Asparagine availability controls B cell homeostasis

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Abstract

Germinal centre (GC) B cells proliferate at some of the highest rates of any mammalian cell, yet the metabolic processes which enable this are poorly understood. We performed integrated metabolomic and transcriptomic profiling of GC B cells, and found that metabolism of the non-essential amino acid asparagine (Asn) was highly upregulated. Asn was conditionally essential to B cells, and its synthetic enzyme, asparagine synthetase (ASNS) was upregulated following their activation, particularly more markedly in the absence of Asn, through the integrated stress response sensor general control non-derepressible 2 (GCN2). When *Asns* is deleted B cell survival and proliferation in low Asn conditions were strongly impaired, and removal of environmental Asn by asparaginase or dietary restriction markedly compromised the GC reaction, impairing affinity maturation and the humoral response to influenza infection. Using stable isotope tracing, we found that metabolic adaptation to the absence of Asn requires ASNS, and that oxidative phosphorylation, mitochondrial homeostasis, and synthesis of nucleotides was particularly sensitive to Asn deprivation. Altogether, we reveal that Asn metabolism acts as a key regulator of B cell function and GC homeostasis.

Introduction

The germinal centre (GC) reaction is essential for effective humoral immunity[1]. Following encounter with antigen, B cells organise in secondary lymphoid tissue and enter the GC reaction, a cyclic process in which somatic hypermutation (SHM) in a microanatomic region known as the dark zone (DZ) leads to random mutation of immunoglobulin genes. B cells then compete to interact with and receive help from T follicular helper (T_{FH}) cells in the

microanatomic light zone (LZ). This process continues as affinity maturation occurs and memory B cells or plasma cells are generated.

GC B cells have some of the highest proliferation rates of all mammalian cells, yet their metabolism is unusual and incompletely understood[2]. Unlike most rapidly dividing immune cells, GC B cells predominantly use fatty acid oxidation and OxPhos rather than glycolysis[3–7], despite residing in a hypoxic and poorly vascularised micro-environment[8]. The key metabolic pathways that are important in their homeostasis have not been fully defined.

Amino acid availability is a critical regulator of T cell responses, demonstrated either by alteration of environmental abundance, synthesis, or interference with cellular import through solute transporters[9–15]. Amino acids are fundamentally required for protein synthesis, but are central to other metabolic processes[16], and it is apparent in T cells that availability of specific amino acids can have profound effects on their homeostasis.

In contrast, although it is known that B cell-derived lymphomas are sensitive to amino acid deprivation[17–20], and whilst loss of the capacity to synthesise serine, or deletion of CD98, a component of large neutral amino acid transporters, impairs GC formation, the importance of amino acid availability more broadly in B cell metabolism and humoral immunity is poorly understood[21].

Here, we find that GC B cells have high levels of amino acid uptake and protein synthesis in vivo. Using integrated multiomic analysis we identify asparagine (Asn), a non-essential amino acid, as a critical regulator of B cell homeostasis and GC formation. Mice with conditional deletion of asparagine synthetase (ASNS) in B cells have selective impairment of GC formation upon depletion of environmental Asn, compromising humoral immunity. ASNS is regulated by sensing through the integrated stress response, and whilst most Asn in B cells is obtained from the extracellular environment following activation, mechanistically, loss of ASNS leads to profound metabolic dysfunction characterised by impairment of OxPhos and failure of nucleotide synthesis, which can be rescued by nucleotide supplementation.

Results

GC B cells have highly active protein synthesis and asparagine metabolism

To evaluate protein synthesis rates in GC B cells, we immunised mice with sheep red blood cells (SRBC) and first examined expression of CD98, which forms heterodimers with the amino acid transporter protein SLC7A5 to form L-type amino acid transporter 1 (LAT1), at day 9 following immunisation. As previously reported, CD98 was markedly upregulated in GC B cells compared with IgD$^+$ naïve B cells (Extended Data Fig. 1A-B)[21], suggesting high amino acid uptake. To more directly estimate protein synthesis rates in GC B cells in vivo and in their spatial context, we used bio-orthogonal non-canonical amino acid tagging (BONCAT), with the amino acid analogues L-azidohomoalanine (L-AHA), and O-propargyl-puromycin (OPP) visualised in situ using Click chemistry[22]. OPP is a puromycin analogue which enters the acceptor site of ribosomes and is incorporated into nascent proteins. L-AHA is an unnatural amino acid, which does not lead to ribosomal stalling once included in polypeptides. To label GC B cells, we immunised mice expressing Rosa26^{STOP}tdTomato under the control of *Aicda-Cre*, with SRBC. At day 14, we first injected a pulse of L-AHA and then after four hours a pulse of OPP, with analysis one hour later (Fig. 1A). We then used distinct Click chemistry reactions to fluorescently co-label OPP and L-AHA in splenic sections. We found that incorporation of L-AHA and OPP were markedly upregulated in the GC compared with the surrounding follicle of naïve B cells, indicating highly active protein

synthesis (Fig. 1B). We also labelled GC B cells with OPP ex vivo, and quantified its incorporation by flow cytometry. This confirmed a significant increase in OPP signal in GC B cells compared with naïve IgD^+ B cells (Fig. 1C-D). Within the GC B cell population, signal was highest in dark zone ($CXCR4^{hi}$ $CD86^{low}$) GC B cells, which are undergoing proliferation and SHM. GC B cells therefore have high rates of both amino acid transporter expression and protein synthesis.

We next directly profiled the metabolite content of GC B cells. To do so, we isolated GC and naïve B cells from pools of wild type mice following immunisation with SRBC and quantified key metabolites using liquid chromatography-mass spectrometry (LC-MS) (Fig. 1E). This revealed a broad increase in the levels of metabolites in GC B cells, with overrepresentation of intermediates of glycolysis, the TCA cycle, and most amino acids (Fig. 1F). Notably however, nucleotide precursor molecules (e.g.adenosine and hypoxanthine) were depleted in GC B cells, potentially reflective of their consumption during the nucleic acid synthesis required for high rates of proliferation. To refine our approach, we fused metabolite and transcriptional datasets[23] and performed integrated pathway analysis on the combined data using hypergeometric set testing (Fig. 1G). We found that there was enrichment of amino acid metabolic pathways in GC B cells, most notably the Kyoto Encyclopedia of Genes and Genomes (KEGG) Alanine, Aspartate, and Glutamate (AAG) metabolism pathway. Examination of the leading-edge genes of the AAG pathway in an independent transcriptional dataset revealed an approximately 40-fold increase in the expression of asparagine synthetase (*Asns*) and a 15-fold increase in glutamic oxaloacetic transaminase-2 (*Got2*) in GC B cells compared with naïve follicular B cells (Fig. 1H)[24]. We confirmed upregulation of *Asns* and *Got2* in sorted GC B cells by qPCR (Fig. 1I) and by immunoblotting (Extended Data Fig. 1C).

ASNS synthesises Asn from aspartate (Asp) in an ATP-dependent reaction, and GOT2 synthesises Asp from the TCA cycle intermediate oxaloacetate[25] (Fig. 1J). The relative levels of both Asn and Asp were markedly increased in GC compared with naïve B cells, which had equivalent glutamate (Glu) levels (Fig. 1K).

To examine ASNS expression in its spatial context, we performed multiplex imaging of human tonsils (Fig. 1L and Extended Data Fig. 1D). ASNS was strongly expressed in GCs and $MZB1^+$ plasmablasts. There was negligible expression in the IgD^+ B cell follicle or in the $CD3^+$ T cell zone. ASNS levels were spatially regulated within the GC, with higher expression in the DZ, where proliferation and SHM occurs (Extended Data Fig. 1D-E). We also performed immunostaining for the amino acid transporter ASCT2, the main transporter of Asn and which also transports L-AHA[13,26], and found high expression in GC B cells, more pronounced in the DZ like ASNS (Fig. 1M and Extended Data 1F). Asn metabolism is therefore one of the most upregulated pathways in GC B cells and plasmablasts, suggesting that it may play an important role in their physiology.

B cells require ASNS when the availability of Asn is limited

Asn is a non-essential amino acid and can be either acquired exogenously or synthesised by ASNS. To understand these relative contributions to Asn metabolism, we first functionally evaluated Asn uptake in ex vivo GC B cells, using an indirect assay in which the bio-orthogonal amino acid analogue homopropargylglycine (HPG) competes for transport through ASCT2 with Asn (Fig. 2A). HPG is then fluorescently labelled using Click chemistry, and its uptake measured by flow cytometry[26]. We found that there was clear dose-dependent competition between HPG and Asn in GC B cells and plasmablasts (Fig. 2B). This was most marked in DZ GC B cells, in keeping with their high levels of OPP incorporation and ASNS

expression, and indicating substantially elevated Asn uptake compared to naïve B cells (Fig. 2C).

To understand the dynamics of Asn uptake following B cell activation, we then stimulated wild type B cells in vitro with either the TLR9 agonist CpG and agonistic anti-CD40 antibody, or IL-4 and agonistic anti-CD40, for 24h in the presence of ^{15}N-labelled Asn, and quantified amino acids by mass spectrometry. We found a generalised increase in the intracellular concentrations of most amino acids, but in particular proline (Pro), glutamate (Glu), and Asn (Fig. 2D). The majority of Asn was labelled with ^{15}N in all conditions, indicating high exogenous uptake, and in preference to endogenous synthesis, which is bioenergetically demanding (Fig. 2E).

Next, to determine the requirements of B cells for exogenous Asn, we stimulated them with agonistic anti-CD40 and IL-4 in RPMI-1640 media with varying concentrations of Asn. Standard RPMI-1640 contains supraphysiologic concentrations of Asn (378µM) compared with those found in plasma (~40µM) and cerebrospinal fluid (CSF) (~4µM)[27,28]. When B cells were deprived of Asn or supplemented with a low concentration of Asn (4µM), and stimulated with IL-4 and anti-CD40 for 72h, there was a severe reduction in cell viability and proliferation, compared with supplementation of Asn at 40µM or 400µM concentrations (Fig. 2F-G). We noted no decrease in cell proliferation or viability on withdrawal of exogenous aspartate (Asp) and/or Glu (Fig. 2H and Extended Data Fig. 2A). There was a reduction in the expression of the activation markers CD86 and MHCII following Asn deprivation, nascent protein synthesis measured by OPP incorporation was lower at 24h (Extended Data Fig. 2B-D), and apoptosis was increased (Extended Data Fig. 2E). ASNS levels in B cells increased substantially over time following stimulation, and the absence of Asn led to higher ASNS expression at 24h (Fig. 2I). ASNS was increased with synergistic IL-4 and anti-CD40 stimulation (Extended Data Fig. 2F).

To determine the role of Asn synthesis in B cell homeostasis, we generated *Cd79a-Cre* × *Asns* ^{LoxP} mice (B-Asns hereafter), in which Cre recombinase is expressed at earliest stages of B cell development[29]. There was no evident B cell developmental defect and the mature B cell compartment was normal, with effective deletion of *Asns*. (Fig. 2J, Extended Data Fig. 2G-I).
Survival, proliferation, and activation of B-Asns B cells were severely compromised when Asn was absent in the culture medium, to a greater extent than B-WT B cells, but normal in its presence (Fig. 2K-L). In vitro culture systems lack the spatial organisation and cellular complexity of lymphoid tissue, and may therefore reproduce normal immune dynamics less well. We used a live ex vivo lymph node slice platform to investigate how Asn deprivation affected GC B cells from mice immunised with 4-hydroxy-3-nitrophenylacetyl-chicken gamma globulin (NP-CGG) in alum (Fig. 2M)[30]. We found that GC B cells from B-Asns lymph nodes were much reduced after 20h of culture without Asn, but total B cell proportions were unaffected (Fig. 2N, Extended Data Fig. 2J). Since GC B cells express ASNS, we reasoned that it conferred protection against Asn deprivation. We therefore pre-stimulated B-Asns or B-WT B cells in the presence of Asn, which was then withdrawn. In contrast to when Asn was initially absent, B-WT B cells were minimally affected by the absence of Asn (Fig. 2O). However in B-Asns B cells this protective effect was attenuated. Plasmablast differentiation induced by LPS and IL-4 was completely abolished in B-Asns B cells when Asn was absent or at low concentrations (10µM), but remained unchanged in the presence of Asn (Fig. 2P-Q, Extended Data Fig. 2K).

Asn uptake and synthesis is therefore specifically required during initial B cell activation, and loss of synthetic capacity leads to severely compromised cell division and survival in B cells in low Asn conditions.

The GC reaction is sensitive to Asn deprivation

To understand the effect of Asn deprivation on the GC reaction in vivo, we treated B-Asns and B-WT mice with asparaginase (ASNase), an enzyme of bacterial origin which rapidly and effectively hydrolyses Asn and is a well-established treatment for leukaemia[31]. We administered ASNase from one day prior to immunisation with SRBC, and then every two days over a nine day period before analysis (Fig. 3A).

We found that following ASNase administration, the GC reaction was markedly compromised, with reduced GC B cell proportions, numbers, and GC area, to a much greater extent in B-Asns mice (Fig. 3B-D), but with minimal effects on naïve B cells (Extended Data Fig. 3A). There was also a substantial reduction in plasma cell numbers with treatment, independent of genotype (Fig. 3E, Extended Data Fig. 3B).

Within the GC B cell compartment, there was a reduction in DZ phenotype cells in B-Asns mice, in keeping with our finding of increased Asn uptake, and elevated ASNS and amino acid transporter expression suggesting higher dependence on Asn metabolism (Fig. 3F-G). We did not detect a defect in proliferation in GC B cells, as measured by incorporation of the thymidine analogue EdU in vivo (Extended Data Fig. 3C-D).

T_{FH} cells, which provide help to GC B cells were not affected proportionally or numerically (Fig. 3H, Extended Data Fig. 3E-F), which may have been expected if they shared a common metabolism due to their microenvironment. The FoxP3$^+$ T follicular regulatory cell (T_{FR}) population also remained unchanged (Extended Data Fig. 3G-H). When considered in relation to T_{FH} proportions, there was a severe mismatch between GC B cells and T_{FH} in ASNase treated B-Asns mice, suggesting a selective impact on GC B cells (Fig. 3I).

Deprivation of Asn with ASNase therefore strongly compromises the GC reaction and plasmablast formation, an effect amplified when the capacity to synthesise Asn is lost.

Asn is required for GC B cell affinity maturation

In order to examine the requirement for Asn in a more physiologically relevant setting, and over a longer time course, we switched to dietary limitation of Asn. To minimise confounding due to cage effects or food intake, we first generated mixed chimeras with CD45.1 wild type and CD45.2 B-Asns or B-WT bone marrow. After 8 weeks, mice were given either an Asn-free or normal diet for 12 days, immunised with SRBC, and analysed after a further 9 days of dietary modification (Fig. 4A).

The relative proportion of GC B cells in CD45.2 B-Asns B cells was much lower in mice fed an Asn-free diet, and the DZ/LZ ratio was reversed compared to B-WT cells (Fig. 4B-C), which reproduced our findings with ASNase. Also reduced were splenic plasma cells of B-Asns origin (Fig. 4D). We found a marked reduction in the frequency of SRBC-binding B-Asns-derived GC B cells in mice fed an Asn-free diet (Fig. 4E, Extended Data Fig. 4A), suggesting impaired development of antigen binding clones.

We then examined the effect of dietary Asn deprivation on the humoral response to the protein-hapten conjugate immunogen NP-CGG in a non-chimeric setting, in B-Asns and B-

WT mice (Fig. 4F). This allowed us to follow the GC response over time by measuring antibody affinity maturation,

At day 14, levels of IgG1 anti-NP antibodies binding to high conjugation NP-BSA (NP_{20}), were lower in B-Asns mice deprived of Asn, and there was substantially reduced binding to low conjugation NP_2 in sera, also seen in B-WT mice given an Asn-free diet (Fig. 4G-J), which was maintained over time. This indicated that affinity maturation was defective in this setting.We did not observe a large difference in anti-NP IgM, which is of likely extrafollicular origin (Extended Data Fig. 4B). Forty days following immunisation, we found that there was a substantial decrease in the NP-binding memory B cell compartment (Fig. 4K, Extended Data Fig. 4C).

Having shown that Asn deprivation leads to compromised GC output, we then asked if supplementation with exogenous Asn would enhance it. We administered Asn in drinking water to wild type mice fed a standard diet for 14 days before immunisation with NP-CGG, and 14 days later quantified IgG1 anti-NP antibodies. We did not observe an increase in antibody concentration or affinity maturation (Extended Data Fig. 4D-E). This suggested that the amount of Asn provided by a standard diet was fully sufficient for the GC reaction over this timescale.

Asn synthesis in B cells by ASNS therefore acts to maintain GC B cell function in the face of dietary Asn deprivation.

Asn metabolism controls the humoral response to influenza infection

The GC reaction is integral to humoral immune responses following flu infection. Therefore, we investigated whether Asn metabolism could affect influenza-induced GC and antibody generation. Mice were assigned to either an Asn-free or normal diet for 12 days, as previously described, and then intranasally infected with the X31 H3N2 influenza A strain (Fig. 5A). Starting from day 3, characteristic weight loss was observed, peaking around day 7. Viral clearance during primary influenza infection is largely mediated by the activity of T cells and other non-B cell immune populations, which coincides with the initiation of recovery in mice[32]. We found that the rate of weight recovery under all conditions remained comparable, suggesting that there was no important defect in the non-B cell compartment of experimental mice (Fig. 5B).

We initially examined the antibody response to X31 influenza in mouse sera. At day 14 post-infection, levels of anti-X31 IgG antibodies were substantially lower in B-Asns mice fed an Asn-free diet (Fig. 5C-D). However, we did not observe a significant difference in anti-X31 IgM levels, consistent with our findings from NP-CGG immunization (Fig.5C, Extended Data Fig. 5A). Affinity maturation in GCs is essential for the development of influenza specific high-affinity antibodies. We therefore assessed the functional affinity (avidity) of X31-specific antibodies by ELISA, using the chaotropic agent urea at increasing concentrations to elute weakly binding antibodies[33]. We observed that anti-X31 IgG antibodies from B-Asns mice fed an Asn-free diet were significantly more sensitive to urea treatment, suggesting markedly impaired affinity (Extended Data Fig. 5B, Fig. 5E). As expected, anti-X31 IgM antibodies exhibited greater sensitivity to urea, to a similar extent across all experimental groups, indicating lower avidity compared to anti-X31 IgG antibodies, owing to the lack of affinity maturation in their extrafollicular origin (Extended Data Fig. 5C). At day 21 post-infection, levels of anti-X31 IgG antibodies remained significantly lower in B-Asns mice fed an Asn-free diet, reproducing the findings at day 14 (Fig. 5F).

