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Cell Reports

Mitochondrial respiration in B lymphocytes is essential for humoral immunity by controlling the flux of the TCA cycle

Graphical abstract



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In brief

Urbanczyk et al. report that dominantnegative Twinkle prevents mtDNA replication in B cells. Intact mtDNA fosters OxPhos and restricts glycolysis in LPS- and BCR-activated B cells, enabling the competition of developing plasma cells. OxPhos secures the flux of the TCA cycle and mTOR activity, which is required for plasmablast proliferation and differentiation.

Highlights

- mtDNA in B cells is required for humoral immunity and plasma cell maturation
- mtDNA maintains OxPhos in LPS and antigen receptorstimulated B cells
- OxPhos drives the TCA cycle in LPS-activated B cells
- OxPhos preserves phosphatidic acid and mTOR activity in activated B cells



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Article

Mitochondrial respiration in B lymphocytes is essential for humoral immunity by controlling the flux of the TCA cycle

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SUMMARY

To elucidate the function of oxidative phosphorylation (OxPhos) during B cell differentiation, we employ CD23Cre-driven expression of the dominant-negative K320E mutant of the mitochondrial helicase Twinkle (DNT). DNT-expression depletes mitochondrial DNA during B cell maturation, reduces the abundance of respiratory chain protein subunits encoded by mitochondrial DNA, and, consequently, respiratory chain super-complexes in activated B cells. Whereas B cell development in DNT mice is normal, B cell proliferation, germinal centers, class switch to IgG, plasma cell maturation, and T cell-dependent as well as T cell-independent humoral immunity are diminished. DNT expression dampens OxPhos but increases glycolysis in lipopolysaccharide and B cell receptor-activated cells. Lipopolysaccharide-activated DNT-B cells exhibit altered metabolites of glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle and a lower amount of phosphatidic acid. Consequently, mTORC1 activity and BLIMP1 induction are curtailed, whereas HIF1 α is stabilized. Hence, mitochondrial DNA controls the metabolism of activated B cells via OxPhos to foster humoral immunity.

INTRODUCTION

Humoral immunity depends on the development of antibody (Ab)-secreting long-lived plasma cells. All plasma cells have undergone profound anabolic and morphologic changes during several cell divisions, such as cell growth and expansion of the ER (Schuh et al., 2020). BLIMP1 is required for the fully secretory phenotype of plasma cells (Kallies et al., 2007), which appears to involve oxidative phosphorylation (OxPhos) (Price et al., 2018). Plasma cells also depend on glucose uptake for Ab glycosylation and import of pyruvate into mitochondria (Lam et al., 2018).

Resting B cells increase OxPhos, glucose uptake, and glycolysis when activated by the B cell receptor (BCR) or the innate stimulus lipopolysaccharide (LPS) within 6 h, switching to glucose and glutamine oxidation (Caro-Maldonado et al., 2014). The mammalian target of rapamycin (mTOR) complex, specifically the rapamycin-sensitive mTORC1 complex (lwata et al., 2017), integrates the metabolic fate of glucose, glutamine, and lipids (Foster et al., 2014). Intriguingly, mTORC1 is required for the generation but not the maintenance of plasma cells (Benhamron et al., 2015; Jones et al., 2016), suggesting an anabolic control of plasma cell development via mTORC1 (Lam et al., 2018). In







Figure 1. Genetic inhibition of mtDNA replication in transitional B cells by dominant-negative Twinkle (DNT)

(A) Abundance of mtDNA encoded *nd1* and 16s *rRNA* relative to nuclear *hk2* in B cells of SRBC-immunized mice; N = 1, n = 4, mean ± SD; n.s. unless indicated, by two-way ANOVA.

(B) Mitochondrial mass (Mitotracker Green) in the indicated populations measured by flow cytometry; N = 1, n = 4, mean \pm SD. Representative data on the right, numbers indicate MFI; by one-way ANOVA.

(C) Mitochondrial membrane potential ($\Delta m\psi$) was measured by tetramethylrhodamine (CCCP-treated control subtracted) and flow cytometry; N = 1, n = 4, mean \pm SD. Representative original data are shown on the right. Numbers indicate MFI; by one-way ANOVA.

(D) Splenic B cells were stimulated with LPS for 3 days. mtDNA was quantified as in (A). Symbols represent individual mice; by one-way ANOVA.

fact, early mTORC1 activity precedes plasma cell generation. Yet, it is unclear how mTORC1 is regulated at these early time points (Gaudette et al., 2020). While plasma cell function is accompanied by OxPhos upregulation and OxPhos is maintained by interferon-regulatory factor 4 (IRF4) in plasma cells (Low et al., 2019), a causal role for OxPhos during B cell activation and plasma cell generation has been questioned (Milasta et al., 2016). Hence, the role that OxPhos plays during plasma cell ontology remains puzzling. OxPhos is fueled by the Krebs/ tricarboxylic acid cycle (TCA) in mitochondria (Kennedy and Lehninger, 1949). The TCA cycle progressively transforms oxaloacetate into intermediates such as citrate, α-ketoglutarate, succinate, fumarate, or malate. In some of these reactions, net energy is transferred to reduction equivalents, such as nicotinamide adenine dinucleotide (NAD), to yield NADH (Krebs, 1948). In turn, complex I oxidizes NADH, and the electrons move along the electron transport chain (ETC) complexes. The protons are pumped across the inner mitochondrial membrane (IMM) by complex III and IV while transferring electrons to O_2 , the essential terminal electron acceptor, which is reduced to H₂O. Complex V utilizes the chemiosmotic gradient to produce ATP. An upregulation of nucleus-encoded genes of the OxPhos complexes I-V during late plasma cell differentiation has been proposed to be controlled by BLIMP1 (Price et al., 2018). Yet, only complex II is encoded entirely by nuclear genes, whereas essential subunits of the OxPhos complexes I, III, IV, and V are encoded by mitochondrial DNA (mtDNA) (Gustafsson et al., 2016). In total, mtDNA codes for these 13 crucial subunits and also contains the genes for 22 tRNAs and two ribosomal RNAs necessary for their synthesis in the mitochondrial matrix. Expression of OxPhos complex subunits requires continuous replication and transcription of mtDNA, which needs to be unwound by the essential mitochondrial helicase PEO1/TWINKLE (TWINKLE) (Milenkovic et al., 2013). Dominant-negative mutants of TWINKLE impair mtDNA replication, thereby causing mitochondrial disease in humans due to multiple mtDNA deletions and/or depletion (Spelbrink et al., 2001). Those large mtDNA deletions, even in the presence of wild-type (WT) molecules (called a heteroplasmic state), reduce the amount of newly synthesized subunits. Truncated or fused subunits further impair ETC function and ATP production (Hornig-Do et al., 2012). Nothing is known about the physiologic function of mtDNA in B cells and how OxPhos shapes B cell metabolism.

We hypothesized that depletion of mtDNA in mature B cells would compromise OxPhos in activated B cells. This approach would allow us to examine specifically the role of OxPhos during B cell activation and differentiation and thereby bring new insights on its function in this cell type. The approach of mtDNA depletion circumvents the use of inhibitors in mice and in cell culture. The murine dominant-negative TWINKLE K320E (DNT) variant (Baris et al., 2015), equivalent to the K319E human muta-



tion (Hudson et al., 2005), leads to massive mtDNA depletion when expressed in rapidly dividing cells such as keratinocytes (Weiland et al., 2018). To determine the role of mtDNA and OxPhos in rapidly dividing B cells in vivo, we specifically expressed DNT in B cells to induce mtDNA depletion. We found increased replication of mtDNA during WT B cell activation. Reduced mtDNA replication in the presence of DNT hindered germinal center (GC) B cell development and BLIMP1 expression as well as plasma cell generation in vivo and in vitro. This was due to lowered OxPhos activity, reduced mTORC1 activity in early plasmablasts, and a disturbed TCA cycle that reduced generation of phosphatidic acid (PA). Inhibition of mTORC1 prevented plasma cell generation. In vivo, plasma cells with depleted mtDNA were outcompeted. Hence, our results place OxPhos upstream of mTORC1 and BLIMP1 during plasma cell differentiation to ensure mitochondrial fitness.

RESULTS

Characterization of mtDNA and mitochondrial abundance in B cells

To determine the amount of mitochondrial DNA (mtDNA) we sorted B cells by flow cytometry (Figures S1A and S2B). MtDNA-encoded 16s rRNA and nd1 genes were quantified by gPCR (Figure 1A). Marginal zone (MZ) B cells and splenic and bone marrow (BM) plasma cells (CD138⁺TACI⁺) (Pracht et al., 2017) exhibited higher amounts of mtDNA than follicular (FO) B cells. These data suggest that mtDNA replicates during development of transitional B cells to MZ B cells (Loder et al., 1999) and from resting B cells to plasma cells. GC B cells showed only a slight increase of mtDNA relative to FO B cells. To compare the mtDNA content to mitochondrial mass and membrane potential ($\Delta m \psi$), we stained the aforementioned B cells (Figure 1A) with Mitotracker Green and tetramethylrhodamine (TMRM) (Figures 1B and 1C). Neither mitochondrial mass nor $\Delta m\psi$ correlated with mtDNA. Instead, MZ B cells and plasma cells exhibited less mitochondrial mass and $\Delta m \psi$ than FO B cells. Thus, mtDNA content rather than mitochondrial mass or $\Delta m \psi$ may correlate with increased OxPhos activity observed during plasma cell differentiation (Price et al., 2018). To determine whether proliferation and differentiation indeed increase mtDNA copy number, we quantified mtDNA in LPS-activated B cells. This revealed an approximately two-fold increase in mtDNA between d0 and d3 of activation (Figure 1D). These data suggested that there is a need for mtDNA-encoded proteins of OxPhos complexes during B cell proliferation and/or differentiation.

