Research Article

miR-148a controls metabolic programming and survival of mature CD19-negative plasma cells in mice

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Long-lived antibody-secreting plasma cells are essential to establish humoral memory against pathogens. While a regulatory transcription factor network has been established in plasma cell differentiation, the regulatory role of miRNAs remains enigmatic. We have recently identified miR-148a as the most abundant miRNA in primary mouse and human plasma cells. To determine whether this plasma cell signature miRNA controls the in vivo development of B cells into long-lived plasma cells, we established mice with genomic, conditional, and inducible deletions of miR-148a. The analysis of miR-148a-deficient mice revealed reduced serum Ig, decreased numbers of newly formed plasmablasts and reduced CD19-negative, CD93-positive long-lived plasma cells. Transcriptome and metabolic analysis revealed an impaired glucose uptake, a reduced oxidative phosphorylation-based energy metabolism, and an altered abundance of homing receptors CXCR3 (increase) and CXCR4 (reduction) in miR-148a-deficient plasma cells. These findings support the role of miR-148a as a positive regulator of the maintenance of long-lived plasma cells.

Keywords: CXC receptors · Glut-1 · miR-148a · Oxidative phosphorylation · Plasma cells

See accompanying Commentary by Tellier

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

The formation of long-lived plasma cells (PCs) along with memory B cells is fundamental in establishing an adaptive humoral memory after vaccination or recovery from infection [1,2]. However, PCs can harm the organism by producing pathogenic autoantibodies or transforming into cells that cause a leukemic disease; for example, multiple myeloma (MM) [3,4]. Therefore, it is crucial to decipher the mechanisms that control the development and maintenance of long-lived PCs.

The majority of activated naïve B cells differentiate in secondary lymphatic organs at the boundary of the T cell zone and the follicular B cell zone into low-affinity IgM-secreting short-lived PCs. However, some activated B cells that receive T cell help migrate into a B cell follicle and start to proliferate, which results in the formation of a germinal center (GC). In GCs, B...
Small microRNAs (miRNAs) are ideal candidates for fine tuners of the GC/PC transcription factor network and the gene expression program of long-lived PCs at the post-transcriptional level [33]. Few miRNAs have been shown to modulate the differentiation of B cells into PCs by interfering with the GC B cell transcription factor network [34]. We could demonstrate that ectopic expression of miR-148a, the most abundantly expressed transcription factor network [35]. Here, in multiple miR-148a-deficient mouse models, we demonstrated the importance of miR-148a for forming and maintaining CD19-negative late PCs in vivo. Furthermore, we show that miR-148a favors glucose consumption and oxidative phosphorylation (OxPhos) while enhancing the surface expression of chemokine receptor CXCR4 and survival receptor CD93 in PCs, suggesting that the PC signature miR-148a attenuates the mobility and enhances the maintenance of long-lived PCs in vivo.

Results

B cell-specific miR-148a-deletion reduces numbers of antibody-secreting cells and mature PCs

We previously identified miR-148a as a PC signature miRNA that targets the GC transcription factors Bach2 and Mitf [35]. To test our hypothesis that miR-148a favors the formation of PBs, we established a mouse line with loxP-flanked (floxed) miR-148a alleles (miR-148α, Supporting information Fig. S1A-C). We crossed this line with B cell-specific deleter mice that expressed the Cre-recombinase under the control of the BCR co-receptor component CD19 (CD19-Cre, Fig. 1A) [36]. The floxed miR-148α allele (148αβ) was efficiently deleted in magnetically sorted CD19-positive B cells from the spleen and BM of CD19-Cre+/- x miR-148αfl/wt mice (148a-bKO mice or bKO) (Supporting information Fig. S1D and E). CD19-Cre-mediated miR-148α-deficiency did not alter numbers of pro-B cells, pre-B cells, immature B cells, and recirculating mature B cells in the BM (Supporting information Fig. S2A).

Amounts of serum IgM, IgG, and IgA were significantly reduced by roughly 50% in non-immunized 148a-bKO mice when compared to CD19-Cre+/+ x miR-148α+/-/wt (CD19-Cre or Cre) control mice (Fig. 1B). ELISpot assays (ESA) revealed that the reduction in serum IgA and IgG could be explained by a significant reduction between 30 and 50% in IgA- (in spleen and BM) and IgG-secreting (in BM) cells (Fig. 1C). To determine which PC subsets are affected in 148a-bKO mice, we used a four-color flow cytometry staining protocol recently developed in our lab [37,38]. Based on the abundance of the PC marker CD138 [39], the survival receptor transmembrane activator and CAML interactor (Taci) [40] and the B cell markers CD19 and B220, this protocol detects three antibody-secreting cell (ASC) subsets: proliferating early PBs (P1; CD138+‘Taci’+CD19+‘B220’), early CD19-positive and B220-negative PCs (P2; CD138+‘Taci’+CD19+‘B220’), and late resting mature CD19-negative PCs (P3; CD138+‘Taci’+CD19+‘B220’), the latter of which contain long-lived PCs [19,20,38]. In line with the reduced serum Ig titer levels, B cell-specific miR-148a deficiency resulted in a significant reduction of the splenic CD138/Taci-positive PB/PC numbers by half (Fig. 1D). The decrease in the CD138/Taci-positive PB/PC population in 148a-bKO mice can largely be attributed to the significant decrease of approximately 60% in the late CD19-negative mature PC subset (P3), indicating that miR-148a controls the formation or maintenance of late PCs. Similarly, the number of late CD19-negative P3-PCs was also reduced in the BM (~50%), but only in the Taci-positive PC subset with a high abundance of surface CD138 (Fig. 1E). As CD138 is upregulated in long-lived PCs [30], this result supports our hypothesis that miR-148a fine tunes the establishment or maintenance of long-lived PCs. We conclude from these findings that
miR-148a is one of the controllers of PC generation and the maintenance of late CD19-negative PCs in the spleen and BM.

B cell-specific miR-148a deficiency reduces antigen-specific antibody responses

148a-bKO mice showed reduced numbers of CD138/Taci-positive cells (PB/PC) in the spleen (Fig. 1D). In addition, miR-148a reduced the expression of Bach-2, a transcription factor that prevents the premature differentiation of GC B cells into PCs by attenuating Blimp-1 expression [35]. Therefore, less GC B cells should enter the PC differentiation pathway in 148a-bKO mice compared to Cre control mice. To test this hypothesis, we immunized CD19-Cre control and 148a-bKO mice with the thymus-dependent model antigen TNP-KLH (Fig. 2A). As predicted, the TNP-specific IgG titers were reduced at all analyzed time points in the serum of 148a-bKO mice compared to CD19-Cre control mice (Fig. 2B). Furthermore, the number of TNP-specific IgG- or IgM-secreting cells dropped by half in the spleen and BM of 148a-bKO mice 70 days after the primary immunization (28 days after boost) (Fig. 2C).

Flow cytometry analysis 70 days after antigen-priming confirmed that the drop in TNP-specific serum antibody titers (Fig. 2B) and TNP-specific ASCs (Fig. 2C) was accompanied by a reduction in the CD138/Taci-positive PB/PC population in the spleen and BM of 148a-bKO mice (Fig. 2D). As in non-immunized mice (Fig. 1D and 1E), we detected significantly less mature P3-PCs in the spleen and BM of 148a-bKO mice, while the number of P1-PBs and early P2-PCs was not reduced this late after immunization (Fig. 2D). Although we did not analyze the frequencies of TNP-specific cells in sorted P1, P2, and P3 subpopulations, these data support our conclusion that the drop in TNP-specific antibody titers and TNP-specific ASCs can be attributed to a reduction in late P3-PC numbers.