Influenza infection induces GCs in the mediastinal lymph nodes and spleen in mice[34]. At day 21, we noted a severe numerical and proportional reduction in mediastinal GC B cells in B-Asns mice deprived of Asn (Fig. 5G-H). Additionally, there was a substantial reduction in the relative ratio of GC B cells to T_{FH} cells and the ratio of DZ to LZ GC B cells, resembling the phenotype observed with ASNase treatment (Extended Data Fig. 5D-E). Defects in splenic GC B cells were also present in B-Asns mice following treatment with an Asn-free diet, largely reproducing the phenotype observed in the mediastinal lymph nodes (Fig. 5I, Extended Data Fig. 5F).

Asn metabolism therefore regulates GC-derived humoral immune responses during influenza infection, and *Asns* plays an indispensable role in this process when Asn availability is restricted.

Disruption of Asn availability alters B cell metabolism

To understand how Asn withdrawal affects B cell metabolism, we performed stable isotope resolved LC-MS on B cells from B-WT or B-Asns mice, stimulated for 72h in Asn-replete media, before switching for 24h into media with or without Asn, and [U-^{13}C]-glutamine, or $^{15}N_1$-glutamine, labelled at either the amine or amide nitrogen position (Fig. 6A). Since ASNS transfers the amide nitrogen group of glutamine to that of asparagine (acting as an amidotransferase), positional labelling can provide information on ASNS activity[25].

Intracellular Asn levels were greatly reduced in Asn-deprived conditions in both B-WT and B-Asns B cells, confirming our previous observation that exogenous uptake is dominant, but to significantly greater degree in those from B-Asns mice (Fig. 6B). B-WT cells did not engage in significant Asn synthesis when Asn was present, with negligible labelling of Asn with Gln-derived ^{15}N from either the amino or amide position, despite ASNS expression (Fig 6C-D). Following Asn deprivation, around half of the intracellular Asn pool was ^{15}N-labelled in B-WT B cells, reflecting de novo synthesis by ASNS. As expected, B-Asns B cells were incapable of Asn synthesis and no Asn was ^{15}N labelled. There was minimal labelling of Asn from ^{13}C glutamine, but around a quarter of Asp was labelled, with no difference between B-WT and B-Asns B cells, suggesting very little of the Gln-derived Asp pool was diverted to Asn synthesis (Fig. 6E, Extended Data Fig. 6A).

In B-WT cells, Asn deprivation had subtle effects on metabolite relative abundance, but significantly reduced fractional labelling from [U-^{13}C]-glutamine and $^{15}N_1$-glutamine. Despite small differences in intracellular Asn, B-Asns B cells exhibited profound metabolic dysfunction upon Asn withdrawal, with large reductions in metabolite relative amounts and glutamine-derived ^{15}N incorporation (Fig. 6F).

We next performed pathway analysis to systemically describe these results. Comparing B-WT and B-Asns B cells, both deprived of Asn, there were a common set of significantly different pathways (Fig. 6G), including AAG, tRNA biosynthesis, glutathione synthesis, sphingolipid metabolism and glyoxylate. We noted that B-Asns B cells, when deprived of Asn, substantially downregulated metabolites in the TCA cycle, nucleotide biosynthesis. pentose phosphate pathway, and β-alanine metabolic pathways (Fig. 6G). Asn is therefore essential to regulate metabolic homeostasis in B cells.

Mitochondrial function in B cells requires Asn

Given the abnormalities in TCA cycle metabolism we observed following Asn withdrawal, we next performed extracellular flux analysis to explore OxPhos and glycolysis in a dynamic

setting. We found that Asn concentration was strongly associated with oxygen consumption rate (OCR) and extracellular acidification (ECAR), reflecting OxPhos and lactate production respectively (Fig. 7A-E, Extended Data Fig. 7A-B), and the deleterious effect of low Asn was more pronounced in B-Asns B cells (Fig 7C-D). In keeping with our metabolite profiling, this effect was only seen when Asn was limited. Surprisingly, the maximal respiration induced by the mitochondrial uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) remained unchanged between B-WT and B-Asns B cells across all Asn concentrations, resulting in a significantly higher maximal-to-basal respiration ratio, known as the spare respiratory capacity (SRC), in B-Asns B cells (Fig. 7E, Extended Data Fig. 7B).

Supplementation with a cell-permeable analogue of the TCA cycle metabolite alpha-ketoglutarate (α-KG), dimethyl 2-oxoglutarate (DM-OG) has been reported to increase cell viability in T cells treated with L-asparaginase[35]. We therefore hypothesized that supplementation with DM-OG could potentially restore defective mitochondrial metabolism and improve B cell homeostasis in Asn-deficient conditions. However, DM-OG failed to rescue the defective B cell survival in B-WT and B-Asns B cells under Asn-free conditions (Extended Data Fig. 7C).

We next directly imaged mitochondria in B-WT and B-Asns B cells using 3D Lattice structured illumination microscopy (SIM) (Fig. 7F). Mitochondrial volume and count were not different when Asn was present, but were much reduced in the absence of Asn, and again this was seen to a greater extent in B-Asns B cells (Fig. 7G, Extended Fig. 7D).

The electron transport chain (ETC) is crucial for OxPhos activity. B cells undergo profound mitochondrial remodelling upon activation associated with ETC protein expression[2]. Using flow cytometry, we characterised the ETC protein abundances in activated B cells and found that Asn deprivation led to a substantial reduction in the expression of ETC proteins cytochrome c oxidase subunit I (COX I), cytochrome c oxidase subunit IV (COX IV), succinate dehydrogenase A (SDHA), and cytochrome C (Cyt C) in B-WT and to a greater extent in B-Asns B cells (Fig. 7H). The relative abundance of mitochondrially-encoded COX I to nuclear-encoded subunit COX IV was also substantially lowered, suggesting impaired mitochondrial protein translation upon Asn restriction (Fig. 7H).

Taken together, these findings indicate that Asn availability maintains mitochondrial ETC homeostasis, and ASNS regulates mitochondrial respiration in low Asn conditions.

Asn regulates nucleotide metabolism and the integrated stress response

Mitochondrial function is intimately linked to nucleotide metabolism, and in our GC B cell metabolomics experiment we found low levels of nucleotides, suggesting that their availability may be limiting, even in normal physiology (Fig. 1F)[36]. When examining the effect of Asn on B cell metabolite abundance, we found that nucleotide synthesis pathways were selectively impaired in B-Asns B cells following Asn deprivation (Fig. 6G). Detailed metabolite pathway analysis revealed significant downregulation of intermediate metabolites in both de novo purine and pyrimidine biosynthesis branches (Extended Data Fig. 8A). Orotate and uridine monophosphate (UMP), a common precursor of pyrimidine end-products cytosine and thymidine, were downregulated in B-Asns B cells.

The enzyme catalysing the oxidation of dihydroorotate to orotate is dihydroorotate dehydrogenase (DHODH), which is located in the mitochondria and forms a functional link between de novo pyrimidine biosynthesis and ETC activity[37,38]. We quantified DHODH levels and found it markedly reduced with Asn deprivation (Fig. 8A). Notably, B-Asns B cells had

the lowest DHODH levels in the absence of Asn, consistent with their lower mitochondrial mass and significantly downregulated pyrimidine metabolites. Interestingly, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC), which is responsible for the synthesis of inosine monophosphate (IMP) in the de novo purine synthesis pathway was not altered at the protein level (Fig. 8A).

We next performed a rescue experiment by supplementing B-WT and B-Asns B cells with nucleosides including guanosine, adenosine, thymidine, cytidine, and uridine, which bypasses the activity of nucleotide biosynthetic enzymes, including DHODH.
In conditions of Asn restriction, nucleoside supplementation substantially increased viability in both B-WT and B-Asns B cells following 72h culture (Fig. 8B), but was unable to restore normal proliferative capacity (Extended Data Fig. 8B). Importantly, nucleoside supplementation did not affect B cell viability or proliferation when Asn was unrestricted.

Asns is known to be upregulated following activation of the integrated stress response (ISR), which occurs as a consequence of a variety of cellular stressors, including amino acid withdrawal[39,40]. A key event in the amino acid response ISR is the phosphorylation of elongation initiation factor 2 alpha (eIF2α) by the kinase general control non-repressible-2 (GCN2). Phosphorylated eIF2α then controls transcription and translation of the ISR effector molecules activating transcription factor-4 (ATF4) and C/EBP homologous protein (CHOP)(Fig. 8C).

We examined the temporal dynamics of eIF2α phosphorylation in B cells, and found that p-eIF2α was increased at 6 hours following stimulation with IL-4 and agonistic anti-CD40 in the absence of Asn (Extended Data Fig. 8C). *Atf4* transcription was elevated with Asn deprivation, and this effect was significantly enhanced in B-Asns B cells (Fig. 8D). CHOP was highly expressed in B-Asns B cells deprived of Asn, and to a lesser extent in B-WT B cells, following in vitro activation with IL-4 and agonistic anti-CD40 (Fig. 8E). We observed a reduction in mTORC1 activation assessed by phosphorylation of 4E-BP1 when B cells were stimulated without Asn, which was more pronounced in those from B-Asns mice (Fig. 8F).

To directly assess the importance of the GCN2-mediated ISR in Asn synthesis, we next examined *Gcn2*$^{-/-}$ B cells. *Gcn2*$^{-/-}$ B cells were unable to upregulate either ATF4 or ASNS following stimulation (Fig. 8F), an effect seen even in Asn-replete conditions. Stimulation of *Gcn2*$^{-/-}$ B cells resulted in a similar phenotype to B-Asns B cells, albeit with a paradoxical increase in viability in the absence of Asn (Fig 8G-J). To further investigate this finding, we then treated B cells with the GCN2 activator halofuginone[41], and found that even when Asn was present, very low concentrations led to decreased survival (Extended Data Fig. 8D). This suggested that the extent of the ISR was carefully balanced in activated B cells, and excessive ISR activation was harmful. The GCN2 branch of the ISR is therefore essential for ASNS expression and cellular homeostasis in B cells. Finally, we examined whether nucleotide supplementation affected survival in *Gcn2*$^{-/-}$ B cells deprived of Asn. We found that unlike B-WT and B-Asns B cells, addition of nucleosides did not improve viability (Fig. 8K). This suggests that the effects seen with loss of GCN2 are distinct to those with ASNS alone, and nucleoside availability is not a major limiting factor in this setting.

These data show that *Asns* therefore essential to regulate B cell metabolic homeostasis, and if it cannot be obtained in sufficient amounts from the environment or synthesised by ASNS in response to activation of the ISR, there is impairment of nucleotide biosynthesis.

Discussion

Here we show using integrated multiomics that Asn metabolism is upregulated in GC B cells and required for their homeostasis, and that deprivation of Asn either from the external environment or by loss of its synthesis strongly alters cellular metabolism, and in particular impairs OxPhos and nucleotide synthetic capacity.

We found that GC B cells exhibited high levels of protein synthesis in vivo, expressed the amino acid transporter ASCT2, and avidly took up Asn. Initial stimulation in the presence of Asn provided a protective effect against subsequent Asn withdrawal, as has also been observed in $CD8^+$ T cells[42], which we found was mediated in part by upregulation of ASNS, which was almost undetectable in naïve B cells. Although the effects of amino acid uptake on T cells is increasingly well described[11,43], how this influences B cell biology is still poorly understood and requires further study.

Lack of Asn severely affected cellular metabolism in B cells, and in particular OxPhos. This was in striking contrast to reports in $CD8^+$ T cells[13,15], in which Asn deprivation leads to activation of OxPhos and increased nucleotide synthesis, associated with enhancement of proliferative capacity and enlarged mitochondrial mass, although others have noted reduction of cell division without Asn in this cell type, and also in cancer cells[12,14,44]. We found that Asn-deprivation led to a substantial drop in mitochondrial volume, and we observed that there was imbalance of expression of ETC proteins. It is unclear why B cells should be so much more vulnerable to the lack of Asn than $CD8^+$ T cells, but may reflect more profound cellular remodelling following activation, or other differences in core metabolism, which are more similar to cancer cells. We did not observe a defect in T_{FH} cells with Asn depletion, despite their shared microenvironment with GC B cells, highlighting their divergent metabolism.

An important consideration in all studies of metabolism in vitro is how to approximate the concentrations of metabolites found within tissue. Whilst this has received attention in the context of the tumour microenvironment, very little is known about normal tissues and especially within complex structures like GCs. GCs are hypoxic and poorly vascularised, but local concentrations of Asn remain unclear. In our study we noted impaired B cell homeostasis at Asn concentrations below 10μM in vitro, which is intermediate between venous blood and CSF. Advances in mass spectrometry imaging may allow more accurate quantification of metabolites in their spatial context in the future[45]. A potential limitation of our study is the use of ASNase, which may have off-target effects, including also acting as a glutaminase. To mitigate this, we selected a dose regime previously shown to have minimal glutaminase effect, whilst also highly effectively depleting Asn[20].

A key finding from our work is that Asn availability acts to gate nucleotide synthesis in B cells, a role previously demonstrated in cancer cell lines[46]. GC B cells have high rates of nucleic acid synthesis, in keeping with their active translational programming, and our LC-MS data revealed they had low levels of nucleosides, suggestive of their consumption. Analysis of Asn synthesis from glutamine showed that even following activation, which was associated with upregulation of ASNS, the great majority of Asn was exogenously acquired. Nonetheless ASNS was essential for metabolic homeostasis and nucleotide synthesis when Asn is restricted, despite seemingly modest rates of Asn synthesis. This raises the interesting question of whether ASNS might have other, non-synthetic functions, as has been recently demonstrated for the enzyme phosphoglycerate dehydrogenase (PHGDH), whose canonical function is to synthesise serine[47]. The relationship between amino acid availability and nucleotide synthesis has been previously defined through mTORC1

signalling, acting via phosphorylation of the enzyme carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, and dihydroorotase (CAD), or the tetrahydrofolate cycle[48,49]. It is therefore possible that in GC B cells, Asn availability tunes these nucleoside synthetic pathways, either through mTORC1 or other mechanisms. An important node in B cell nucleoside metabolism downregulated with Asn deprivation involved the enzyme which oxidises dihydroorotate, DHODH.

ASNase has been a cornerstone of the treatment of leukaemia for decades, but given the results of our work, ASNS may also be a novel therapeutic target in non-malignant, autoimmune disease mediated by the GC reaction, and is deserving of future study in this context. Another aspect of ASNase is its role in promoting bacterial infection. It is interesting to speculate that its capacity to reduce environmental Asn is also effective against the protective humoral response, which is protected against by the expression of ASNS.

Figures and legends

Figure 1

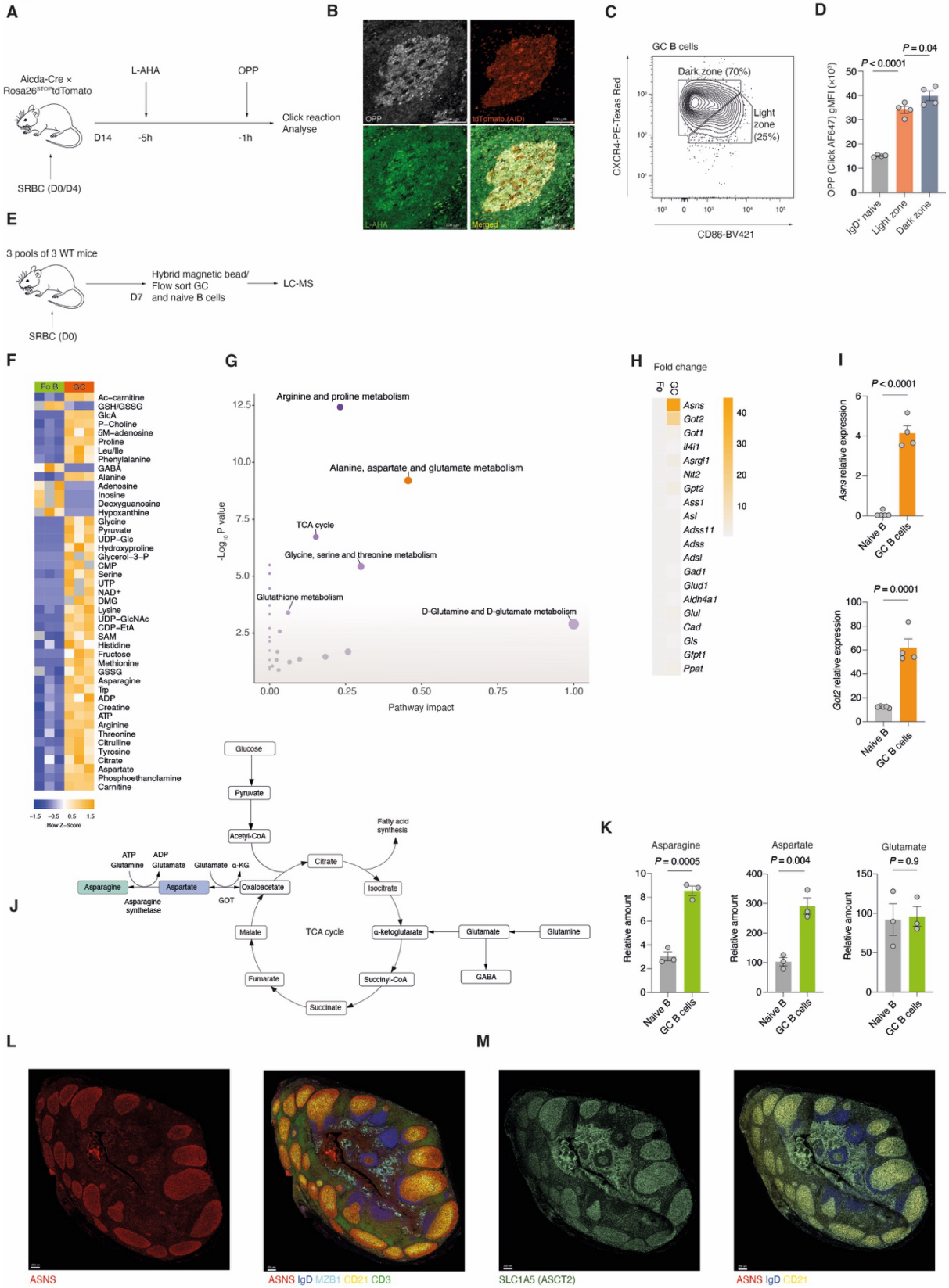


Figure 1. GC B cells have highly active protein synthesis and asparagine metabolism

- **A.** Schematic of in vivo bio-orthogonal non-canonical amino acid tagging. Aicda-cre × Rosa26^{STOP}tdTomato mice were immunised with SRBC and then boosted at day 4. At day 14, they were injected with L-AHA, and then four hours later with OPP. They were sacrificed one hour later, and non-canonical amino acid incorporation into splenic tissue was revealed using Click chemistry.
- **B.** Representative immunofluorescence images of splenic GCs labelled with OPP, tdTomato (AID), and L-AHA. Representative of two independent experiments. Scale bar: 100µm.
- **C.** Gating strategy of light zone (CD86^{hi}CXCR4^{lo}) and dark zone (CD86^{lo}CXCR4^{hi}) GC B cells
- **D.** Quantification of OPP incorporation in naïve, LZ GC and DZ GC B cells from n=4 mice. Representative of two independent experiments.
- **E.** Schematic of experimental design. Three pools of three WT mice were immunised with SRBC, and at day 7 GC and follicular B cells were isolated by a hybrid magnetic bead and flow sorting approach, before analysis of polar metabolites by LC-mass spectrometry (LC-MS).
- **F.** Heatmap of significantly differentially abundant metabolites (P_{adj} <0.05) in naïve and GC B cells at day 7 from wild type mice immunised with SRBC, measured by LC-MS (n=3 pools of 3 mice/pool). Representative of two independent experiments.
- **G.** Integrated pathway analysis of differentially abundant metabolites from **F** and expressed genes from Immgen in GC compared to naïve B cells.
- **H.** Heatmap of relative gene expression of KEGG Alanine, Aspartate, and Glutamate pathway in GC vs naïve B cells, represented as fold change. Data are from GSE133971[24].
- **I.** Relative expression of *Asns* and *Got2* in flow-sorted GC and naïve B cells from wild type mice immunised with SRBC at day 7, normalised to *Ubc* (n=4 mice). Representative of two independent experiments.
- **J.** Diagram of asparagine synthesis and its association with the TCA cycle.
- **K.** Relative amounts of asparagine, aspartate, and glutamate in GC and naïve B cells measured by LC-MS (n=3 pools of 3 mice) as in **F**. Representative of two independent experiments.
- **L.** Multiplexed CellDIVE images of normal human tonsil section, with immunofluorescence staining for ASNS, CD21, IgD, MZB1 and CD3. Representative of 3 separate tonsils. Scale bar = 250µm.
- **M.** Multiplexed CellDIVE images of normal human tonsil serial section of **L**, with immunofluorescence staining for SLC1A5 (ASCT2), CD21 and IgD. Representative of 3 separate tonsils. Scale bar = 250µm.