Expression of the dominant-negative mitochondrial helicase twinkle (DNT) in B cells

We therefore sought to interfere with mtDNA homeostasis in activated B cells. To avoid metabolic checkpoints of early B cell

⁽E) Schematic of the construct encoding a dominant-negative form of TWINKLE (K320E; DNT; Baris et al., 2015) knocked into the ROSA26 locus. Top: inactive conformation. Bottom: CRE-mediated recombination with excised *neo*/STOP cassette (*neo* | WSS), activation of the composite cytomegalovirus, chicken beta-actin, rabbit beta-globin (CAG) promoter, and expression of DNT and IRES-GFP. Expression of DNT impairs replication of mtDNA (right).

⁽F) Splenic B cells from CD23CRE and DNT mice were stimulated with LPS for 3 days, and mtDNA was quantified on day 0 and day 3 as in (A). Symbols represent individual mice. N = 1; n = 3; by two-way ANOVA. Data for day 3 are representative of three independent experiments.

p values <0.05. *p < 0.05, **p < 0.01, ***p < 0.002, ****p < 0.0004. N indicates the number of experiments, n indicates the sample number per experiment.







d10 SRBC

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development (Urbanczyk et al., 2018), we crossed mice carrying a loxP-flanked STOP cassette upstream of the dominant-negative Twinkle variant K320E (DNT) coupled to IRES-GFP (Baris et al., 2015) with CD23CRE (for short: CRE) mice (Kwon et al., 2008) to obtain DNT×CD23CRE (for short: DNT) mice (Figure 1E). DNT-IRES-GFP was expressed in B cells but not in T-cells or innate immune cells, except of a very minor CD11b^{int}F4/80^{int} population (Figures S1C-S1E). To determine the onset of GFP expression, we analyzed B cells in the bone marrow (BM) (Figure S2), revealing that \sim 20% of transitional and \sim 90% of recirculating B cells express GFP. These data are consistent with the onset of CD23 expression in transitional type 2 B cells (Kwon et al., 2008). In keeping with DNT expression in immature/transitional BM B cells (Figure S2), mtDNA was reduced about 10-fold in resting splenic B cells of DNT mice, and B cell activation by LPS did not increase the amount of mtDNA in DNT-B cells (Figure 1F). Because CREmediated excision of the floxed STOP cassette of DNT mice was not 100% (Figures S1C and S2), we reasoned that this could lead to a competition of STOP cassette-deleted vs. -non-deleted B cells in vivo. By monitoring GFP fluorescence, this would allow us to define the checkpoints where DNT-B cells are potentially outcompeted.

Loss of B cells with depleted mtDNA during plasma cell differentiation

To determine the effect of DNT expression, we analyzed B cells in spleens and BM of DNT and CD23CRE mice (representative FACS plots in Figure 2A). Whereas numbers and frequencies of B cells and plasma cells were comparable (Figures 2A, 2B and 2C), the GFP⁺ fraction was specifically reduced in GC B cells, plasmablasts (PBs), and plasma cells (Figure 2B). In particular, the more plasma cells matured from P1 to P3 (P1: CD138^{high}, TACI^{high}, CD19^{int}, B220^{int}, BLIMP1^{low}; P2: CD138^{high}, TACI^{high}, CD19^{int}, B220^{low}, BLIMP1^{med}; P3: CD138^{high}, TACI^{high}, CD19^{low}, B220^{low}, BLIMP1^{high}) (Cossarizza et al., 2019; Pracht et al., 2017), the lower was the GFP⁺ frequency. This was more pronounced in the BM than in the spleen (Figure 2D). We conclude that DNT-GFP+ cells are outcompeted during GC development and plasma cell differentiation. DNT expression conferred the strongest disadvantage to the most mature plasma cells, the P3 cells. Consequently, non-mutant (i.e., GFP⁻) P2 and P3 plasma cells filled up the plasma cell compartments. Unexpectedly, in regard of the normal total numbers of



plasma cells (Figure 2C), class-switched serum Ig antibodies were reduced, whereas IgM was not (Figure 2E), indicating that T cell-dependent (TD) immune responses are impaired by mtDNA depletion. To test this hypothesis directly, we immunized mice with sheep red blood cells (SRBCs). GC formation assessed by histology was severely impaired in SRBC-immunized DNT mice (Figure 2F), pointing to a requirement of OxPhos for the GC reaction.

TI2 and TD responses as well as class switch recombination to IgG require mtDNA

To characterize TD Ab responses, mice were immunized with nitrophenol-keyhole limpet hemocyanin (NP-KLH). This revealed a reduction in primary and secondary NP-specific IgG in DNT mice, whereas NP-specific IgM was not reduced (Figure 3A). We hypothesized that non-recombined GFP⁻IgM⁺ plasma cells catch up. To show this directly, we stained antigen-specific plasma cells with NP-phosphatidylethanolamine (PE) (Reimer et al., 2020), which detects surface IgM- and IgA-expressing plasma cells (Blanc et al., 2016). NP-specific, CD138^{high} plasma cells were diminished by \sim 40% in DNT mice (Figures 3B and 3C). Strikingly, the cells binding surface NP-PE in DNT mice were not GFP⁺ (Figures 3B and 3C). These data suggested that GFP⁺IgM⁺DNT plasma cells are outcompeted during TD immune responses and that non-mutant GFP-IgM+ cells catch up. To determine why IgG after immunization was reduced we analyzed CSR that has been linked to mitochondrial function (Jang et al., 2015). CSR to IgG was reduced by 40% but CSR to IgA was not affected (Figures 3D and 3E) as DNT-B cells were only slightly underrepresented in the IgA⁺ fraction (Figure 3F). This result is consistent with the observation that homeostasis of IgA⁺ cells is mediated through glycolysis (Kunisawa, 2017). To further follow the idea that specifically the IgM⁺ plasma cells are most vulnerable to mtDNA depletion by DNT, we immunized mice with the type 2 T independent (TI2) antigen NP-Ficoll which targets both FO and marginal zone (MZ) B cells (Garcia de Vinuesa et al., 1999). There was virtually no NP-specific IgM or IgG3 after immunization of DNT mice with NP-Ficoll (Figure 3G). This experiment provides strong evidence that mtDNA is required for TI antibody responses in vivo (Figure 3G). We conclude that mtDNA deletion in B cells by DNT reduces GC responses, class switch recombination (CSR), TD=induced IgG, and TI2-induced IgM/IgG3. Hence, mtDNA homeostasis in B cells is essential for humoral immunity.

Figure 2. Outcompetition of DNT-expressing plasma cells

p values <0.05. *p < 0.05, **p < 0.01, ***p < 0.002, ****p < 0.0004.

⁽A) Representative dot plots (merged from three mice) of splenic B cells; of germinal center (GC) B cells in the Peyer's patches, and of plasmablasts/plasma cells (TACI⁺CD138⁺) from spleen and bone marrow. Plasmablasts/plasma cells were further analyzed by CD19 and B220 staining. Numbers depict frequencies in indicated gates.

⁽B) Total cell numbers of follicular (FO) B cells, marginal zone (MZ) B cells, and plasmablasts/plasma cells, frequencies of GC B cells of Peyer's patches from CD23CRE mice and GFP-negative and -positive cells from DNT mice. Data are presented as mean ± SD. Each dot represents one mouse, three experiments; by one-way ANOVA.

⁽C and D) (C) Total cell numbers of P1, P2, and P3 cells (see A) in the spleen and bone marrow and (D) frequencies of GFP-expressing B cells, plasma cells, and , P2, and P3 cells (see A) in the spleen and bone marrow. Each dot represents one mouse; three experiments; by one-way ANOVA. Data are presented as mean ± SD.

⁽E) Serum antibody concentrations from unimmunized mice (8–15 weeks old) were determined by ELISA, mean ± SD. Each dot represents one mouse; three experiments. Outliers were excluded after Routs test; by Student's t test.

⁽F) Mice were immunized with SRBC. Spleen sections were analyzed by histology and confocal microscopy: 10× scale bars, 100 μm; 20× scale bars, 50 μm; blue, IgD-AF647; green, GL7-PacificBlue; red, PNA-rhodamine.







Figure 3. Impaired NP response and class switch recombination by DNT expression in B cells

(A) Mice were immunized with NP-KLH in alum and at day 42 with NP-KLH in PBS i.p. NP(20)- and NP(4)-specific serum antibodies were quantified by ELISA. The line represents the simple moving average. Each dot represents one mouse, two experiments each with three mice; by two-way ANOVA of area under curve. (B) Plasma cells in the bone marrow were analyzed at day 70. Plasma cells were marked using surface staining with NP-PE. Numbers depict frequencies in the indicated gates.

(C) Frequencies of NP⁻GFP⁺, NP⁺GFP⁺, or NP⁺GFP⁻ cells in the TACI⁺CD138⁺ bone marrow cell population (shown in B); by two-way ANOVA. Data are presented as mean ± SD.

(D) Splenic B cells were stimulated with LPS, anti CD40 antibody, IL-4, IL-5, TGFβ, and retinoic acid for 4 days. Cells were analyzed by intracellular flow cytometry of IgA, IgG, and IgM. Representative dot blots; numbers depict frequencies in the indicated gates.

