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PGC-1 β maintains mitochondrial metabolism and restrains inflammatory gene expression

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Metabolic programming of the innate immune cells known as dendritic cells (DCs) changes in response to different stimuli, influencing their function. While the mechanisms behind increased glycolytic metabolism in response to inflammatory stimuli are well-studied, less is known about the programming of mitochondrial metabolism in DCs. We used lipopolysaccharide (LPS) and interferon- β (IFN- β), which differentially stimulate the use of glycolysis and oxidative phosphorylation (OXPHOS), respectively, to identify factors important for mitochondrial metabolism. We found that the expression of peroxisome proliferator-activated receptor gamma co-activator 1 β (PGC-1 β), a transcriptional co-activator and known regulator of mitochondrial metabolism, decreases when DCs are activated with LPS, when OXPHOS is diminished, but not with IFN- β , when OXPHOS is maintained. We examined the role of PGC-1 β in bioenergetic metabolism of DCs and found that PGC-1 β deficiency indeed impairs their mitochondrial respiration. PGC-1 β -deficient DCs are more glycolytic compared to controls, likely to compensate for reduced OXPHOS. PGC-1 β deficiency also causes decreased capacity for ATP production at steady state and in response to IFN- β treatment. Loss of PGC-1 β in DCs leads to increased expression of genes in inflammatory pathways, and reduced expression of genes encoding proteins important for mitochondrial metabolism and function. Collectively, these results demonstrate that PGC-1 β is a key regulator of mitochondrial metabolism and negative regulator of inflammatory gene expression in DCs.

Numerous studies in recent years have established cellular metabolism as an essential regulator of immune cell function and activation^{1–3}. Two of the main ATP-generating pathways are glycolysis, which occurs in the cytosol, and oxidative phosphorylation (OXPHOS), which takes place in the mitochondria. During mitochondrial metabolism, the tricarboxylic (TCA) cycle produces reducing agents that fuel the electron transport chain (ETC) with oxygen as the final electron acceptor, thus consuming oxygen in the process of OXPHOS.

The peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) group of transcriptional co-activators are major regulators of mitochondrial metabolism. This group comprises PGC-1 α and PGC-1 β , which are close homologs, and a less closely related member PGC-related coactivator (PRC)⁴. PGC-1 proteins interact with and promote the activity of several transcription factors—including but not limited to PPAR α / β / δ / γ , NRF1/2, ERR α / β / γ , and SREBP1a/1c/2—that regulate mitochondrial metabolism, lipid metabolism, and other aspects of cellular metabolism (reviewed in⁴). PGC-1 α and PGC-1 β regulate the expression of overlapping sets of genes but can have distinct functions in different tissues. For example, in the liver, PGC-1 α enhances the expression of genes for gluconeogenesis in response to fasting, while PGC-1 β has a key role in lipogenesis and lipoprotein secretion in response to dietary fats^{5,6}. In addition, PGC-1 β promotes cellular respiration that is more highly coupled to energy production in myoblasts compared to PGC-1 α ⁷.

Existing studies on PGC-1 proteins in the context of immunity have largely been focused on PGC-1 α , with a relatively small number on PGC-1 β . For example, PGC-1 α is important for promoting anti-tumor immunity by enforcing mitochondrial biosynthesis and metabolic fitness in T cells^{8,9}. In exhausted virus-specific T cells, overexpression of PGC-1 α similarly helps reverse some of their metabolic defects and counteract exhaustion¹⁰.

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PGC-1 β is known, however, to promote oxidative metabolism to drive alternative activation (required for modulating inflammation and tissue repair) in response to IL-4 in macrophages¹¹.

Dendritic cells (DCs) are innate immune cells that are found in most tissues throughout the body, constantly patrolling and sampling their microenvironment. At steady state, DCs engage both glycolysis and oxidative metabolism¹. Upon Toll-like receptor (TLR) stimulation, DCs are known to rapidly upregulate their glycolytic activity, which is required for optimal DC activation¹². At late-stage activation, glycolysis maintains survival of TLR-activated DCs¹. Conversely, an increase in fatty acid oxidation (FAO) drives the generation of tolerogenic DCs^{13,14}. While the role of glycolysis in regulating DC function has been well-studied^{1,12,15}, the role of mitochondrial metabolism in DCs requires more extensive investigation. Here, we characterized the metabolic and transcriptional changes that occur in DCs treated with lipopolysaccharide (LPS), a bacterial component that induces glycolysis in DCs^{1,12}, or interferon- β (IFN- β), an antiviral mediator. We found that PGC-1 β gene expression dramatically decreases with LPS stimulation, but levels are largely maintained in IFN- β -treated cells. PGC-1 β deficiency impairs ATP production by oxidative metabolism and shifts the bioenergetic balance towards glycolysis. Gene expression analyses reveals that PGC-1 β loss in DCs leads to an increase in inflammatory genes, and reduced expression of genes encoding proteins important for optimal mitochondrial function, including the TCA cycle, respiratory electron transport, and cristae formation. In all, we demonstrate the importance of PGC-1 β in the maintenance of oxidative metabolism and bioenergetic capacity, and for limiting the activation state of DCs.

Results

Comparing metabolite and gene expression profiles of metabolically distinct DCs. To compare metabolite levels and gene expression in metabolically distinct cells, we used the bacterial component, LPS, and the antiviral mediator, IFN- β . We measured the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of these cells using the Seahorse bioanalyzer, with sequential treatments with mitochondrial drugs to determine parameters of mitochondrial function. The results show that LPS- and IFN- β -treated DCs have contrasting bioenergetic profiles (Fig. 1a), with LPS-activated cells not using OXPHOS and therefore not responding to inhibitors and activators of mitochondrial respiration, as previously established¹⁶ and IFN- β -treated DCs having greater maximal respiration than unstimulated DCs (Fig. 1a, left). ECAR for LPS-activated cells was greater than unstimulated cells, which is in line with previous results^{1,12}, while ECAR was not changed with IFN- β treatment (Fig. 1a, right).

We compared the metabolite profiles of cells treated with LPS or IFN- β using LC-MS (Fig. 1b) and analysis of genes encoding factors involved in metabolic pathways (Fig. 1c). LPS induced significant changes in metabolites involved in glycolysis, the TCA cycle, pentose phosphate pathway, and amino acid metabolism, while changes induced by IFN- β were minimal (Fig. 1b). Despite their contrasting OCR, ECAR, and metabolite profiles, similar genes encoding proteins involved in major metabolic pathways, including glycolysis, the TCA cycle, and fatty acid metabolism, showed changes in expression in response to LPS or IFN- β treatment (Fig. 1c). However, the overall magnitude of change for a given gene induced by IFN- β was less. Some notable exceptions include the maintenance of *Sdha* and *Sdhb* expression, which are members of complex II of the ETC, and the induction of *Cpt1a* expression by IFN- β treatment, which is consistent with previous studies^{17,18}. *Ppargc1b*, which encodes PGC-1 β , a transcriptional co-activator important for mitochondrial metabolism, was also maintained in IFN- β -treated DCs but decreased in LPS-stimulated DCs. *Ppargc1a* was not detected at baseline, nor was its expression induced by either LPS or IFN- β treatment (Fig. 1d). We validated the RNA sequencing results using RT-qPCR and confirmed that *Ppargc1b* is downregulated by LPS but maintained during IFN- β treatment compared with untreated cells (Supplementary Fig. S1).

PGC-1 β -deficient DCs have impaired oxidative metabolism and increased glycolysis. To determine how PGC-1 β affects mitochondrial metabolism, we first examined bioenergetic metabolism in DCs, with cells expressing an shRNA targeting *Ppargc1b* (sh*Ppargc1b*). Knockdown efficiency of two different shRNA for *Ppargc1b* were determined by RT-qPCR (Supplementary Fig. S2a,b). sh*Ppargc1b* #1 is used for most experiments presented unless stated otherwise. Majority of both control and sh*Ppargc1b* expressed human CD8 (marker of transduced cells), CD11c, and CD11b (Supplementary Fig. S2c,d), indicating high transduction efficiency and nominal changes in DC development. DCs transduced with sh*Ppargc1b* have reduced basal OCR and spare respiratory capacity (SRC; the difference between maximal OCR and basal OCR) compared to the control (Fig. 2a; Supplementary Fig. S2e). In contrast, the basal ECAR of PGC-1 β -deficient DCs is higher (Fig. 2b). FCCP treatment further elevates ECAR but fails to substantially raise OCR in PGC-1 β -deficient DCs (Fig. 2b), suggesting that at basal rates, these cells are already operating at maximal oxidative capacity.