Statistical significance was determined by two-way ANOVA with Tukey's multiple testing correction (F), hypergeometric test (G), unpaired two-tailed t test (I,K) or one-way ANOVA with Tukey's multiple testing correction (D). Data are presented as the mean +/- SEM.

Figure 2

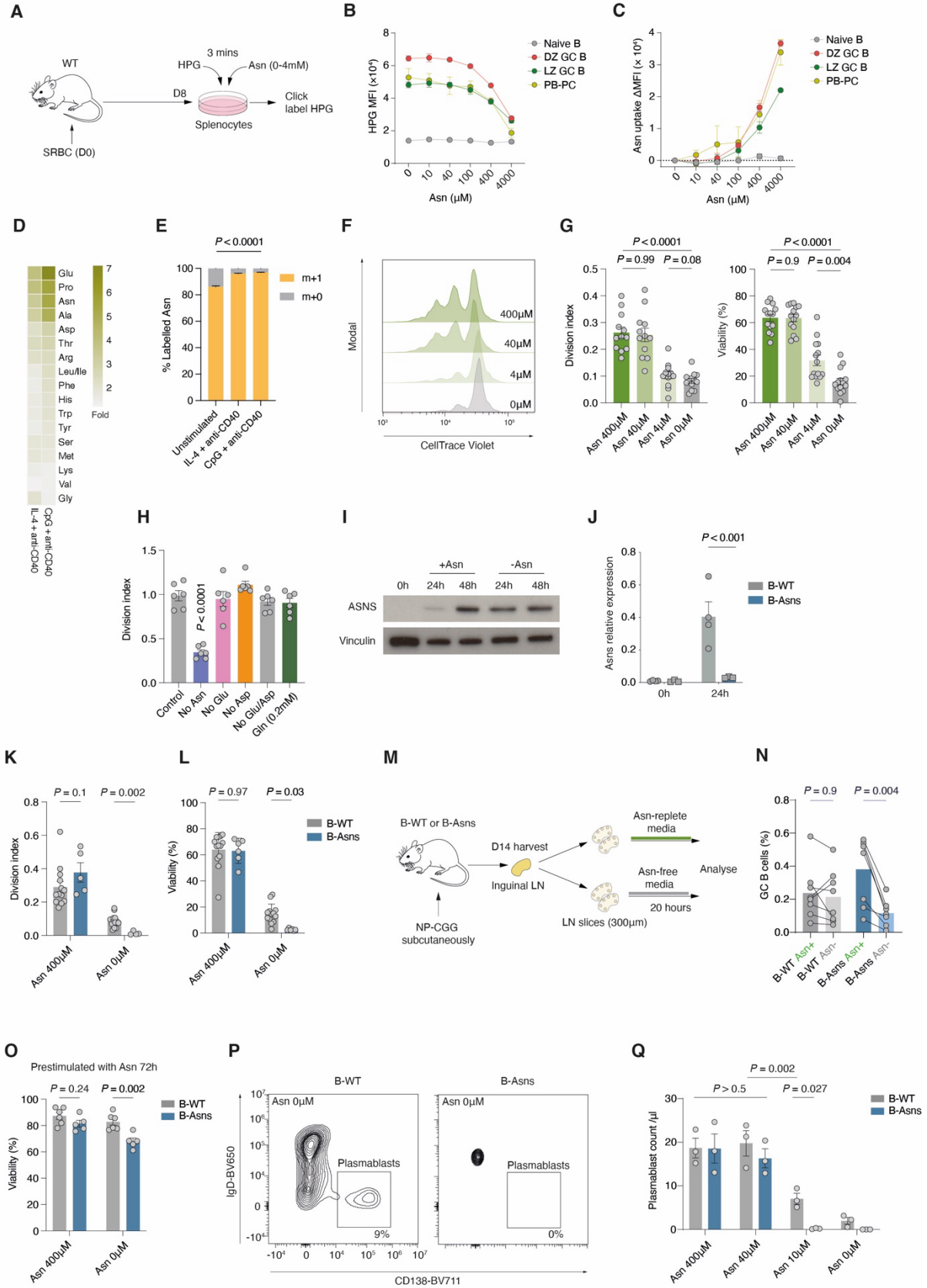

A
WT
SRBC (D0)
D8
3 mins
HPG
Asn (0-4mM)
Splenocytes
Click label HPG
B
HPG MFI (x10⁴)
Asn (μM)
Naive B
DZ GC B
LZ GC B
PB-PC
C
Asn uptake ΔMFI (x 10⁴)
Asn (μM)
Naive B
DZ GC B
LZ GC B
PB-PC
D
Glu
Pro
Asn
Ala
Asp
Thr
Arg
Leu/Ile
Phe
His
Trp
Tyr
Ser
Met
Lys
Val
Gly
Fold
IL-4 + anti-CD40
CpG + anti-CD40
E
P < 0.0001
% Labelled Asn
m+1
m+0
Unstimulated
IL-4 + anti-CD40
CpG + anti-CD40
F
Modal
400μM
40μM
4μM
0μM
CellTrace Violet
G
P < 0.0001
P = 0.99
P = 0.08
Division index
P < 0.0001
P = 0.9
P = 0.004
Viability (%)
Asn 400μM
Asn 40μM
Asn 4μM
Asn 0μM
H
Division index
P < 0.0001
Control
No Asn
No Glu
No Asp
No Glu/Asp
Gln (0.2mM)
I
+Asn
-Asn
0h
24h
48h
24h
48h
ASNS
Vinculin
J
P < 0.001
Asns relative expression
B-WT
B-Asns
0h
24h
K
P = 0.1
P = 0.002
Division index
Asn 400μM
Asn 0μM
L
P = 0.97
P = 0.03
Viability (%)
B-WT
B-Asns
Asn 400μM
Asn 0μM
M
B-WT or B-Asns
NP-CGG subcutaneously
D14 harvest
Inguinal LN
Asn-replete media
Asn-free media
20 hours
LN slices (300μm)
Analyse
N
P = 0.9
P = 0.004
GC B cells (%)
B-WT Asn+
B-WT Asn-
B-Asns Asn+
B-Asns Asn-
O
Prestimulated with Asn 72h
P = 0.24
P = 0.002
Viability (%)
B-WT
B-Asns
Asn 400μM
Asn 0μM
P
B-WT
B-Asns
Asn 0μM
Asn 0μM
Plasmablasts
9%
Plasmablasts
0%
IgD-BV650
CD138-BV711
Q
P = 0.002
P > 0.5
P = 0.027
Plasmablast count /μl
B-WT
B-Asns
Asn 400μM
Asn 40μM
Asn 10μM
Asn 0μM

Figure 2. B cells require ASNS when the availability of Asn is limited

- **A.** Schematic of ex vivo SLC1A5-mediated competitive Asn uptake assay.
- **B.** Quantification of HPG (400µM) uptake at increasing concentration of Asn (0-4000µM) in naïve B, plasmablast/plasma cells (PB-PC), LZ and DZ GC B cells harvested from n=4 mice at day 8 following SRBC immunisation. Background signal subtraction was performed based on 4°C and no-HPG controls. Representative of two independent experiments.
- **C.** Quantification of the shift in the HPG signal intensity (ΔMFI) as in **B** reflecting Asn uptake calculated as following: Asn uptake at x concentration = HPG $MFI_{[Asn0]}$ – HPG $MFI_{[Asn\ x]}$.
- **D.** Heatmap of fold change of indicated amino acids in B cells, measured by LC-MS following stimulation for 24h with IL-4 and anti-CD40, or CpG and anti-CD40, relative to unstimulated condition (n=3 mice). Representative of two independent experiments.
- **E.** Fraction of labelling of intracellular Asn with ^{15}N in B cells stimulated as in **D**, in the presence of $^{15}N_1$-Asn (n=3 mice). Representative of two independent experiments.
- **F.** Representative histogram of dilution of Celltrace Violet in B cells stimulated with IL-4 and anti-CD40 and cultured for 72h with the indicated concentration of Asn.
- **G.** Division index and viability of B cells stimulated with IL-4 and anti-CD40 and cultured for 72h with the indicated concentration of Asn. Each dot represents a single mouse. Representative of and pooled from three independent experiments.
- **H.** Division index of B cells stimulated for 72h with IL-4 and anti-CD40 in the presence or absence of the indicated amino acids (n=6 mice). Representative of three independent experiments.
- **I.** Representative immunoblot of ASNS in B cells stimulated with IL-4 and anti-CD40 with or without Asn, at the indicated timepoints. Representative of two independent experiments.
- **J.** Relative expression of *Asns* by qPCR in freshly isolated, and stimulated (IL-4 and anti-CD40 for 24h) B cells (n=4 B-WT and n=4 B-Asns mice). Representative of two independent experiments.
- **K.** Division index of B cells from B-Asns and B-WT mice stimulated for 72h with IL-4 and anti-CD40 in the presence or absence of Asn (n=13 B-WT and n=5-6 B-Asns mice). Representative of >4 independent experiments.
- **L.** Viability of B cells stimulated with IL-4 and anti-CD40 with 400µM Asn for 72h, then restimulated for an additional 24h in the presence or absence of Asn (n=6-10 mice). Representative of two independent experiments.
- **M.** Schematic of lymph node slice culture system. B-WT and B-Asns mice were immunised with NP-CGG in alum subcutaneously and inguinal lymph nodes were harvested at day 14. 300µm thick slices were cultured for 20h in the presence or absence of Asn in duplicates and analysed by flow cytometry.
- **N.** Quantification of $CD38^-GL\text{-}7^+$ GC B cell proportions by flow cytometry in lymph node slices cultured for 20h in the presence or absence of Asn (400µM). Each dot represents single lymph node (n=8 lymph nodes from n=4 mice). Pooled from two independent experiments.
- **O.** Viability of B cells stimulated with IL-4 and anti-CD40 with 400µM Asn for 72h, then restimulated for an additional 24h in the presence or absence of Asn (n=5-6 mice). Representative of and pooled from two independent experiments.
- **P.** Representative flow cytometry plots of IgD^-CD138^+ plasmablasts generated following stimulation of B cells from B-WT and B-Asns mice with LPS and IL-4 for 72h at the indicated concentration of Asn. Representative of three independent experiments.

Q. Quantification of plasmablast counts as in **P**. Representative of three independent experiments.

Statistical significance was determined by one-way ANOVA with Tukey's multiple testing correction (E), repeated measures one way ANOVA with Tukey's multiple testing correction (G-H), paired two-tailed t test (N) or two-way ANOVA with Šidák's multiple testing correction (J-L, O,Q). Data are presented as the mean +/- SEM.

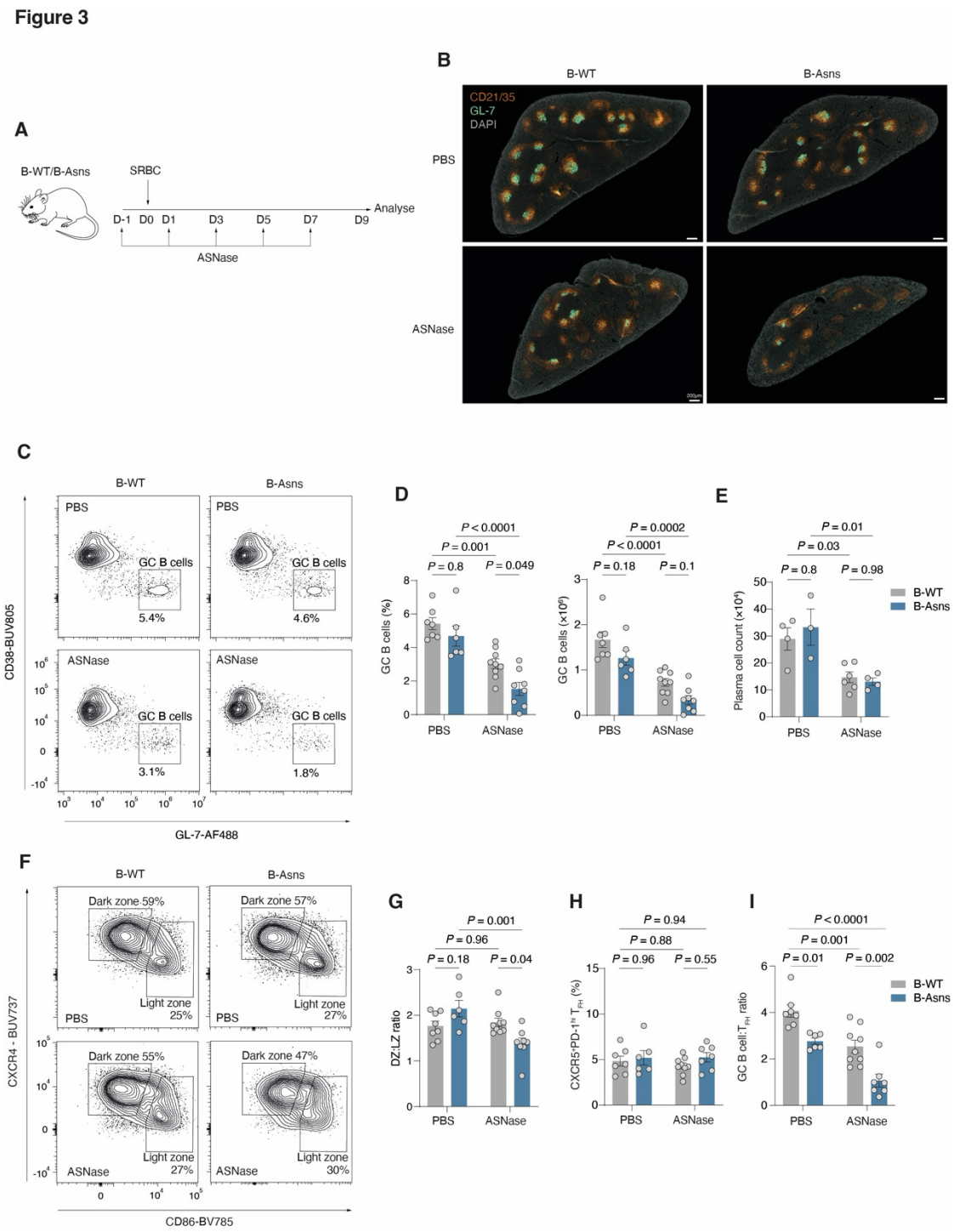


Figure 3: The GC reaction is sensitive to Asn deprivation

A. Schematic of in vivo ASNase administration following SRBC immunisation. B-WT or B-Asns mice were intraperitoneally injected with ASNase (500U/kg) or PBS vehicle one day prior to immunisation with SRBC (day 0) and given repeated ASNase injections every two days until day 7. Spleens were harvested at day 9 and analysed by immunohistochemistry and flow cytometry.

B. Immunohistochemistry of representative spleen sections (8µm) at day 9. GL-7 and CD21/35 highlights GCs and B cell follicles respectively. DAPI is used as background staining. Representative of two independent experiments.

C. Representative flow cytometry plot of GC B cells ($CD19^{+}CD38^{-}GL\text{-}7^{+}$) at day 9 post-immunisation with SRBC, from B-WT and B-Asns mice treated with PBS or ASNase.

D. Quantification of splenic GC B cell proportions (% of $CD4^{-}CD19^{+}$ B cells) and counts. Each data point represents a single mouse. Representative of four independent experiments.

E. Quantification of IgD^{-} $IRF4^{+}$ $CD138^{+}$ splenic plasma cell counts. Each data point represents a single mouse. Representative of three independent experiments.

F. Representative flow cytometry plots of light zone ($CD86^{hi}CXCR4^{lo}$) and dark zone ($CD86^{lo}CXCR4^{hi}$) GC B cells

G. Quantification of DZ:LZ ratio. Representative of three independent experiments.

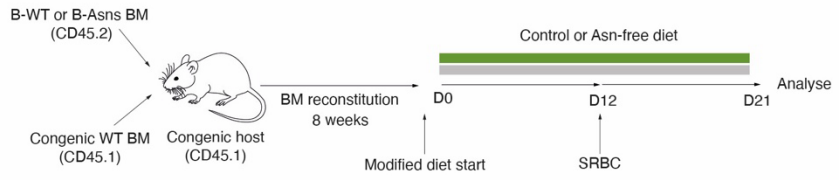
H. Quantification of the proportion of T_{FH} as $CXCR5^{hi}$ $PD\text{-}1^{+}$ within $CD19^{-}$ $CD4^{+}$ $FoxP3^{-}$ T cells. Each data point represents a single mouse. Representative of three independent experiments.