As the GC reaction in mice peaks around 14 days after immunization [41] and the TNP-IgG serum titers were diminished in 148a-bKO mice at this time point (Fig. 2B), we analyzed the effect of miR-148a deficiency on GCs and PC subsets by flow cytometry 14 days after the primary immunization with TNP-KLH (Supporting information Fig. S2B). While numbers of splenic GC B cells and TNP-specific ASCs were not significantly reduced, we detected lower numbers of early P1-PBs in the blood of 148a-bKO mice, indicating that miR-148a favors the differentiation of GC B cells in PB/PCs.

Interestingly, flow cytometric analysis of immunized 148a-bKO mice revealed a more pronounced shift to CD19-positive cells in CD138/Taci-positive populations compared to Cre control animals than seen under homeostatic conditions (Fig. 2D). While the reduction in mature CD138high P3-PCs in the BM of immunized 148-bKO mice was comparable to that found in non-immunized mice, TNP-KLH immunization also affected the CD138low P3-PC population. Surprisingly, the number of CD138low early P2-PCs in the BM was significantly elevated in immunized 148a-bKO mice. Although we do not have a good explanation for the increase, this finding questions whether mature P3-PCs originate from the CD19-positive early P2-PCs and whether miR-148a is enhancing their final maturation in the BM.

miR-148a promotes the differentiation of germinal center B cells into plasmablasts

The thymus-dependent immunization experiments with 148a-bKO mice revealed that miR-148a favors antigen-specific humoral immune responses. However, it is still unclear whether this effect was caused by a reduced output of newly formed ASCs from the GC reaction or by enhanced maintenance of mature PCs in the survival niches of the spleen and BM.

If the reduction in antigen-specific IgG-switched ASCs in the spleen of 148a-bKO mice were a consequence of an attenuated PC differentiation during a GC reaction, we would expect lower numbers of newly generated PBs (i.e., those that still carry typical GC surface makers) [42]. To address this point, we analyzed GC populations in CD19-Cre controls and 148a-bKO mice 14 days after immunization with NP-KLH in Alum (Fig. 3A). As revealed by flow cytometry, the number of GC B cells was comparable in 148a-bKO and control animals. However, CD138/Taci-positive PBs were significantly reduced by half in the dark zone (DZ) and light zone (LZ) populations of the GC B cell compartment in 148a-bKO mice when compared to CD19-Cre control animals (Fig. 3A). Moreover, the number of surface IgM/IgA-negative (IgM–IgA–) CD138/Taci-positive PB/PCs—the majority of which are IgG-positive PBs/PCs [38]—were substantially diminished in the spleens of 148a-bKO mice (Fig. 3B).

As Bach 2 is a confirmed miR-148a target [35], we wondered whether Bach2-favored memory BC (B<sub>mem</sub>) differentiation was elevated in 148a-bKO mice. When we analyzed mice 42 days after primary NP-KLH immunization, we detected a trend of increased numbers of NP-specific B<sub>mem</sub> (CD38<sup>hi</sup> slgG<sup>-</sup>) in the spleens of 148a-bKO mice when compared to those of CD19-Cre controls (Fig. 3C). Interestingly, the same tendency was observed in the BM of the analyzed mice (Supporting information Fig. S2C).

Based on these findings, we conclude that miR-148a shifts the GC output of B effector populations in favor of PCs, likely by inhibiting GC-identity transcription factors, for example, Bach2 [35].

Induced deletion of miR-148a reduces the number of mature resting plasma cells in the bone marrow

Our findings that late mature P3-PCs are reduced in 148a-bKO mice (Figs. 1E and 2E) and that miR-148a inhibits both the proapoptotic factor Bim (Bcl2l11) and the tumor suppressor, PTEN, in <i>in vitro</i> cultured B cells [35], suggest that miR-148a could also regulate the lifespan of mature PCs. To address this hypothesis, we first established a mouse model to delete the miR-148a gene in already established PCs. Such a miR148a-inducible KO mouse (148a-iKO) was established by crossing tamoxifen (Tam)-inducible Rosa26CreERT2<sup>+/−</sup> mice with our miR-148awt/wt mice (Fig. 4A). 148a-iKO mice and Rosa26CreERT2<sup>+/−</sup> x miR-148awt/wt
A B cell-specific miR-148a KO mice

CD19-Cre\textsuperscript{+/-} \quad \text{miR-148a\textsuperscript{flox}} \quad \rightarrow \quad \text{CD19-148a\textsuperscript{bKO}}

Cre \quad \text{X} \quad \text{Floxed miR-148a} \quad \text{bKO}

B Quantitation of serum Ig (ELISA)

\begin{itemize}
  \item \text{IgG} \quad \text{IgM} \quad \text{IgA}
  \item \text{Cre} \quad \text{bKO} \quad \text{Cre} \quad \text{bKO} \quad \text{Cre} \quad \text{bKO}
\end{itemize}

C Enumeration of ASC in spleen and bone marrow (ELISpot)

\begin{itemize}
  \item \text{Spleen} \quad \text{IgG} \quad \text{IgM} \quad \text{IgA}
  \item \text{BM} \quad \text{IgG} \quad \text{IgM} \quad \text{IgA}
\end{itemize}

D Analysis of PB/PC populations in spleen (flow cytometry)

\begin{itemize}
  \item \text{Cre} \quad \text{bKO}
  \item \text{P1} \quad \text{P2} \quad \text{P3}
\end{itemize}

E Analysis of PB/PC populations in bone marrow (flow cytometry)

\begin{itemize}
  \item \text{CD138\textsuperscript{hi} Tac1\textsuperscript{+}}
  \item \text{Cre} \quad \text{bKO}
\end{itemize}
mice (CD19Cre

Bcell-specific miR-148a-deletion reduces numbers of antibody-secreting cells & mature plasma cells. (A) Experimental setup. Cre control mice (Fig. 4B), thus, confirming the positive effect of positive mature P3-PCs in the BM of 148a-iKO mice compared to days after Tam treatment detected a significant reduction in EdU-

12 day—EdU-pulse should maintain EdU in their DNA [43]. This the fluorescence intensity of labeled EdU diminishes in prolifer-

mice with 5-Ethinyl-2'-deoxyuridine (EdU), a nucleotide analog after the second TNP-KLH boost of 148a-iKO mice and Cre control animals after Tam treatment. (Supporting information Fig. S3).

Tam-induced deletion of miR-148a in TNP-KLH immunized mice resulted in a significant reduction in the CD138/Taci-positive PB/PC population—notably the CD19-negative mature resting P3-PCs in the spleen and BM of 148a-iKO mice compared to Cre control animals (Fig. 4A, left and middle panel). In addition, the number of P1-PBs was diminished in the spleen. Surprisingly, the number of P3-PCs in the blood was significantly elevated after the loss of miR-148a (Fig. 4A, right panel), which suggests that miR-148a maintains the retention of late PCs in sur-

vival niches.

As expected, the reduction in BM P3-PCs was accompanied by a significant decrease in the total number of ASCs in the BM of Tam-induced 148a-iKO mice compared to Cre control mice (Supporting information Fig. S4A). Surprisingly, numbers of TNP-specific ASCs in spleen and BM remained unchanged in Cre control and 148a-iKO mice after Tam treatment.

To determine the turnover of PCs in BM before and after the Tam-induced deletion of miR-148a, we labeled proliferating cells after the second TNP-KLH boost of 148a-iKO mice and Cre control mice with 5-Ethenyl-2'-deoxyuridine (EdU), a nucleotide analog that integrates into the DNA of proliferating cells (Fig. 4B). While the fluorescence intensity of labeled EdU diminishes in prolifer-

ating cells, non-proliferating PCs that were generated during the 12 day—EdU-pulse should maintain EdU in their DNA [43]. This setup allowed for a determination of the PC turnover rate after Tam-induced deletion of miR-148a.