LPS (TI1)-induced proliferation and plasmablast development require mtDNA

Next, we sought to explore the mechanism of the blunted TI response in DNT mice. To this end, we switched to another TI antigen, LPS (a TI1 antigen), and in vitro stimulation. LPS elicits proliferation and differentiation of mouse B cells in vitro and allows the generation of large cell numbers eligible for biochemical analyses. In accord with the TI2 immunization, IgM or IgG antibodies in the supernatants of LPS activated DNT-B cells, and the proportions of CD138⁺TACI⁺ plasmablasts were diminished (Figures 4A-4C). DNT-B cells were outcompeted by 40% at the CD138⁺TACI⁺ stage (Figures 4D and 4E), whereas TACI⁺CD138⁻ cells that arose earlier in those cultures were proportionally increased (Figures 4B-4E), providing more evidence for a competitive disadvantage of DNT-expressing plasma cell numbers (Figure S3E). However, apoptosis was unaltered (Figures S4A and S4B). Despite proliferation rates being lower, there were DNT-B cells that had divided five or six times, that is, as many times as the control B cells (Figure S3D). To separate proliferation and differentiation, we used a dye dilution assay and CD138 staining (Nutt et al., 2011). This experiment revealed that even the DNT cells that had divided four to six times exhibited a lower frequency of CD138⁺ cells, i.e, plasma cells (Figures 4F and 4G). We conclude that mtDNA is required for plasma cell differentiation at least partially independently of cell proliferation. In confirmation, western blot analysis of day 3 LPS blasts revealed that factors defining plasma cells (BLIMP1, XBP1) (Nutt et al., 2011) as well as IgM were reduced, whereas the amount of IRF4 was comparable (Figures 4H and 4l). Of note, prdm1 RNA (encoding BLIMP1) was lower in DNT-B cells as well (Figure 4J). To exlude the possibility that BLIMP1 was repressed by high abundance of BCL6 (Nutt et al., 2011) we also examined BCL6 protein (Figures 4H and 4I), showing that diminished BLIMP1 was not due to elevated BCL6. To corroborate these findings in vivo and to exclude potentially confounding effects of non-mutant B cells, we labeled 48-h LPS-activated B cells with a proliferation dve and transferred them into rag2^{-/-} mice (Figure S4C). After an additional 72 h, B cells and plasma cells (Figure S4D), proliferation (Figure S4E), and apoptosis (via staining of activated caspase-3; Crowley and Waterhouse, 2016) were assessed (Figure S4F). There were fewer plasma cells in rag2^{-/-} mice that had received purified GFP⁺ DNT-B cells in both spleen and BM (Figure 4K), and the rate of activated-caspase-3-positive B cells was higher in DNT-B cells (Figure S4F). In line with the in vitro stimulation, proliferation of DNT-B cells was also reduced (Figures S4G-S4I; please note that the number of cell divisions appears lower than in vitro because we had labeled the cells 48 h later). Cumulatively, IgM and IgG serum antibodies were strongly reduced in $rag2^{-/-}$ hosts having received DNT-B cells (Figure 4L). We conclude that DNT-B cells have a strong cell-intrinsic developmental disadvantage toward a TI1-antigenic challenge.



Specific reduction of mtDNA-encoded COXI and mitochondrial super-complexes in B cells expressing DNT

To delineate the effect of mtDNA deletion by DNT on mitochondria, we examined cellular and mitochondrial morphology of resting and LPS-activated B cells by electron microscopy (Figure 5A). Resting splenic B cells of DNT mice with already 10-fold-reduced mtDNA (Figure 1E) did not contain fewer mitochondria, and their shape also did not differ grossly from that of the controls, but they were less electron dense, indicating a looser cristae structure (Figure 5A, top; Figure 5B). However, LPS-activated DNT-B cells had fewer but larger mitochondria. Their cristae structure was almost completely disrupted (Figure 5A, bottom; Figure 5B). Of note, we had to focus on activated B cells without obvious plasma cell morphology because only roughly 60% of CD138-expressing plasmablasts were GFP positive (Figures 4E and 4F), hindering the unambiguous identification of DNT-expressing plasmablasts. To determine whether the low density of the DNT-mitochondria corresponds to respiratory chain super-complex formation (Schägger and Pfeiffer, 2000) we analyzed digitonin-solubilized mitochondrial membranes from activated B cells by Blue-Native-PAGE (Schägger and von Jagow, 1991) (Figure 5C). We also analyzed abundance of a mtDNA-encoded protein (COX1) versus nucleus-encoded mitochondrial proteins and the mitochondrial import receptor TOM20 (Hanson et al., 1996) (Figure 5D). Whereas TOM20, ATP5A, COX7A, and UQCRC2 were unaltered (Figures 5D and 5F), COX1 expression in DNT-B cells was reduced, leading to less mitochondrial super-complex formation (Figure 5E). Nevertheless, at least after 3-day LPS stimulation, there was no drop in mitochondrial mass or $\Delta m \psi$ in DNT-expressing cells (Figure 5G), which is in line with unaltered apoptosis (Figures S4A and S4B).

Intact mtDNA prevents the LPS-stimulated switch to glycolysis

Next, we wished to confirm that mtDNA controls OxPhos during B cell activation and plasma cell differentiation. Extracellular flux analyses of 3-day LPS cultures in normoxic conditions revealed reduced basal and maximal oxygen consumption as well as ATP production coupled to respiration in DNT-B cells, while the basal extracellular acidification rate (ECAR), glucose consumption, and lactate secretion were enhanced (Figures 6A-6D). Shortterm activation for 6 h via either LPS or the BCR showed that DNT-B cells performed OxPhos to a similar extent to that of controls (Figure S5A). These results can explain the normal B cell development. In line, the ATP content was normal in resting DNT-B cells, but we measured less ATP in 3-day LPS-stimulated DNT-B cells (Figure 6E). The fact that DNT-B cells were still able to increase their intracellular ATP content upon activation (Figure 6E) indicated compensatory pathways for ATP production, most probably glycolysis. To delineate the contribution of

⁽E) Frequencies of IgA, IgG, or IgM B cells (see D). Mean ± SD. Each dot represents one mouse; two experiments, each with three mice; by two-way ANOVA. (F) GFP-expressing cells in the indicated populations (see E) of DNT mice at day 4, mean ± SD. Each dot represents one mouse; two experiments, each with three mice; by one-way ANOVA.

⁽G and H) Mice were immunized with NP-Ficoll i.p. or PBS, and NP(20)-specific IgM and IgG3 antibody concentrations were determined by ELISA. Data are shown as mean \pm SD. Each dot represents one mouse; two experiments, each with 3 mice; by two-way ANOVA. p values <0.05. *p < 0.05, **p < 0.01, ***p < 0.002, *****p < 0.0004.





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OxPhos and glycolysis to ATP production longitudinally, we performed a long-term Mito Stress Test and determined the ATP rate index. The ATP rate index indicates the proportion of ATP generated by OxPhos or glycolysis. If the ATP rate index is >1, more ATP is produced by OxPhos than by glycolysis. LPS-stimulated WT (CRE) B cells increased both OxPhos and glycolysis over time, with OxPhos dominating. However, the ATP rate index was lower in DNT-B cells already at day 0 and dropped even below 1 after 48 h of LPS activation (Figure 6F), confirming that DNT-B cells switch strongly to glycolysis. Still, only after 72 h was glycolysis not able to compensate fully for ATP generation anymore in DNT-B cells (Figure 6F).

Intact mtDNA prevents the BCR-stimulated switch to glycolysis

For immunization we had used TI antigens that function in a B cell-intrinsic manner via Toll-like receptor 4 (LPS) or via the BCR (NP-Ficoll), and the TD antigen NP-KLH. During TD reactions, B cells receive T cell help via CD40 stimulation and IL-4 (among others) (Vallé et al., 1989). We therefore addressed how DNT-B cells react to BCR stimulation with or without CD40 activation and IL-4. LPS was included as control. Notably, more ATP appears to generated via OxPhos in 3-day BCR-activated WT (CRE) B cells than in 3-day LPStreated cells (Figures 6F and 6G). In stark contrast, activation of DNT-B cells via the BCR elicited an overwhelming switch of ATP generation via glycolysis (Figure 6G), which appeared even stronger than with LPS stimulation (Figure 6F). However, when CD40 stimulation and IL-4 were included, there was no difference between CRE and DNT-B cells, because most cells had switched to glycolysis (Figure 6G). These results show that different B cell activation modes have different requirements for OxPhos, which has strong implications for TI and TD immune responses and the GC reaction. Moreover, these experiments reveal how flexibly B cells can handle ATP production.



OxPhos coordinates the TCA cycle, lipid synthesis, and mTORC1 activity in plasmablasts

OxPhos and the TCA cycle are intertwined (Kennedy and Lehninger, 1949). To elucidate the metabolic consequences of reduced OxPhos in DNT-B cells, we measured glycolytic and TCA intermediates by liquid chromatography-mass spectrometry (LC-MS) (Hofmann et al., 2011) in day 3 LPS cultures (Figure 7A). In line with the extracellular glucose measurements (Figure 6D), intracellular glucose was slightly increased together with erythrose 4-phosphate (E4P). Phosphoenolpyruvate (PEP) and uridine diphosphate N-acetylglucosamine (UDPNAG) were less abundant in DNT-B cells. Metabolites upstream of succinate dehydrogenase (SDH), citrate, isocitrate, and itaconate, were also reduced (see Figure S5B). Contrastingly, there was an accumulation of the TCA metabolites fumarate and malate downstream of SDH, the only OxPhos complex whose subunits are not encoded by mtDNA (Gustafsson et al., 2016). These data show that depletion of mtDNA in B cells diverts the TCA cycle by reducing OxPhos. We confirmed these data by a second independent approach (Figure 7B), showing decreased UDPNAG (0.56-fold, p = 0.004) but increased malate (1.8-fold; p = 0.026) in DNT-B cells.