I. Quantification of the ratio of GC B cell to T_{FH} counts. Each data point represents a single mouse. Representative of three independent experiments

Statistical significance was determined by two-way ANOVA with Šidák's multiple testing correction multiple testing correction. Data are presented as the mean +/- SEM.

Figure 4

A



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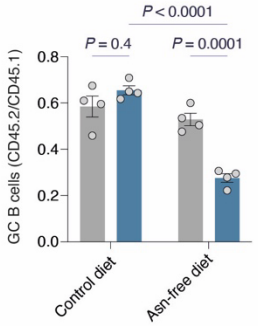

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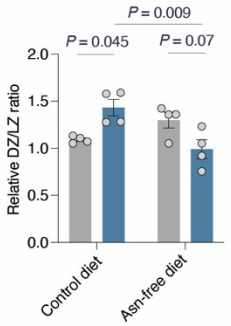

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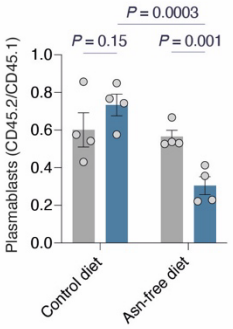

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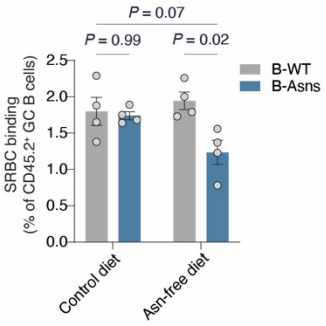

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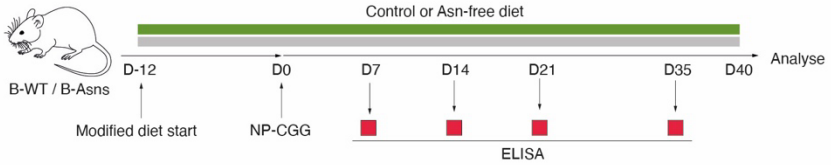

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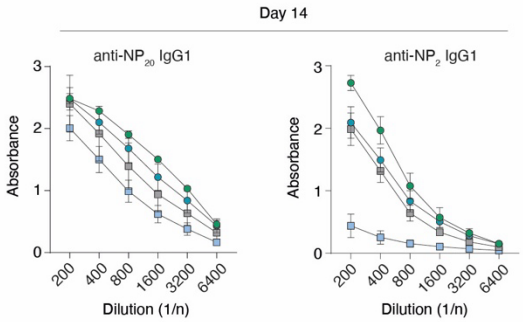

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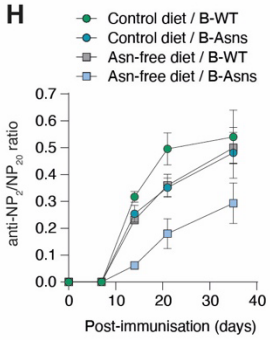

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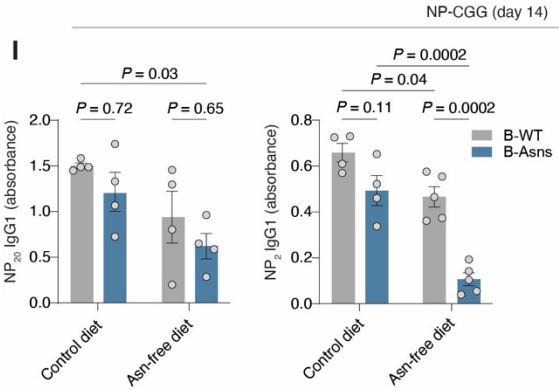

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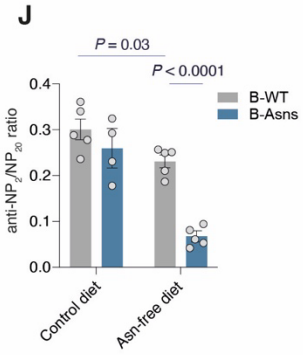

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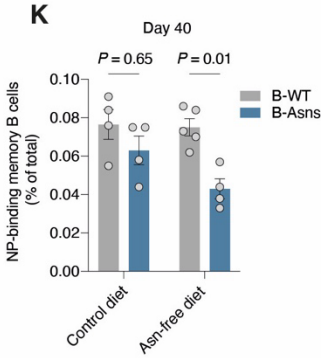


Figure 4: Asn is required for GC B cell affinity maturation

- **A.** Schematic of bone marrow (BM) chimera/dietary modification experiment. Mixed BM chimeras were generated with B-WT (CD45.2) or B-Asns (CD45.2) and CD45.1 wild type BM, with n=4 congenic CD45.1 recipient mice for each condition. After an 8 week reconstitution period, mice were transferred to a control (Asn-replete) or Asn-deficient diet for 12 days, before immunisation with SRBC. Spleens were analysed 9 days after immunisation.
- **B.** Ratio of splenic CD45.2:CD45.1 GC B cells from bone marrow chimeric mice as described in **A**. Each data point represents a single mouse. Data pooled from two independent experiments.
- **C.** Relative DZ:LZ ratio of splenic CD45.2:CD45.1 GC B cells from bone marrow chimeric mice as described in **A**. Data pooled from two independent experiments.
- **D.** Ratio of splenic CD45.2:CD45.1 plasma cells ($CD138^+IRF4^+$) from bone marrow chimeric mice as described in **A**. Data pooled from two independent experiments.
- **E.** Ratio of splenic $CD45.2^+$ SRBC-binding (high-binding as described in **Extended Fig. 4A**) GC B cells from bone marrow chimeric mice as described in **A.** Data pooled from two independent experiments.
- **F.** Schematic of non-chimeric NP-CGG/dietary modification experiment. B-WT or B-Asns mice were assigned to either an Asn-free or control diet (Asn-replete) for 12 days, before immunisation with 50µg NP-CGG precipitated in Alum hydrogel (1:1) subcutaneously on both flanks. Mice were bled on day 7, 14, 21 and 35 post-immunisation and spleens were analysed following sacrifice at day 40.
- **G.** Dilution curves of IgG1 anti-NP antibodies ($NP_{>20}$-BSA and NP_2-BSA respectively) at day 14, n=4 mice each condition. Data pooled from two independent experiments.
- **H.** Comparison of the ratio of IgG1 NP-specific high-affinity antibodies to low-affinity antibodies detected by binding to NP_2 and $NP_{>20}$ antigens respectively, from B-WT/control diet (n=4), B-Asns/control diet (n=4), B-WT/Asn-free diet (n=5), B-Asns/Asn-free diet (n=5) across different time points. Data pooled from two independent experiments.
- **I.** Quantification of IgG1 anti-NP antibodies ($NP_{>20}$-BSA at 1:1600 and NP_2-BSA at 1:200 dilution respectively) at day 14, n=4-5 mice each condition. Data pooled from two independent experiments.
- **J.** Quantification of NP_2:NP_{20} ratio of IgG1 anti-NP antibodies at day 14, n=4-5 mice each condition. Data pooled from two independent experiments.
- **K.** Comparison of the proportions of splenic NP-binding memory B cells gated as $CD19^+$ $B220^+$ IgD^- $GL\text{-}7^-$ $CD138^-$ $CD38^+$ within total cells at day 40. Data pooled from two independent experiments.

Statistical significance was determined by two-way ANOVA with Šidák's multiple testing correction. Data are presented as the mean +/- SEM.

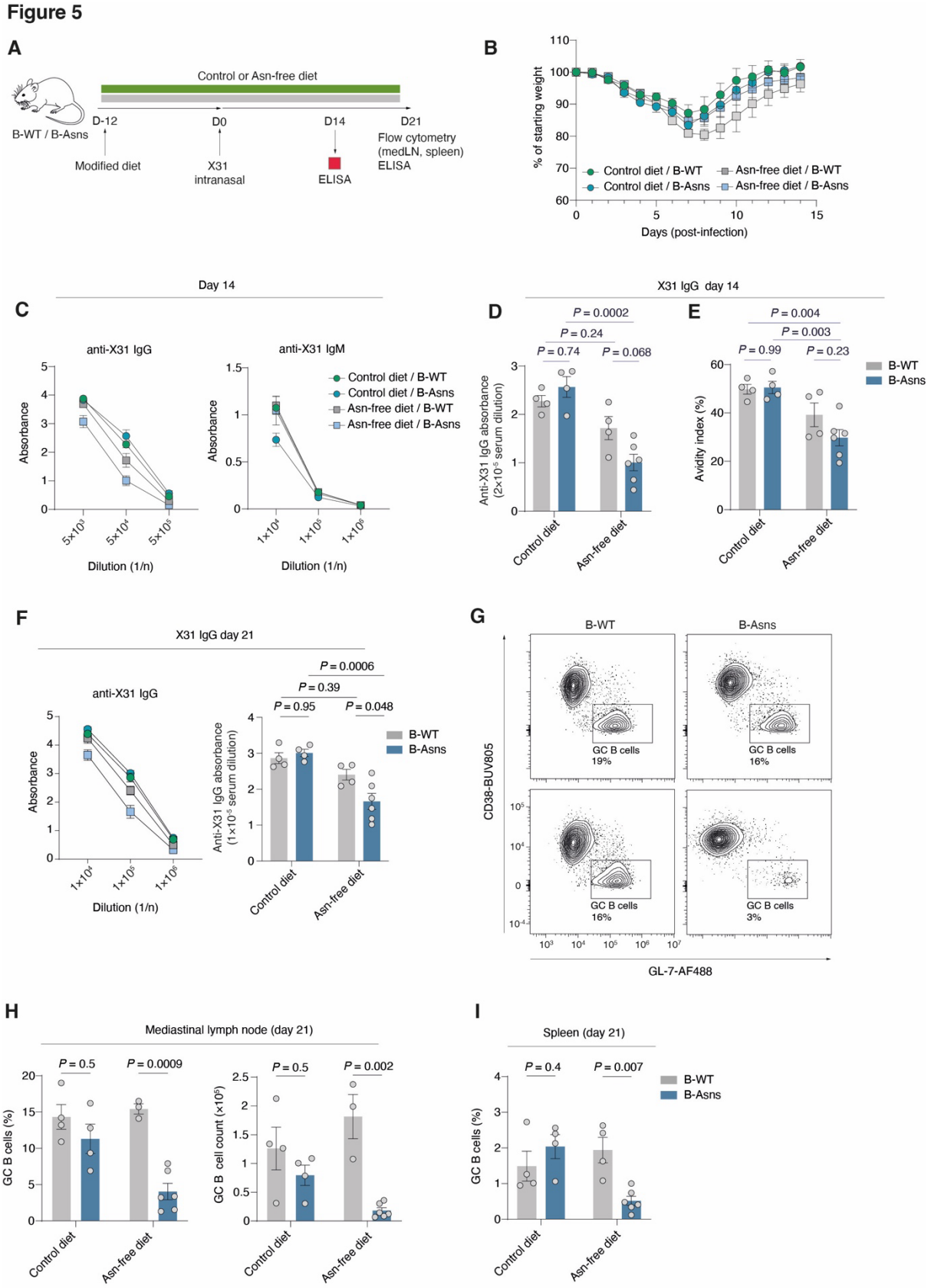


Figure 5. Asn metabolism controls the humoral response to influenza infection

A. Schematic of influenza/dietary modification experiment. B-WT and B-Asns mice were transferred to a control (Asn-replete) or Asn-deficient diet for 12 days, before intranasal infection with X31 influenza A strain. Mice were weighed daily for 14 days and bled at days 14 and 21. Mediastinal lymph node and spleen were harvested for flow cytometry analyses following sacrifice at day 21.

B. Quantification of weight loss as % of initial weight over 14 days. Control diet/ B-WT (n = 4), control diet/B-Asns (n = 4), Asn-free diet/B-WT (n = 4), Asn-free diet/B-Asns (n=6). Data pooled from two independent experiments.

C. Dilution curves of X31-specific IgG and IgM antibodies at day 14. n=4-6 mice each condition. Data pooled from two independent experiments.

D. Comparison of X31-specific IgG (at $1/5\times10^4$ dilution) at day 14. n=4-6 mice each condition. Data pooled from two independent experiments.

E. Comparison of avidity (functional affinity) of X31-specific IgG at day 14 which was calculated as following: Absorbance [5M urea] / Absorbance [0M urea] × 100 also indicated in **Extended Data Fig 5B**. Data pooled from two independent experiments.

F. Dilution curves and quantification of X31-specific IgG antibodies at day 21. n=4-6 mice each condition. Data pooled from two independent experiments.

G. Representative flow cytometry plots of GC B cells in mediastinal lymph node at day 21.

H. Quantification of GC B cell ratio and counts in mediastinal lymph nodes at day 21. n=3-6 mice each condition. Data pooled from two independent experiments.

I. Quantification of splenic GC B cell proportions at day 21. n=4-6 mice each condition Data pooled from two independent experiments.

Statistical significance was determined by two-way ANOVA with Šidák's multiple testing correction. Data are presented as the mean +/- SEM.

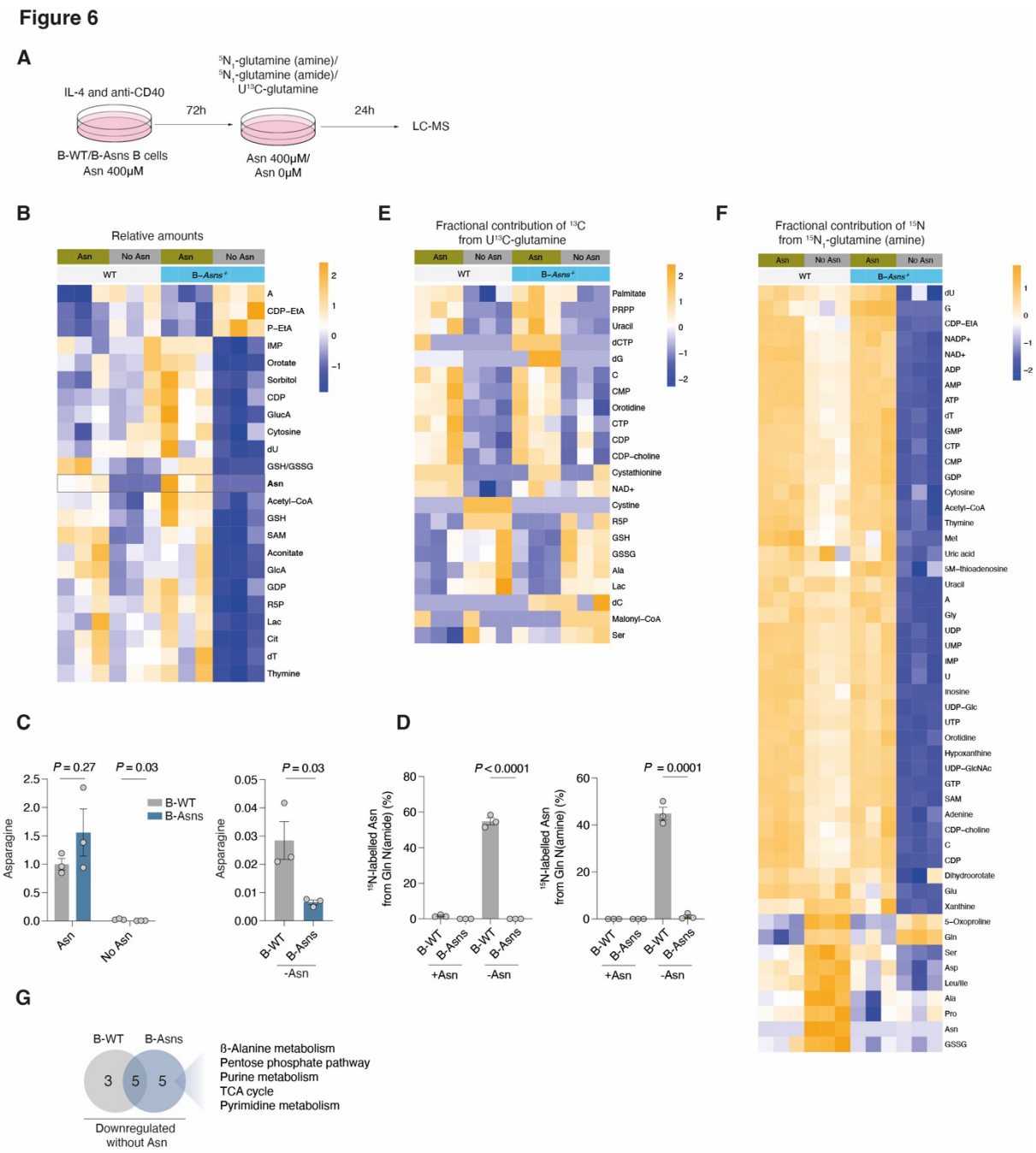


Figure 6. Disruption of asparagine availability alters B cell metabolism

- **A.** Schematic of experimental design for stable isotope-resolved metabolomics. B-WT or B-Asns B cells were stimulated with IL-4 and agonistic anti-CD40 for 72 hours in the presence of Asn (400µM), then stimulated for an additional 24h with or without Asn (400µM), and either $^{15}N_1$(amine)-glutamine, $^{15}N_1$(amide)-glutamine, U^{13}C-glutamine, or unlabelled glutamine, before analysis by LC-MS.
- **B.** Heatmap of total relative amounts of significantly different metabolites in B cells from B-WT or B-Asns mice as in **A**. Scale represents row Z-score. Significantly differentially abundant metabolites ($P_{adj} < 0.05$) are shown. Representative of two independent experiments.

C. Abundance of intracellular Asn in B cells cultured as in **A**, and rescaled plot of Asn-deprived condition. Representative of two independent experiments.

D. Fractional contribution of ^{15}N to Asn pool, derived from $^{15}N_1$-amino or amide Gln, following culture as described in **A**.

E. Heatmap of fractional contribution of ^{13}C to indicated metabolites, derived from U-^{13}C-Gln following culture as described in **A**. Scale represents row Z-score. Significantly differentially labelled metabolites ($P_{adj} < 0.05$) are shown.

F. Heatmap of fractional contribution of ^{15}N to indicated metabolites, derived from $^{15}N_1$-amine Gln following culture as described in **A**, with labelled compound added for final 24h of culture. Scale represents row Z-score. Significantly differentially labelled metabolites ($P_{adj} < 0.05$) are shown.

G. Venn-diagram indicating number of significantly differentially-regulated pathways in B-WT and B-Asns B cells following Asn-withdrawal, based on relative metabolite abundance as in **B**. Listed pathways are those specifically downregulated in B-Asns B cells.

Statistical significance was determined by two-way ANOVA with Šidák's multiple testing correction (B, C, D-F), or hypergeometric test (G). Data are presented as the mean +/- SEM.