The increase in ECAR in PGC-1 β -deficient DCs is likely due to increased glycolytic activity to compensate for the reduction in oxidative metabolism. To test the glycolytic capacity of DCs, we used sequential addition of glucose, oligomycin, and 2-deoxyglucose (2-DG) using the Seahorse bioanalyzer (Fig. 2c). Non-glycolytic ECAR (after 2-DG addition) was not different following PGC-1 β deletion, but ECAR was higher when glucose was added (glycolysis; Fig. 2c). We also found that PGC-1 β -deficient DCs have increased glycolytic capacity, measured by the maximal ECAR after addition of oligomycin, which inhibits mitochondrial respiration. This suggests that PGC-1 β -deficient cells are programmed to use glycolysis for bioenergetic metabolism (Fig. 2c). In addition, gene expression of glucose transporter 1 (*Slc2a1*) is increased in PGC-1 β -deficient DCs compared to control DCs under conditions of glucose withdrawal (Fig. 2d), suggesting increased dependence by the cells on glucose. Together, these results demonstrate that deletion of PGC-1 β results in decreased mitochondrial oxidative capacity, and therefore a shift to glycolytic metabolism.

Since elevated glycolysis is required for optimal DC activation^{12,19}, we next examined whether PGC-1 β -deficient DCs display enhanced activation levels. First, we determined that PGC-1 β -deficient DCs express higher

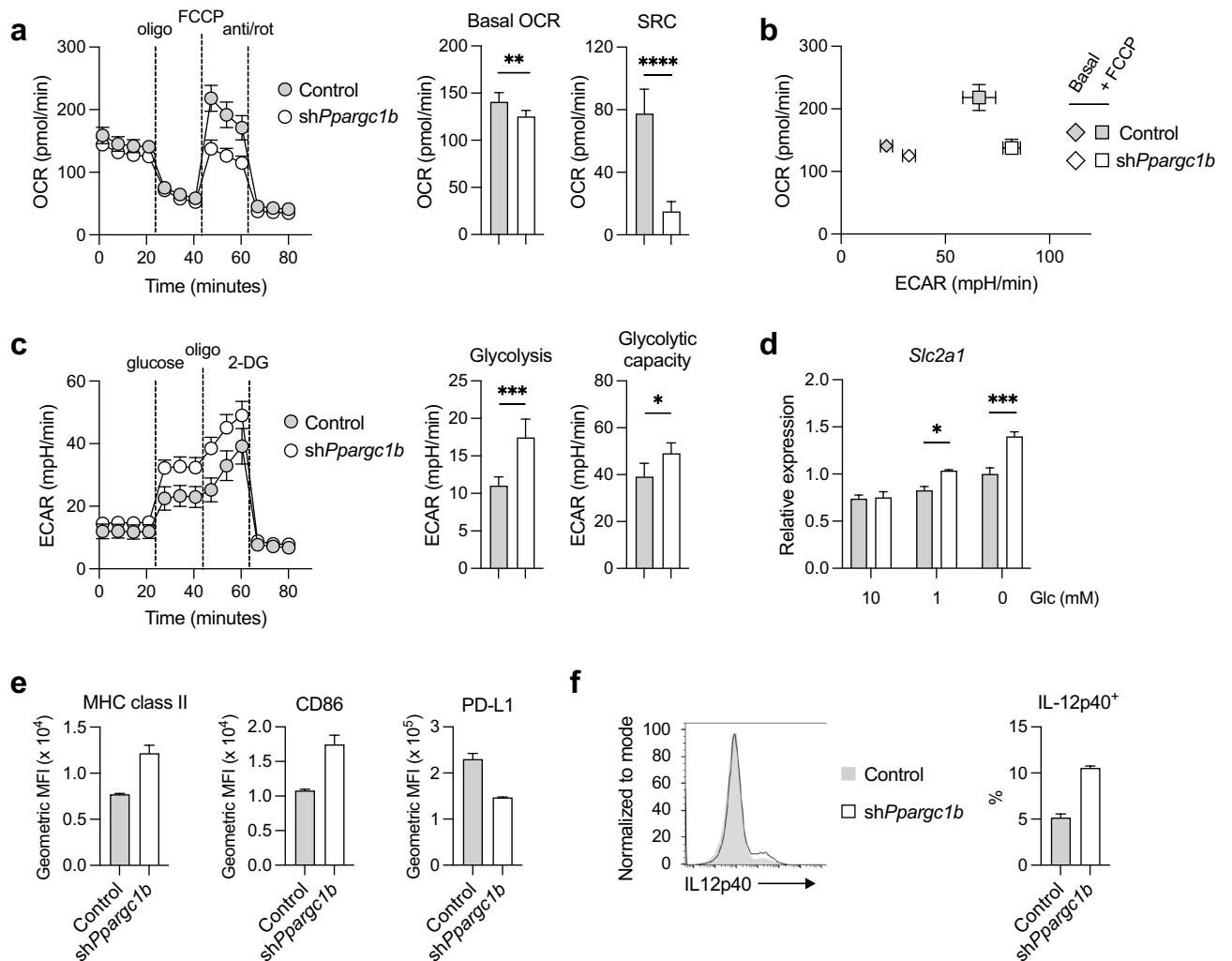


Figure 2. PGC-1 β -deficient DCs have impaired oxidative metabolism and increased glycolysis. DCs were transduced with control shRNA or *Ppargc1b* shRNA and (a) OCR was measured by Seahorse bioanalyzer over time with sequential treatments with oligomycin (oligo), FCCP, and antimycin and rotenone (anti/rot). Basal oxygen consumption rate (OCR) and spare respiratory capacity (SRC) were determined from these measurements (right). (b) Basal and maximal (with FCCP) levels of OCR and ECAR from experiment in (a). (c) ECAR was measured over time with sequential treatments with glucose, oligomycin, and 2-deoxyglucose (2-DG). From this graph (left), glycolysis (difference between ECAR after glucose addition and non-glycolytic ECAR before glucose addition) and glycolytic capacity (maximal ECAR after oligomycin treatment) were determined. (d) Gene expression of GLUT1 gene *Slc2a1* relative to *Hprt* levels in DCs cultured in 10, 1, or 0 mM glucose for 6 h. (e) Geometric MFI of MHC class II, CD86, and PD-L1, and (f) intracellular levels of cytokine IL-12p40 (histogram on left; IL-12p40⁺ cells expressed as a percentage of transduced CD11c⁺ DCs on right) were determined by flow cytometry. Data are of one experiment representative of (a,b) five experiments, (c) three experiments (mean and s.d. of five to six replicates per condition), (d) two experiments (mean and s.e.m. of triplicates), and (e,f) three experiments (mean and s.d. of duplicates). Statistical significance was determined by (a,c) unpaired t-test or (d) two-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

levels of MHC class II, a molecule for antigen presentation, and CD86, a co-stimulatory molecule, and reduced levels of PD-L1, an immunomodulatory ligand, compared to control DCs (Fig. 2e). In addition, at both steady state and with IFN- β treatment, PGC-1 β -deficient DCs express higher levels of the pro-inflammatory cytokine IL-12p40 (Fig. 2f; Supplementary Fig. S3a). However, expression of CCR7, a chemokine receptor essential for directional DC migration typically elevated with activation¹⁹, is generally reduced with PGC-1 β deficiency (Supplementary Fig. S3b). Further, intracellular IL-6 expression in DCs at steady state or with IFN- β treatment was not altered by PGC-1 β loss (Supplementary Fig. S3c), suggesting that not all pro-inflammatory markers are elevated. Thus, PGC-1 β deficiency leads to a selective increase in activation marks in steady-state DCs and IFN- β -treated DCs.

