and 15 days later, tumors were extracted for metabolic analysis of γδ T cells using SCENITH™. **b.** SCENITH™ assesses the impact of metabolic inhibitors on protein synthesis. Mean fluorescence intensity (MFI) of puromycin is analysed in each condition (Co: control-no inhibition; DG: 2-deoxyglucose inhibiting glycolysis; O: oligomycin inhibiting OXPHOS; and DGO: DG + O inhibitors). Glucose dependence, fatty acid and amino acid oxidation capacity, mitochondrial dependence and glycolytic capacity are calculated as detailed in the Methods and reference #23. Error bars show mean±SEM. Data are representative of 3 independent experiments (n=3 mice in triplicates per group and per experiment).
Extended Data Fig. 2 | In vitro expanded γδ^17 and γδ^IFN γδ T cells retain their mitochondrial and lipid phenotypes. 

**a**, Representative flow plots of CD3 and TCRγδ expression on γδ^17 and γδ^IFN T cells expanded in vitro from total spleen/LN cells. **b**, CD27 expression on in vitro expanded γδ^17 and γδ^IFN T cells. 

**c**, IL-17 and IFNγ production by in vitro expanded γδ^17 and γδ^IFN T cells respectively, following activation with PMA/ionomycin. 

**d**, Vγ4 and Vγ1 expression on in vitro expanded γδ^17 and γδ^IFN T cells. 

**e**, Representative staining of in vitro expanded γδ^17 and γδ^IFN T cells for mitotracker, TMRM, lipidTOX and Bodipy-FL-C16. 

**f**, MFI of mitotracker, TMRM, lipidTOX and Bodipy-FL-C16 staining in vitro expanded γδ^17 and γδ^IFN T cells. n=3, data representative of 3 independent experiments. Mitotracker p=0.0026; TMRM p=0.0003; LipidTOX p<0.0001; Bodipy FL-C16 p=0.036. Error bars show mean+SD, **p < 0.01, ***p < 0.001, ****p < 0.0001, using two-tailed unpaired Student’s t-test.
Extended Data Fig. 3 | γδTN cells can generate γδ17 and γδIFN T cells. Flow cytometry profiles of thymic γδ T cells from E15 thymic lobes that had been cultured for 7 days in fetal thymic organ culture (E15 + 7dFTOC). CD24+ (γδ24+) precursors downregulate CD24 to become a CD24-CD44-CD45RB- (γδTN) population. γδTN cells are able to become either IL-17-secreting CD44+CD45RB-γδ17 cells, or IFN-γ-producing CD44+CD45RB+γδIFN cells.
Extended Data Fig. 4 | Thymic γδT cells are increased upon inhibition of glucose uptake. Flow cytometry profiles of thymic γδTN (CD44-CD45RB-), γδIFN (CD44+CD45RB+) and γδ24- (CD44-CD45RB-) cells in γδ24- cells from E15 thymic lobes in 7-day FTOC with media containing or not Fasentin. Histograms show the number of γδTN T cells (p<0.0001) and γδIFN/γδTN ratio (p=0.0028). Data are representative of 2 independent experiments (at least 4 lobes pooled per group per experiment). Error bars show mean±SEM, **p<0.01, ****p<0.0001, using two-tailed unpaired Student's t-test.
Extended Data Fig. 5 | Mitochondrial activity identifies Vγ4+ progenitors with distinct effector fates at very early stages. **a**, Flow cytometry plots pre-sort, and after sorted TMRElo and TMREhi Vγ4+γδ24+ cells were cultured for 5-days on OP9DL1 cells. Percentage of thymic γδ17 and γδIFN cells generated are displayed in the graph on right. Data are representative of 3 independent experiments (cells sorted from n = 4 independent mice pooled per group per experiment). **b**, Flow cytometry plots for pre- and post-sort TMREhi and TMRElo Vγ4+γδTN cells that were cultured on OP9-DL1 cells for a further 5-days (plots on right). Histogram shows the percentage of each γδ T cell subset generated from cultured TMREhi and TMRElo Vγ4+γδTN cells. Error bars show mean ± SD. Data are representative of 2 independent experiments (at least 4 lobes pooled per group per experiment). Error bars show mean ± SD, *p < 0.05, **p < 0.01, using two-tailed unpaired Student’s t-test.
Extended Data Fig. 6 | Distinct mitochondrial activities underlie effector fate of thymic γδ T cell progenitors. 