Plasma cell differentiation involves *de novo* lipogenesis via glucose (Dufort et al., 2014). Since we observed a disequilibrium in the TCA cycle and glycolytic intermediates in DNT-B cells, we reasoned that lipid synthesis could be altered. We first corroborated that glucose incorporates into phosphatidylethanolamine (PE) in LPS-activated B cells (Figure 7C). To explore whether DNT-B cells exhibit an altered lipid composition, we exploited glycerophospholipids (GPL) of LPS blasts by mass spectrometry (Kumar et al., 2015) (a representative spectrum is shown in Figure 7D). We observed a specific reduction of total PA (Figures 7D and 7E) but no major quantitative differences for phosphatidylglycerol (PG), phosphatidylserine (PS) (Figure 7E). As to length and saturation of the fatty acid (FA) side chains,

- (C) Frequencies of TACI⁺ and TACI⁺CD138⁺ cells shown in (B), mean ± SEM. Each dot represents one mouse; three experiments; by two-way ANOVA.
- (D) Representative histograms of GFP expression in resting or 3-day LPS-activated DNT-B cells.
- (E) GFP-expressing cells in the indicated populations of DNT mice shown in (D), mean ± SEM. Each dot represents one mouse; two experiments each with three mice; by one-way ANOVA.
- (F) Efluor 450-marked splenic B cells were stimulated with LPS for 3 days, stained with anti CD138 antibodies, and analyzed by flow cytometry.
- (G) Frequencies of 4–6×-divided CD138⁺GFP⁺ and CD138⁺GFP⁻-expressing cells shown in (F). Two experiments; each dot represents one mouse; by one-way ANOVA. Data are presented as mean ± SD.
- (H) Splenic B cells were stimulated with LPS for 3 days. Cell lysates were analyzed by western blot with antibodies indicated on the right. Molecular-mass standards are on the left (kDa). Representative western blots showing lysates of two mice each are shown. The brackets indicate a different western blot.
- (I) IRF4, BLIMP1, XBP1s, IgM, and BCL6 protein expressions relative to actin of 3-day LPS-activated splenic B cells. Symbols indicate individual mice. Two experiments each with two to four mice; by Student's t test. Data are presented as mean \pm SD.
- (J) qPCR quantification of *prdm1* RNA relative to *actin* of splenic B cells stimulated with LPS for 3 days. Symbols indicate individual mice; two experiments each with two to four mice; by Student's t test. Data are presented as mean ± SD.
- (K) 48-h LPS-activated B cells were transferred into $rag2^{-/-}$ mice and analyzed 72 h later. TACI⁺ and CD138⁺ cells in the spleen and bone marrow, mean ± SEM. Each dot represents one mouse; one experiment; by two-way ANOVA.
- (L) 48-h LPS-activated B cells were transferred into $rag2^{-/-}$ mice and analyzed 72 h later. IgM and IgG abundance in the serum of recipient mice (mean \pm SEM) normalized to the number of transferred cells. Each dot represents one mouse; one experiment; by t test.

 $p \ values < \! 0.05. \ ^*\!p < 0.05, \ ^{**}\!p < 0.01, \ ^{***}\!p < 0.002, \ ^{****}\!p < 0.0004.$

Figure 4. Decreased proliferation and plasmablast differentiation in LPS-activated B cells from DNT mice

⁽A) Splenic B cells were stimulated with LPS for 3 days. Supernatants were analyzed by ELISA. Bars represent the mean ± SEM; each dot represents one mouse; two experiments, each with four mice; by Student's t test.

⁽B) Splenic B cells were stimulated with LPS for 3 days and analyzed by flow cytometry. Merged dot plots (three mice per genotype) are shown. Numbers depict frequencies in the indicated gates.







Figure 5. Mitochondrial phenotypes of resting and activated DNT-B cells

(A) Splenic B cells were left unstimulated or stimulated with LPS for 3 days and analyzed by transmission electron microscopy. Top: resting cells (\times 10,000, scale bar 10 µm; enlargements, \times 21.560, scale bar 4.4 µm). Bottom: blasts (\times 6,000, scale bar 21 µm; enlargements, \times 16.700, scale bar 7.5 µm).

(B) The optical density of mitochondria (20–40) from resting and activated B cells was quantified; by Student's t test.

(C) Splenic B cells from each of six mice were stimulated for 12 h with LPS and pooled if necessary. Super-complexes (indicated by roman letters) were separated by BlueNative PAGE and analyzed by western blotting with indicated antibodies. Each lane represents one mouse or one distinct pool.

(D) Splenic B cells were stimulated for 48 h with LPS and pooled if necessary. Lysates were analyzed by western blotting with indicated antibodies. Molecularmass standards are shown on the left (kDa). Each lane represents a different pool.

(E) Abundance of super-complexes relative to the protein concentration of the total mitochondrial membrane fraction. Each dot represents one lane (bottom blot); by two-way ANOVA.

(F) Abundance of the indicated proteins relative to Tom20. Each dot represents one lane; by two-way ANOVA.

(G) Analysis of mitochondrial mass with Mitotracker Red and $\Delta m \psi$ with tetramethylrhodamine of GFP⁺ and GFP⁻ cells from 3-day DNT LPS cultures. CCCPtreated controls were subtracted. One experiment; dots represent individual mice; numbers depict MFI; by Student's t test.

 $p \text{ values } < 0.05. \ ^*p < 0.05, \ ^{**}p < 0.01, \ ^{***}p < 0.002, \ ^{****}p < 0.0004. \ Data \text{ in (B), (E), and (G) are presented as mean } \pm SD.$







Figure 6. Impaired oxygen consumption and flexible ATP production of T-independently activated DNT-B cells

(A) Representative extracellular flux analysis of 3-day LPS-activated splenic B cells. The basal oxygen consumption rate (OCR) was measured before and after injection of oligomycin, FCCP, and rotenone plus antimycin A. Symbols represent means of two mice, each of three replicate wells, and error bars are ±SEM.
(B) Calculated OCR values of basal and maximal respiration, spare respiratory capacity, and proton leak. Means ± SEM from two experiments each with two mice; by two-way ANOVA.

(C) Calculated extracellular acidification rate (ECAR) under conditions of basal and maximal respiration. Means ± SEM from two experiments performed each with two mice; by two-way ANOVA.

(D) Lactate and glucose concentrations in the supernatants on day 3 after LPS activation. Each dot represents one mouse; two experiments each with two or three mice; by two-way ANOVA. Data are presented as mean \pm SEM.

(E) Luminometric ATP detection of resting and 3-day -activated B cells. RLU, relative light units. Symbols represent individual mice; by two-way ANOVA.

(F) B cells were activated with LPS over 3 days. ATP rate assays were performed. Top: mean ± SD. Bottom: ATP rate index (relative contributions of OCR and ECAR to ATP production). Each dot represents one mouse; by two-way ANOVA.

(G) B cells were activated with anti-BCR antibodies alone, in combination with anti-CD40 antibody and IL-4, or with LPS for 3 days. ATP rate assays were performed at day 3. Top: mean ± SD. Bottom: ATP rate index (relative contributions of OCR and ECAR to ATP production). Each dot represents one mouse; by twoway ANOVA.

p values <0.05. *p < 0.05, **p < 0.01, ***p < 0.002, ****p < 0.0004.







(legend on next page)



there were no obvious changes for PG but PA, PCH, PI, PE, and PS differed remarkably. Especially, GPL species with shortchain and saturated or monounsaturated FAs such as 32:0, 34:0, 32:1, or 34:1 were reduced within the pools of PA, PI, PE, and PS, with the exception of 30:0, 32:0, or 32:1 PCH (Figure S6). Although there were not always statistical differences, many GPLs with long-chain, polyunsaturated FAs such as 36:3-36:7, or 40:3-40:7 were increased continuously regardless of the head group (Figure S6). Since many GPL species with polyunsaturated FAs-except within the class of PG-were increased, we consider this effect cumulatively to be biologically relevant. Although there were elevated GPL with long-chain and polyunsaturated FAs within almost all classes, PA was overall the only quantitatively reduced GPL (Figure 7E). PA is a central precursor for many lipids, such as PE, by integrating anaplerotic products of both glycolysis and the TCA cycle (Figure S5B) (Athenstaedt and Daum, 1999), acts directly on mTORC1 in a cell intrinsic manner (Menon et al., 2017), and is required for assembly and stability of mTOR complexes (Foster, 2013). Genetic evidence has shown that mTORC1 is required for plasma cell development (Benhamron et al., 2015; Jones et al., 2016). Hence, the reduced PA in DNT-B cells might affect mTORC1 activity, which could explain the defective plasma cell development. In fact, phosphorylation of the ribosomal protein RPS6, which is a substrate of the p70RPS6 kinase downstream of mTOR (lwata et al., 2017) was reduced in DNT LPS blasts at d2 and d3 (Figures 7F and 7H; please note that the western blot is an overestimation or pRPS6 in the DNT-B sample because DNT-B cells become outcompeted; Figure 4E; Figures S7A and S7C). These data suggested that OxPhos and/or the TCA cycle can influence metabolic signaling in B cells (Martinez-Reyes and Chandel, 2020). For instance, succinate, fumarate, and malate can inhibit prolyl hydroxylase domain (PHD)-containing enzymes that normally target the von Hippel-Lindau protein (vHL), which ubiquitinylates hypoxia-inducible factor 1a (HIF1a), leading to its degradation (Harnoss et al., 2015; Martinez-Reyes and Chandel, 2020). In line with the increased fumarate and malate there was a stabilization of HIF1 a in DNT-B cells at day 3 of LPS stimulation under normoxic conditions (Figures 7G and S7B). Taking these together, we propose that OxPhos in LPS-activated B cells controls metabolic signaling pathways, namely HIF1 α and mTORC1.