PGC-1 β -deficient DCs have reduced ATP production by oxidative metabolism. Given the shifts in bioenergetic preferences we observed, we next sought to understand whether PGC-1 β deficiency impacts global cellular ATP production. ATP production rates attributed to OXPHOS and glycolysis were calculated using Seahorse assay measurements (see “Methods”). As expected, the basal ATP production rate from OXPHOS is reduced in PGC-1 β -deficient cells, whereas ATP production from glycolysis is increased (Fig. 3a), demonstrating that in the absence of PGC-1 β , cells can pivot to use glycolysis to support their bioenergetic needs. At maximal oxidative capacity (with FCCP treatment), the estimated ATP production by OXPHOS decreased in PGC-1 β -deficient cells (Fig. 3a, right). However, compensation by glycolysis is not observed, resulting in overall decreased ATP production rates (Fig. 3b). These results demonstrate that while PGC-1 β -deficient cells can engage glycolysis to compensate for loss of OXPHOS-generated ATP, this compensation is not complete.

We next investigated how PGC-1 β deficiency affects the metabolic phenotypes of activated DCs. Since stimulation by LPS results in downregulation of *Ppargc1b* expression (Fig. 1d), we hypothesized that PGC-1 β deficiency would not have a considerable effect on the metabolism of LPS-activated DCs. IFN- β -treated DCs, in contrast, maintain PGC-1 β expression, and therefore were predicted to be affected by PGC-1 β deficiency. Both basal and maximal ATP production rates by LPS-activated DCs were not significantly affected by PGC-1 β deficiency (Fig. 3c). Total ATP production rates by LPS-activated DCs were also not significantly different (Fig. 3d). ATP production rates by oxidative metabolism in IFN- β -treated DCs lacking PGC-1 β , however, were lower at both basal metabolism and maximal respiration (Fig. 3e). Basal ATP production rates by glycolysis were higher in PGC-1 β -deficient DCs treated with IFN- β (Fig. 3e). Like that of unstimulated DCs, total ATP production rates at maximal oxidative capacity by IFN- β -treated DCs were lower in PGC-1 β -deficient DCs (Fig. 3f). These data confirm that PGC-1 β is required for optimal bioenergetic output in activated DCs that use oxidative metabolism.

Disruption of energy homeostasis can activate the energy sensor 5' adenosine monophosphate-activated protein kinase (AMPK), which in turn activates metabolic pathways such as glycolysis and OXPHOS to promote the maintenance of ATP levels^{20,21}. Since the ATP production rate by oxidative metabolism is blunted by PGC-1 β loss, we examined AMPK activation by measuring the levels of phosphorylation at residue threonine-172 (T172). We confirmed that phospho-AMPK at T172 increases when glucose is restricted (Fig. 3g). We found that PGC-1 β -deficient DCs have higher levels of phosphorylation at T172 compared to control DCs (Fig. 3g), indicating that the cells sense diminished ATP and AMPK is activated to counteract this energy loss resulting from PGC-1 β deficiency. Despite the energy stress, we confirmed that PGC-1 β deficiency does not lead to a significant change in cell death at steady state (Supplementary Fig. S4a).

AMPK is also known to promote autophagy, which can make available more nutrients for cells to catabolize²²; we therefore measured markers of autophagy, sequestosome 1 (SQSTM1 or p62) and microtubule-associated protein 1 light chain 3 (LC3). Cells were cultured in either full glucose conditions or in the absence of glucose to induce nutrient stress. Chloroquine treatment was used to determine the flux of autophagy, as it blocks autophagosome-lysosome fusion, resulting in accumulation of autophagosome proteins. Gene expression of SQSTM1 was greater with glucose deprivation (Supplementary Fig. S4b), and the protein levels of autophagy-related proteins SQSTM1 and LC3 was elevated in PGC-1 β -deficient DCs (Supplementary Fig. S4c,d). LC3 is involved in autophagosome formation, with LC3-I as the cytoplasmic form and LC3-II as the modified form that associates with autophagosomes. The relative intensity of LC3-II normalized to β -actin levels shows that LC3-II levels are greater in PGC-1 β -deficient cells (Supplementary Fig. S4d). Autophagic flux, measured by determining the difference in intensity of LC3-II in the presence or absence of chloroquine for a given condition, is elevated in PGC-1 β -deficient cells as well (Supplementary Fig. S4d). This suggests that the metabolic stress resulting from PGC-1 β deficiency also stimulates autophagy as cells try to maintain energy homeostasis.

Impaired oxidative metabolism in PGC-1 β -deficient DCs is not due to net changes in mitochondrial mass nor is it substrate-specific. Since PGC-1 co-activators are known to be important for mitochondrial biogenesis in many tissues²³, and we have shown that autophagy increases with PGC-1 β deficiency (Supplementary Fig. S4b–d), we examined whether reduced mitochondrial content is responsible for the impaired OCR by PGC-1 β -deficient DCs. By flow cytometry, we determined that the mean fluorescence intensity (MFI) of dyes to measure mitochondrial mass is not reduced in PGC-1 β -deficient DCs (Fig. 4a). The levels of voltage-dependent anion channel (VDAC), typically used as a loading control for mitochondrial content, was also unchanged (Fig. 4b). Despite PGC-1 β -deficient DCs having reduced gene expression of many TCA cycle and ETC-related proteins, protein levels of the ETC complexes were not noticeably different (Fig. 4b) suggesting there is a functional defect in the mitochondria.

Processing of different substrates, including fatty acids, can contribute to the OCR. Since PGC-1 β is known to target transcription factors that regulate lipid metabolism^{6,24}, we examined whether components involved in FAO were affected by PGC-1 β deficiency. We determined that gene expression of *Hadha* and *Cpt1a*, which encode enzymes involved in key steps of FAO, were reduced in PGC-1 β -deficient DCs (Fig. 4c). In addition, control DCs more readily utilize fatty acids as fuel after nutrient starvation compared to PGC-1 β -deficient DCs, as determined by OCR when the cells are in the presence of BSA-conjugated palmitate (Fig. 4d). Together, these data suggest that the reduced oxidative metabolism by PGC-1 β -deficient DCs is in part due to impaired FAO, and not due to diminished mitochondrial biosynthesis.

We also assessed how nutrient limitation of other carbon sources affects OCR. In control cells, removing glucose results in an increase in basal OCR (likely to compensate for the loss of ATP production by glycolysis), but a decrease in maximal respiration, whereas removal of glutamine results in a decrease in both basal OCR and maximal respiration (Fig. 4e). These data demonstrate that glutamine is required for optimal maximal respiration even in the presence or absence of glucose. For all conditions, PGC-1 β -deficient cells have lower basal and maximal respiration compared to control DCs (Fig. 4e,f). Altogether, these data indicate that PGC-1 β -deficient