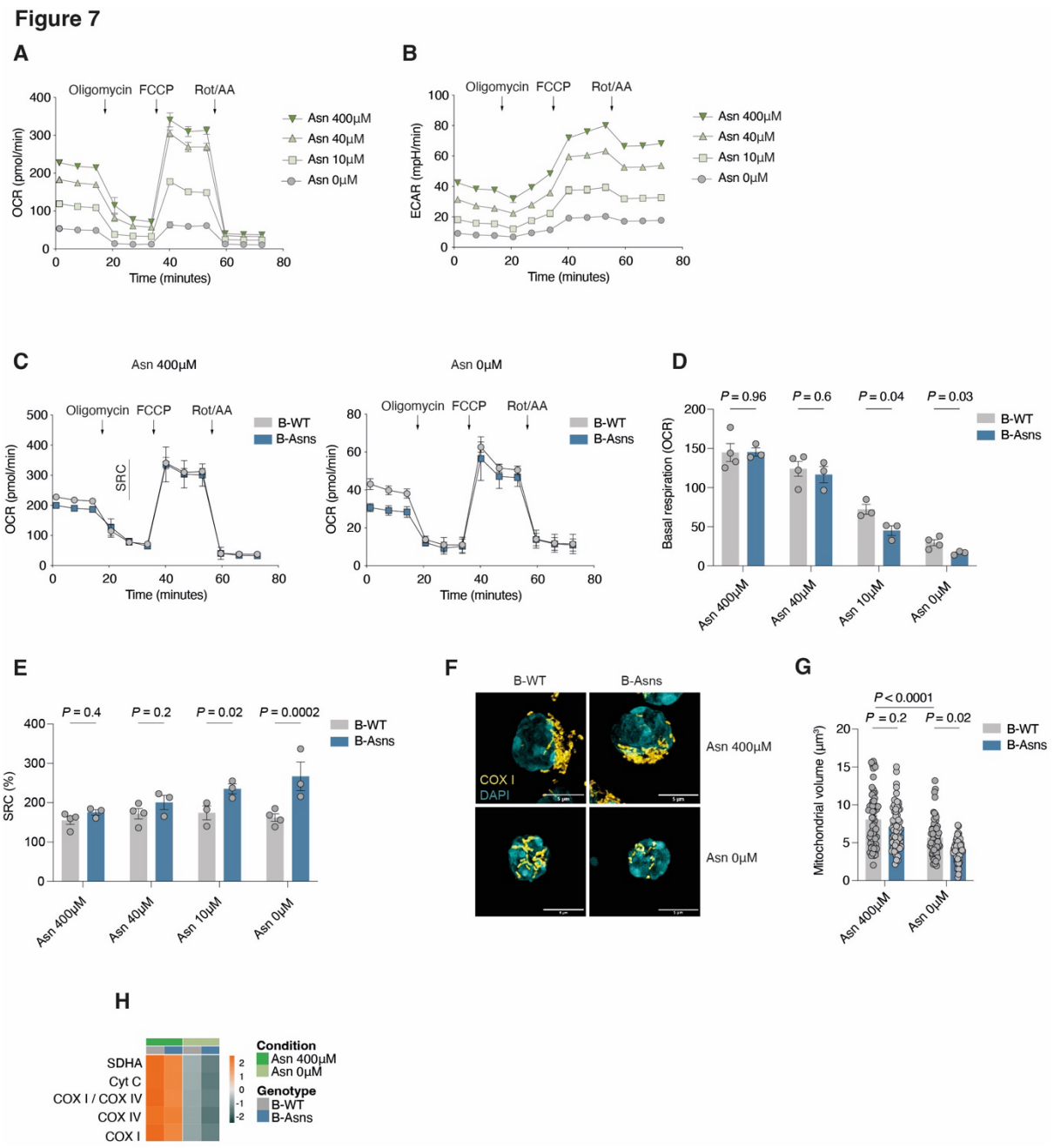


Figure 7. Mitochondrial function in B cells requires Asn

- **A.** Seahorse extracellular flux analysis of B cells stimulated for 24h with IL-4 and agonistic anti-CD40 at the indicated concentrations of Asn. Oxygen consumption rate (OCR) was measured during the MitoStress test. Representative of three independent experiments with 5-6 technical replicates.
- **B.** Seahorse extracellular flux analysis of B cells as in **A**. Extracellular acidification rate (ECAR) was measured during the MitoStress test. Representative of three independent experiments with 5-6 technical replicates.
- **C.** Seahorse OCR quantification in B-WT (n=4 mice) and B-Asns (n=3 mice) B cells stimulated for 24h with IL-4 and agonistic anti-CD40 at the indicated concentrations of Asn. Spare respiratory capacity (SRC) is illustrated. Representative of three independent experiments.

D. Quantification of basal OCR in B-WT and B-Asns B cells stimulated for 24h with IL-4 and agonistic anti-CD40 at the indicated concentrations of Asn. Each data point represents a mouse. Representative of three independent experiments.

E. Quantification of SRC (%) based on quantifications in **Extended Fig.7B** and **Fig. 7D**.

F. 3D lattice SIM images of COX I and DAPI in B-WT and B-Asns B cells stimulated for 24h with IL-4 and agonistic anti-CD40 in the presence or absence of Asn (400µM).

G. Quantification of mitochondrial volume as in **F**. Each data point represents a cell pooled from n=3 mice B-WT or B-Asns mice. Representative of three independent experiments.

H. Heatmap of row z-scores for the gMFI of indicated ETC proteins, measured by flow cytometry in B-WT or B-Asns B cells stimulated for 24h with IL-4 and agonistic anti-CD40 in the presence or absence of Asn (400µM) (mean of n = 2), Results are representative of two independent experiments with n = 3 mice per group in total.

Statistical significance was determined by two-way ANOVA with Šidák's multiple testing correction (D-E, G). Data are presented as the mean +/- SEM.

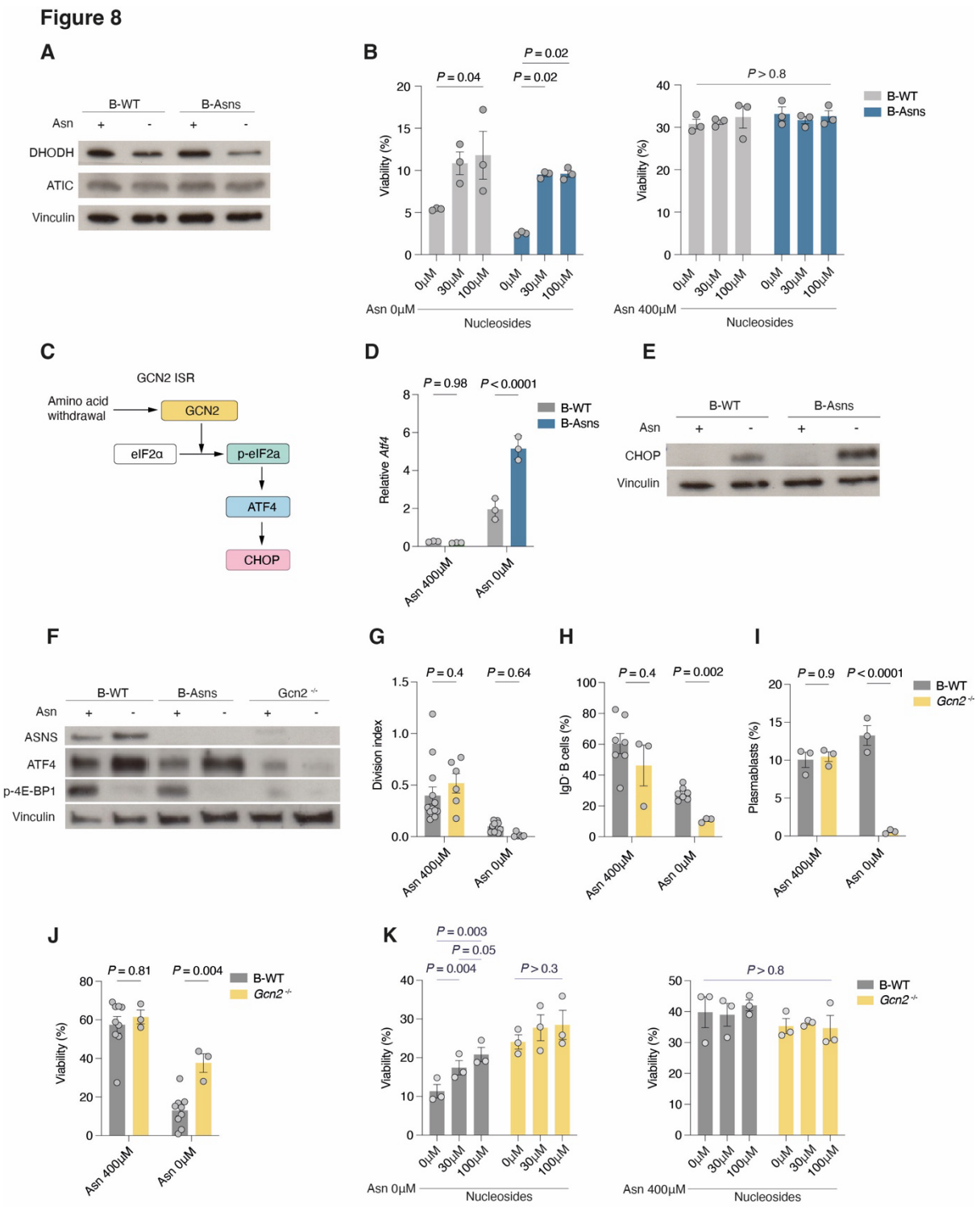


Figure 8. Asn regulates nucleotide metabolism and the integrated stress response

A. Representative immunoblot of DHODH and ATIC in B-WT or B-Asns B cells stimulated with IL-4 and anti-CD40 for 24h, in the presence or absence of Asn (400μM). Representative of two independent experiments. Vinculin used as loading control.

B. Viability of B cells from B-Asns and B-WT mice (n=3 mice) stimulated for 72h with IL-4 and anti-CD40 in the presence or absence of Asn, supplemented with

adenosine, thymidine, uridine, cytidine, and guanosine at 0µM, 30µM or 100µM each. Data pooled from two independent experiments.

C. Schematic of the GCN2 branch of the integrated stress response.

D. Relative expression of *Atf4* in B-Asns and B-WT B cells stimulated for 72h in the presence or absence of Asn (n=3 B-WT and n=3 B-Asns mice). Representative of two independent experiments.

E. Representative immunoblot of CHOP in B-WT or B-Asns B cells stimulated with IL-4 and anti-CD40 for 24h, in the presence or absence of Asn (400µM). Representative of two independent experiments. Vinculin used as loading control.

F. Representative immunoblot of ASNS, ATF4, phospho-4E-BP1 in B cells from B-Asns, *Gcn2*$^{-/-}$, and B-WT mice, stimulated for 24h with anti-CD40 and IL-4 in the presence or absence of Asn (400µM). Representative of two independent experiments. Vinculin used as loading control.

G. Division index of *Gcn2*$^{-/-}$ and wild type B cells stimulated with IL-4 and agonistic anti-CD40 for 72h, in the presence or absence of Asn (400µM). Each data point represents a mouse. Representative of four independent experiments.

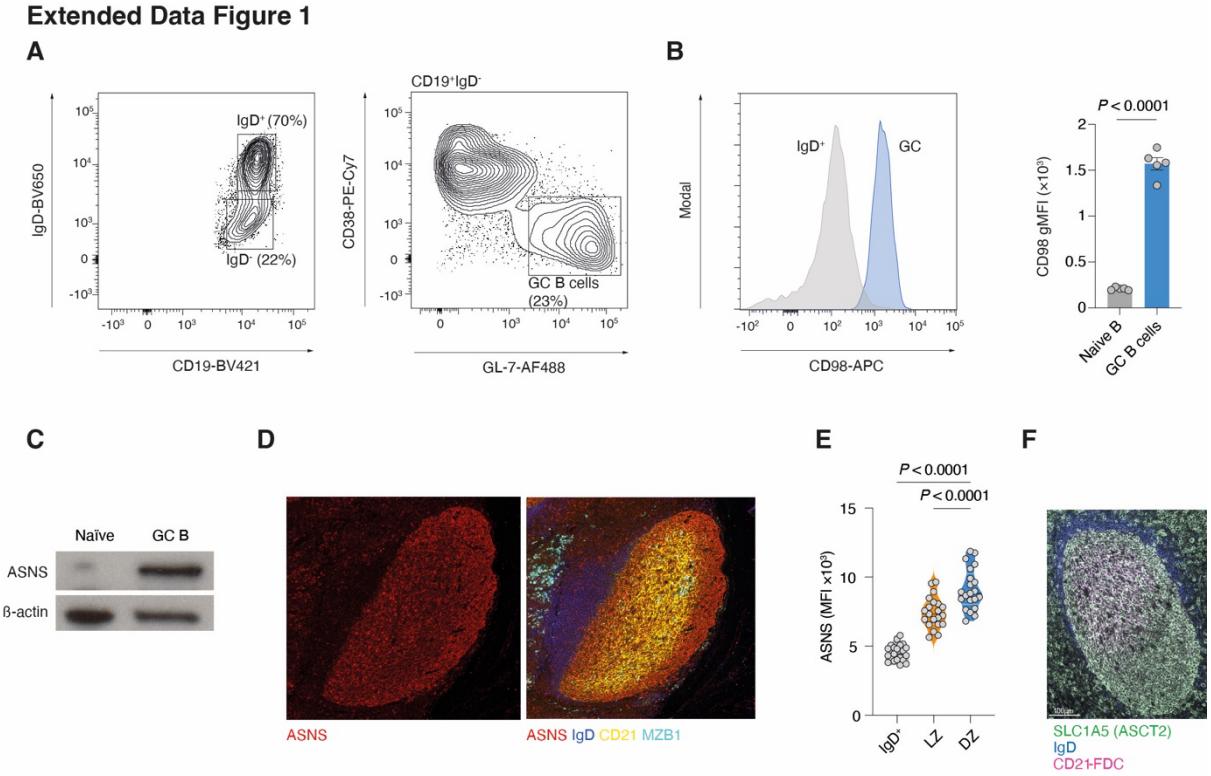
H. Flow cytometry quantification of activated B cell ratio based on IgD downregulation (IgD^{-}) in *Gcn2*$^{-/-}$ and wild type B cells as in **G**. Representative of two independent experiments.

I. Representative flow cytometry quantification of plasmablast (IgD^{-} CD138^{+}) differentiation of *Gcn2*$^{-/-}$ and wild type B cells following stimulation with LPS (10µg/ml) and IL-4 (50ng/ml) for 72h in the presence or absence of Asn (400µM) (n=3 *Gcn2*$^{-/-}$ mice and n=3 wild type mice). Data pooled from two independent experiments.

J. Viability of *Gcn2*$^{-/-}$ and wild type B cells stimulated with IL-4 and agonistic anti-CD40 for 72h, in the absence or presence of Asn (400µM) (n=3 *Gcn2*$^{-/-}$ mice and n=9 wild type mice). Representative of three independent experiments.

K. Viability of B cells from B-WT or *Gcn2*$^{-/-}$ mice (n=3 mice) stimulated for 72h with IL-4 and agonistic anti-CD40 in the presence or absence of Asn, supplemented with adenosine, thymidine, uridine, cytidine, and guanosine at 0µM, 30µM or 100µM each. Data pooled from two independent experiments.

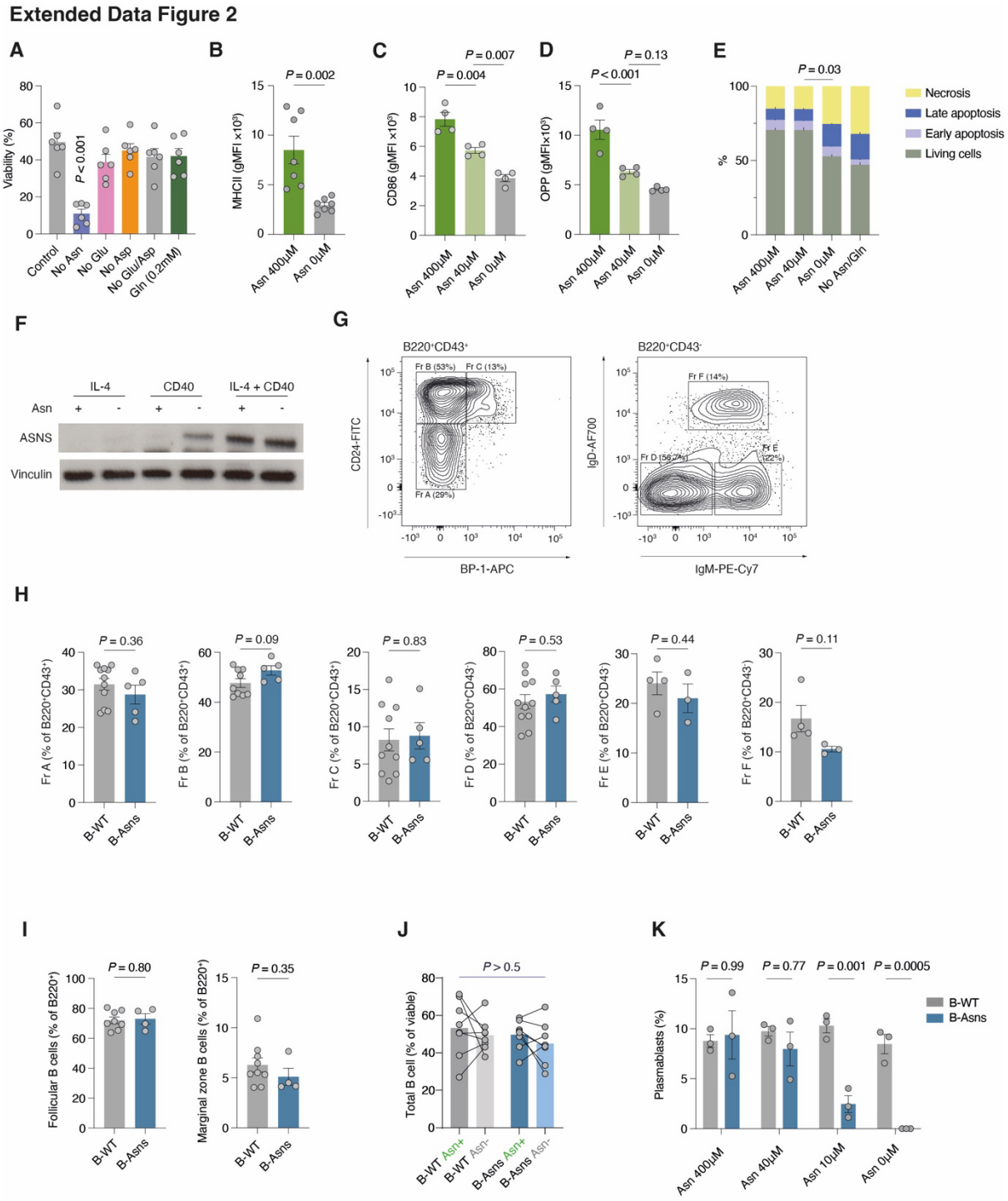
Statistical significance was determined by repeated measure two-way ANOVA with Šidák's multiple testing correction. Data are presented as the mean +/- SEM.