PA increases OxPhos-dependent mTORC1 activity in LPS-activated B cells

To confirm that mTORC1 is involved in plasma cell development in our system, we treated LPS cultures of WT B cells with 10 nM rapamycin over 3 days. Measuring pRPS6 via flow cytometry revealed the efficacy of rapamycin (Figures S7D and S7E). By analogy to DNT-B cells, there were fewer plasma cells in rapamycintreated cultures (Figures S7F and S7G), and BLIMP1 expression was reduced (Figure S7H). B cell cultures treated with rapamycin proliferated less (Figure S7I), and the total cell number was diminished by one-half after 3 days (Figure S7J), which was not due to increased apoptosis (Figure S7K; not significant, n.s.). These data mirror the phenotype of DNT LPS cultures. Hence, mTORC1 activation downstream of OxPhos could be responsible for proliferation and differentiation effects observed with DNT-B cells.

To reverse mTORC1 activity and potentially proliferation and plasma cell development in DNT-B cells, we reasoned that the lowered PA in day-3 DNT LPS blasts is responsible for suppressed mTORC1 activity. Ergo, we added PA liposomes to LPS-activated DNT B cells (Figure 7H; Figures S8A and S8B). We chose PA 32:0, which was reduced the most in DNT-B cells (Figure 7E). Addition of PA for 24 h to LPS-activated B cells increased the frequency of pRPS6-expressing cells and the pRPS6 level in DNT-B cells and controls slightly after 24 h (Figure 7H; S8C). Polymersomes that served as control had no effect (Figure S8D), and the PA-elicited increase in pRPS6 was reversed by mTOR inhibition via rapamycin (Figure S8E). pRPS6 increased further after 48 h in LPS-activated control B cells independently of PA addition but declined in DNT-expressing B cells (Figure 7H; compare Figure 7F) and, after 72 h, also in control B cells (compare Figure 7F). PA was not able to rescue proliferation or plasma cell development (data not shown). We

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Figure 7. DNT expression alters the TCA cycle, lipid composition, and signaling in LPS-activated B cells
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(A) HPLC/MS analysis of intracellular metabolites (experimental setup I; see STAR Methods) of FACS-sorted B cells (DNT, viable GFP⁺; CD23CRE, viable) stimulated with LPS for 72 h. Data show the relative abundance. Symbols indicate the mean of each independent experiment. Each experiment was performed with two or three mice (total data points, 7). Data are presented as mean ± SEM.

(C) Isotopolog distribution of ¹³C in phosphatidylethanolamine in 3-day LPS-activated B cells cultivated for 48 h in medium containing [U-¹³C]glucose.

(D) Splenic B cells were stimulated with LPS for 3 days. Glycerophospholipids of FACS-sorted B cells (DNT, viable GFP⁺; CD23CRE, viable) were analyzed via direct-infusion MS/MS (shotgun lipidomics). A representative neutral-loss mass spectrum of phosphatidic acid from CRE and DNT-B cells (overlay) is shown. Small letters indicate masses of the $[M + NH_4]^+$ precursor ions, and large letters indicate the total number of carbon atoms and the total number of double bonds within the two fatty acyl chains (two representative species, 32:0 and 34:1, are shown).

(E) Absolute abundance (nMol/mg protein) of phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylcholine (PCH), phosphatidylethanolamine (PE), and phosphatidylserine (PS). Each symbol represents one mouse. One experiment of two (each with three to five mice) with identical results is shown; by two-way ANOVA.

(F) Splenic B cells were stimulated with LPS for 0–72 h. Lysates were analyzed by western blot, with antibodies indicated on the right. Molecular-mass standards are shown on the left (kDa). Quantification of pRPS6 relative to RPS6 is shown on the right, mean ± SEM. All cutouts are from the same blot and exposure. (G) Splenic B cells were stimulated with LPS for 0, 24, or 72 h. Cell lysates were analyzed by western blot, with antibodies indicated on the right. Molecular-mass

(a) Spienic B cells were stimulated with LPS for 0, 24, or 72 n. Cell lysates were analyzed by western blot, with antibodies indicated on the right. Molecular-mass standards are shown on the left (kDa).

(H) Splenic B cells from CRE or DNT mice were stimulated with LPS in the presence or absence of 32:0 PA liposomes or PBS for the indicated time points, and pRPS6 abundance was analyzed by flow cytometry, as depicted.

p values <0.05. *p < 0.05, **p < 0.01, ***p < 0.002, ****p < 0.0004.

⁽B) Confirmation of representative metabolites by an independent experiment (setup II). One experiment with B cells each from two to three mice stimulated with LPS for 72 h. Diagrams show the abundance and retention times on a C₁₈ column.



conclude that mTORC1 activity is transient in LPS-activated B cells and that PA can activate mTORC1 in primary B cells; OxPhos is upstream of mTORC1 in LPS-activated B cells, and mTORC1 controls plasma cell differentiation. Taken together, OxPhos and mTORC1 activity depends critically on mtDNA homeostasis in B cells, thereby, being essential for humoral immunity.

DISCUSSION

Here, we investigated the function of OxPhos in mature B cells by expressing the dominant-negative mitochondrial helicase, Twinkle K320E (DNT) (Sarzi et al., 2007), via CD23CRE (Kwon et al., 2008). DNT expression in B cells strongly reduced mtDNA, as expected (Baris et al., 2015; Holzer et al., 2019; Weiland et al., 2018). Whereas B cell development was normal, TI1, TI2, and TD immune responses were abrogated due to impaired proliferation and plasma cell differentiation of DNT-B cells. Albeit DNT expression distorted mitochondrial super-complexes, resting and briefly BCR- and LPS-activated B cells could still perform normal OxPhos. However, prolonged stimulation via the BCR and LPS elicited an overwhelming switch to glycolysis in DNT-B cells. Hence, mtDNA is essential to maintain normal OxPhos during TI immune responses and to prevent an aberrant glycolytic switch. We have further established that maintenance of OxPhos in B cells via replication of mtDNA prevents HIF1a accumulation, drives the TCA cycle, maintains the amount of PA, and fosters mTORC1 activity, which is required for plasma cell development.

Our data support the finding that development of FO B cells does not require optimal expression of mitochondrial complexes I, III, and IV proteins (Milasta et al., 2016) but they stand in contrast to data showing that hypoxia increases IgG1 class switch and plasma cell differentiation (Abbott et al., 2016). The discrepancies may relate to the fact that we used a B cell-intrinsic model. Unexpectedly, mtDNA was not increased in GC B cells. Since GC B cells are the most rapidly dividing cells in the mammalian body (MacLennan, 1994), we speculate nevertheless that B cell proliferation in GC is still accompanied by replication of mtDNA to maintain OxPhos.

Another surprising observation was that mtDNA copy number did not always correlate with $\Delta m\psi$ or mitochondrial mass. $\Delta m\psi$ and mitochondrial mass were even unchanged in 3-day LPSactivated DNT-B cells. One explanation could be that mtDNA mutations induce a retrograde signaling pathway that actually increases mitochondrial biogenesis, thereby, fostering metabolic fitness and the tumorigenic potential of Reed-Sternberg cells (Haumann et al., 2020). Hence, quantifying mtDNA, and its mutation rate, of human B cell populations in health and disease will be important (Frasca et al., 2019).

One advantage of our model is that mutant B cells express GFP (Baris et al., 2015), which helped to reveal that DNT-B cells become outcompeted. This affected particularly the most mature plasma cells, the P3 cells, and confirms the function of miRNA 148a in the regulation of OxPhos in plasma cells (Pracht et al., 2020). Furthermore, we have established a causal relation between OxPhos and TI plasma cell differentiation *in vivo* and *in vitro* and place OxPhos-mediated generation of TCA interme-

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diates upstream of mTORC1 and BLIMP1. We propose that an initial increase in OxPhos, which presupposes replication of mtDNA, drives the TCA cycle to generate PA and activates mTORC1 and consecutive BLIMP1 expression (Brookens et al., 2020; Gaudette et al., 2020; Jones et al., 2016). BLIMP1 may subsequently increase expression of OxPhos genes encoded in the nucleus, thereby boosting OxPhos progressively proportional to the number of cell divisions (Price et al., 2018; Tsui et al., 2018). In support, our long-term ATP rate assay confirms that OxPhos increases progressively during LPS-induced plasma cell differentiation. The suggested model could explain how BLIMP1 is able to sustain mTORC1 activity (Tellier et al., 2016), namely via OxPhos and the TCA cycle. In confirmation, OxPhos produces more than half of the ATP during TI activation of B cells in vitro by the BCR and LPS. Therefore, DNT expression elicits a prominent glycolytic shift, a so-called pseudohypoxic state (Shanmugasundaram et al., 2014), exemplified by lactate secretion, fumarate and malate elevation, and HIF1 a stabilization under normoxic conditions. This forces immediate outcompetition of DNT-B cells during their differentiation phase. We suggest that this outcompetition is due to (1) reduced proliferation and (2) differentiation. Increased apoptosis involving caspase-3 as a consequence of mitochondrial dysfunction may contribute as well in vivo (Berard et al., 1999).