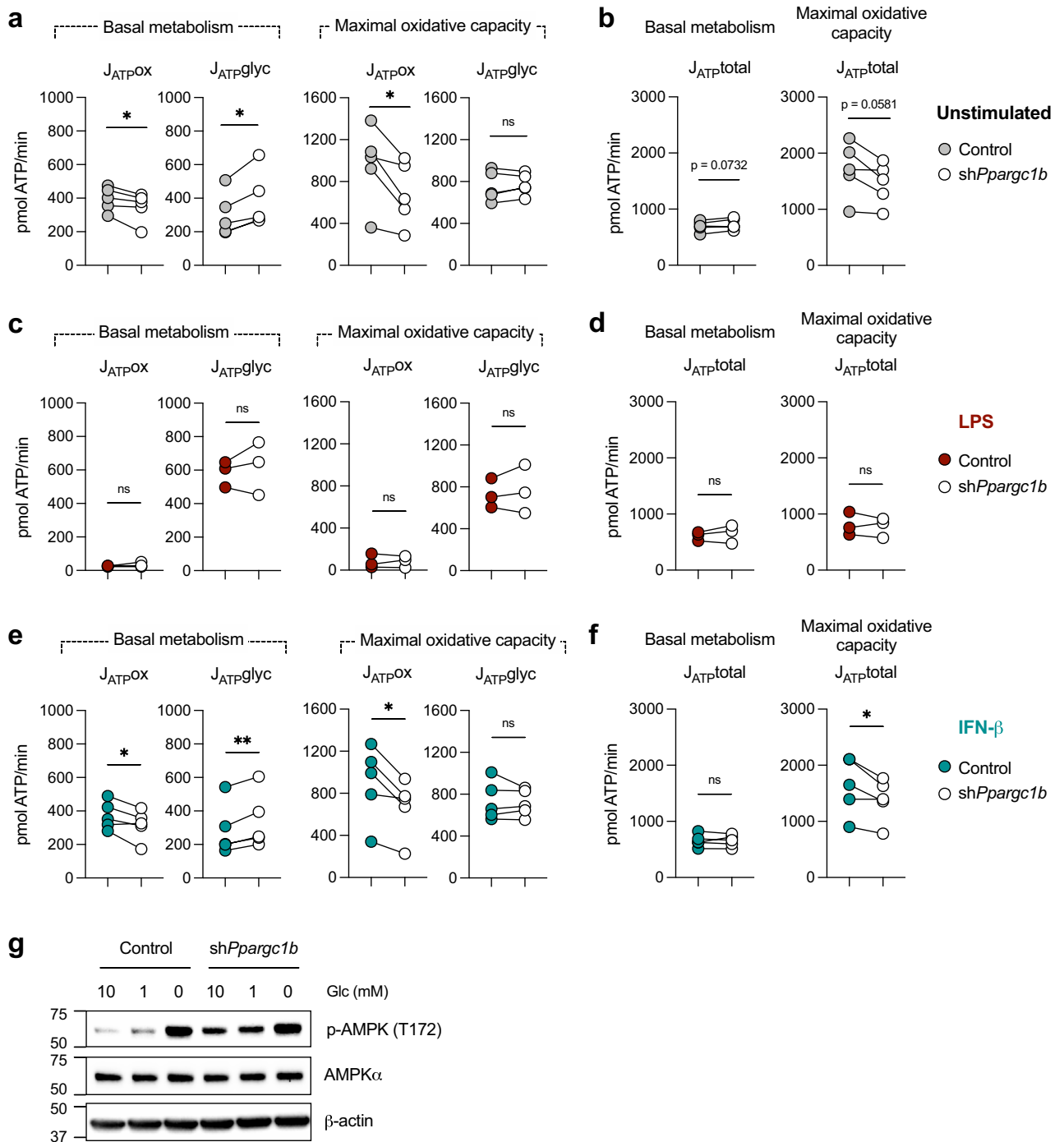


Figure 3. Effect of PGC-1 β on metabolic phenotype of differentially-activated DCs. DCs were transduced with control shRNA or *Ppargc1b* shRNA and (a) ATP production rates by oxidative metabolism (J_{ATPox}) and glycolysis ($J_{ATPglyc}$) were calculated from Seahorse assay measurements (see Methods) at basal (left) and maximal (right) respiratory rates. (b) Total ATP production rates ($J_{ATPtotal}$) at basal metabolism and maximal respiration were determined by adding J_{ATPox} and $J_{ATPglyc}$ from (a). ATP production rates by oxidative metabolism (J_{ATPox}) and glycolysis ($J_{ATPglyc}$) were calculated from Seahorse assay measurements in DCs stimulated with (c,d) LPS (100 ng/mL) or (e,f) IFN- β (1000 U/mL). Total ATP production rates of stimulated DCs in (d,f) at basal metabolism and maximal respiration were determined by adding J_{ATPox} and $J_{ATPglyc}$ from (c,e). Colored circles are control DCs and open circles are DCs transduced with *Ppargc1b* shRNA. (g) AMPK phosphorylated at threonine 172 (p-AMPK T172), AMPK α , and β -actin, were visualized by western blot for DCs that were cultured in media containing 10, 1, or 0 mM glucose. Data are of (c,d) three or (a,b,e,f) five experiments pooled together, with each individual experiment represented by a circle and values from the same experiment joined by a line. Data in (g) are of one experiment representative of three experiments. Statistical significance was determined by paired *t*-test. **p* < 0.05, ***p* < 0.01.