Flow cytometric analysis 51 days after the EdU pulse and 21 days after Tam treatment detected a significant reduction in EdU-positive mature P3-PCs in the BM of 148a-iKO mice compared to Cre control mice (Fig. 4B), thus, confirming the positive effect of miR-148a on the maintenance of late CD19-negative PCs in BM. Interestingly, the EdU-positive P2-PC subset was not significantly

changed after Tam treatment of 148a-iKO mice, further highlight-

ing the specific effect of mir-148a on the mature PC compart-

ment. While it remains unclear why the P2-PCs were not affected by the loss of miR-148a, the increase in the expression of mir-148b (Supporting information Fig. S1F) could, at least in part, compensate for the loss of miR-148a. The unaltered number of early P2-PCs could also explain why we did not detect a change in the frequencies of newly formed TNP-specific ASCs in immun-

ized and Tam-treated 148a-iKO mice (Supporting information Fig. S4A).

The reduction of resting CD19-negative late P3-PCs and Ig-

secreting cells in the BM and the spleen after the loss of miR-148a confirms our hypothesis that this miRNA controls the maintenance of long-lived PCs in BM survival niches, which is further supported by the analysis of EdU-labeled PCs in Tam-induced miR-148a-deficient mice. Considering the appearance of mature P3-PCs in the blood of Tam-treated 148a-iKO mice, it is likely that miR-148a is one of the controllers that contribute to the maintenance of BM PCs not only by regulating intrinsic survival factors but also the retention of PCs in their BM survival niches.

RNASeq analysis identifies miR-148a-regulated target genes in bone marrow plasma cells

To identify miR-148a-regulated transcripts in PCs, we performed RNASeq analysis of sorted CD138/Taci-positive BM cells from non-immunized mice with a homozygous germline deletion of miR-148a (148a-gKO) and wildtype (WT) littermates. 148a-gKO mice are also an excellent model to study the effects of miR-148 at the molecular levels because these mice display the same reduction in late CD19-negative P3 PCs in the BM and spleen as 148a-bKO mice and Tam-induced 148a-iKO mice (Supporting information Fig. S5A).

RNASeq analysis revealed many upregulated genes (82 genes > twofold significantly increased) in 148a-gKO BM PCs compared to WT control cells including potential direct targets of miR-148a (Fig. 5A). Nevertheless, 30 genes are significantly downregulated > twofold in 148a-gKO BM PCs, indicating a multilayered negative regulatory mechanism controlled by miR-148a. We further examined the RNASeq results for predicted mir-148a targets from the mirDB (V3.0) database [44] (Fig. 5A). As expected, putative

Figure 1. Bcell-specific miR-148a-deletion reduces numbers of antibody-secreting cells & mature plasma cells. (A) Experimental setup. Cre control mice (CD19Cre

Bcell-specific miR-148a-deletion reduces numbers of antibody-secreting cells & mature plasma cells. (A) Experimental setup. Cre control mice (CD19Cre

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A  Immunization scheme bKO mice with boost

CD19-Cre (Cre)  
148a-bKO (bKO)

100μg TNP-KLH in Alum s.p.
50μg TNP-KLH in PBS s.p.

B  Serum TNP-IgG level of bKO mice

C  Enumeration of TNP-specific ASC (ELISpot)

TNP-IgM d70  
TNP-IgG d70

Spleen  |  BM

D  Analysis of PB/PC populations in spleen and bone marrow (flow cytometry)

Spleen  
BM

CD138^ Tac^  
CD138^ Tac^  
CD138^ Tac^
mir-148a target genes were enriched in BM PCs of 148a-gKO mice compared to WT controls, indicated by the accumulation of genes at the right side in the volcano blot (red dots) and the barcode blot. An interesting mir-148a target gene that is upregulated in BM PCs of 148a-gKO mice is the G Protein-Coupled Receptor 183 (Gpr183), also named Epstein–Barr virus-induced receptor 2 (EBI2). This protein interferes with chemokine (C-X-C motif) receptor 5 (CXCR5)-mediated migration of activated B cells in the B cell follicle [45]. Further analysis using a PC signature gene set identified by Shi and colleagues [23] also revealed that miR-148a-deficient CD138/Taci-positive BM cells show a less prominent PC phenotype than WT control cells (Fig. 1B, upper row). Therefore, genes that were described as upregulated in PCs by Shi and colleagues are enriched in CD138/Taci-positive BM cells (Fig. 1B, lower row). Therefore, genes that were described as upregulated in PCs by Shi and colleagues are enriched in CD138+ Taci+ cells from WT BM (red lines), while genes identified as negatively regulated in PCs (B cell signature) are enriched in the CD138/Taci-positive cells from 148a-gKO BM (blue lines). Interestingly, one of these signature genes identified by Shi et al as downregulated in PCs is CD19 [23]. In contrast, the abundance of CD19 transcripts was increased in 148a-gKO BM PCs as detected by RNAseq (Supporting information Fig. SSB), which perfectly fits the reduced numbers of BM CD19-negative CD138/Taci-positive cells in 148a-gKO mice (Supporting information Fig. SSA). The analysis also revealed a reduced expression of other PC signature genes [23] in 148a-gKO BM PCs, such as the amino acid transporter CD98 (Slc3a2) and the survival receptors CD93 and BCMA (Tnfrsf17) (Fig 5A and Supporting information Fig. SSD), which places mir-148a at the center of PC identity control [24,26,46,47]. Importantly, we could confirm by flow cytometry that the surface abundance of the survival receptor CD93 (AA4.1) was significantly reduced by circa 30% in both the early CD19-positive P2-PC and late CD19-negative P3-PC populations (Fig. 5C). These findings support the idea that 148a-gKO BM PCs have a survival deficit.

We also identified elevated expression of the validated miR-148a target CXCR3, but an unaltered expression of the BM homing receptor CXCR4 (Fig. 5A and Supporting information Fig. SSB). Circulating PCs or PBs that express CXCR3 on their surface migrate toward the chemokine (C-X-C motif) ligand 9 (CXCL9) and home into inflamed tissues [48,49], posing a possible mechanism for the observed egress of PCs from the BM after miR-148a deletion (Fig. 4A). An elevated surface expression of CXCR3 on PCs could interfere with their CXCR4-mediated homing or maintenance in the BM survival niche. To confirm the mir-148a-controlled expression of the above-mentioned chemokine receptors at the protein level, we stained BM cells from 148a-gKO and WT littermates and analyzed the early CD19-positive (P2) and late CD19-negative (P3)-PC populations for the surface abundance of the respective surface receptor (Supporting information Fig. S6A). We found a threefold increase in the frequencies of CXCR3-high expressing cells in early CD19-positive P2 and late CD19-negative P3-PC populations in the BM of 148a-gKO mice when compared to WT control mice (Fig. 5D). Most interestingly, the frequency of CXCR4-high expressing cells in the CD19-negative P3-PC population was reduced by ∼25% in 148a-gKO mice compared to WT controls. Based on the surface abundance of CXCR3 and CXCR4, we divided P2- and P3-PC populations into four subpopulations (A–D). While the frequencies of subpopulation A (CXCR4++CXCR3−) were only slightly diminished in P2-PCs, but significantly reduced by ∼30% in the resting P3-PC population in 148a-gKO mice, the frequencies of subpopulation D (CXCR4−CXCR3++) were significantly elevated in both analyzed PC populations (∼twofold in P2-PCs and ∼threefold in P3-PCs). In addition to the altered cell frequencies of subpopulations A–D in CD19-positive P2-PCs and late CD19-negative P3-PCs, the mean surface abundance of the miR-148a target CXCR3 was significantly increased (∼twofold) in the miR-148a-deficient P2- and P3-PC subpopulation D (CXCR4−CXCR3++) as CXCR4 expression was not significantly reduced in RNAseq analysis of CD138/Taci-positive BM cells of 148a-gKO mice (Fig. 5A), the drop in CXCR4-positive cells (subpopulation A) in BM P3-PCs of 148a-gKO mice was surprising (Fig. 5D). Therefore, reduction in frequencies of subpopulation A cannot be explained by the absence of CXCR4-high-expressing cells in the BM of 148a-gKO mice alone but is instead a result of a miR-148-induced change in synthesis or surface transport of CXCR4 in the remaining P3-PCs.