(a) Experimental design for single-cell RNAseq (10x Genomics) on TMRE<sup>+</sup> and TMRE<sup>−</sup> gd<sup>24+</sup> cells from E15 + 2d FTOC. (b) Heatmap of differentially upregulated genes from comparison of TMRE<sup>+</sup> and TMRE<sup>−</sup> gd<sup>24+</sup> cells. Genes are grouped in relation to their function in either OxPhos or glucose metabolism.
Extended Data Fig. 7 | Enriched lipid metabolism and higher lipid uptake in γδT cells. a, Experimental setup for bulk RNA-sequencing of PLZF+ (gd17) and PLZF− (gdIFN) cells isolated from PLZF-GFP (Zbtb16GFP) mice. b, LipidTOX MFI in γδT (CD27-) and γδIFN (CD27+) T cells from LN cells activated in vitro with IL-1β+IL-23 and IL-12+IL-18 respectively. n=9, data pooled from 3 independent experiments. c, Representative plots of LipidTOX staining and IL-17A, IL-17F or RORγt expression in γδT T cells from LNs activated in vitro with IL-1β+IL-23 for 6h. Data representative of 3 independent experiments. d, Bodipy-FL-C16 MFI in γδT (CD27-CD27+) and γδIFN (CD27+) T cells T cells unstimulated or stimulated in vitro with IL-12+IL-18 or IL-1β+IL-23. (n=3, data from 1 experiment; γδT p=0.0044; γδIFN p=0.8035). Error bars show mean±SD, **p<0.01, ***p<0.001, ****p<0.0001 using one-way ANOVA.
Extended Data Fig. 8 | Inhibition of dietary fat uptake reduces tumour growth and γδ17 cells in the tumour. B16F10-tumour bearing mice were given daily injections of either vehicle or orlistat on days 6-9, and tumours were analysed on day 10. a, Percentage body weight following tumor cell injection; arrows indicate when orlistat or vehicle were administered. b, Tumor volume on days 8-10 following B16F10 inoculation. Absolute numbers c, and LipidTOX staining d, of tumor-infiltrating γδ17 cells on day 10. n=8 biologically independent animals, data from 1 independent experiment. Data represents mean±SD, *p<0.06, **p<0.01 using unpaired Student’s t-test or one-way ANOVA.
Extended Data Fig. 9 | Glucose supplementation diminishes γδ T cell numbers and proliferation. a, Flow cytometry profiles of peripheral γδ T cells cultured with media containing low (5mM) or high (50mM) doses of glucose. Graph depicts total numbers of γδ T cells (p=0.0028). b, Number of proliferating Ki-67+ γδ T cells cultured with low or high glucose (p=0.0034). n=6 biologically independent animals, data from 2 independent experiments. Error bars show mean±SEM, **p < 0.01, using unpaired two-tailed Student’s t-test.
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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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

Software and code

Policy information about availability of computer code

Data collection

- Flow cytometry data was acquired using BD FACSDiva software (BD Bioscience).
- Seahorse metabolic flux data was collected using XF-96 / Seahorse XFp analyser softwares (Seahorse Bioscience).
- Imaging data was collected using INSPIRE acquisition software (Amnis) and Zen 2.3 software (Zeiss).
- CLAMS data was collected using metascreen (Sable Systems)
- For triglyceride quantification data was collected using FLUOstar OPTIMA [BMG Labtech]

Data analysis

- All analysis were described in the relevant section of Methods.
- Flow cytometry data was analysed using Flowio v10 software.
- Seahorse metabolic flux data was analysed using Seahorse XFp analyser software (Seahorse Bioscience).
- Imaging data was analysed using IDEAS software (Amnis) and ImageJ version 2.0.0.
- Single-cell RNAseq data was analysed using the R package Seurat v2.3.
- Cufflinks and cuffdiff was used for bulk RNA sequencing analysis.
- CLAMS data was analysed using SpeData (Sable Systems) and CallR Version 1.2 (doi: http://www.sciencedirect.com/science/article/pii/S155041318304017)
- Statistical analysis were done with GraphPad Prism 8.4.2

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Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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The accession code for single cell RNA sequencing data is GSE150585 and for bulk RNA sequencing data is GSE156782

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Life sciences study design

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

Sample size

No sample size calculation were performed. Pilot studies were used for estimation of the sample size required. In some instances, three replicates had to be performed due to the larger signal-to-noise ratio, while conclusions could be made from other experiments where variability was less.

Data exclusions

Obvious outliers were excluded. We also excluded data from failed experiments. Data exclusions concern SCENITH experiments and in vitro experiments.

Replication

All experiments were reproduced to reliably support conclusions stated in the manuscript. Each experiment was repeated in multiple animal and at least twice unless otherwise stated.

Randomization

Samples were randomly allocated into a control or experimental group. In the 816 tumor model, mice were randomized into groups after tumor cell injection.

Blinding

No blinding was used. However, the outcomes were measured as objective as possible. We also performed duplicates, triplicates and we repeated experiments many times to limited biases introduced by the lack of blinding.