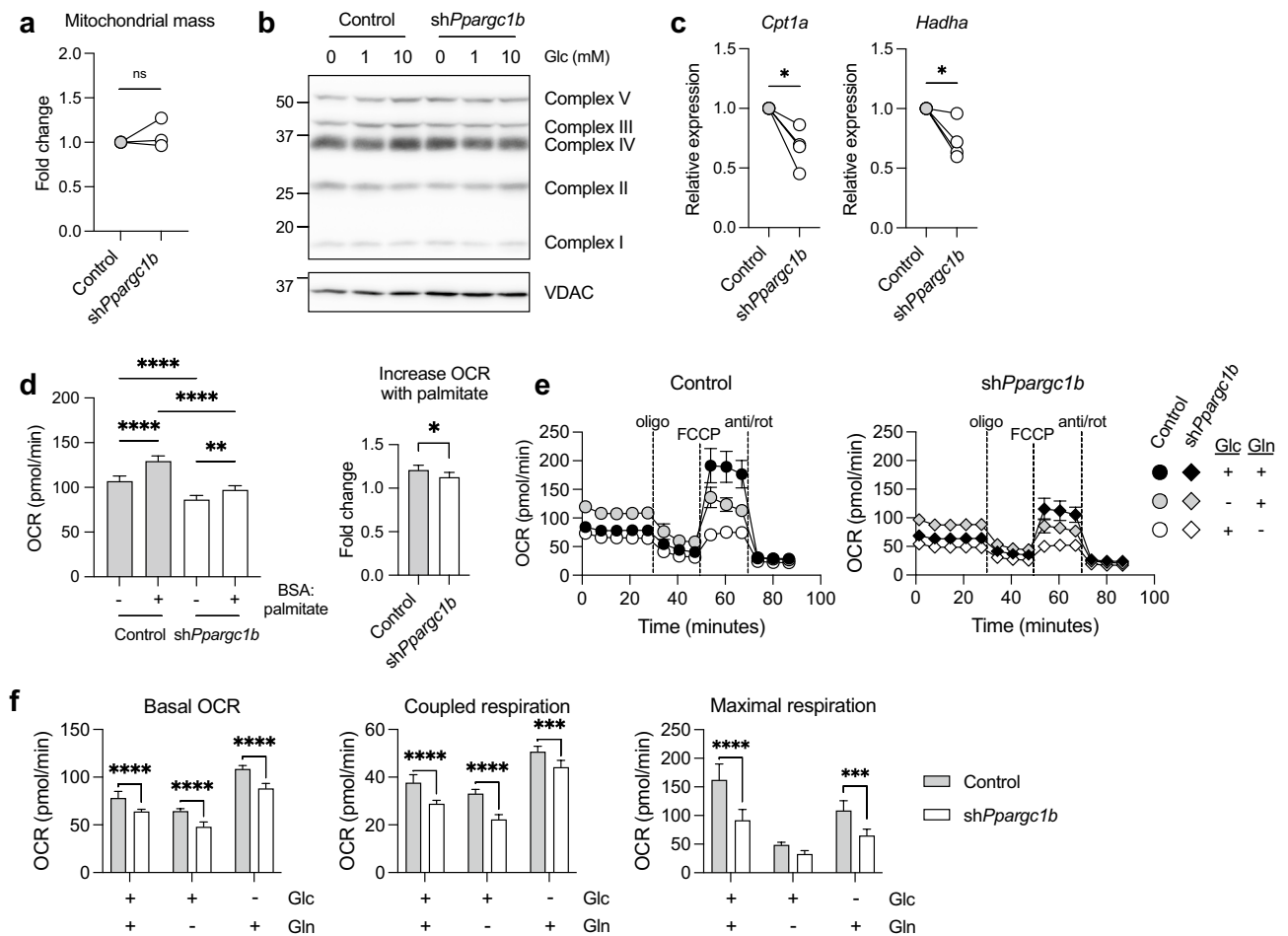


Figure 4. Impaired oxidative metabolism in PGC-1 β -deficient DCs is not due to net changes in mitochondrial mass nor is it substrate-specific. DCs were transduced with control shRNA or *Ppargc1b* shRNA. **(a)** Mitochondrial mass of DCs stained with MitoSpy Green or MitoTracker Deep Red, with geometric MFIs represented as fold changes relative to the control condition. **(b)** Protein expression of complexes I-V of the ETC and VDAC of control or PGC-1 β -deficient DCs cultured with 0, 1, or 10 mM glucose for 6 h. **(c)** Gene expression fold changes of *Cpt1a* (left) and *Hadha* (right) relative to *Hprt*. **(d)** DCs were cultured in nutrient-limiting media for 6 h and OCR was measured immediately following addition of either BSA or BSA-conjugated palmitate. **(e)** OCR was measured by Seahorse bioanalyzer over time with sequential treatments with oligomycin (oligo), FCCP, and antimycin and rotenone (anti/rot), in assay medium with or without 10 mM glucose or 2 mM glutamine. **(f)** Basal OCR, coupled respiration, and maximal respiration determined from the experiment in **(e)**. Data from **(a,c)** are of **(a)** three and **(c)** four experiments pooled together, with each individual experiment represented by a circle and conditions from the same experiment joined by a line. Data from **(b)** is one experiment representative of three experiments. Data from **(d–f)** are of one experiment representative of two experiments (s.d. of five to six replicates per condition). Statistical significance was determined by **(a,c)** paired *t*-test, **(d, left)** one-way ANOVA, **(d, right)** unpaired *t*-test, or **(e,g)** two-way ANOVA. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

cells have reduced oxidative metabolism compared to the control regardless of the substrate, suggesting that the mitochondria-specific dysfunction is substrate-independent.

PGC-1 β loss results in succinate accumulation. While PGC-1 β -deficient DCs have increased glycolysis (Fig. 2c), they also have reduced glucose-supported maximal respiration (Fig. 4e,f). We therefore examined whether there is a difference in the proportions of glucose-derived TCA cycle metabolites (Fig. 5a) by stable isotope labeling and metabolomics using ^{13}C -glucose. We found that the proportion of glucose-derived TCA cycle metabolites is smaller in PGC-1 β -deficient DCs (Fig. 5b), which is in line with our data showing less oxidation of glucose in these cells (Fig. 4e). Furthermore, the relative abundance of lactate (m + 3 isotopologue) is elevated in PGC-1 β -deficient DCs (Supplementary Fig. S5a), supporting the earlier results in Fig. 2 that these cells are more glycolytic. We also observed that the relative abundance of succinate is significantly elevated in the PGC-1 β -deficient DCs (Fig. 5c). Relative abundances of several other TCA intermediates, including citrate, aconitate, and malate, also trend higher, while levels of α -ketoglutarate (α -KG) are unchanged (Fig. 5c). The ratio of succinate

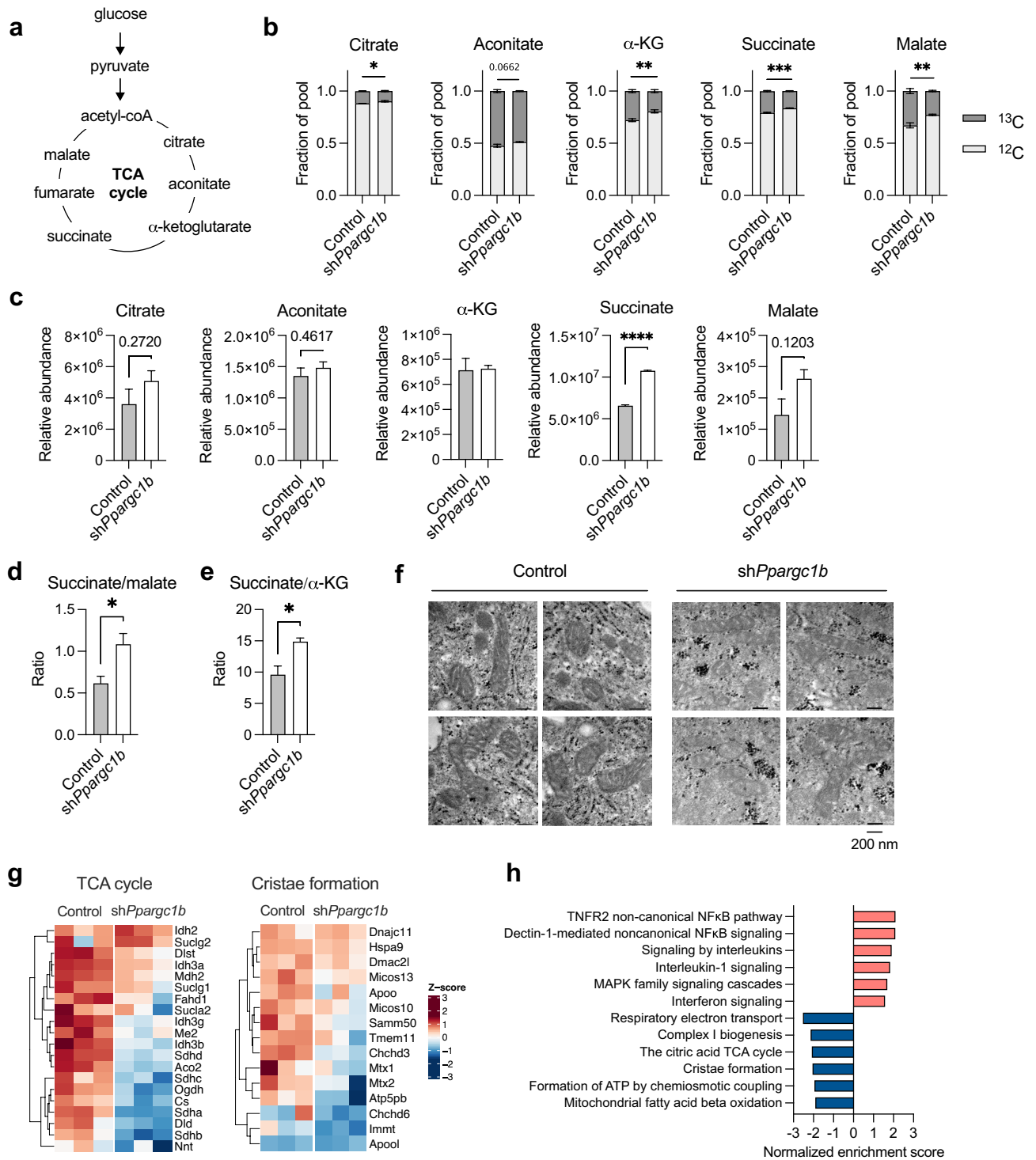


Figure 5. Succinate accumulates in PGC-1 β -deficient DCs. **(a)** Schematic of glucose metabolism and TCA cycle. DCs were transduced with control shRNA or *Ppargc1b* shRNA and **(b)** fractional enrichment of select glucose-derived TCA cycle metabolites was measured following culture with U-[¹³C]-glucose for 6 h. **(c)** Relative abundance of metabolites were measured by LC-MS (aconitate, α -KG, and succinate) or GC-MS (citrate and malate). **(d)** Ratio of succinate to malate from measurements in **(c)**. **(e)** Ratio of succinate to α -KG from measurements in **(c)**. **(f)** Mitochondria imaged by transmission electron microscopy (dark dots are microbeads used for sorting). **(g)** Heat maps of the expression of genes from the TCA cycle (left) and cristae formation (right) Reactome pathways. **(h)** Select inflammatory and metabolic Reactome pathways that are significantly enriched in PGC-1 β -deficient versus control DCs. Data from **(b–e)** are of one experiment representative of two experiments (s.e.m. of three replicates). Statistical analysis in **(b)** was determined by 2-way ANOVA and represents the comparison of the ¹³C-labeled fraction between each condition. Statistical significance for **(c–e)** was determined by unpaired *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

to malate (downstream of succinate in the TCA cycle) (Fig. 5d) and succinate to α -KG (upstream of succinate in the TCA cycle) is higher in PGC-1 β -deficient DCs (Fig. 5e), demonstrating that there is an accumulation of succinate in PGC-1 β -deficient cells.