Therefore, miR-148a regulates the expression of surface receptors necessary for the establishment and maintenance of BM PC numbers. As the expression of the PC key regulator Blimp-1 is not altered in 148a-gKO BM PCs, mir-148a-regulated pathways are downstream of the Blimp-1-initiated transcriptional regulatory network. 

**Figure 2.** B cell-specific mir-148a deficiency reduces antigen-specific antibody responses. (A) CD19-Cre (Cre) control animals and mice with a B cell-specific mir-148a deletion (NKO) were immunized intraperitoneally with TNP-KLH in Alum and boosted intraperitoneally with TNP-KLH in PBS as indicated. (B) Titters of serum TNP-specific IgG were determined at indicated time points by ELISA (N = 3 experiments, n = 8–11 mice/genotype). Each dot on the graphs represents one individual mouse, and the bars are the median from the indicated number of analyzed mice. Grubb’s test was used to identify significant outliers (max. one per genotype/analysis). No further statistical analysis was performed due to the lack of a fitting test. (C) ELISpot analysis was used to determine the number of TNP-specific ASCs. 2 × 10^5 cells from the spleen and the BM of mice treated in (A) were seeded in triplicates in 96-well plates in a serial 1:3 dilution. The number of ASCs per well was extrapolated to 2 × 10^5. One representative well from each analysis is depicted under the corresponding graph, and the numbers next to the pictures indicate the number of seeded cells in that well. (D) Single-cell suspensions were prepared from the indicated organs of mice treated in (A), stained with fluorochrome-conjugated antibodies (CD138-PE, Taci-APC, B220-PerCP/Cy5.5, CD19-Brilliant Violet 421), and analyzed by flow cytometry, as described in Fig. 1D. One representative flow cytometric result per genotype is depicted for each organ. Frequencies of cells in the respective gates of flow cytometry plots are % of gated cells. Pre-gating strategies are depicted in the Supporting information Fig. S7. Cell numbers were calculated for the whole spleen or both tibiae and femurs (BM), (C–D) N = 3 experiments, n = 8–11 mice/genotype. To identify significant outliers (max. one per genotype/analysis), Grubb’s test was used before the statistical analysis by the Mann–Whitney U test. *p < 0.01, **p < 0.001, ***p < 0.0001. i.p. = intraperitoneal, PC/PB = CD138+ Taci+ plasma cells/plasmablasts, TNP-KLH = trinitrophenol (TNP)hapten conjugated to Keyhole Limpet Hemocyanin; ASC = antibody-secreting cell; BM = bone marrow.
A  Analysis of plasmablasts in the GC population (flow cytometry)

B  Analysis of splenic Ig-switched plasma cell populations (flow cytometry)

C  Analysis of splenic memory B cells (flow cytometry)
miR-148a supports the metabolic capability of bone marrow plasma cells

To identify pathways that are regulated by miR-148a, we performed gene set enrichment analysis (GSEA) using Molecular Signature Database (MSigDB) collection for curated gene sets (C2) with the above described RNASeq data from 148a-gKO BM PCs [50]. The 20 most significant regulated pathways are depicted in Supporting information Fig. S5C.

Interestingly, some pathways that are downregulated in BM PCs from 148a-gKO mice describe protein translation processes. As PCs produce high amounts of proteins, mostly antibodies, miR-148a-mediated support of mRNA-translation could be essential for proper PC functionality. In addition, genes of the KEGG pathway “Ribosome” are also downregulated in miR-148a-deficient BM PCs, supporting the hypothesis that this miRNA maintains the high-throughput of the antibody production machinery in mature PCs.

The GSEA analysis further revealed that 148a-gKO BM PCs show a reduced expression of genes involved in mitochondrial function and OxPhos (Supporting information Fig. S5C). Especially long-lived PCs need the energy provided by mitochondria to continuously secrete correctly glycosylated antibodies and maintain their survival [46,51]. A recently published study by Price and colleagues demonstrates that activated B cells increase oxidative processes and that Blimp-1 expression is essential for their maximal metabolic activity in vitro [52]. Our finding that miR-148a transcripts are reduced by half in lipopolysaccharide (LPS)-activated B cells from heterozygous Blimp-1:GFP-reporter mice that lack one prdm1-allele due to a GFP-knock-in (Fig. 6A) suggests that the effect of Blimp-1 on metabolic activity could at least in part be mediated by miR-148a. This is further supported by our earlier finding that miR-148a correlates with Blimp-1 expression in vitro [35]. Further, the pathway that is associated with OxPhos processes and with Blimp-1 [52] is also the most significantly downregulated pathway in miR-148a-deficient BM PCs (Fig. 6A).

Based on these findings, we hypothesize that miR-148a expression is regulated by Blimp-1 and is the missing link between Blimp-1 expression and alterations in OxPhos gene expression patterns.

To further support our conclusion, we examined the mitochondrial capacity of miR-148a-deficient PBs in vitro by extracellular flux assay using the Cell Mito Stress Test (Seahorse technology, Fig. 6B). This analysis revealed that the loss of miR-148a significantly reduced the maximal respiratory capacity of LPS-activated B cell blasts. Interestingly, the non-mitochondrial oxygen consumption rate was also significantly reduced in miR-148a-deficient cells, suggesting that other oxygen-consuming pathways, such as the partial oxidation of long-chain fatty acids in the peroxisomes [53], are affected. These results establish a function of miR-148a in fine tuning the mitochondrial respiration in ASCs.

In addition, we previously demonstrated that miR-148a reduces the expression of the tumor suppressor PTEN in LPS-activated B cells in vitro [35]. Apart from its pro-apoptotic function in tumor cells, PTEN negatively regulates the glucose import into human cancer cells in vitro by controlling the phospho-Akt1-dependent translocation of the glucose transporter Glut-1 to the cell surface [54]. Interestingly, Glut-1-mediated fueling with glucose was identified as a critical survival mechanism for long-lived BM PCs [51]. Therefore, we expected that the deletion of the PTEN repressor miR-148a would result in a reduced uptake of glucose and metabolic changes in PCs, which could be one explanation for why PC homeostasis in BM is disturbed in the absence of miR-148a.

To investigate whether miR-148a controls the uptake of glucose into splenic and BM PCs, we measured the in vivo uptake of the fluorescent glucose-derivate 6-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-Deoxyglucose (6-NBDG) [55] in PC subsets from 148a-gKO mice (Fig. 6C and Supporting information Fig. S6B). 6-NBDG is a fluorescent glucose derivative, transported through the cell membrane into the cell cytoplasm by Glut-1, and can be used to quantify Glut-1 activity by flow cytometry [55]. As expected from studies by Lam and colleagues [51], CD138/Taci-positive BM cells showed a twofold higher 6-NBDG uptake than the splenic ones (6-NBDG total uptake in Fig. 6C). In contrast to published data [46], we further subdivided CD138/Taci-positive cells based on the CD19/B220-expression described before (P1-P3). Notably, the late CD19-negative PC population P3 in the spleen and BM of WT mice exhibited the highest uptake of 6-NBDG when compared to P1-PBs and early CD19-positive P2-PCs or B cells from the same tissues (6-NBDG total uptake in Fig. 6C), implying a functional difference between the two described B220-negative PC subsets. This observation outlines the importance of
**A** Analysis of PB/PC populations in spleen, BM and blood of iKO mice (flow cytometry)

- **Spleen**
  - Cre: P1 1.2, P2 4.2, P3 9.7
  - iKO: P1 0.4, P2 4.2, P3 9.7

- **Bone marrow**
  - Cre: P1 0.45, P2 3.2, P3 47.6
  - iKO: P1 0.39, P2 7.7, P3 38.0

- **Blood**
  - Cre: P2 0.06, P3 38.6
  - iKO: P2 0.17, P3 55.2

**B** Analysis of EdU-labeled PC populations in BM of iKO mice (flow cytometry)

- **Cre**
  - PC: 0.38
  - B220^−: P2 2.5, P3 3.2

- **iKO**
  - PC: 0.16
  - B220^−: P2 2.4, P3 1.3

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a more detailed definition of PC subsets for reliable analyses. In addition, the P2 and P3-PC populations from miR-148a-deficient BM revealed a striking reduction in 6-NBDG uptake when compared to the same populations in WT littermates (~10-30% in the spleen, 40-50% in BM). Furthermore, we observed considerable heterogeneity in NBDG-uptake within the P1-P3 PB/PC populations and the reduction observed in miR-148a-deficient cells is mainly a consequence of a diminished 6-NBDG MFI detected in the "6-NBDG high" populations (Fig. 6C). Based on these findings, we conclude that miR-148a is involved in the control of glucose uptake in mature PCs.