In line with TI immunizations, TD immunization also triggered outcompetition of NP-specific IgM⁺ DNT cells. Hence, the TD immunization elicited IgM in DNT mice stems likely for the most part from escaped B cells, which, on top, may even over-compensate (Durandy and Honjo, 2001). Because Ig catabolism is proportional to Ig abundance (Morell et al., 1970; Waldmann and Strober, 1969), Ab half-life could be prolonged in DNT mice.

If non-mutant cells compensate, how can these findings be reconciled with the reduced IgG responses in DNT mice? TD immune responses involve the GC reaction and CSR (MacLennan, 1994). B cells require T cell help (at the minimum, CD40L and IL-4) at least at two checkpoints: (1) GC entry (Yeh et al., 2018) and (2) affinity selection (Victora et al., 2010). The GC reaction and affinity selection seem to be coupled to OxPhos and mTOR (Chen et al., 2021; Ersching et al., 2017; Weisel et al., 2020), while others have shown that glycolysis also plays an important role (Boothby and Rickert, 2017; Jellusova et al., 2017; Boothby et al., 2021). GC did form in DNT mice, albeit to a lesser extent. We propose that the metabolism of B cells having received T cell help in vitro (anti CD40 and IL-4) differs from exclusive BCR stimulation in vitro: BCR/CD40/IL-4-activated CRE B cells had the same low ATP rate index as the DNT-B cells; that is, most of the ATP was generated by glycolysis. Hence, DNT-B cells may not have a significant energetic disadvantage at the GC entry point and could form GC competitively. However, during affinity selection, the requirement for OxPhos may be forced in light-zone GC B cells (Chen et al., 2021). Consequently, DNT-B cells would have a strong disadvantage, the GC would collapse, and IgG-secreting cells would wane.

The survival of BM plasma cells, defined as CD138⁺B220^{low} (Lam et al., 2016), appears to require mitochondrial pyruvate, which is essential to generate citrate in the TCA cycle and, consequently, lipids. In that sense, the reduced OxPhos and diverted TCA cycle in LPS-activated DNT-B cells shifted the

overall equilibrium to glycerophospholipids (GPLs) with polyunsaturated fatty acyl chains and lowered the total abundance of PA, in particular PA species with saturated FAs such as palmitic acid (32:0). One reason for decreased saturated GPLs could be their facilitated β-oxidation (Schulz, 2008). It seems also plausible that lipid synthesis pathways are different in DNT-B cells, which requires further investigation. As for lipid synthesis, the responsiveness of mTORC1 to PA has likely evolved to sense lipid precursors for membrane biosynthesis prior to doubling the mass of a cell (Foster, 2013). In our hands, the addition of 32:0 PA enhanced mTORC1 activity only transiently but did not rescue proliferation or plasma cell development. This can be due to experimental conditions, or a function of acyl side chains of PA (Tei et al., 2021). Because TCA alterations can impact the microtubule cytoskeleton, which might prevent proper localization and activity of mTORC1 at the lysosomes (Wu et al., 2020), it may not even be possible to reverse mTORC1 activity in DNT-B cells. Another limitation of our work is that many results were obtained with short-lived LPS blasts and not with long-lived plasma cells.

Many tumor cells adjust to defective OxPhos and rely on reductive carboxylation of glutamate to produce citrate (Mullen et al., 2011). Unlike stochastically mutated tumor cells the DNT-B cells do have a functional complex II, SDH (Gustafsson et al., 2016). SDH is likely the driving enzymatic reaction in the TCA cycle of DNT-B cells due to loss of function in the other complexes. This shifts the equilibrium to more downstream (fumarate, malate) and fewer upstream metabolites (citrate, isocitrate, itaconate). Exactly this shift is seen in human B cells with gain-of-function SDH that show a proinflammatory phenotype (Burgener et al., 2019). Alternatively, the argininosuccinate shunt may be overactive in DNT-B cells similar to LPS-triggered M1 macrophages (Fall et al., 2020). Moreover, the stalled TCA cycle in DNT-B cells and HIF1a stabilization are remarkably similar to LPS-activated proinflammatory M1 macrophages that disassemble their OxPhos complexes during late differentiation (Kieler et al., 2021). Our mouse model will therefore enable future work that targets mechanisms of immunodeficiency and inflammation. In conclusion, we show that OxPhos, which requires the continuous replication and transcription of mtDNA, supplies B cells with TCA intermediates needed for mTORC1 activity and humoral immunity.

STAR*METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j. celrep.2022.110912.

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AUTHOR CONTRIBUTIONS

S.U., O.R.B., F.G., U.S-S., J.H., S.B., W.S., T.S., N.G., L.W., J.T., and V.T. performed experiments. S.U. and D. Mielenz designed the study. S.U., F.G., U.S-S., J.H., S.B., R.W., N.G., V.T., and D. Mielenz analyzed data. S.R.S. performed bioinformatics analyses. S.U., R.W., and D. Mielenz wrote the paper. D. Mougiakakos, U.S., K.C., and R.W. provided intellectual and infrastructural help.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community.

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STAR***METHODS**

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti CD16/32, clone 93	Biolegend	# 101302, RRID:AB_312801
Anti TACI/CD267 (APCH, clone eBio8F10-3)	eBioscience	Cat# 17-5942-82, RRID:AB_842758
Anti CD19 (APCHFire750, clone 6D5)	Biolegend	Cat# 115558, RRID:AB_2572120
Anti CD38 (PerCPCHy5.5, clone 90)	Biolegend	Cat# 102722, RRID:AB_2563333
Anti CD138 (PECy7, clone 281–2)	Biolegend	Cat# 142513, RRID:AB_2562197
Anti CD8a, (APCH, clone 53–6.7)	ebioscience	Cat# 17-0081, RRID:AB_469335
Anti CD4 (PE, clone gk1.5)	ebioscience	Cat# 12-0043-82, RRID:AB_464900
Anti GL-7 (AF647, clone GL-7)	BD Biosciences	Cat# 561529
Anti CD95 (PECy7, clone Jo2),	BD Biosciences	Cat# 554254, RRID:AB_395326
Anti CD21/35 (BV421, clone 7E9)	BD Biosciences	Cat# 558768, RRID:AB_397114
Anti CD23 (PE, clone B3D4)	eBioscience	Cat# 12-0232-82, RRID:AB_4655593
Anti IgM (PE)	Southern Biotech	Cat# 1021-09
Anti IgA (AF647)	Southern Biotech	Cat# 1040-31
Anti IgG (Cy5)	Southern Biotech	Cat# 1030-15
Anti GL7 (Pacific Blue)	Biolegend	Cat# 144613, RRID:AB_2563291
Anti IgD (AF647)	Southern Biotech	Cat# 1120-31
Anti F4/80 (APC, clone bm8)	eBioscience	Cat# 51-4801-80, RRID:AB_469791
Anti CD11b (BV510, M1/70)	Biolegend	Cat# 101245, RRID:AB_2561390
Anti CD11c (APC.Cy7, N418)	Biolegend	Cat# 117317, RRID:AB_493569
Anti Phospho-S6 Ribosomal Protein (Ser235/236) (PE, clone cupk43k)	eBioscience	Cat# 12-9007-42, RRID:AB_2573857
Goat anti mouse IgM-HRP	Southern Biotech	Cat# 1021-05
Goat anti mouse IgG-HRP	Southern Biotech	Cat# 1030-05
Goat anti-IgM AffiniPure F(ab')_2 μ chain specific	Jackson ImmunoResearch	Cat# 115-006-020, RRID:AB_2338469
Anti CD40 (clone FGK45)	Rolink et al., 1996	RRID:AB_2490239
Anti IRF4 (clone P173)	Cell Signaling	Cat# 4948, RRID:AB_2208963
Anti BLIMP1 (clone 6D3)	Santa Cruz	Cat# sc-47732, RRID:AB_628168
Anti Actin	Sigma-Aldrich	Cat# A2066
Anti XBP1s	Cell Signaling	Cat# 12782S
Anti HIF1a	Cayman	Cat# 10006421, RRID:AB_409037
Anti Phospho-S6 Ribosomal Protein (Ser235/236)	Cell Signaling	Cat# 2211S, RRID: AB_1658222
Anti S6 Ribosomal Protein	Cell Signaling	Cat# 64106, RRID: AB_2238583
Anti BCL6	Cell Signaling	Cat# 4242,RRID: RRID: AB_10694770
Anti cleaved Caspase-3	Cell Signaling	Cat# 9664, RRID:AB_2070042
Anti MT-CO1	Abcam	Cat# ab90668, RRID:AB_204999
Anti Tomm20	Abcam	Cat# ab78547, RRID:AB_2043078
Anti COXVa	Abcam	Cat# ab110262, RRID:AB_10861723
Anti NDUFS1	Abcam	Cat# ab22094, RRID:AB_2151098
Anti ATP5a	Abcam	Cat# ab103431, RRID:AB_10710826
Anti UQCRC2	Abcam	Cat# ab14745, RRID:AB_2213640
Goat Anti-Mouse IgG H&L (Alexa Fluor® 680)	Abcam	Cat# ab175775
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 790)	Abcam	Cat# ab175781