Since succinate is known to induce IL-1 β production in macrophages²⁵, we examined whether PGC-1 β -deficient DCs also have elevated IL-1 β levels. Baseline gene expression of IL-1 β is higher in PGC-1 β -deficient DCs (Supplementary Fig. S5e), which is in agreement with previous literature showing that increased succinate levels drives IL-1 β gene expression²⁵. However, this did not translate into detectable IL-1 β protein by ELISA or western blot (Supplementary Fig. S5b,c). The NLRP3 inflammasome, which promotes the cleavage of pro-IL-1 β into its biologically active form, and other measures of NLRP3 activation such as ASC and cleaved IL-1 β , generally had reduced protein expression in PGC-1 β -deficient DCs across conditions (Supplementary Fig. S5c). This data indicates that the increased succinate levels in PGC-1 β -deficient DCs was not sufficient to increase inflammasome activation.

We examined the mitochondria by electron microscopy and found that mitochondria in PGC-1 β -deficient DCs are less electron dense (Fig. 5f), suggesting reduced density of ETC components. Because we observed normal protein levels of ETC components by western blot (Fig. 4b), we performed RNA-seq to determine the expression levels of other components of the TCA cycle and ETC. We found that at steady state and in response to IFN- β treatment, PGC-1 β deficiency results in less expression of genes important for the TCA cycle and respiratory electron transport (Fig. 5g,h; Supplementary Fig. S5d–f). Gene expression of succinate dehydrogenase components *Sdha*, *Sdhb*, *Sdhc*, and *Sdhd*, are reduced in PGC-1 β -deficient DCs (Supplementary Fig. S5e), explaining the accumulation of succinate we observed with PGC-1 β loss (Fig. 5). Interestingly, genes important for cristae formation were also decreased in the absence of PGC-1 β (Fig. 5g). One of the affected genes, *Chchd3*, has been shown to be critical for preserving the structural integrity of mitochondrial cristae, as its loss induces mitochondrial dysfunction²⁶. In addition, *Opa1* and *Mfn2*, genes encoding factors important for mitochondrial fusion and OXPHOS²⁷, are downregulated in PGC-1 β -deficient DCs, while *Dnm1l*, which encodes mitochondrial fission marker DRP1, has unchanged expression (Supplementary Fig. S5g). PGC-1 β -deficient DCs also exhibit an increase in gene expression in several inflammatory pathways (Fig. 5h; Supplementary Fig. S6), consistent with their increased activation phenotype (Fig. 2e,f). Together, these data demonstrate that PGC-1 β is necessary for optimal oxidative metabolism and a functional ETC, which restrains the inflammatory phenotype of DCs.

Discussion

Previous studies have demonstrated that cellular metabolism is crucial for the regulation of immune cell function and activation^{2,3}. In this study, we found that in bone marrow-derived DCs, *Ppargc1a* is not appreciably expressed while *Ppargc1b* expression is dynamically changed in response to stimulation. We showed that LPS, but not IFN- β , induces substantial downregulation of *Ppargc1b* expression, which we found notable, since in certain tissues such as skeletal muscle, PGC-1 α expression is typically more dynamic, while PGC-1 β expression is largely unchanged^{28,29}. PGC-1 β deletion resulted in higher baseline activation in DCs, which supports previous studies that associate PGC-1 β expression with an anti-inflammatory state^{30–32}.

We found that loss of PGC-1 β expression leads to impaired oxidative metabolism and increases glycolytic activity to compensate. PGC-1 β is necessary to maintain ATP levels in IFN- β -treated DCs, but not the ATP levels of LPS-activated DCs, which solely depend on glycolysis. In response to this energy deficiency in PGC-1 β -deficient DCs, AMPK is activated (Fig. 3g), which likely leads to the upregulation of glycolysis and glucose uptake. Therefore, the increase in glycolytic rates in these cells is a compensatory mechanism to maintain intracellular ATP levels in response to the drop in ATP production by the mitochondria. While gene expression of glucose transporter *Slc2a1* in PGC-1 β -deficient DCs is comparable to that of control DCs in full glucose conditions, glucose deprivation results in a greater fold change in *Slc2a1* expression, suggesting an increased reliance on glucose. These results highlight the impaired oxidative metabolism of PGC-1 β -deficient DCs and their increased reliance on glycolysis.

Stress signals due to bioenergetic insufficiencies in PGC-1 β -deficient DCs lead to AMPK activation, which is known to promote autophagy²². Our data show that autophagy is indeed elevated in PGC-1 β -deficient DCs (Supplementary Fig. S4). This result is in contrast with a few studies demonstrating positive regulation of autophagy by PGC-1 α and PGC-1 β in muscle cells: PGC-1 α deficiency results in a reduction in autophagy, and PGC-1 β overexpression upregulates autophagy-related genes^{33,34}. Similarly, another study describes that the upregulation of autophagy-related genes induced in skeletal muscle cells by exercise is attenuated in PGC-1 α -deficient mice³⁵. The most likely explanation of these differences is that in our study, autophagy is induced by AMPK activation, and this is not due to specific transcriptional effects of PGC-1 β loss.

To determine how PGC-1 β deficiency leads to impaired oxidative metabolism, we first assessed mitochondrial content since PGC-1 proteins are important for mitochondrial biogenesis in certain contexts. Considering the elevated autophagy, we examined whether this increased autophagy lead to reduced mitochondria in PGC-1 β -deficient DCs. However, mitochondrial mass was not consistently different between control and PGC-1 β -deficient DCs, nor were the levels of select ETC components (Fig. 4a,b). We found that multiple substrates, including fatty acids, glucose, and glutamine, contribute to supporting oxidative metabolism in DCs (Fig. 4d–f). PGC-1 β -deficient DCs, however, have reduced capacity to use each of these substrates for oxidative metabolism, suggesting there is a mitochondria-specific defect at the ETC or TCA cycle. Metabolite analysis shows a specific accumulation of succinate (Fig. 5), which indicates that the activity of succinate dehydrogenase, which is part of both the ETC and the TCA cycle, may also be defective. Elevated succinate levels are known to promote IL-1 β production^{25,36} and we observed increased gene expression of IL-1 β with PGC-1 β loss (Supplementary Fig. S5e). This corresponds to the reduced gene expression of succinate dehydrogenase components in PGC-1 β -deficient DCs (Supplementary Fig. S5e). The protein levels of NLRP3 inflammasome, IL-1 β , and ASC (a marker

of inflammasome assembly) suggest that the elevated succinate levels found with PGC-1 β deficiency are not sufficient to stimulate inflammasome activity (Supplementary Fig. S5c). However, we observed that PGC-1 β loss in DCs elevates other inflammatory markers and enriches for gene expression of proteins that participate in inflammatory pathways such as interleukin signaling and MAPK family signaling cascades (Fig. 2; Supplementary Fig. S6). This observation is in line with previous studies that establish a link between dysregulated PGC-1 proteins and NF- κ B, a central factor in inflammation. Multiple indirect and direct effects may be involved, such as perturbed redox homeostasis, dysfunctional mitochondrial metabolism, and impaired regulation of NF- κ B family member expression^{11,30,37}.