If miR-148a-deficient PCs import less glucose than WT PCs, we expect that intracellular pathways that utilize glucose are affected. When we analyzed the intracellular glucose metabolism in LPS-activated splenic B cells of 148a-gKO mice and WT littermates by extracellular flux analysis with the Seahorse technology (Agilent), we detected a small but non-significant reduction in the extracellular acidification rate (ECAR) of miR-148a-deficient LPS blasts compared to WT control cells (Supporting information Fig. S6C).

In summary, Blimp-1-induced miR-148a controls the energy metabolism in ASCs by fine tuning the Glut-1-mediated glucose uptake, as well as the mitochondrial and non-mitochondrial respiration. These data and the work by Lam and colleagues support our idea that miR-148a regulates the establishment and the maintenance of BM PCs, at least in part, by fine tuning their glucose metabolism and respiratory capacities.

Discussion

In this study, we demonstrate in mice with conditional and inducible miR-148a deletions that miR-148a favors both the formation and maintenance of mature CD19-negative PCs in vivo by controlling their metabolic activity and PC signature gene expression. In contrast to already published data describing a function of miR-148a in early B cell development and B cell tolerance [56], we could not detect any differences in the numbers of major B cell populations in the spleen (follicular, marginal zone, transitional) or BM (pre-B, pro-B, immature, recirculating) of 148a-bKO mice (Supporting information Fig. S2A). Therefore, our miR-148a-deficient mice are prime models to study the effect of miR-148a on the antigen-induced activation phase of B cells as well as on the maintenance of established PCs.

The reduced capability of mice with a B cell-specific deletion of miR-148a (148a-bKO) to mount a robust T-dependent antibody response points to an essential function of miR-148a in establishing and sustaining humoral immune responses. As miR-148a negatively regulates the GC transcription factors Bach2 and Mift [35], the increased abundance of splenic B<sub>mem</sub> and the reduced number of newly generated class-switched (IgG<sup>+</sup>) PBs in the GCs of 148a-bKO mice confirms our model that miR-148a, similarly to miR-155, miR-361, or miR-181b, favors the bi-furcation of GC B cells into the PC differentiation pathway by dampening factors that prevent the initiation of the PC transcription factor network [34]. Reduced numbers of P1 PBs in inducible miR-148a-deficient (148a-iKO) mice after Tam-mediated miR-148a-ablation supports this hypothesis.

The major finding of this study is that the deletion of miR-148a in already established PCs led to an apparent reduction in late CD19-negative PCs (P3) in the spleen and BM of 148a-iKO mice. As expected, the reduction in total CD19-negative PCs was accompanied by a reduction in the total number of Ig-secreting PCs, confirming the flow cytometric results on a functional level. We also detected diminished serum IgA titers and significantly reduced numbers of IgA-secreting cells in the spleen and BM of 148a-KO mice. Most IgA-secreting cells in peripheral lymphatic organs migrated very likely from the gut-associated lymphoid tissue [57], the primary source of IgA-secreting PCs [58]. Also, long-lived IgA-positive PCs can be found in the lamina propria [59,60]. Therefore, miR-148a could also be part of the molecular circuit that controls the maturation of gut IgA-producing PCs.

To our knowledge, miR-148a is the first factor whose deletion selectively targets a defined subset of mature antibody-secreting...
A miR-148a target gene enrichment

B PC signature gene enrichment

C miR-148a deficiency and surface CD93

D Effect of miR-148a deficiency on surface CXCR3 and CXCR4 abundance (flow cytometry)
PCs in mice, that is, late CD19-negative PCs. Most published studies that address the longevity of PCs do not provide a set of surface markers that would define the long-lived subset in the ASC population, especially in the BM. However, some authors described CD138-positive and CD19-negative PCs in humans as long-lived [19,20]. Our detailed analysis of the miR-148a-dependent CD19-negative subpopulation P3 supports a similar conclusion in mice, that is, the heterogeneous CD19-negative PC subpopulation contains most of the long-lived PCs. This hypothesis is supported by our previously published studies defining new PC subsets [38]. Therefore, miR-148a is part of a molecular circuit that controls the maintenance of long-lived PCs in the BM and spleen. Most interestingly, the absence of CD19 on human PCs is used as one of the biomarkers to diagnose MM [61], indicating that most of these cancer cells would likely fall in the P3-PC compartment in mice. As miR-148a is also among the three most abundantly expressed miRNAs in human MM cells (data not shown), the results described here strongly suggest miR-148a as a target gene for the treatment of MM patients.

Even though total PC numbers and Ig titers were reduced in miR-148a-deficient mice, the antigen-specific humoral responses were not always altered. Kinetic studies of the GC reaction in a Tam-inducible AIDCreERT2 reporter mouse model showed an ongoing and long-lasting activation of B cells in secondary lymphoid organs, which resulted in a long-lasting pool of antigen-specific GC B cells [62]. Therefore, TNP-specific PCs could continuously originate in our miR-148a-KO mice from the long-lasting TNP-specific GC B cell pool and either displace “old” PCs in the niches [63] or replenish free spaces in survival niches that have been generated by the constant loss of long-lived PCs in the absence of miR-148a. This idea is further supported by our observation that the number of Edu-labeled CD19-negative PCs was reduced after Tam-pulses in 148a-iKO mice, while the number of TNP-specific ASCs was comparable in 148-iKO and Cre control mice. As the application of Edu in a one-pulse setup limits Edu-incorporation into the DNA to a short-time frame and thereby does not allow labeling of continuously antigen-activated naive or memory B cells after the pulse, the miR-148a-dependent loss of Edu-positive CD19-negative P3-PCs supports our model of miR-148a as a regulator of the maintenance of long-lived PCs.

Regardless of the miR-148a KO mouse model used in this study, we always detected a clear reduction in the number of CD19-negative late PCs, confirming that the changes in 148a-KO mice are B cell intrinsic. Therefore, we used mice with a genomic deletion of miR-148a (148a-gKO) and WT controls to address the molecular mechanisms by which miR-148a could control the maintenance of PCs. RNA-Seq analysis revealed clear differences in the transcriptome of CD138/Taci-positive BM PCs from 148a-gKO mice and WT controls. In general, analysis using the PC signature gene set from Shi and colleagues [23] convincingly demonstrated that miR-148a-deficiency results in a less prominent PC phenotype of CD138/Taci-positive BM PCs. While the expression of B cell marker genes like CD19, CD38 or Siglec-g are significantly upregulated in BM PCs from 148a-gKO mice, long-lived PC signature genes like CD93, CD98 or BCMA showed a reduced expression. The effects on CD19 and CD93 expression were confirmed on the protein level by flow cytometry. These data indicate that miR-148a-deficient BM PCs do not develop into a stage with a transcriptome signature of a long-lived PC, or newly formed PCs continuously replace miR-148a-deficient PCs with a defective survival mechanism.