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

Materials & experimental systems

| n/a | Involved in the study |
|-----|------------------------|
| ☑   | Antibodies             |
| ☑   | Eukaryotic cell lines  |
| ☒   | Palaeontology          |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |
| ☑   | Clinical data          |

Methods

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

Antibodies

From Abcam - Anti-CD3e (145-2C11) #78-0031-82, CD24 (M1/69) #48-0242-82, CD25 (PC61) #25-0251-82, CD27 (LG.7F9) #11-0271-82, CD73 (T1/11.8) #25-0731-82, Vy2 [UC3-10A6] #25-5828-82, IFNγ (XMG1.2) #12-7311-41, Ki-67 (SolA15) #56-5698-82, RO961 [B2D] #25-6981-82, TCRβ (GL3) #17-5711-82 and Tbet (B4.10) #17-5825-82

From BD Bioscience - Anti-CD44 (IM7) #560569, anti-CD16/32 (2.4G2) #553140

From Biolegend - Anti-TCRβ (GL3) #118119, CD45RA (C363.16A) #103319, CD45 (5D11) #103149, Vy1 (2.11) #114107, Vy4 (UC3-10A6) #137711, Vy5 (S56) #137505 and CD24 (M1/69) #101815, IL-17F (FD13.1C8) #517004, IL-17A (TC11-18+10.1) #506929, CD16/32 (Tristar) #101320

Antibodies used

From Abcam - Anti-CD3e (145-2C11) #78-0031-82, CD24 (M1/69) #48-0242-82, CD25 (PC61) #25-0251-82, CD27 (LG.7F9) #11-0271-82, CD73 (T1/11.8) #25-0731-82, Vy2 [UC3-10A6] #25-5828-82, IFNγ (XMG1.2) #12-7311-41, Ki-67 (SolA15) #56-5698-82, RO961 [B2D] #25-6981-82, TCRβ (GL3) #17-5711-82 and Tbet (B4.10) #17-5825-82

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Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) The E0771 murine breast adenocarcinoma cancer cells, MC38 murine colon adenocarcinoma cells and B16.F10 melanoma cells were purchased from ATCC (Manassas, VA).

Authentication None of the cell lines used were authenticated.

Mycoplasma contamination All cell lines are tested negative for mycoplasma contamination.

Commonly misidentified lines (See ICLAC register) Name any commonly misidentified cell lines used in the study and provide a rationale for their use.

Animals and other organisms

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Laboratory animals CS78/6J WT mice and Myc-GFP mice [B6;129-Mycm1Skeletal] were purchased from Charles River and Jackson Laboratories, PLZF-GFP (Zbtb16GFP) mice were generated in the laboratory of Dr. Sant’Angelo with a modified bacterial artificial chromosome transgene expressing eGFP under the control of PLZF regulatory elements. Males and females were used at the foetal (embryonic day 14-18), neonatal (1-5 days old) or adult (6-12 weeks) stages. Mice were housed in 12-hour/12-hour light/dark cycle at an ambient temperature of 22±1°C with humidity 52-55%.

Wild animals No wild animals were used in the study.

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

Ethics oversight All mouse experiments performed in this study were evaluated and approved by the institutional ethical committee (Instituto de Medicina Molecular Orbea), the national competent authority (DGAV) under the license number 019069, UK Home Office regulations and institutional guidelines under license number 70/8758 and by the Institutional Animal Care and Use Committee of Brigham and Women’s Hospital and Harvard Medical School, the Trinity College Dublin ethics committee.

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

Flow Cytometry

Plots

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

Methodology

Sample preparation

Tumours were collected and digested with 1mg/ml collagenase Type I, 0.4mg/ml collagenase Type IV (Worthington) and 10 μg/ml DNase I (Sigma-Aldrich) for 30 minutes at 37°C. Cell suspension was then filtered through a 100μm nylon cell strainer (Falcon/Corning). y6 T cells were isolated by scratching thymus, spleen and lymph node on a 70μm mesh. Lungs were minced then homogenized in RPMI 1640 using a TissueLyser (Qiagen) and filtered through 70μm mesh wire mesh. Adipose tissue was processed as described previously (3). Red blood cells were lysed using RBC Lysis Buffer (Biolegend) or ammonium chloride lysis buffer (made in-house). Single-cell suspensions of foetal and neonatal thymocytes were obtained by gently homogenizing thymic lobes followed by straining through 40μm strainers (BD).

Cells were incubated for 15 min at 4°C with Fc-block (anti-CD16/CD32, 2.4G2, BD biosciences) before surface staining. For intracellular staining, cells were fixed, permeabilized and stained with the Foxp3 staining kit or with BD Cytofix/CytoPerm and Perm/Wash buffers.

Instrument

Sorbs were performed using a FACS AriaIII cell sorter (BD) and analysis were done with Fortessa, LSRII or Cantoll (BD Biosciences).

Software

Flow cytometry data was collected using BD FACSDiva software (BD Bioscience) and analysed using FlowJo software.

Cell population abundance

Post-sort sample purity was examined by flow cytometry. Samples containing at least 95% of desired population were used for further analysis.

Gating strategy

Using the FSC/SSC, debris were removed. We excluded doublet by using FSC-A/FSC-H and SSC-A/SSC-H. We used viability dye.
Positive threshold was applied to all samples within cell populations. γδ T cells are defined as CD3+TCRδ+. γδ IFN were gated on CD27+, IFNγ+ or CD44+CD45RB+. γδIL-17+ were gated on CD27−, IL-17+ or CD44+CD45RB−.

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