Images of mitochondria acquired by electron microscopy reveal that mitochondria from PGC-1 β -deficient DCs are less electron dense than those from control DCs (Fig. 5f), suggesting there is reduced electron flow through the ETC. Gene expression analyses reveal that several components of the ETC and the TCA cycle, and genes involved in cristae formation, show decreased expression. Together these data suggest that the mitochondria of PGC-1 β -deficient DCs have a dysfunctional crista and ETC, leading to general impairment of mitochondrial metabolism. The ETC has crucial roles in the inflammatory phenotype or differentiation of many immune cell types. For example, macrophages undergo remodeling of their ETC upon LPS activation that disrupts OXPHOS. This leads to production of reactive oxygen species mainly by complex I and III, and the consequent stabilization of the transcription factor HIF-1 α and expression of proinflammatory gene targets such as IL-1 β and genes related to glycolysis³⁶. For T cells, complex II activity is required for optimal T helper (Th) 1 cell differentiation and IFN- γ production, while also suppressing their proliferation³⁸. ATP synthase (complex V) activity is necessary for Th17 differentiation and function, and inhibition of ATP synthase shifts CD4⁺ T cell differentiation into suppressive regulatory T cells³⁹. These and other studies illustrate that there are specific requirements of ETC complexes in immune cell function.

Since we found that the protein expression levels of several ETC components are not affected by PGC-1 β loss in DCs, other mechanisms may be responsible for the dysfunctional ETC. Each of the ETC complexes are comprised of several protein subunits and protein expression of only select subunits were examined in this study. Optimal enzymatic activity of the ETC complexes requires precise assembly of their components, which involves not only proper expression of the subunits, but also a myriad of nuclear-encoded assembly factors⁴⁰. These assembly factors have a range of functions, including stabilization of the complexes and incorporation of necessary co-factors⁴⁰, several of which were expressed lower in PGC-1 β deficiency. Larger structures of ETC complexes known as supercomplexes enhance electron flux⁴¹ and defective assembly of supercomplexes could provide an explanation for the reduced oxidative metabolism yet unchanged protein levels of individual ETC complexes. Many NADH:ubiquinone oxidoreductase subunit (NDUF) proteins, which are generally important for the assembly and maintenance of complex I and supercomplexes and for mitochondrial health^{42,43}, were reduced in expression in PGC-1 β -deficient DCs (Supplementary Fig. S5e), potentially resulting in reduced or compromised complex assembly. Further, reducing agents such as NADH and FADH₂ are generated by the TCA cycle and donate electrons to the ETC, fueling OXPHOS. Diminished levels of these electron donors could result in reduced electron flow through the ETC. In addition, since gene expression for several proteins involved in cristae formation was decreased in PGC-1 β deficiency (Fig. 5g), efficient organization and packing of the ETC components may also be affected. However, the precise mechanism by which PGC-1 β supports efficient ETC activity in DCs requires additional investigation. Further studies to address these questions will help expand our findings that highlight the importance of PGC-1 β in maintaining an oxidative metabolic program and bioenergetic balance in DCs.

Methods

Mice. C57BL/6 were bred and maintained at the Van Andel Research Institute in a specific pathogen-free environment. Up to four mice per cage were housed in microisolator cages on 12 h light/dark cycles in a temperature-controlled environment with enviro-dry enrichment and free access to water and standard chow. Mice were subject to regular cage changes, veterinary inspection, and sentinel monitoring. Female mice were used at 8 to 20 weeks of age. All animal studies were carried out under protocols approved by the Van Andel Research Institute Institutional Animal Care and Use Committee and in accordance with ARRIVE guidelines⁴⁴. Mice were euthanized according to American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals.

In vitro differentiation and retroviral transduction of dendritic cells. Bone marrow from the tibia and femur of C57BL/6 mice was extracted and seeded at day 0 in 6-well non-tissue culture-treated plates at 7.5×10^5 non-erythrocytes/well or at 1×10^6 non-erythrocytes/well if the cells were to undergo retroviral transduction. The cells were cultured in the presence of 20 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF; from Peprotech) in complete DC medium (CDCM): RPMI-1640 medium (Corning) containing 100 U/mL penicillin-streptomycin (Gibco), 2 mM L-glutamine (Gibco), 55 μ M β -mercaptoethanol (Gibco), and 10% heat-inactivated Nu-Serum (Corning), with media changes at days 3 and 6. For retroviral transductions, the sequences for the *Ppargc1b* short hairpin RNA (shRNA) were obtained from RNAi Codex and cloned into the LMP retroviral vector expressing a human CD8 reporter⁴⁵. Retrovirus containing a control shRNA (firefly luciferase) or the *Ppargc1b* shRNA were produced by 293 T cells transfected using Lipofectamine 2000 (Invitrogen). After 48 h, supernatant containing retrovirus was collected and applied to day 2 DC culture and spun at 2500 rpm 30 $^{\circ}$ C for 90 min. At day 8 or 9, DCs were harvested and sorted on human CD8 (for retrovirally transduced DCs) using a biotin anti-human CD8 antibody (clone SK1; BioLegend) and anti-biotin microbeads (Miltenyi). Sorted cells were seeded at a final density of 1×10^6 cells/mL and were left unstimulated or stimulated

with 100 ng/mL LPS (*Escherichia coli* O111:B4; Invivogen), 1000 U/mL IFN- β (PBL Interferon Source), and/or 250 μ g/mL of aluminium hydroxide (alum; Invivogen) for the indicated length of time.

Transmission electron microscopy. Bone marrow-derived dendritic cells retrovirally transduced with control shRNA or *Ppargc1b* shRNA were sorted on human CD8 as described above. Cells were fixed in 2.5% glutaraldehyde in PBS and shipped to the Center for Advanced Microscopy at Michigan State University for analysis.

Metabolic assay. DCs were analyzed by Seahorse XFe96 Analyzer (Agilent) to measure ECAR and OCR in real-time. DCs were seeded at 7×10^4 cells/well in CDCM and stimulated for the indicated length of time. For the XF Cell Mito Stress Test (Agilent), the cell medium was replaced with XF RPMI base medium containing 10 mM glucose, 2 mM glutamine, and 5% FBS, with pH adjusted to 7.4. The DCs were sequentially treated with oligomycin (1.5 μ M), FCCP (1.5 μ M), antimycin/rotenone (both 1 μ M), and monensin (20 μ M). For the XF Glycolysis Stress Test (Agilent), the cell medium was replaced with XF RPMI base medium containing 2 mM glutamine and 5% FBS, but no glucose. The DCs were sequentially treated with glucose (10 mM), oligomycin (1.5 μ M), and 2-deoxyglucose (50 mM). For the XF Palmitate Oxidation Stress Test (Agilent), DCs were cultured for 6 h in nutrient-limiting media (RPMI-1640 containing 0.5 mM glucose, 1 mM L-glutamine, 1% FBS, 55 μ M β -mercaptoethanol, 100 U/mL penicillin–streptomycin, and 0.5 mM carnitine), followed by replacing this with XF RPMI base medium containing 2 mM glucose and 0.5 mM carnitine. BSA or BSA-conjugated palmitate was added immediately prior to starting the run. For all assays, cells were placed at 37 °C in a non-CO₂ incubator for approximately 1 h prior to the start of each run. After each run, cells were stained with 20 μ M of Hoescht stain (ThermoFisher Scientific) for 15 min at 37 °C and imaged using a Cytation imaging reader (BioTek). Measurements from the assay were then normalized to cell number. ATP production rates (J_{ATP}) were calculated based on protocols developed by Mookerjee et al., and adapted by Ma et al.^{46,47}. J_{ATP} by glycolysis (J_{ATP}^{glyc}) and oxidative metabolism (J_{ATP}^{ox}) were calculated by ECAR or OCR, respectively, before any drug treatment for basal metabolism, and after FCCP treatment for maximal respiration. J_{ATP}^{total} represent the sum of J_{ATP}^{glyc} and J_{ATP}^{ox} .

Stable isotope labeling (SIL) and metabolomics. DCs were cultured for the indicated times in medium containing 10% dialyzed FBS, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 55 μ M β -mercaptoethanol, and 10 mM ¹³C-labeled glucose (Cambridge Isotope Laboratories). Cells were washed with ice-cold saline and snap frozen on dry-ice and stored at – 80 °C.