RNA-Seq analysis also revealed that the direct miR-148a target gene CXCR3 [64,65] was amongst the most significantly upregulated genes in miR-148a-deficient BM PCs. Flow cytometry of 148a-gKO BM confirmed RNA-Seq data at the protein level and revealed an increased number of surface CXCR3-positive PCs with an elevated CXCR3 surface abundance. These data and the fact that CXCR3-positive PCs are directed to migrate into inflamed tissues [48] further support our finding that miR-148a fine tunes the persistence of PCs in the BM. EBV-induced molecule 2 (EBI2; GPR183), another miR-148a target gene, was also among the top upregulated genes in miR-148a-deficient BM PCs (Fig. 5A). EBI2 is a migration receptor for T cell homing to sites of inflammation and B cell movements in the follicle by counteracting the CXCR4-guided exit of the GC DZ [66]. Most interestingly, while the amount of CXCR4 transcripts was unaltered (Supporting information Fig. S5D), flow cytometry clearly revealed diminished numbers of CXCR4-expressing mature CD19-negative BM PCs in 148a-gKO mice, while this was not the case for early CD19-positive PCs. To complete the picture of miR-148a as one of the regulators of long-lived PC persistence in BM, mature CD19-negative PCs appeared in the circulation after the Tam-induced deletion of miR-148a, while the same subset was severely diminished in the BM. This implies an expulsion of these PCs from their BM survival niches. The altered surface expression of the survival receptor CD93 on miR-148a-deficient PCs in the BM could also contribute the reduction in CD19-negative long-lived PCs by inducing
**A** OxPhos pathway gene enrichment in BM PCs and Blimp-1

| LPS-blasts d3 | miR-148a | RNASEq of BM PCs |
|---------------|----------|-----------------|
| Blimp-1 WT    |          | WT              |
| Blimp-1 gKO   |          | gKO             |

Oxidative phosphorylation

0 Enrichment 29

**B** In vitro LPS-blasts: Cell Mito Stress Test

- Oligom.
- FCCP
- Rot. & Ant.A

OCR

|                | WT  | gKO |
|----------------|-----|-----|
| 0               | 500 | 500 |
| 80              | 50  | 50  |

max. replicating OCR

**C** In vivo 6-NBDG uptake in splenic cells of gKO mice (flow cytometry)

| P1 | P2 | P3 | B cells | 6-NBDG uptake in PC subpopulations |
|----|----|----|---------|----------------------------------|
| CD138+ Tac1+ |       |     |        |                                  |
| WT  |     |     |        |                                  |
| MFI 1.5 | MFI 2.3 | MFI 3.8 | MFI 0.8 |                                  |
| gKO |     |     |        |                                  |
| MFI 0.7 | MFI 1.4 | MFI 2.2 | MFI 0.6 |                                  |

In vivo 6-NBDG uptake in bone marrow cells of gKO mice (flow cytometry)
Mitochondrial activity, Blimp-1 regulates the unfolded protein response (UPR), regulating OxPhos, which has to be further tested. In addition, Blimp-1 regulates the unfolded protein response (UPR), autophagy and the mTOR pathway [67, 68], which could also support mitochondrial genesis and clearance of mitochondria independently of miR-148a. However, the metabolic analysis demonstrated that miR-148a-deficient in vitro-generated PBs show indeed reduced mitochondrial respiratory capacities. Furthermore, miR-148a-deficient CD19-negative late PCs take up less glucose. The diminished uptake can be explained by an increased expression of the direct miR-148a target PTEN [35] (Fig. 5A) that negatively controls glucose transport via Glut-1 [54].

The reduced glucose import and diminished OxPhos activity in miR-148a-deficient CD19-negative PCs likely affects their ability to adapt to a fluctuating nutrient abundance by, for example, a metabolic switch from OxPhos to glycolysis. This survival strategy has recently been elucidated by Lam and colleagues, who demonstrated that long-lived PCs require a Glut-1-mediated glucose uptake and functional glucose metabolism for survival and antibody glycosylation [51]. The decrease in CD19-negative PCs in miR-148a-KO mice can, therefore, at least in part be attributed to a defective glucose uptake of miR-148a-deficient PCs. Future experiments will investigate whether the antibodies in miR-148a-deficient mice exhibit an altered glycosylation pattern and whether this alters their function in vivo.

In summary, we demonstrate that miR-148a promotes the formation and maintenance of mature CD19-negative PCs by reducing their mobility and maintaining their bioenergetic metabolism. In support, the inhibition of miR-148a in a multiple myeloma cell line resulted in reduced growth of the tumor cells [69]. Therefore, miR-148a is a promising potential drug target to reduce long-lived PCs to treat PC-associated diseases such as autoimmunity or multiple myelomas [70].

**Material and methods**

**Experimental models—mice**

All mice were bred and maintained under specific-pathogen-free (SPF) conditions in the Franz-Penzoldt-Center or the Nikolaus-Fiebiger-Center animal facility of the University of Erlangen-Nürnberg, Erlangen, Germany. All animal experiments were performed according to institutional and national guidelines. All mice were with C57BL/6-background and between 10–16 weeks old at the date of immunization. All analyzed miR-148a-deficient mice (conditional or total knock-out) were sex- and age-matched to the proper control animals in the same experiment; if possible, littermates were used. All tamoxifen-treated mice were males. CD19-Cre mice were initially obtained from the Rajewsky lab [36], and Rosa26CreERT2 mice were purchased from Taconic Artemis GmbH. Both Cre Deleter lines were bred to the miR-148a Δ/Δ mice (Taconic Artemis GmbH commercially established the line). Mice with genomic deletion of miR-148a (148a-gKO or miR-148a Δ/Δ) were generated in our facility by breeding the miR-148a Δ/Δ-mouse to an E2Acre-deleter mouse [B6.PVB-Tg(Ella-cre)CS3791mgd/J, Jackson Laboratory; kindly provided by Dr. Michael Wegner, FAU Erlangen]. The transgenic Cre gene was removed from the mouse.
line by back-crossing the offspring to C57BL/6-WT mice. The total number of the animals (n) used per experiment and the number of independent experiments (N) is stated in the figure legends accordingly.

**Immunizations with T-cell-dependent hapten-protein conjugates**

Mice were initially immunized with either 100 μg TNP17-20-KLH (Biosearch Technologies, Cat# T-5060) or 100 μg NP20-KLH (Biosearch Technologies, Cat# N-5060-25) in Alum (ThermoScientific, Cat# 77161). Briefly, 100 μL thawed (T)NP-KLH stocks (1 mg/mL PBS; stored at −20°C) were sonicated in a water bath for 15 min, mixed with 100 μL Alum under sterile conditions, rotated at room temperature (RT) for 30 min and injected intraperitoneally into a mouse. For booster immunizations, 50 μL of the thawed and sonicated TNP-KLH stocks were mixed with 150 μL PBS under sterile conditions and immediately injected intraperitoneally. Mice injected with 100 μL PBS in 100 μL Alum instead of the NP-KLH solution were used as a negative control for the analysis of NP-specific memory B cells.

**Tamoxifen treatment**

For the activation of CreERT2, mice were treated with three applications of 4.55 mg tamoxifen (Tam) in 50 μL (1:10 ethanol/sunflower seed oil) on three consecutive days. Briefly, 100 mg Tam (Sigma Aldrich, Cat# TS648) was suspended in 100μL pure ethanol by sonication in a water bath at RT. To dissolve the Tam, 1000 μL sterile sunflower seed oil (Sigma Aldrich, Cat# S5007) was added to the solution that was again sonicated in a water bath at RT. The Tam solution was stored at −20°C in aliquots for a maximum of 14 days. Before the application, Tam aliquots were thawed and pre-heated for 15 min at 56°C in a thermomixer (1400 rpm), and per mouse, 100 μL of the Tam solution (4.55 mg) was administered per os with a dispensable feeding needle (20G; AgnTho’sAB, Cat# 424). Control mice received only an ethanol and sunflower seed oil mix (1:10) without Tam. Control-treated mice were always handled with separate equipment and housed in separate cages in the same animal room than the Tam-treated animals.