Extended Data Figure 1.

- **A.** Gating strategy for GC B cells ($CD19^+IgD^{lo}CD38^-GL\text{-}7^+$) and naïve IgD^+ B cells ($CD19^+IgD^+$).
- **B.** Representative flow cytometry histogram and quantification of CD98 geometric mean fluorescence intensity (gMFI) in IgD^+ naïve and GC B cells (n=5 mice). Data representative of two independent experiments.
- **C.** Immunoblot of ASNS in magnetic bead sorted GC B cells and naïve B cells, from wild type mice immunised with SRBC at day 0 and day 4, with analysis at day 12. Representative of two independent experiments. Beta-actin is used as a loading control.
- **D.** CellDIVE immunofluorescence images of a GC from a tonsil highlighting ASNS in $CD21^+$ LZ, $CD21^-$ DZ, IgD^+ naïve, and $MZB1^+$ plasma cell niches.
- **E.** Quantification of mean fluorescence intensity (MFI) of ASNS in areas shown in **D**. Each dot represents a randomly-selected GC with a representative DZ, LZ and surrounding IgD^+ naïve B cell follicle (n=21 GCs in total, pooled from three tonsils).
- **F.** CellDIVE immunofluorescence images of a GC from a tonsil highlighting SLC1A5 (ASCT2) expression in $CD21^+$ LZ, $CD21^-$ DZ, and surrounding IgD^+ naïve B cell follicle.

Statistical significance was determined by unpaired two-tailed t test (B) or one-way ANOVA with Tukey's multiple testing correction (E). Data are presented as the mean +/- SEM.



Extended Data Figure 2.

- **A.** Viability of B cells stimulated for 72h with IL-4 and anti-CD40, in the presence or absence of the indicated amino acids (n=6 mice). Representative of three independent experiments.
- **B.** MHCII gMFI in B cells stimulated for 24h with IL-4 and anti-CD40 with the indicated concentrations of Asn (n=7 mice). Representative of three independent experiments.
- **C.** CD86 gMFI in B cells stimulated for 24h with IL-4 and anti-CD40 with the indicated concentrations of Asn (n=4 mice). Representative of three independent experiments.
- **D.** OPP incorporation determined by flow cytometry in B cells stimulated for 24h with IL-4 and anti-CD40 with the indicated concentrations of Asn (n=4 mice). Representative of two independent experiments.

E. Quantification of stages of apoptosis in B cells stimulated with IL-4 and anti-CD40 at 18h, defined by annexin V labelling and propidium iodide staining, in the indicated concentrations of Asn of Asn or Asn and Gln (n=3 individual mice). Representative of two independent experiments.

F. Representative immunoblot of ASNS in B cells stimulated for 48h with IL-4, anti-CD40, or IL-4 and anti-CD40, in the presence/absence Asn. Representative of two independent experiments.

G. Representative gating strategy for indicated Hardy stages of B cell development in bone marrow.

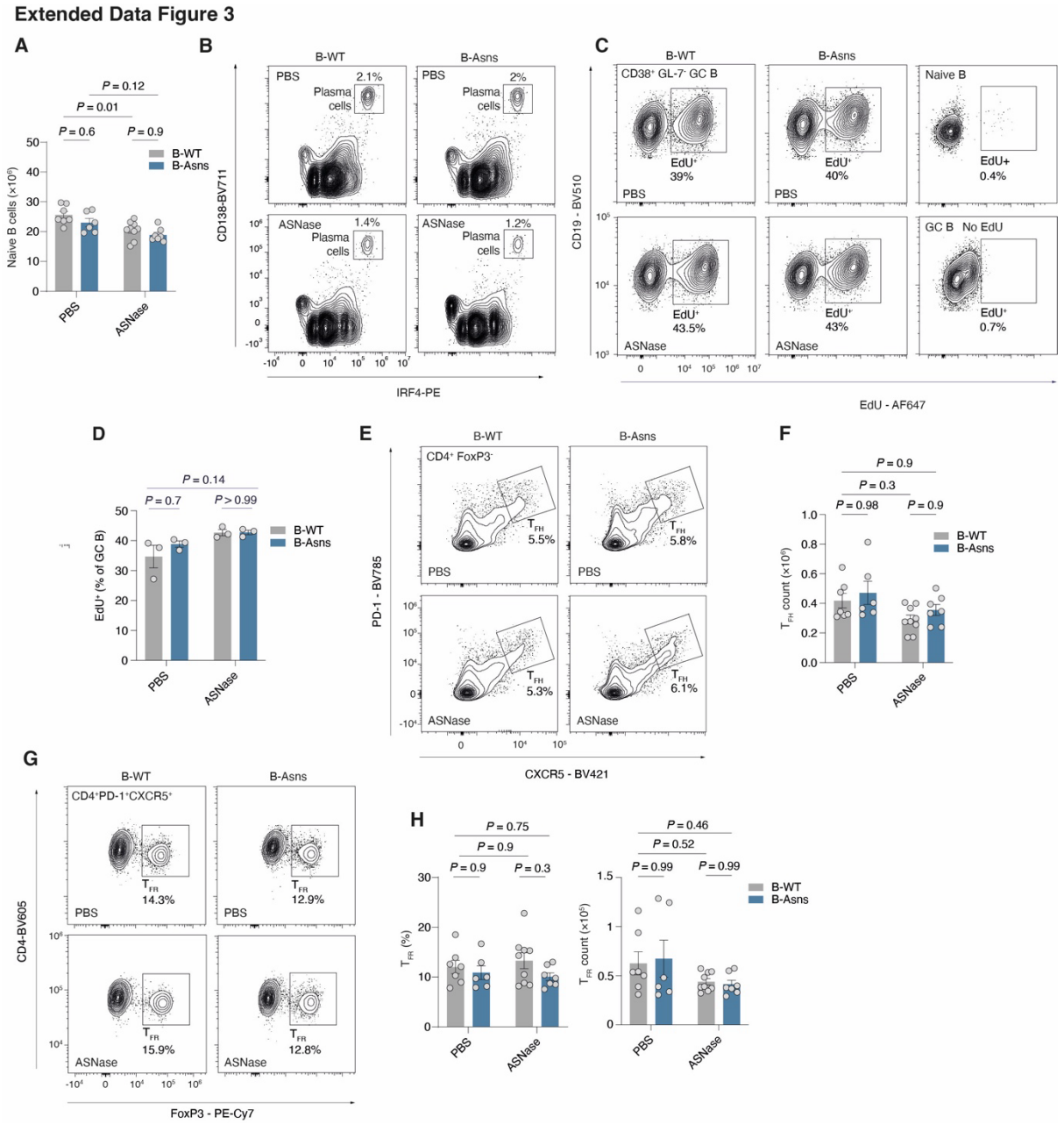
H. Quantification of indicated Hardy stages of B cell development in bone marrow. Each data point represents a mouse. Data pooled from two independent experiments.

I. Marginal zone and follicular B cell quantification in spleen. Each data point represents a mouse. Data pooled from two independent experiments.

J. Quantification of $CD19^+$ B cell proportions by flow cytometry in lymph node slices (300µm thick sections) cultured for 20h in the presence or absence of Asn (400µM) obtained from inguinal lymph nodes of B-WT and B-Asns mice immunised with NP-CGG (day 14). Each dot represents a single lymph node. Data pooled from two independent experiments.

K. Quantification of IgD^- $CD138^+$ Plasmablast proportions in B cells from B-WT and B-Asns mice stimulated with LPS and IL-4 for 72h in the absence of presence of the indicated concentrations of Asn. Representative of three independent experiments.

Statistical significance was determined by one-way ANOVA with Tukey's multiple testing correction (A, C-E), unpaired two-tailed t test (B,H-I), paired two-tailed t test (J), or two-way ANOVA with Šidák's multiple testing correction (K). Data are presented as the mean +/- SEM.



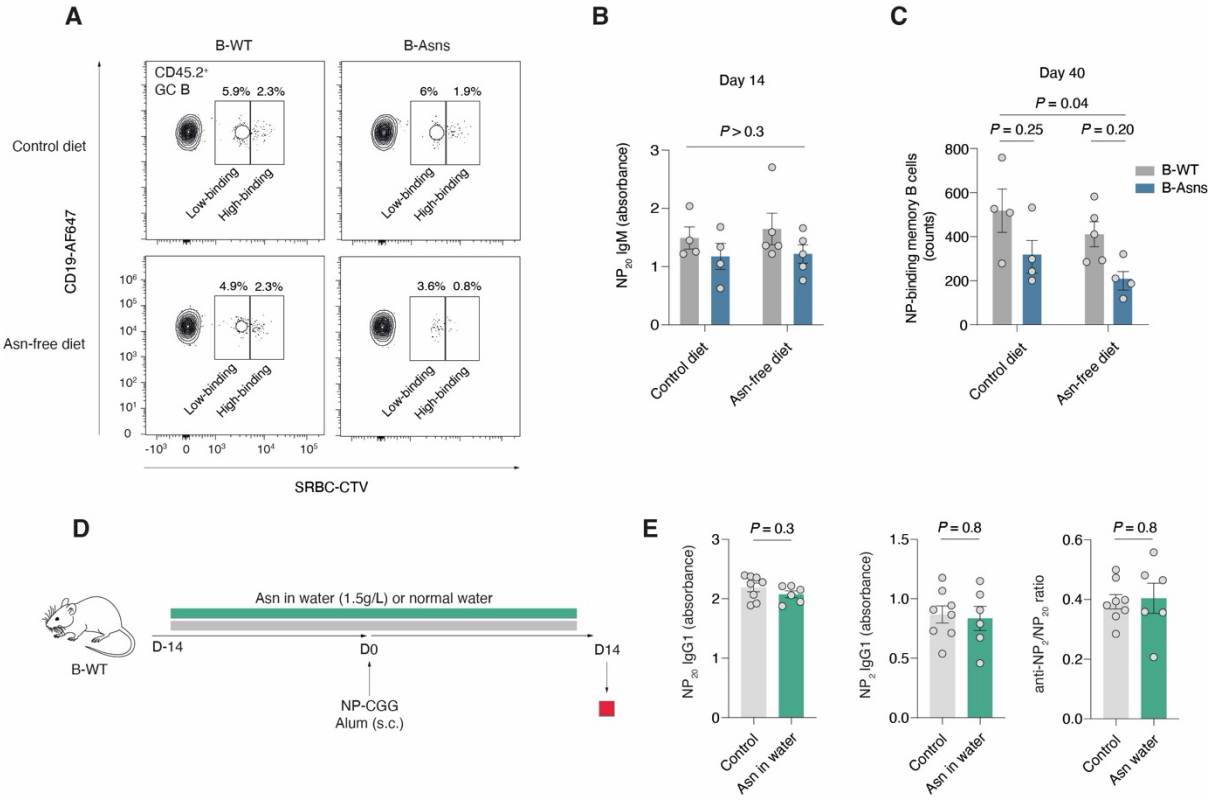
Extended Data Figure 3.

- **A.** Quantification of splenic naïve B cell counts from B-WT or B-Asns mice treated with ASNase or PBS. Each point represents a single mouse. Data pooled from two independent experiments.
- **B.** Representative gating strategy for plasma cells as IgD^- $IRF4^+$ $CD138^+$
- **C.** B-WT or B-Asns mice were treated with ASNase or PBS every two days as described in **Fig. 3A** and immunised with SRBC at day 0. On day 8, mice were pulsed with 1mg EdU intraperitoneally and two hours later EdU incorporation in splenic GC B cells were analysed by flow cytometry. Representative gating strategy EdU^+ GC B cell fraction with negative controls.
- **D.** Quantification of **C.** Data representative of two independent experiments.
- **E.** Representative gating strategy for $CD19^-$ $CD4^+$ $Foxp3^-$ $CXCR5^{hi}$ $PD\text{-}1^+$ T_{FH} cells

F. Quantification of T_{FH} numbers at day 9 post SRBC immunisation. Each point represents a single mouse. Data pooled from three experiments.
G. Representative gating strategy for $CD19^{-}CD4^{+}CXCR5^{+}PD\text{-}1^{+}Foxp3^{+}$ T_{FR} cells
H. Quantification of T_{FR} ratio and counts at day 9 post SRBC immunisation. Each point represents a single mouse. Data pooled from three experiments.

Statistical significance was determined by two-way ANOVA with Šidák's multiple testing correction. Data are presented as the mean +/- SEM.

Extended Data Figure 4

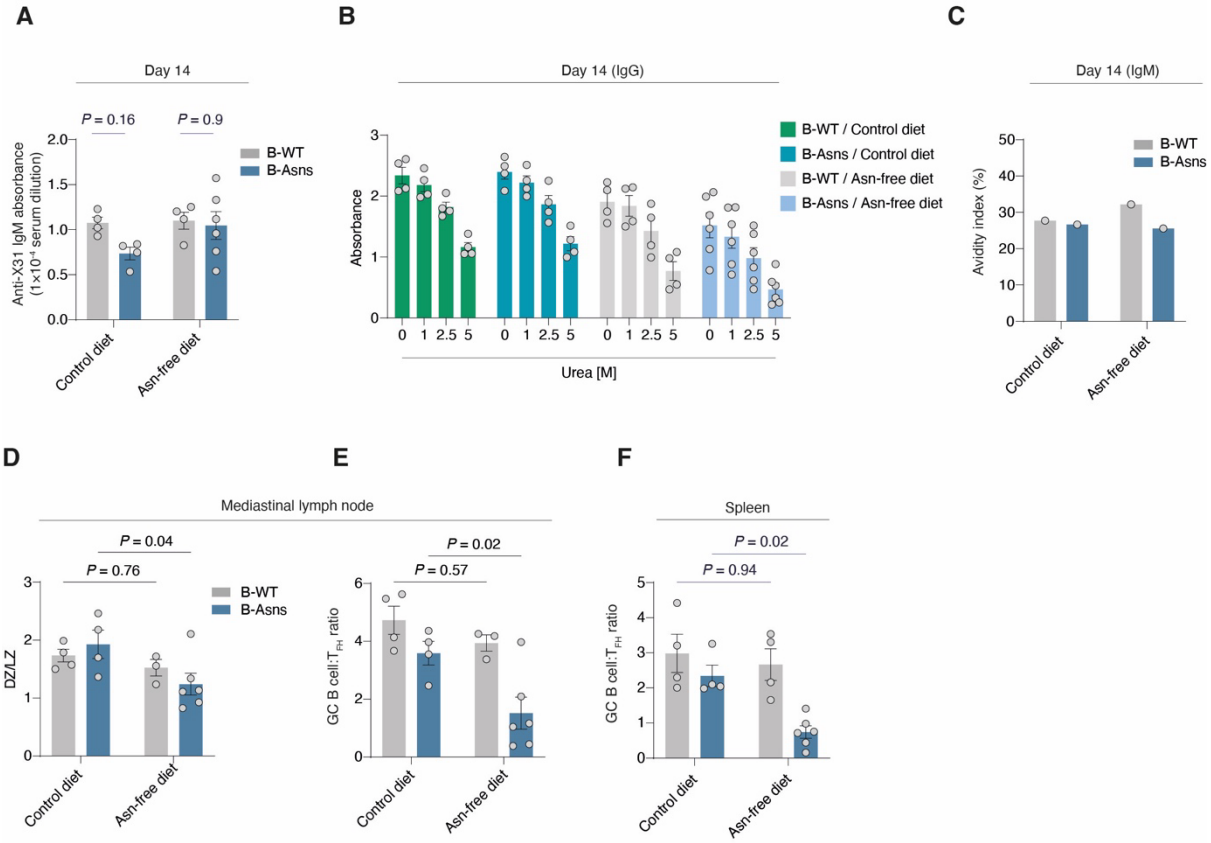


Extended Data Figure 4.

A. Representative high and low binding fractions of CD45.2 GC B cells from BM chimeric recipient mice to SRBCs labelled with Celltrace Violet (CTV).
B. ELISA quantification of Anti-NP_{20} IgM antibodies at day 14 post-NP-CGG immunisation. Each point represents a single mouse. Representative of two independent experiments.
C. Quantification of NP-binding memory B cell counts at day 40 post-NP-CGG immunisation as in Fig. **4K**. Data pooled from two independent experiments.
D. Schematic of NP-CGG/Asn supplementation in water experiment. B-WT mice were administered normal or Asn supplemented-water (1.5g/L) for 14 days before subcutaneous immunisation with NP-CGG. Sera were collected at day 14 post-immunisation for the detection of NP-specific antibodies by ELISA.
E. ELISA quantification of anti-NP_2 and anti-NP_{20} IgG1 antibodies along with the NP_2/NP_{20} ratio (affinity maturation index) at day 14 post-NP-CGG immunisation as in **D.** Each point represents a single mouse. Representative of two independent experiments.

Statistical significance was determined by two-way ANOVA with Šidák's multiple testing correction (B,C) or unpaired two-tailed t test (E). Data are presented as the mean +/- SEM.

Extended Data Figure 5

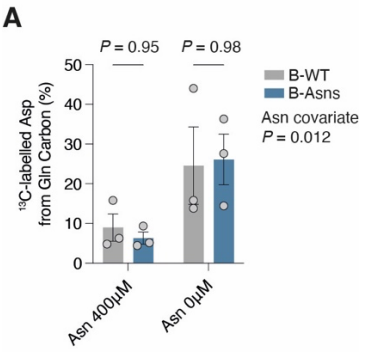


Extended Data Figure 5.

- **A.** Quantification of anti-X31 IgM at day 14 following influenza infection, as depicted in **Fig. 5A**
- **B.** ELISA quantification at day 14 for anti-X31 IgG absorbance at $1/5\times10^4$ dilution following increasing concentration of urea treatment (at 0, 1, 2.5 or 5M). Each point represents a single mouse. Data pooled from two independent experiments.
- **C.** Avidity index quantification for anti-X31 IgM based on 5M Urea treatment calculated as in **Fig. 5E**. Each point represents a pooled sera from n=4-6 mice per condition. Data representative of two independent experiments.
- **D.** Quantification of DZ/LZ ratio in $CD38^-$ $GL\text{-}7^+$ GC B cells in mediastinal lymph node at day 21 post-influenza infection. Each point represents a single mouse. Each point represents a pooled sera from n=4-6 mice per condition. Data pooled from two independent experiments.
- **E.** Quantification of GC B:T_{FH} ratio in mediastinal lymph node at day 21 post-influenza infection. Each point represents a pooled sera from n=4-6 mice per condition. Data pooled from two independent experiments.
- **F.** Quantification of splenic GC B:T_{FH} ratio at day 21 post-influenza infection. Data pooled from two independent experiments.