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Untargeted metabolomics and glycerophospholipid analysis	This manuscript	https://dx.doi.org/10.21228/M8K70K
¹³ C glucose flux analysis	This manuscript	https://www.ebi.ac.uk/ metabolights/MTBLS4647
Experimental models: Cell lines		
R26-K320E-TWINKLE loxP+/-	Baris et al. (2015)	N/A
Tg(Fcer2a-cre)5Mbu	Kwon et al. (2008)	N/A
Oligonucleotides		
16s rRNA fwd CCGCAAGGGAAAGATGAAAGAC	Invitrogen, Quiros et al., 2017	N/A
16s rRNA rev TCGTTTGGTTTCGGGGTTTC	Invitrogen, Quiros et al., 2017	N/A
ND1 fwd CTAGCAGAAACAAACCGGGC	Invitrogen, Quiros et al., 2017	N/A
ND1 rev CCGGCTGCGTATTCTACGTT	Invitrogen, Quiros et al., 2017	N/A
HK2 fwd GCCAGCCTCTCCTGATTTTAGTGT	Invitrogen, Quiros et al., 2017	N/A
HK2 rev GGGAACACAAAAGACCTCTTCTGG	Invitrogen, Quiros et al., 2017	N/A
Chemicals, peptides, and recombinant proteins		
4-Hydroxy-3-nitrophenylacetyl (Phycoerythrin)	Biosearch Technologies.	Cat# N-5070
4-Hydroxy-3-nitrophenylacetyl (keyhole limpet hemocvanin)	Biosearch Technologies.	Cat# N-5060
4-Hydroxy-3-nitrophenylacetyl (bovine serum albumin)	Biosearch Technologies.	Cat# N-5050
4-Hydroxy-3-nitrophenylacetyl (ficoll)	Biosearch Technologies.	Cat# F-1420
eFluor450	eBioscience	Cat# 65-0842-85
Imject Alum	Thermo Scientific	Cat# 77161
Glutaraldehyde EM grade	Carl Roth	Cat# 4995.1
Poly-L-Lysine	Sigma	Cat# P8920
Interleukin-4, premium grade	Miltenyi Biotech	Cat# 130-097-761
Interleukin-5, premium grade	Prepro Tech	Cat# 215-15
Lipopolysaccharide	Sigma	Cat# L3024
Transforming growth factor β	R & D systems	Cat# 7666-MB
Retinoic acid	Sigma	Cat# R2625
Mitotracker Green	Thermo Scientific	Cat# M7514
Tetramethylrhodamine	Thermo Scientific	Cat# T668
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	Sigma	Cat# C2759
Streptavidin (Cy5)	Jackson ImmunoResearch	Cat# 016-170-084
1,2-dipalmitoyl-sn-glyero-3-phosphate	Sigma	Cat# P4393
D- ¹³ C-Glucose	Sigma	Cat# 389374
Staurosporin	Sigma	Cat# S4400
Rapamycin	Sigma	Cat# R8781
Software and algorithms		
GraphPad Prism	Graphpad	https://www.graphpad.com/ scientific-software/prism/
Seahorse Wave Desktop Software	Agilent	https://www.agilent.com/en/product/ cell-analysis/real-time-cell-metabolic- analysis/xf-software/seahorse-wave-

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desktop-software-740897



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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Kaluza	Beckman Coulter	https://www.beckman.de/flow- cytometry/software/kaluza
R & Pheatmap Plugin		https://rdrr.io/cran/pheatmap/ (Kolde, 2015)
Other		
Sheep red blood cells	Fiebig Nährstofftechnik	Cat# 31100100

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Dirk Mielenz (dirk.mielenz@fau.de).

Materials availability

All reagents will be made available on request after completion of a Materials Transfer Agreement.

Data and code availability

- Metabolomic and lipidomic data are available at the NIH Common Fund's National Metabolomics Data Repository website. (https://dx.doi.org/10.21228/M8K70K) Flux measurement data are deposited at the EMBL-EBI MetaboLights database (https://www.ebi.ac.uk/metabolights/MTBLS4647)
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the Lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All experimental procedures were done in agreement with animal protocols approved by the government of Lower Franconia, Bavaria, Germany. Both female and male mice at 8–12 weeks of age were used in the experiments. Mice were maintained on a 12-h light/dark cycle with free access to food and water according to governmental rules. K320E-TWINKLE floxed mice (Baris et al., 2015) were crossed to CD23 CRE mice (Kwon et al., 2008; kindly provided by Meinrad Busslinger) to generate DNT animals. DNT mice used in these experiments had the genetic background DNT^{+/-} CRE^{+/-} and CRE control mice were DNT^{-/-} CRE^{+/-}. WT animals used in this study were DNT^{-/-} CRE^{-/-} littermates. All mice were on the C57BI/6 background.

METHOD DETAILS

Blue Native-PAGE

Mitochondria were enriched by using differential centrifugation, according to (Desquiret-Dumas et al., 2019). Mitochondria-enriched fractions were then treated with DNase I (ThermoFischer) at room temperature for 30 min, washed in 1 mL cold PBS, centrifuged (10000g, 4°C), and the pellet was finally resuspended in AC/BT buffer (1.5 M aminocaproic acid and 75 mM Bis–Tris/HCl, pH 7.0, supplemented with Complete Mini Protease Inhibitor (Roche Diagnostics, Stockholm, Sweden)) and kept frozen at −80°C until analysis. For super-complex assembly analysis, 20 µg of the samples were diluted at 1 mg/mL in AC/BT buffer and mitochondrial membrane proteins were solubilized by 1% digitonin for 10 min at 4°C. After centrifugation for 20 min, 20.000g at 4°C, the supernatant was collected, and 5% Serva Blue G dye (Biorad, Marnes-la-Coquette, France) in 1 M aminocaproic acid/50 mM Bis–Tris/HCl, pH 7 was added (1/20 v/v) prior to loading. Respiratory chain super-complexes were separated on Native PAGE Novex 3–12% Bis-Tris gels (Invitrogen) for approximatively 3 h and transferred on PVDF membranes (GE Healthcare, Velizy-Villacoublay, France) in cold blotting buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol). Membranes were indubated using dedicated monoclonal antibodies (NDUFS1, SDHA, UQCRC2, MT-CO1, Abcam). Acquisitions were performed using the Odyssey FC imaging system and analysed using Image StudioTM Lite (LI-COR Biosciences).

DNA extraction and RT-qPCR

Cell pellets were resuspended in PBS and DNA was isolated using the DNeasy Blood & Tissue Kit (Quiagen), according to manufacturer's instructions. qPCR was performed using SYBR Green I-dTTP (Eurogentec) using the Applied Biosystems 7500 real-time PCR system. Samples were analyzed in triplicates and *16s rRNA* and *nd1* copies, representing mtDNA, were normalized to the single copy nuclear *hk2* gene (Quiros et al., 2017).





Electron microscopy

Viable cells were collected by ficoll density gradient centrifugation and were fixed overnight in 2.5% glutaraldehyde (Carl Roth, 4995.1) in PBS. Cells were further processed and analyzed, as described previously (Steinmetz et al., 2020).

Enzyme-linked immunosorbent assay (ELISA)

NP-specific antiobodies were quantified in duplicates serially diluted on 96-well flat-bottom microtiter plates (Greiner bio-one) coated with 1 µg/mL NP₄-BSA or NP₂₀-BSA conjugates (Biosearch Technologies) in 50 µL/well coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃) overnight at 4°C. Captured NP-specific Abs were detected with goat anti-mouse IgM and IgG specific horseradish peroxidase (HRP)-coupled Abs (1:1000, Southern Biotech). Plates were normalized using ELISA IgM and IgG standards as internal reference. For analysis of total Ig, microtiter plates were coated with 1 µg/mL goat anti-IgM/anti-IgG in coating buffer overnight at 4°C and then blocked for 1h at room temperature with 275µL/well of PBS, 2 % FCS. Samples were analyzed in duplicates diluted serially 2-fold in PBS, 2% FCS for 1h at room temperature. Plates were washed 3 times with PBS, 0.05% Tween 20 and incubated with goat antimouse IgM-HRP/IgG-HRP in PBS, 2% FCS. After washing, plates were developed using developed using TMB substrate reagent (BD OptEIA) and acid stop (0.5 M H₂SO₄). Optical density (OD) was measured at 450nm on a FLUOstar Omega Microplate Reader (BMG Labtech). Plates were normalized using ELISA IgM and IgG standards as internal reference.

Extracellular flux analysis

The day before cell plates were coated with 10 μ g/mL Poly-L-Lysine in 1x TE buffer. Day 3 LPS blasts were seeded at a density of 2.5 × 10⁵ cells/well, measured at least in triplicates. Extracellular flux analysis was performed as described (Stein et al., 2017). Mito Stress Test and ATP rate assays were performed according to manufacturer's instructions (Agilent).

Flow cytometry

 2×10^{6} -4 × 10^{6} cells were pelleted in FACS tubes (Micronic) at 300 x g for 5 min at 4°C and blocked with anti-CD16/32 Ab (10 µg/mL in FACS-buffer (PBS containing 2% FCS, 0.05% sodium azide)) for 15 min on ice. Cells were washed once with FACS-buffer, resuspended in 50µL FACS-buffer containing the respective fluorochrome-coupled Abs and incubated for 20 min on ice in the dark. Cells were washed with FACS-buffer and measured in a Gallios flow cytometer (Beckman Coulter) or cells were sorted with various cell sorters (https://www.facs-core-erlangen.de/zellsortierung/geraete/). Analyses were performed using Kaluza version 1.3 and 2.1 (Beckman Coulter). Abs and other reagents are described in the key resources table. Mitotracker and Tetramethylrhodamine staining were performed as described previously (Cossarizza et al., 2019; Stein et al., 2017). Cleaved Caspase-3 was stained intracellularly using a commercial fixation and permeabilization kit and secondary antibody (Biolegend). Data presentation and analysis was performed according to (Cossarizza et al., 2019). Frequencies in the depicted gates are indicated by numbers.