**In vivo labeling of cells with EdU**

EdU was administered to mice via the drinking water. Briefly, EdU was dissolved in sterile tap water (1 mg/mL) and stored at −20°C in the dark. EdU stocks protected from light by wrapping the vials with aluminum foil were thawed in a water bath (20°C) and diluted to a 0.4 mg/mL EdU suspension with 1% glucose in water. Water bottles were protected from light by wrapping with aluminum foil, EdU solutions were freshly prepared every second day, and mice were pulsed for a total of 12 days.

**Analysis of in vivo uptake of the Glut-1 substrate 6-NBDG**

For the analysis of the Glut1-specific glucose uptake in PCs under in vivo conditions, mice received 200 μg of the fluorescence-labeled glucose analog 6-NBDG in 100 μL PBS intravenously. Mice were sacrificed 15 min after the injection. Single-cell suspensions were prepared from the BM and the spleen, stained with fluorochrome-coupled antibodies for the surface markers CD19, B220, CD138, and Taci, and analyzed by flow cytometry as described below. 6-NBDG has fluorescent characteristics comparable to GFP or FITC.

**Antibodies**

Antibodies used in this study are listed in a table provided in the supplementary material.

**Isolation of murine cells**

BM cells were obtained by opening (remove joints) and flushing the hollow bones (mostly femur and tibia, sometimes humerus), spleens were minced and passed through a cell strainer (70 μm, Falcon, Cat# 352350), and blood was obtained by cardiac puncture. Cells were washed with PBS/2% FCS or completely supplemented R10 medium (Gibco: 500 mL RPMI1640 Cat# 31870-25; 2 mM l-glutamine Cat# 25030-24; 1 mM sodium pyruvate Cat# 11360-039; 100 U/mL penicillin/streptomycin Cat# 15140-122; 0.05 μM β-mercaptoethanol Cat# 31350-010; 50 mL heat-inactivated fetal bovine serum (FCS) Cat# 10270-106) and centrifuged at 470 g for 7 min at 4°C. Red blood cells (RBC) in the cell pellets from all analyzed organs (except mesenteric lymph nodes) were lysed in RBC lysis buffer (either 0.15 M NH4Cl/0.02 M Hepes solution or RBC lysis buffer from Biolegend, Cat# 420301) for 6 min at RT. The lysis was stopped by adding PBS/2% FCS or R10 medium. Cells were filtered to remove clotted substances (30 μm, Sysmex, Cat# 04-0042-2316), centrifuged (470 g, 7 min, 4°C), and suspended in the desired volume PBS/2% FCS or R10 medium. Cell concentrations were determined in a Neubauer counting chamber or a Nucleocounter NC3000 (ChemoMetec, Allerød, Denmark).

**Flow cytometry analysis**

Flow cytometry analysis was performed in accordance with Cossarizza et al [37]. Single-cell suspensions were incubated with unlabeled monoclonal antibodies against IgG Fc receptors CD16/32 (1:100; 25 μL per 1-2 million cells) in PBS/2% FCS/0.05% sodium azide (FACS buffer) to block unspecific binding of antibodies to Fc receptors (RT, 5 min or 15 min on ice). Cells were washed with FACS buffer and centrifuged. Cell pellets were suspended in FACS buffer supplemented with fluorochrome-coupled antibodies in the appropriate concentrations (25 μL per 1-2 million cells; 15-30 min on ice, in the dark). Biotin-conjugated antibodies were detected with the respective fluorochrome-coupled antibodies.
conjugated streptavidin conjugates. Cells were resuspended in FACS buffer at 10-16 × 10^6 cells/mL and analyzed in a Gallios flow cytometer (Beckman Coulter). To detect NP-specific surface receptors, cells were stained with fluorochrome-coupled NP (load 28 = 28 NP molecules were coupled to one fluorochrome molecule). For the detection of Edu-positive cells, samples were processed using the “Click-IT Plus Edu Alexa Fluor 647 Flow Cytometry Assay Kit” (Thermo Fisher) according to the manufacturer’s instructions. Raw data obtained with the Gallios flow cytometer were analyzed with Kaluza software. Cells were always gated for singlets using properties in forward scatter INT versus TOF and “viable cells” or “lymphocytes” using properties in the forward scatter and side scatter blot before detailed analysis shown in the figures. If necessary, channels that were not needed for fluorescence analysis (“empty channels”) were used to exclude autofluorescence.

**Cell isolation by flow cytometry (FACS) or magnetic cell sorting (MACS)**

“Untouched” naive splenic B cells were enriched from lymphatic organs by the magnet sorting technique using the “EasySep Mouse B cell isolation Kit” (Stemcell, Cat# 19854), according to the manufacturer’s manual. Splenic CD19-positive B cells were isolated with the “CD19MicroBeads-mouse” from MACS Miltenyi Biotec (Cat# 130-052-201, according to the manufacturer’s manual). CD138+Taci+—PBs/PCs and PC subpopulations (P2, P3) were isolated by FACS. Briefly, single-cell suspensions from mouse tissues were surface stained for CD138, Taci, CD19, and B220 as described under “Flow cytometry analysis,” but in sodium azide-free FACS buffer and sorted in our FAU cell sorting core facility using either the “MoFlo XDP” (Beckman Coulter) or the “MoFlo Astrios EQ” (Beckman Coulter).

**In vitro stimulation of isolated B cells**

Isolated “untouched” murine splenic B cells (EasySep) were cultured in R10 medium supplemented with 10 μg/mL LPS (Sigma-Aldrich, Cat# L3012) at 37°C (5% CO₂) at a starting concentration of 0.25 × 10^6 cells/mL. Cell aliquots were analyzed over a period of 3 days.

**Metabolic seahorse assays**

Metabolic activity of in vitro cultured B cells was analyzed using the “Seahorse XF Cell Mito Stress Test Kit” and the “Seahorse XF Glycolysis Stress Test Kit” with the Seahorse XFe96 Analyzer (Agilent). Briefly, murine splenic B cells were isolated with the “EasySep Mouse B cell isolation Kit” and cultured with LPS in vitro as described above. LPS-blasts were harvested on day 3, and cell concentrations were determined in a ChemoMetec Nucleocounter NC3000. Cells were processed following the manufacturer’s instructions. Analyses were performed with 0.2 × 10^6 cells per well in a Seahorse XFe96 Analyzer (Agilent). Metabolic inhibitors and activators were used in the following concentrations: 1.5 μM oligomycin; 0.5 μM Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP); 3 μM rotenone; 3 μM antimycin A; 10 mM glucose; 50 mM 2-Desoxy-D-glucose (2-DG).

**RNA isolation**

RNA, including miRNAs, were isolated using the “miRNeasy Mini Kit” (cell numbers ≥ 5 × 10^5, Qiagen, Cat# 217004) or the „miRNeasy Micro Kit” (cell numbers ≤ 5 × 10^5, Qiagen, Cat# 217084), respectively. Briefly, cells harvested from cell cultures were centrifuged and resuspended in 700 μL Qiazol Lysis Reagent; FACS-isolated cells were directly sorted into the Qiazol Lysis Reagent (700 μL final volume). The samples were stored at −70°C until further processing. Thawed samples were vortexed for 1 min and incubated at RT for 5 min before further processing following the manufacturer’s manual. RNA concentrations and purity (absorption at 260 nm and a ratio of 260/280 of > 2.0, respectively) were determined using the NanoDrop ND-1000 (Peqlab).

**RNA-Seq analysis**

BM PCs (CD138+ Taci+) were FACS-purified and RNA was isolated as described above (RIN > 7). Sequencing libraries were prepared with the Clontech SMART-Seq v4 kit and sequenced on an Illumina HiSeq X instrument (2 × 150 bp) by Admera Health LLC. Reads were aligned to the mouse reference genome (GENCODE M21) with STAR [71] and summarized to gene-wise counts with feature Counts [72]. Differential expression was analyzed with the R package edgeR [73]. Genes with low expression of fewer than 0.5 counts per million in at least three libraries and immunoglobulin genes were removed. The exactTest function was used to test for differential expression, and genes with a false discovery rate ≤ 0.05 were determined as significant. Competitive gene set tests were performed with camera [74] and the curated C2 gene set collection from MSigDB [50].