Statistical significance was determined by two-way ANOVA with Šidák's multiple testing correction. Data are presented as the mean +/- SEM.

Extended Data Figure 6

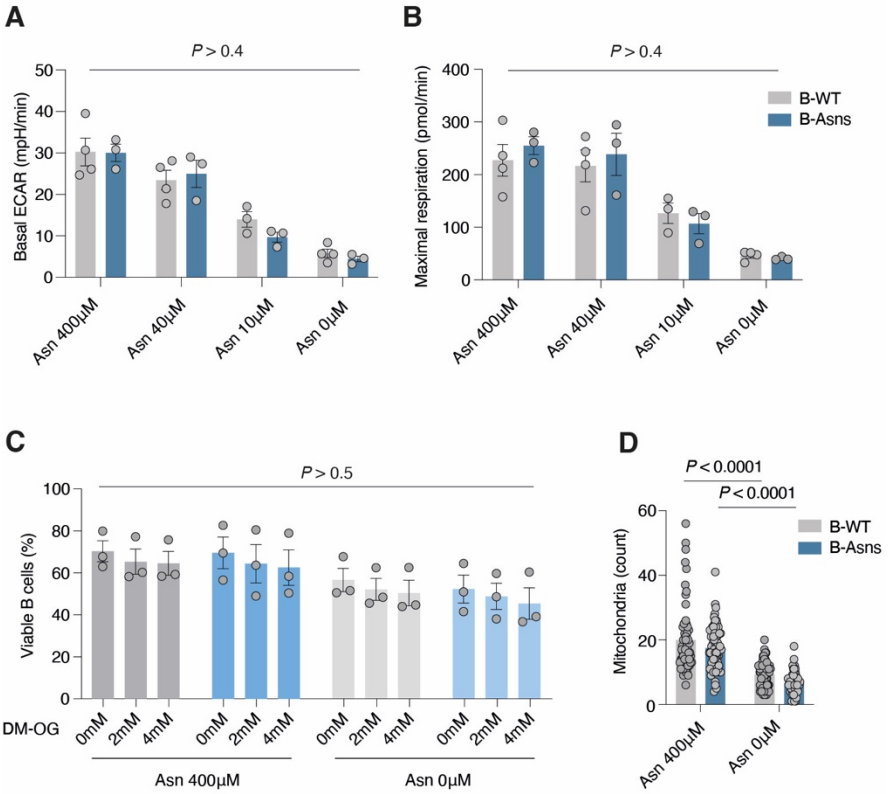


Extended Data Figure 6.

- **A.** Percentage of aspartate labelled with ^{13}C, indicating derivation from U^{13}C-glutamine, as in **Fig. 6E**.

Statistical significance was determined by two-way ANOVA with Šidák's multiple testing correction, with Asn concentration as a co-variate. Data are presented as the mean +/- SEM.

Extended Data Figure 7



Extended Data Figure 7.

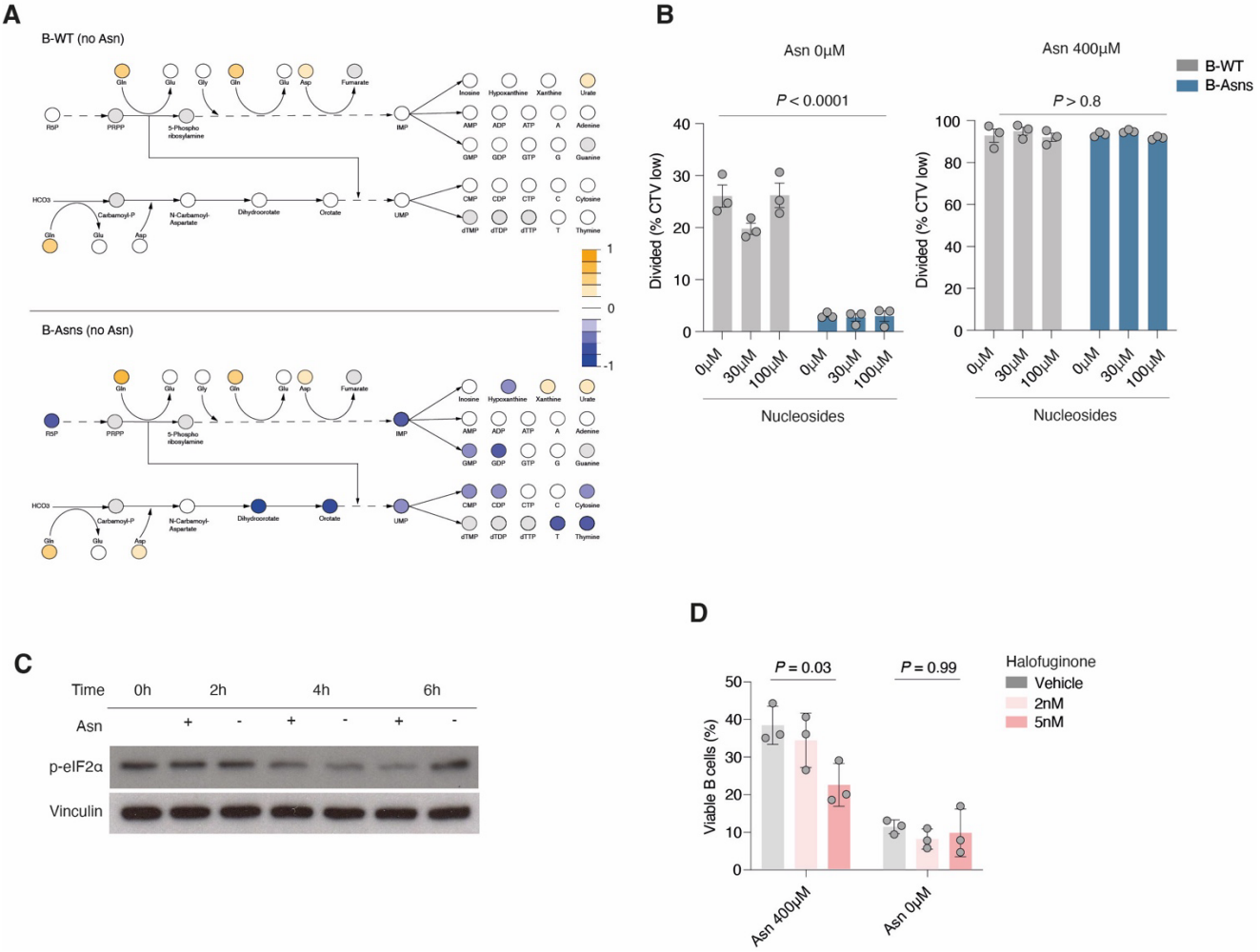
- **A.** Quantification of basal ECAR as in Fig. **7B**.
- **B**. Quantification of maximal respiration as in Fig. **7D**.

C. Viability of B cells from B-WT and B-Asns stimulated with anti-CD40 and IL-4 for 24h in the presence or absence of Asn (400µM), supplemented with vehicle (DMSO) or DM-OG at the indicated concentrations (0mM, 2mM or 4mM). Data pooled from two independent experiments (n=3 B-WT, n=3 B-Asns mice).

D. Quantification of mitochondrial count as in Fig. **7G**. Each data point represents a cell pooled from n=3 mice B-WT or B-Asns mice. Representative of three independent experiments.

Statistical significance was determined by two-way ANOVA with Šidák's multiple testing correction (A-B,D), one way ANOVA with Tukey's multiple testing correction (C). Data are presented as the mean +/- SEM.

Extended Figure 8



Extended Data Figure 8.

A. Relative abundance of metabolites in nucleotide synthesis pathway in B-WT and B-Asns B cells deprived of Asn. Grey-filled circles indicate that the metabolite was not detected. Scale represents abundance ratio.

B. Quantification of the fraction of B cells from B-Asns and B-WT mice (n=3 mice) having diluting Celltrace Violet after stimulation for 72h with IL-4 and anti-CD40 in the

presence or absence of Asn supplemented with adenosine, thymidine, uridine, cytidine, and guanosine at 0µM, 30µM or 100µM. Each data point represents a mouse. Data pooled from two independent experiments.

- **C.** Representative immunoblot time course of phospho-eIF2α in wild type B cells stimulated with IL-4 and agonistic anti-CD40 in the absence or presence of Asn (400µM). Representative of two independent experiments.
- **D.** Viability of wild type B cells stimulated with IL-4 and agonistic anti-CD40 for 72h, in the presence or absence of Asn (400µM), and with the indicated concentration of halofuginone or vehicle (DMSO). Representative of two independent experiments.

Statistical significance was determined by two-way ANOVA with Šidák's multiple testing correction (B,D). Data are presented as the mean +/- SEM.

Material and methods

Mice

C57BL/6J mice were obtained from Envigo. C57BL/6N-*Asns*$^{tm1c(EUCOMM)Wtsi}$/H (EM:05307) mice were obtained from the Mary Lyon Centre, MRC Harwell, UK. B6.129S6-*Eif2ak4*$^{tm1.2Dron}$/J (JAX: 008240), B6.C(Cg)-*Cd79a*$^{tm1(cre)Reth}$/EhobJ (JAX: 020505), B6.129P2-*Aicda*$^{tm1(Cre)Mnz}$/J (JAX: 007770), and B6;129S6-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J (JAX: 007905) mice were obtained from Jackson Laboratories. B6.SJL.CD45.1 mice were provided by the central breeding facility of the University of Oxford. Male and female mice between the ages of 6-15 weeks were used. *Asns* $^{LoxP/+}$ × *Cd79a*-Cre$^{+/-}$, *Asns*$^{+/+}$ × *Cd79a*-Cre$^{+/-}$, *Asns* $^{LoxP/+}$ × *Cd79a*-Cre$^{-/-}$, and *Asns* $^{LoxP/ LoxP}$ × *Cd79a*-Cre$^{-/-}$ mice were used as controls (B-WT). All phenotypes were reproduced with all control genotypes.

Mice were bred and maintained under specific pathogen-free conditions at the Kennedy Institute of Rheumatology, University of Oxford. They were housed in cages that had individual ventilation and were provided with environmental enrichment. The temperature was kept between 20-24°C, with a humidity level of 45-65%. They were exposed to a 12-hour cycle of light and darkness (7 am to 7 pm), with a thirty-minute period of dawn and dusk. All procedures and experiments were performed in accordance with the UK Scientific Procedures Act (1986) under a project license authorized by the UK Home Office (PPL number: PP1971784).

In vivo modification of asparagine levels

Wild type mice were intraperitoneally injected with ≈10U (500U/kg) of asparaginase from *E. coli* (Abcam, ab277068) diluted in PBS every two days.

Asn-free diet or control chow (Research Diets, A05080216i and A10021Bi) was administered for 12 days before immunogenic challenges and maintained for the duration of experiments.

Drinking water was supplemented with L-asparagine monohydrate (Merck, cat: A7094-25G) at 1.5 g/L and administered to mice two weeks prior to immunisation and maintained for the whole duration of the experiment. Drinking water was replaced with freshly prepared Asn every 2-3 days.

Immunisation

1ml of sterile SRBCs in Alsever's solution (ThermoFisher or EO Labs or TCS bioscience) were washed twice with 10ml of ice-cold PBS and reconstituted in 3ml of PBS, and 200µl injected intraperitoneally or intravenously. In some experiments, an enhanced SRBC immunisation method was used to maximise GC B cell yield by immunising mice with 0.1ml SRBC on day 0 followed by a second injection of 0.2ml on day 4-5[50]. For protein antigen immunisations, 50µg $NP_{(30-39)}$-CGG (Biosearch Tech, cat: N-5055D-5) in PBS was mixed with Imject Alum (ThermoFisher) or Alum Hydrogel (InvivoGen, cat: vac-alu-50) at a 1:1 ratio (vol:vol) and rotated at room temperature for 30 mins before intraperitoneal injection in 100µl volume, or mixed vigorously with a pipette for 5 mins before subcutaneous injection on the flanks or hock.

Influenza infection

A/HK-x31 (x31, H3N2) strain was used. Mice were anaesthetised using isoflurane and intranasally inoculated with $5x10^3$ PFU of X31 influenza A virus in PBS. Mice were closely monitored and weighed for 14 days following infection, characteristic weight loss was confirmed in all infected mice.

Bone marrow chimera generation

B6.SJL.CD45.1 recipient mice were administered two doses of 5.5Gy irradiation four hours apart. Mice were then intravenously injected with 4×10^6 mixed bone marrow (BM) cells at a 1:1 ratio, isolated from age- and sex-matched $CD45.2^+$ B-WT or B-Asns and $CD45.1^+$ WT donor mice. Recipient mice were maintained on antibiotics (Baytril, Bayer corporation) administered in their drinking water for two weeks. Bone marrow reconstitution was confirmed by flow cytometry of peripheral blood at 8 weeks. Asn-free diet or control chow (Research Diets, A05080216i and A10021Bi) was administered for 12 days starting from week 8, maintained for additional 9 days (total of 21 days) during immunisation with SRBC until mice were terminated at 11 weeks.

Cell isolation

Spleens were dissociated by passing through 70µm cell strainers. For ex vivo GC B cell mass spectrometry, GC B cells were first enriched using the mouse Germinal Center B Cell (PNA) MicroBead Kit (Miltenyi), and then further purified by flow sorting (Live/$Dead^-$ $CD19^+IgD^-GL\text{-}7^+CD95^+$). Total B cells from the same mouse pool were pre-enriched using $CD19^+$ Microbeads (Miltenyi), then naïve B cells purified by flow sorting (Live/$Dead^-$ $B220^+IgD^+$).
To isolate cells for RNA extraction, GC B cells (Live/$Dead^-CD19^+IgD^-CD95^+GL\text{-}7^+$) and naïve B cells (Live/$Dead^-CD19^+IgD^+$) were flow sorted into RLT Plus buffer (Qiagen) following pre-enrichment with the Pan B Cell Isolation Kit II (Miltenyi). B cells for culture were isolated using the Pan B Cell Isolation Kit II (Miltenyi). Purity was routinely >90% by flow cytometry.

For some experiments, untouched GC B cells were isolated using a magnetic bead-based protocol as described[51]. Briefly, single cell suspensions were prepared from spleens of SRBC-immunised mice (enhanced protocol) in ice cold MACS isolation buffer (PBS with 0.5% BSA and 2mM EDTA) followed by ACK (Gibco) RBC lysis for 4 mins at 20°C. Following washing, cells were labelled with anti-CD43 microbeads (Miltenyi) and biotinylated antibodies against CD38 and CD11c (both eBioscience, clones 90 and N418 respectively).

Then, cells were incubated with anti-biotin Microbeads (Miltenyi), and subsequently run through an LS column (Miltenyi). GC B cell purity was around 90%.

Cell culture

Total B cells were isolated as described above. B cells were cultured at 1-3×10^6 cells/ml in RPMI1640 (custom product from Cell Culture Technologies, Gravesano, Switzerland, lacking glutamate, aspartate, glutamine, and asparagine), supplemented with 1mM pyruvate, 10mM HEPES, 100 IU/ml penicillin/streptomycin and 50µM 2-mercaptoethanol, and 10% dialysed FBS (Gibco), Asp (150µM), Glu (140µM), GlutaMAX (Thermo or Sigma)(2mM), indicated concentration of Asn, except where mentioned otherwise. B cells were stimulated with agonistic anti-CD40 (5µg/ml, FGK45.4, functional grade, Miltenyi), recombinant IL-4 (1,10 or 50ng/ml, Peprotech), LPS (10µg/ml, Merck) or CpG (100nM, ODN1826, Miltenyi).

For proliferation experiments, cells were labelled with CellTrace Violet (ThermoFisher) according to manufacturer's instructions before stimulation. For stable isotope labelling, ^{15}N(amide)-asparagine (485896, Merck) (400µM), ^{15}N(amide)-glutamine (NLM-557, Cambridge Isotope Laboratories) (2mM), ^{15}N(amino)-glutamine (NLM-1016, Cambridge Isotope Laboratories) (2mM), or $^{13}C_5$-glutamine (CLM-1822, Cambridge Isotope Laboratories) (2mM) were used, replacing their unlabelled molecule in RPMI1640.

For nucleoside rescue experiment, adenosine (Fluorochem cat: F093333), thymidine (Fluorochem cat: F078885), uridine (Fluorochem cat: F078878), cytidine (Fluorochem cat: F226675) and guanosine (Merck, cat: G6264-5G) were prepared freshly in water and added to culture media at a final concentration of 30µM or 100µM.

Halofuginone hydrobromide (Bio-techne, cat no: 1993) was reconstituted in DMSO and used at 2nM and 5nM in the culture. Dimethyl 2-oxoglutarate (DM-OG) was purchased from Merck (cat no: 349631-5G) and added to the culture medium at 2mM and 4mM final concentrations.

Ex vivo lymph node slice culture

Inguinal lymph nodes (LNs) were harvested from B-WT or B-Asns mice immunised with NP-CGG (precipitated in Alum hydrogel) subcutaneously on both flanks. They were then removed from surrounding fat by dissection and mild detergent wash (<1 sec) in 0.01% digitonin solution (Thermo Scientific) and embedded in 6% w/v low melting point agarose (Lonza) in 1x PBS. 300µm thick slices were cut using the Precisionary Compresstome® VF-210-0Z following manufacturer's instructions with a blade speed of 4 and oscillation of 6. The buffer tank was filled with ice cold PBS containing 1% Pen/Strep. Slices were removed from surrounding agarose and placed in a 24 well plate in 500µl of Asn-replete or Asn-free complete RPMI media in duplicate. LN slices were allowed to rest and equilibrate in the media for 1 hour at 37˚C and 5% CO2 before transferral into fresh media and incubation for 20hrs. Following culture, slices were collected, duplicate slices from same lymph nodes pooled, and slices disrupted into PBS containing 2% FBS and 2mM EDTA using the tip of an insulin syringe plunger to generate a cell suspension in a 96-well U bottom plate. Cells were then stained with viability dye and surface antigens. Following washes, cells were fixed in fresh 4% PFA (Cell Signalling) for 15 mins at 20°C and run on an Aurora flow cytometer (Cytek).