Glycerophospholipid analysis

Glycerophospholipids (PCH, PE, PI, PS, PG, PA) in B-cells were analyzed by Nano-Electrospray Ionization Tandem Mass Spectrometry (Nano-ESI-MS/MS) with direct infusion of the lipid extract (*Shotgun Lipidomics*): 14 to 45×10^6 cells were homogenized in 300 µL of Milli-Q water using the Precellys 24 Homogenisator (Peqlab, Erlangen, Germany) at 6.500 rpm for 30 s. The protein content of the homogenate was routinely determined using bicinchoninic acid. To 100 µL of the homogenate 400 µL of Milli-Q water, 1.875 mL of methanol/chloroform 2:1 (v/v) and internal standards (125 pmol PCH 17:0–20:4, 132 pmol PE 17:0–20:4, 118 pmol PI 17:0–20:4, 131 pmol PS 17:0–20:4, 62 pmol PG 17:0/20:4, 75 pmol PA 17:0/20:4 Avanti Polar Lipids) were added. Lipid extraction and Nano-ESI-MS/MS analysis were performed as previously described (Kumar et al., 2015). Endogenous glycerophospolipids were quantified by referring their peak areas to those of the internal standards. The calculated glycerophospolipid amounts were normalized to the protein content of the tissue homogenate. Data were deposited with the Metabolomics Workbench (Sud et al., 2016) (https://dx.doi.org/ 10.21228/M8K70K).

Immunohistology

Mice were immunized with SRBCs, sacrificed after 10 days and spleens were embedded in OCT medium (Tissue-Tek). Cryotome sections of 8 μm were prepared, fixed in acetone, blocked with 20 μg/mL anti-CD16/23 in 1% PBS, 10% FCS, 2% BSA) and stained with anti-IgD-AF647, PNA-Rhodamine and GL7-PacificBlue. Slides were sealed with MOWIOL and cover slips and analyzed by confocal microscopy (Zeiss LSM 700, Optical imaging center Erlangen).

Immunization

Mice (8–12 weeks old) were injected with 100 µg NP₂₉-KLH (Biosearch Technologies) in Alum (ThermoScientific) in a 1:2 ratio (200µL total volume) or in PBS after 6 weeks for boost immunization (*i.p.*). NP-FicoII (50 µg in 200 µL PBS; Biosearch Technologies) was injected *i.p.*

Isolation of primary murine cells from spleen and bone marrow

Spleen of 8–12 week old mice was transferred into cold 2% FCS (in PBS) and gently passed through a 70 µm cell strainer (BD) using the plunger of a 5 mL syringe (BD). Femur and tibia were flushed with cold 2% FCS using a 27 G cannula (BD). Cell suspensions were



pelleted by centrifugation at 300 x g for 5 min at 4°C. Erythrocytes were lysed in red blood cell-lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 100 μ M EDTA) for 5min at room temperature. The reaction was stopped by adding cold 2% FCS before centrifugation at 300 x g for 5 min at 4°C. The final cell suspensions were kept in cold 2% FCS after filtration through 30 μ m mesh filter (Sysmex).

Intracellular ATP measurement

The amount of intracellular ATP/5 \times 10⁴ cells was determined using the ATP Bioluminescence Assay Kit HS II (Roche) according to manufacturer's instructions.

In vitro cultivation of primary murine B-cells

Splenic B-cells were cultured with a starting concentration of 0.5×10^6 cells/mL in R10 medium (RPMI1640, 10% fetal calf serum (FCS), 2 mM glutamate, 1 mM sodium pyruvate, 50 U/mL penicillin G, 50 µg/mL streptomycin, 50 µM β -mercaptoethanol) for 72 h at 37°C and 5% CO₂, supplemented with 10 µg/mL LPS. For *in vitro* class switch recombination cells were seeded at 0.1 × 10⁶ cells/mL in R10 medium for 96 h, supplemented with 5 ng/mL transforming growth factor β , 5 nM retinoic acid, 10 µg/mL anti-CD40 antibody, 10 µg/mL LPS, 100 U/mL IL4 and 10 ng/mL IL5 (Chiu et al., 2019).

Lactate and glucose measurements

Supernatants of LPS activated B-cells were harvested after 3 days and analyzed with a SUPER GL compact analyzer according to the manufacturer's instructions.

Liposomes

Liposome and polymersome production was performed as described (Poschenrieder et al., 2016). 11.4 mL of vesicle buffer (10 mM TRIS-HCl, 150 mM NaCl, pH 8.0) were poured into unbaffled miniaturized stirred tank reactors (BioREACTOR48, 2mag, Munich, Germany) and stirred at 4.000 rpm at 25°C using an S-type stirrer. Then, 600 μ L of the dissolved phospholipid 16:0 PA (1,2-dipalmitoyl-sn-glyero-3-phosphate) (10 mM in chloroform) were injected under stirring, leading to a whitish highly dispersed emulsion. The reactor shell, originally made of polystyrene, has been replaced by borosilicate glass, in order to avoid damaging by the chloroform. The process was continued, until the solution became transparent, indicating the evaporation of the chloroform, which was the case after 6 h. Subsequently, the solution was centrifuged at 13,000 rpm in a tabletop centrifuge to remove precipitates. The liposomes in the supernatant were then concentrated to 1.5 mg mL⁻¹ by centrifugation for 50 min at 50,000 g and resuspending of the resulting pellet in the appropriate amount of vesicle buffer. The quality of the liposomes during and after the production process was monitored via dynamic light scattering using a Zetasizer NS (Malvern, Worcestershire, UK) as reported (Poschenrieder et al., 2016).

Metabolomics

Experimental Setup I: Splenic B-cells were isolated, activated with LPS and viable cells, only GFP⁺ for DNT, were sorted after 3days using flow cytometry. Perchloric acid extraction was performed and metabolic profiles as previously published (Hofmann et al., 2011). Data were deposited with the Metabolomics Workbench (Sud et al., 2016) (https://dx.doi.org/10.21228/M8K70K). Experimental Setup II: B-cells were isolated and activated with LPS. 24 h after activation, half of the cells were switched to custom RPE media containing 1g/L U¹³C glucose (Sigma) and incubated for another 48 h. Cells were lysed in 80% MeOH. After centrifugation, supernatants were transferred into HPLC vials, dried and reconstituted in 200 μ L eluent. Flux measurements were performed via reverse phase HPLC coupled to a high resolution mass spectrometer (Orbitrap, Thermo Fisher Scientific) in negative polarization mode. The results were analyzed and quantified statistically using Compound Discoverer version 3.2 (Thermo Fisher). Data were deposited with the Metabolights tool (Haug et al., 2013) (https://www.ebi.ac.uk/metabolights).

Purification of murine B lymphocytes from spleen

B-cells were enriched from splenic cell suspensions using the EasySep Mouse B-cell isolation negative selection kit (EasySep #19854, Stemcell Technologies) according to the manufacturer's instructions. In short, spleen cells were resuspended in MACS buffer (PBS, 2% FCS, 2 mM EDTA), blocked with rat serum and immuno-magnetically enriched. Purity of isolated B-cells was certified by CD19 staining. Typical enrichments were >95%.

Western Blot

Proteins were electrophoresed on 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes (45 min, 400 mA). Transfer efficiency was determined by Ponceau S. Membranes were blocked in 5% skimmed milk powder in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) and probed with the respective antibody at 4°C overnight diluted in 3% BSA in PBS, containing 0.1% Tween 20 and 0.1% Sodium Azide. Membranes were washed in TBST and incubated with anti-mouse/rabbit/rat IgG HRP conjugates diluted in 5% skimmed milk powder in TBST for 1 h at room temperature. After washing, blots were developed by enhanced chemiluminescence. Scanned X-ray films were converted to 8-bit type and color inverted. Band intensities were measured, and background of X-ray films was subtracted. Quantification was performed by densitometric analysis with ImageJ. Protein expression was normalized to β -Actin.





Western Blot analysis of mitochondrial proteins

Cell were lysed in RIPA buffer 1x (Merck) and 40 µg cellular proteins solubilized in Laemmli buffer were resolved by SDS-PAGE as in (Guillet et al., 2011). The following antibodies were sequentially applied: MT-CO1 (mouse monoclonal, Abcam) with TOMM20 (Rabbit monoclonal, Abcam), COXVa (Mouse monoclonal, Abcam) with ATP5A (Mouse monoclonal, Abcam) and UQCRC2 (Mouse monoclonal, Abcam). Goat Anti-Mouse IgG H&L (Alexa Fluor 680) and Goat Anti-Rabbit IgG H&L (Alexa Fluor 790) (Abcam) were used as secondary antibodies. Near-infrared acquisitions were performed using the Odyssey FC imaging system and analyzed using Image Studio Lite (LI-COR Biosciences).

QUANTIFICATION AND STATISTICAL ANALYSIS

Values were assessed for Gaussian distribution using the Shapiro-Wilk normality test. Mann-Whitney test was used for non-Gaussian distributed datasets. Datasets revealing Gaussian-like distribution were assessed by Student's t test. Differences between the analyzed groups were considered to be statistically significant with p values <0.05. *, p < 0.05, **, p < 0.01, ***, p < 0.002, ****, p < 0.0004. Data were analyzed using Prism (GraphPad) and R using the Pheatmap plugin (Kolde, 2015). N = number of experiments, n = number of replicates per experiment.