**cDNA synthesis and TaqMan® qRT-PCR**

Isolated miRNAs were first transcribed in PCR templates (cDNA) using the “TaqMan MicroRNA Reverse Transcription Kit” (Applied Biosystems, Cat# 4366597). Briefly, 5 μL RNA (2 ng/μL) and 3 μL of 5 x Primer stock (Applied Biosystems, mir-148a: Cat#: 000470; mir-148b: Cat#: 000471; mir-152: Cat#: 000475 or RN6B Cat#: 001093) were added to the 7 μL master mix (0.15 μL dNTP, 1 μL transcriptase, 1.5 μL buffer, 0.2 μL RNase inhibitor, and 4.15 μL RNAase-free water). Mixtures were incubated in a PCR machine for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. The cDNA preparation was then pre-diluted 1:5 with RNAase-free water and quantified using the “TaqMan® qPCl analysis TaqMan Universal Master Mix II” (Invitrogen, Cat# 4427788). Each sample was measured in triplicates. For each
reaction, 5 μL cDNA (1:5), 0.75 μL miRNA-specific probe mix (20×), 7.5 μL master mix, and 1.75 μL RNase-free water were mixed in 96-well plates (Thermo Scientific, Cat# AB-1100) and covered with “adhesive qPCR Plate Seals” (Thermo Scientific, Cat# AB-1170). TaqMan® qRT-PCR analysis was performed in the “7300 Real-Time PCR System” (Applied Biosystems). Reactions without the cDNA template (NTC) served as a negative control to validate the specificity of the reaction. The mean of the Ct-values (cycle threshold) was calculated for the triplicates of each sample. The mean-Ct of the housekeeping gene RNU6B was subtracted from the mean-Ct of the respective miRNA. This value was used to calculate the ΔCt-values.

ELISpot assay

For the detection of ASC, 96-well flat-bottom plates (Greiner bio-one, Cat# 655001) were either coated with TNP-BSA (2 μg/mL; 50 μL per well; TNP load 4-5 molecules per BSA) or unlabeled Immunoglobulin H chain-specific (IgH) goat antiserum against mouse IgG, IgA, or IgM (Southern Biotechnologies) in coating buffer (15 mM Na2CO3, 35 mM NaHCO3) overnight (ON) at 4°C. For the detection of IgH ASC cells, plates were washed three times with PBS and incubated with 200 μL PBS/1% gelatin; for the detection of TNP-specific ASC, plates were washed three times with PBS/0.05% Tween20 and incubated with 200 μL PBS/1% BSA at 37°C for 1 h to block unspecified bindings. Plates were again washed three times with the buffers described before and incubated with single-cell suspensions from the indicated organs ON at 37°C 5% CO2. Cells were plated in triplicates at eight 1:3 dilutions, starting with 0.1 × 10^6 cells in 100 μL R10 (Total IgH) or 2-3 × 10^5 cells (TNP-specific IgH) in 100 μL R10-1% BSA per well. The next day, wells were monitored microscopically, the medium and cells were removed, and the plates were washed once with water/0.1% Tween20 and three times with PBS/0.05% Tween20 and incubated for 10 min with the final washing solution at RT. After repeating this process twice (~30 min washing), the plates were incubated with 50 μL/well of the appropriate AP-conjugated secondary antibodies (1:4000-dilution in 1% gelatin/1% Tween20/PBS solution) for 1 h at 37°C, washed for 30 min as described before, and incubated with the ESA substrate solution (50 μL/ well) diluted in water (4 mL ESA substrate solution +1.5 mL water for one 96-well plate) at 37°C for at least 3 h or ON at 4°C in the dark. ESA substrate solution was prepared using 10 × AMP-substrate buffer pH 10.25 (1 M AMP; 5 mM MgCl2; 0.07% Triton X-405; 0.1% NaN3; stored at 4°C in the dark) and a final concentration of 1 mg/mL BCIP (Sigma-Aldrich, Cat# B8503) and was stored at 4°C in the dark. ELISpot analysis was performed as described in [75].

ELISA

For the detection of TNP-specific antibodies and IgH classes, 96-well flat-bottom plates (Greiner bio-one, Cat# 655001) were either coated with TNP-BSA (2 μg/mL; 50 μL per well; TNP load 4-5 molecules per BSA) or unlabeled Immunoglobulin H chain-specific (IgH) goat antiserum against mouse IgG, IgA, or IgM (Southern Biotechnologies) in coating buffer (15 mM Na2CO3, 35 mM NaHCO3) ON at 4°C. Plates were washed three times with PBS/0.05% Tween20 and blocked with 200 μL of blocking buffer (PBS/2% FCS) at 37°C for 1 h. The blocking buffer was discarded, mice sera (60-100 μL/ well), serially diluted in blocking buffer, were added in duplicates, and plates were incubated for 1-2 h at RT or ON at 4°C. An appropriate standard was used in comparable serial dilutions (starting with 50 ng/mL) on each 96-well plate, as well as a blank-control without mouse sera. After incubation, the plates were washed three times with PBS/0.05% and incubated with the appropriate AP- or HRP-conjugated secondary antibodies in blocking buffer (50 μL/well; diluted 1:1000-1:5000) for 1 h at RT or ON at 4°C. Secondary-antibody solutions were discarded, the plates were washed three times in PBS/0.05% Tween20, and the substrate solutions were added (50 μL/well). For AP-conjugated secondary antibodies, the Alkaline Phosphatase Yellow (pNPP) Liquid Substrate System for ELISA (Sigma-Aldrich, Cat# P7998) was used, and OD was measured at 405 nm in a spectrophotometer. For HRP-conjugated secondary antibodies, the OptEIA-Kit (TM substrate, BD, Cat# 555214) was used, stopped with 0.5M H2SO4 after 1-2 min, and the OD was measured at 450 nm. Alternatively, a home-made substrate buffer (20 mM Na2HPO4/7 mM citric acid/0.001 % OPD/0.035 % H2O2) was used, stopped after 1-2 min with 0.5 M H2SO4, and measured at the spectrophotometer at 490 nm wavelength. For quantification, OD values of wells containing only buffer and coating were first subtracted from OD values of wells containing the serum samples. To compare OD values from different 96-well plates, correction factors were determined for each measured plate by comparing on all plates the OD values of the same IgH standard with a known concentration. To obtain corrected OD values (relative OD), measured ODs of samples from all plates were multiplied with the corresponding correction factor. The Ig concentrations were calculated using standard curves obtained by analyzing appropriate standard Ig samples with a known concentration. There was no applicable standard with known Ig concentrations available to quantify the TNP-specific Ig concentrations in mouse serum.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.0 or 7.0 for Windows (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). Dots in respective diagrams represent individual mice, bars the mean. “N” represents the number of independent experiments and “n” the total number of mice used. Detailed descriptions for each statistical analysis method are stated in respective figure legends.

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Abbreviations: AID: activation-induced cytidine deaminase · ASC: antibody-secreting cell · Bach2: BTB and CNC homology, basic leucine zipper transcription factor 2 · Bim: Bcl-2-like protein 11 · Bmem: memory B cell · CXCL: chemokine (C–X–C motif) ligand · CXCR: C-X-C motif chemokine receptor · ERT2: human estrogen receptor 2 · GSEA: gene set enrichment analysis · miR/miRNA: microRNA · Mitf: Microphthalmia-associated transcription Factor · ON: overnight · OxPhos: oxidative phosphorylation · PB: plasmablast · PC: plasma cell · PTEN: Phosphatase and Tensin homolog · Taci: transmembrane activator and CAML interactor

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