Flow cytometry

Spleens were dissociated by passing through 70µm cell strainers, and red cells lysed with ACK Lysis Buffer (Gibco). Single cell suspensions were incubated with Fixable Viability Dye eFluor™ 780 (eBioscience) or Zombie NIR (BioLegend) in PBS, supplemented with Fc Block and then surface antibodies (30 mins on ice) in FACS buffer (PBS supplemented with 0.5% BSA and 2mM EDTA). For intracellular staining, cells were fixed in 4% paraformaldehyde at 20°C, then permeabilised with ice cold 90% methanol for 10 mins. For FoxP3 staining, eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (ThermoFisher) was used. To measure OPP ex vivo incorporation, cells were incubated in RPMI1640 with the indicated concentration of Asn, in the presence of 20µM OPP for 25 mins at 37°C. Click labelling was then performed with the Click-iT™ Plus OPP Alexa Fluor 647 Protein Synthesis Assay Kit (ThermoFisher, cat: C10458). NP binding was determined by incubation with NP-PE (Biosearch) for 30 mins at 4°C. Apoptosis was quantified by annexin V-FITC binding (Biolegend). The proliferation index was determined using the Proliferation module of FlowJo (BD). SRBC binding assay was performed as previously reported[52]. Briefly, splenocytes were harvested from mice immunised with SRBC. Following surface staining, cells were mixed with SRBCs labelled with 10μM Cell Trace Violet (ThermoFisher) on ice for 20 mins. After washing, cells were resuspended in FACS buffer and acquired on BD Fortessa X20.

Antibody (Catalogue number)	**Clone**	**Manufacturer**
Anti-GL-7 (144612)	GL-7	Biolegend
Anti-CD138(142504)	281-2	Biolegend
Anti-CD19 (115538)	6D5	Biolegend
Anti-IgD (405721)	11-26c-2a	Biolegend
Anti-CD38 (102718)	90	Biolegend
Anti-CXCR4 (146513)	L276F12	Biolegend
Anti-CD98 (128211)	RL388	Biolegend
Anti-IgG1 (406617)	RMG1-1	Biolegend
Anti-MHCII (107605)	M5/114.15.2	Biolegend
Anti-CD23 (101608)	B3B4	Biolegend
Anti-CD43 (12-0431-82)	eBioR2/60	ThermoFisher
Anti-CD86 (105031)	GL-1	Biolegend
Anti-B220 (103247)	RA3-6B2	Biolegend
Anti-IgM (406513)	RMM-1	Biolegend
Anti-CD21/35 (123409)	7E9	Biolegend
Anti-TACI (133403)	8F10	Biolegend
Anti-CD16/32 (Fc Block)	2.4G2	ThermoFisher
Anti-CXCR4 (741783)	2B11	BD Bioscience
Anti-CD38 (741955)	90	BD Bioscience
Anti-CD4 (100547)	RM4-5	Biolegend
Anti-PD-1 (135213)	29F.1A12	Biolegend
Anti-CXCR5 (145511)	L138D7	Biolegend
Anti-CD45.1 (110717)	A20	Biolegend
Anti-CD45.2 (109817)	104	Biolegend
Anti-CD3 (100215)	17A2	Biolegend

Anti-IRF4 (12-9858-82)	3E4	ThermoFisher
Anti-BCL6 (561525)	K112-91	BD Bioscience
Anti-CHOP (NB600-1335AF700)	9C8	Novus Bio
Anti-Foxp3 (25-5773-82)	FJK-16	ThermoFisher

Ex vivo Asn uptake assay

We followed the recently published QUAS-R protocol with certain modifications[26]. Briefly, spleens were harvested 8 days post SRBC immunisation and dissociated through 70µm cell strainers. Red blood cells were lysed with ACK lysis buffer and single cell suspensions were incubated with Zombie NIR (BioLegend) and Fc block in PBS on ice for 30 minutes followed by surface staining in FACS buffer for 30 minutes on ice. Hanks' Balanced Salt Solution HBSS (ThermoFisher) supplemented with Click-iT homopropargylglycine HPG (ThermoFisher) at 400µM and L-asparagine monohydrate (Merck) at increasing concentrations (0, 10, 40, 100, 400, 4000µM) were warmed at 37°C. Cells were resuspended in HBSS and 50µl of suspension was plated and incubated at 37°C for 10 minutes. Control cells incubated at 4°C were kept on ice for background detection. HPG-containing media supplemented with each Asn dilution was added and cells were further incubated for 3 minutes, before immediate fixation with PFA at 4% for 25 minutes at room temperature. Cells were washed and permeabilised with 1× saponin-based permeabilization buffer (ThermoFisher) for 20 minutes at room temperature before adding the click chemistry reaction mix, using the Click-iT™ Plus OPP Alexa Fluor™ 647 Protein Synthesis kit due to the shared Click Chemistry of HPG with OPP and incubating for 30 minutes at room temperature. Cells were washed and resuspended in FACS buffer for flow cytometry acquisition.

Immunofluorescence microscopy

Spleens were fixed overnight in Antigenfix (DiaPath) solution at 4°C. The next day, spleens were washed in PBS, followed by overnight incubation in 30% sucrose (in PBS) at 4°C for cryoprotection. On the following day, spleens were snap frozen in 100% methanol on dry ice and stored at -80°C until cryosectioning at 8µm thickness. Slides were then rehydrated in PBS at 20°C, then blocked in PBS containing 0.05% Tween-20, 10% goat serum, and 10% rat serum at RT for one hour. All primary antibody staining was performed overnight at 4°C or 1 hour at RT in PBS supplemented with 2% rat serum and 0.05% Tween-20.

To visualise protein synthesis in vivo, mice were immunised with SRBC using the enhanced protocol. At day 14, they were injected intraperitoneally with 1.5mg L-AHA (Thermo) and then 4 hours later 1mg OPP (Jena Bioscience) dissolved in 100µl PBS, or PBS alone as a negative control. Mice were sacrificed 1h later, and spleens were harvested for immunofluorescence analysis. L-AHA and OPP were detected by Click Chemistry using the Click-iT™ Plus L-AHA and then Click-iT™ Plus OPP Alexa Fluor™ 488 or 647 Protein Synthesis kits (Thermo) following the manufacturer’s protocols.

Slides were mounted with Fluoromount G (Southern Biotech, cat: 0100-01) and imaged using a Zeiss LSM 980 equipped with an Airyscan 2 module or Zeiss Axioscan 7.

Multiplexed Tissue Imaging using Cell DIVE

Formalin-fixed paraffin embedded (FFPE) human tonsil sections (5µm) were deparaffinised and rehydrated, and then permeabilised in 0.3% Triton X100 for 10 mins followed by

washing in PBS for 5 mins. Antigen retrieval was performed in a Biocare NxGen decloaker by boiling the slides in a citrate-based antigen unmasking solution (Vector, #H3300) at pH 6, and then in a Tris-based antigen retrieval solution, containing 1×Tris, EDTA, 0.05% Tween20 and ddH2O (pH 9), for 20 mins at each step. The slides were blocked overnight in PBS solution containing 3% BSA (Merck, #A7906), 10% donkey serum (Bio-Rad, #C06SB), then incubated with human FcR Blocking Reagent (Miltenyi) at 1:100 at room temperature for 1 hour. After washing in PBS, the slides were stained with DAPI (ThermoFisher), washed again, and cover slips were placed with mounting media (50% glycerol, [Sigma] and 4% propyl gallate [Sigma, #2370]).

All FFPE human tonsil slides were imaged using a GE Cell DIVE system. Firstly, a ScanPlan was performed by acquiring at 10× magnification so that regions of interest could be selected. This was then followed by imaging at 20× to acquire background autofluorescence and generate virtual H&E images. This background was subtracted from all subsequent rounds of staining.

All slides were decoverslipped in PBS before each staining round, which consisted of three antibodies applied in staining buffer (3% BSA in PBS). Unconjugated primary antibodies were used in the first round, incubated at 4°C overnight. The next day, slides were washed in 0.05% PBS-Tween 20, (Sigma, P9416) three times. AlexaFluor 488, 555 or 647 conjugated secondary antibodies were then added to the slides for an hour at room temperature. Directly conjugated antibodies were used in subsequent staining rounds, incubated at 4°C overnight. Antibody conjugation was performed in house for some of the antibodies using the antibody labelling kit (Invitrogen).

Fluorescent dye inactivation was performed by bleaching the slides with 0.1 M NaHCO3 (pH 10.9-11.3) (Sigma, S6297) and 3% H_2O_2 (Merck, #216763,) for 3 rounds (15 mins each) with a 1 min wash in PBS in between. The slides were then re-stained with DAPI for 2 mins before being washed in PBS. The bleached slides were imaged as the new background for subsequent subtraction.

Image analysis was performed in ImageJ. 225µm^2 region of interests (ROIs) were selected randomly in CD21$^-$ IgD$^-$ DZ GC, CD21$^+$ IgD$^-$ LZ GC and surrounding IgD$^+$ B cell follicle areas of individual GCs with representative DZ-LZ distribution. These ROIs were then inspected in the ASNS channel and mean fluorescence was calculated using the Measure function. Seven GCs from each tonsil were pooled for analysis, and a total of 21 GCs from 3 tonsils were quantified.

Antibody list

Antibody	**Catalogue number**	**Manufacturer**
IgD	324502	Biolegend
ASNS	14681-1-AP	Proteintech
CD21	ab202692	Abcam
CD3	ab307335	Abcam
MZB1	NBP2-90320AF647	Novus
SLC1A5/ASCT2	HPA035240-25UL	Atlas

3D Lattice SIM

Cultured B cells were transferred onto poly-L-lysine coated coverslips and incubated at 37°C for 10 minutes. Cells were subsequently fixed in 4% paraformaldehyde for 10 minutes at 37°C, before permeabilisation and blocking at 20°C for 30 minutes in PBS containing 0.2% Triton X-100, 10% goat serum and 10% rat serum. Staining with conjugated antibodies was then performed overnight at 4°C in PBS supplemented with 2% goat serum. Nuclear staining was carried out with DAPI (Sigma) diluted in PBS for 15 minutes at 20°C, prior to coverslip mounting with Fluoromount G (Southern Biotech, catalogue no. 0100-01). Imaging was performed with the Zeiss Elyra 7 lattice SIM using the 63× oil immersion objective and 15 phase SIM. SIM processing was then carried out on ZEN Black.

Image quantification of the mitochondrial network was performed using ImageJ software. Single cells were defined in individual Region of Interest (ROI) based on COX I signal. 3D mitochondrial morphological and network analysis was subsequently performed using an ImageJ plugin[53] with modified parameters: rolling (microns) 1.28 and gamma (0.90).

Extracellular flux analysis (SeaHorse)

Real-time oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using a Seahorse XFe96 Extracellular Flux Analyzer (Agilent). Ex vivo isolated B cells from B-WT and B-Asns mice were stimulated (anti-CD40 at 5µg/ml and IL-4 at 10ng/ml) overnight at 10^6 cells per ml in a 24 well plate in the presence of varying concentrations of Asn. The next day, 2×10^5 B cells were plated on a poly-D lysine (Sigma)-coated XF96 cell culture microplate with 5-6 technical replicate wells and incubated at 37 °C for a minimum of 30 min in a CO_2-free incubator in assay medium (XF DMEM medium pH 7.40 supplemented with 2 mM L-glutamine, 1 mM pyruvate and 10 mM Glucose) before the initiation of the measurements. Basal OCR and ECAR were measured, then followed by the MitoStress test, with oligomycin (1 µM), fluorocarbonyl cyanide phenylhydrazone (FCCP, 2µM) and rotenone + antimycin A (0.5µM) (Agilent). Analyses were performed on Wave (Agilent).

ELISA

96 well EIA/RIA plates (Corning, cat: 3590) were coated with NP conjugated with BSA (NP_2 or $NP_{20>}$, cat: N5050XL-10 and cat: N-5050H-10, both from BioSearch) at 5µg/ml in bicarbonate/carbonate coating buffer overnight at 4°C. The next day, plates were washed with PBS and blocked with 5% skimmed milk in PBS for 2-2.5 hours at 37°C. Sera obtained from mice (NP-CGG-immunisation day 14 subcutaneously or unimmunized control) were serially diluted in 1% skimmed milk and added into blocked plates for exactly 1hr at 37°C. After several washes with PBS-Tween20 (0.05%), alkaline phosphatase-conjugated goat anti-mouse IgG1 (Southern Biotech, cat: 1071-04) or IgM (Southern Biotech, cat: 1021-04) detection antibodies (at 1:2000 dilution) were incubated for exactly 1hr at 37°C. After the final washing step, plates were developed with alkaline phosphatase substrate (Sigma, cat: P7998-100ML) with simultaneous reading on FLUOstar Omega plate reader every 5 mins.

96 well EIA/RIA plates were coated overnight at 4°C with PFA-inactivated X31 virus in bicarbonate/carbonate coating buffer. After blocking with 1% BSA in PBS-Tween (0.05%) at 20°C for 2h, serially-diluted sera added and incubated further 2h at 20°C. After multiple washes, biotinylated goat anti-mouse IgG or IgM detection antibodies (Southern Biotech, 1:1000) were added and incubated at 20C for 1h. Following washes, Horseradish Peroxidase (HRP) conjugated with Streptavidin (Jackson ImmunoResearch, 1:1000) was

added and incubated for additional 30 mins. The 3,3′,5,5′-Tetramethylbenzidine (TMB substrate) (ThermoFisher) added following multiple washes. Reading was performed at two wavelengths (450 and 570) with precise scanning mode after stopping the reaction with 2N H_2SO_4.

For avidity assessment, following serum incubation, there was an additional 15-minute washing step at 20°C with freshly prepared urea (vWr) in PBS at concentrations of 1, 2.5, or 5M. The detection and development procedures remained the same as the aforementioned protocol.

Mass spectrometry

To extract molecules, cell were pelleted and washed twice with cold PBS and once with ice cold ammonium acetate (150mM, pH 7.3). The pellet was then resuspended in 1ml 80% LC-MS grade methanol/ultrapure water chilled on dry ice. The samples were vortexed three times on dry ice, and then centrifuged at 15,000×g for 5 mins. The initial supernatant was then transferred into a 1.8ml glass vial, and the pellet resuspended in 200µl 80% methanol as above, then centrifuged again at 15,000×g for 5 mins. The second supernatant was then combined with the first, dried at 4°C using a Centrivap (Conco), then stored at -80°C until analysis.

Dried metabolites were resuspended in 50% ACN:water and 1/10th was loaded onto a Luna 3µm NH_2 100A (150 × 2.0 mm) column (Phenomenex). The chromatographic separation was performed on a Vanquish Flex (Thermo Scientific) with mobile phases A (5mM NH_4AcO pH 9.9) and B (ACN) and a flow rate of 200µl/min. A linear gradient from 15% A to 95% A over 18 min was followed by 7 min isocratic flow at 95% A and reequilibration to 15% A. Metabolites were detected with a Thermo Scientific Q Exactive mass spectrometer run with polarity switching (+3.5 kV/– 3.5 kV) in full scan mode with an m/z range of 70-975 and 140.000 resolution. Maven (v8.1.27.11) was used to quantify the targeted metabolites by area under the curve using expected retention time (as determined with pure standards) and accurate mass measurements (< 5 ppm). Values were normalized to cell number. Relative amounts of metabolites were calculated by summing up the values for all measured isotopologues of the targeted metabolites. Metabolite Isotopologue Distributions were corrected for natural ^{15}N abundance. Metabolite and transcriptome data were analysed using MetaboAnalyst[54] and statistical analysis was performed in R.

Western blotting

Cells were lysed on ice in RIPA buffer (Merck) supplemented with protease and phosphatase inhibitors (complete™ ULTRA Tablets and PhosSTOP™, Roche) for 30mins with frequent vortexing, and then centrifuged at 15,000×g for 15mins at 4°C and the supernatant recovered. Protein was quantified using the BCA method (Pierce, Thermo). Samples were denatured in Laemmli buffer (BioRad) containing 10% β-mercaptoethanol at 90°C for 5 mins then transferred to ice, before running on a 4-15% gel (Mini-PROTEAN TGX Precast Gels, BioRad). Protein was transferred onto PVDF membranes (BioRad), which were blocked in 2.5% skimmed milk for 1h, and then stained with the primary antibody diluted in 2.5% BSA/0.05% TBS-Tween 20 at 4°C overnight.

Following washing, membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody in 2.5% milk/0.05% TBS-Tween 20 for 1h at RT. Chemiluminescence was used to detect the secondary antibody (Anti-rabbit IgG, HRP-

linked Antibody #7074 and SuperSignal™ West Pico PLUS Chemiluminescent Substrate, ThermoFisher).

Antibody (Catalogue number)	**Dilution**	**Clone**	**Manufacturer**
DHODH (14877-1-AP)	1:1000	Polyclonal	Proteintech
ASNS (14681-1-AP)	1:2000	Polyclonal	Proteintech
CHOP (2895)	1:1000	L63F7	Cell Signaling
Phospho-4E-BP1 (236B4)	1:500	Monoclonal	Cell Signaling
ATF4 (11815)	1:500	D4B8	Cell Signaling
Vinculin (13901)	1:1000	E1E9V	Cell Signaling
β-actin (4970)	1:2000	13E5	Cell Signaling
ATIC (ab188321)	1:500	EPR13243-53	Abcam
Phospho-eIF2a (9721)	1:500	Polyclonal	Cell Signaling

Quantitative PCR

Cells were washed in PBS and lysed in RLT Plus buffer (Qiagen) supplemented with 1% β-mercaptoethanol. RNA extraction was performed according to manufacturer's instructions using an RNeasy Plus Mini Kit (Qiagen). Reverse transcription was performed using the High Capacity RNA-to-cDNA Kit (Thermo). Quantitative qPCR was performed using Taqman Probes (Thermo). Relative expression was calculated with reference to *Ubc*.

List of probes used:

Gene	**Taqman ProbeID**
Asns	Mm00803785_m1
Ubc	Mm02525934_g1
Atf4	Mm00515325_g1
Got2	Mm00494703_m1

Statistical analysis
The use of the statistical tests is indicated in the respective figure legends, with the error bars indicating the mean ± S.E.M. P values ≤ 0.05 were considered to indicate significance. Analyses were performed with GraphPad Prism v9-10 or R 4.1. No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those reported in previous publications. The distribution of data was determined using normality testing to determine appropriate statistical methodology, or otherwise assumed to be normally distributed. For in vivo experiments we matched the age of the mice in experimental batches, but other modes of randomization were not performed. Data collection and analysis were not performed blind to the conditions of the experiments in most of the experiments.

Code availability
This paper does not report novel code.

Author contributions
Y.Y., E.M., and A.C. conceived and designed the study. Y.Y., E.M., K.B., H.A., Z.Y.W, R.M, J.F., J.C.J, M.A, and B.K. performed experiments. A.C. and E.M. analysed metabolomic and transcriptional data. Y.Y. performed image analysis. C.B., M.C. and S.J.D. provided resources. Y.Y. and A.C. wrote the manuscript and A.C. supervised the study.

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