Metabolites were extracted by modified Bligh-Dyer extraction⁴⁸ by the addition of ice-cold methanol (A456, Fisher Scientific) directly to frozen cells, to which one volume of chloroform (A456, Fisher Scientific) was added. The sample was vortexed for 10 s, incubated on ice for 30 min, and then 0.9 parts of LC/MS grade water (W6-4, Thermo Fisher Scientific) was added. The samples were vortexed vigorously and centrifuged at maximum speed to achieve phase separation. The top layer containing polar metabolites was aliquoted into a fresh tube and dried in a speedvac for LC/MS analysis. The bottom layer was retained for fatty acid methyl-ester measurement.

Stable isotope tracing was conducted as previously described⁴⁹. Briefly, two complementary LC/MS methods (basic-pH negative mode and acidic-pH positive mode HILIC separations) were used for broad metabolite coverage using an Orbitrap ID-X mass spectrometer (Thermo). Data were analyzed by Compound Discoverer (v3.2, Thermo). For TCA cycle intermediates, post-LC/MS analysis samples were dried and derivatized with MOX + MSTFA for GC/MS analysis and data analyzed by MassHunter (v8.0, Agilent). Natural abundance isotope correction to unlabeled control samples was completed using fluxfix.science^{50,51}.

RNA sequencing and analysis. RNA was isolated using TRIzol (ThermoFisher Scientific) according to manufacturer's instructions. Libraries were prepared by the Van Andel Genomics Core from 500 ng of total RNA using the KAPA RNA HyperPrep Kit with RiboseErase (v2.17) (Kapa Biosystems, Wilmington, MA USA). RNA was sheared to 300–400 bp. Prior to PCR amplification, cDNA fragments were ligated to IDT for Illumina DNA/RNA UD Indexed adapters (Illumina Inc, San Diego CA, USA). Quality and quantity of the finished libraries were assessed using a combination of Agilent DNA High Sensitivity chip (Agilent Technologies, Inc.) and QuantiFluor® dsDNA System (Promega Corp., Madison, WI, USA). Individually indexed libraries were pooled and 100 bp, paired end sequencing was performed on an Illumina NovaSeq6000 sequencer using an S1, 200 bp sequencing kit (Illumina Inc., San Diego, CA, USA) to an average depth of 50 M reads per sample. Base calling was done by Illumina RTA3 and demultiplexed to FASTQ format with Illumina Bcl2fastq v1.9.0.

Raw reads were first trimmed of adapters with trim_galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and then mapped to mm10 with STAR v2.7.3a using option –quantMode GeneCounts to directly output counts for all features from GENCODE release M20⁵². A negative binomial generalized log-linear model was then fit to the filtered count data with edgeR using the weighted trimmed mean of M-values to normalize for library size and composition biases⁵³. An interactions model was fit to find genes that responded differently depending on the stimulation type ((treatmentIFNb-treatmentcontrol)-(treatmentLPS-treatmentcontrol)). P-values were generated using empirical Bayes quasi-likelihood F-tests, and then adjusted using the BH method; adjusted P-values less than 0.05 were considered significant.

Heatmaps were generated from library-size normalized counts centered across genes (z-scores). Heatmaps were generated using the R package ComplexHeatmap⁵⁴. KEGG^{55–57} and REACTOME pathways were retrieved with the R package msgdbr⁵⁸. Pathway enrichment testing was done with GSEA as implemented in the clusterProfiler v3.14.3 function ‘GSEA.’ Enrichment testing for GO terms was also done with clusterProfiler v3.14.3 using the enrichGO function.

| Gene name | Forward primer | Reverse primer |
|-----------------|---------------------------------|-------------------------------------|
| <i>Ppargc1b</i> | GGC AGG TTC AAC CCC GA | CTT GCT AAC ATC ACA GAG GAT ATC TTG |
| <i>Slea21</i> | CTG GAC CTC AAA CTT CAT TGT GGG | GGG TGT CTT GTC ACT TTG GCT GG |
| <i>Sqstm1</i> | CCT CAG CCC TCT AGG CAT TG | TTC TGG GGT AGT GGG TGT CA |
| <i>Hadha</i> | AGT GGA AAG CGT GAC TCC AG | ACA TCC ACA CCC ACT TCG TC |
| <i>Cpt1a</i> | GGA CTC CGC TCG CTC ATT | ACC TTG ACC ATA GCC ATC CAG |
| <i>Hprt</i> | AGG ACC TCT CGA AGT GTT GG | GGC TTT GTA TTT GGC TTT TCC |

Table 1. List of primers used for qPCR.

Quantitative RT-PCR. RNA was isolated using TRIzol (ThermoFisher Scientific) according to manufacturer's instructions. cDNA was obtained using RT MasterMix (Applied Biological Materials) and used to perform a SYBR-based real-time PCR (BioLine) with primers (Table 1) from Integrated DNA Technologies. Data were generated using the $\Delta\Delta Cq$ method. Relative gene expression was normalized to that of hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Flow cytometry. Cells were washed with wash buffer (PBS containing 1% FBS, 1 mM EDTA, and 0.05% sodium azide) and stained with eFluor 506 Fixable Viability Dye (Invitrogen) and Fc block (anti-CD16/CD32) prior to staining with cell surface markers, which include human CD8 (OKT-8), CD11c (N418), MHC-II (M5/144.15.2), CD86 (GL-1), PD-L1 (10F.9G2), CD11b (M1/70), and CCR7 (4B12). To examine intracellular cytokines IL-12p40 (C17.8) and IL-6 (MP5-20F3), cells were fixed with IC Fixation Buffer (Invitrogen) for 30 min, followed by cell permeabilization using Permeabilization Buffer (Invitrogen), and at least 1 h of intracellular cytokine staining in Permeabilization Buffer. For mitochondrial mass measurements, cells were stained with 50 nM MitoSpy Green (BioLegend) or 50 nM MitoTracker Deep Red (ThermoFisher Scientific) in HBSS at 37 °C for 20 min, followed by viability and surface marker staining as described above. Apotracker Green (BioLegend) was used at 400 nM according to manufacturer's instructions to detect apoptotic cells. Samples were acquired in wash buffer on the Cytoflex (Beckman Coulter) or Aurora (Cytek) and analyzed using FlowJo software.

Western blot. Protein lysates were prepared in RIPA lysis buffer with protease inhibitor and phosphatase inhibitor, then quantified by detergent compatible protein assay (Bio-Rad). Samples were run on 10% or 12.5% gels using SDS-polyacrylamide gel electrophoresis, and proteins were transferred by Turbo transfer system (Bio-Rad) onto methanol-activated polyvinylidene fluoride membranes. After blocking for 1 h with 5% milk, membranes were incubated overnight rocking at 4 °C with antibodies against β -actin (13E5), LC3I/II, SQSTM1 (D1Q5S), NLRP3 (D4D8T), ASC (D2W8U), IL-1 β (D6D6T), and cleaved IL-1 β Asp117 (E7V2A) (all from Cell Signaling Technology), Total OXPHOS Rodent WB Antibody Cocktail (Abcam), and α -tubulin (Proteintech), all at 1:1000 in 4% BSA. Following washes with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T), membranes were incubated with horseradish peroxidase-linked antibody to rabbit or mouse IgG in 5% milk for 45 min. Enhanced chemiluminescence using SuperSignal substrate (ThermoFisher Scientific) and ChemiDoc (Bio-Rad) were used to develop blots. Densitometric analysis was performed using Bio Rad Image Lab software. Original blots are presented in Supplementary Figures.

Statistical analysis. Data were analyzed using GraphPad Prism software (version 9). An unpaired or paired Student's *t*-test was performed as indicated to determine statistical significance between two conditions. A two-way analysis of variance was performed to determine statistical significance between multiple groups. Differences between conditions were considered significant when *P* values were below 0.05.

Data availability

Raw data files for the RNA sequencing analysis in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE204957 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE204957>). Requests for data or reagents can be made to connie.krawczyk@vai.org.

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Author contributions

H.G., J.S.-P., R.G.J., and C.M.K. conceived this study. H.G., R.S., E.H.M., and C.M.K. designed the experiments. H.G., A.V., and M.J.W. performed experiments. H.G., R.S., I.B., E.H.M. and H.S. analyzed data and generated figures. H.G. wrote the manuscript. H.G., J.S.-P., and C.M.K. edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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