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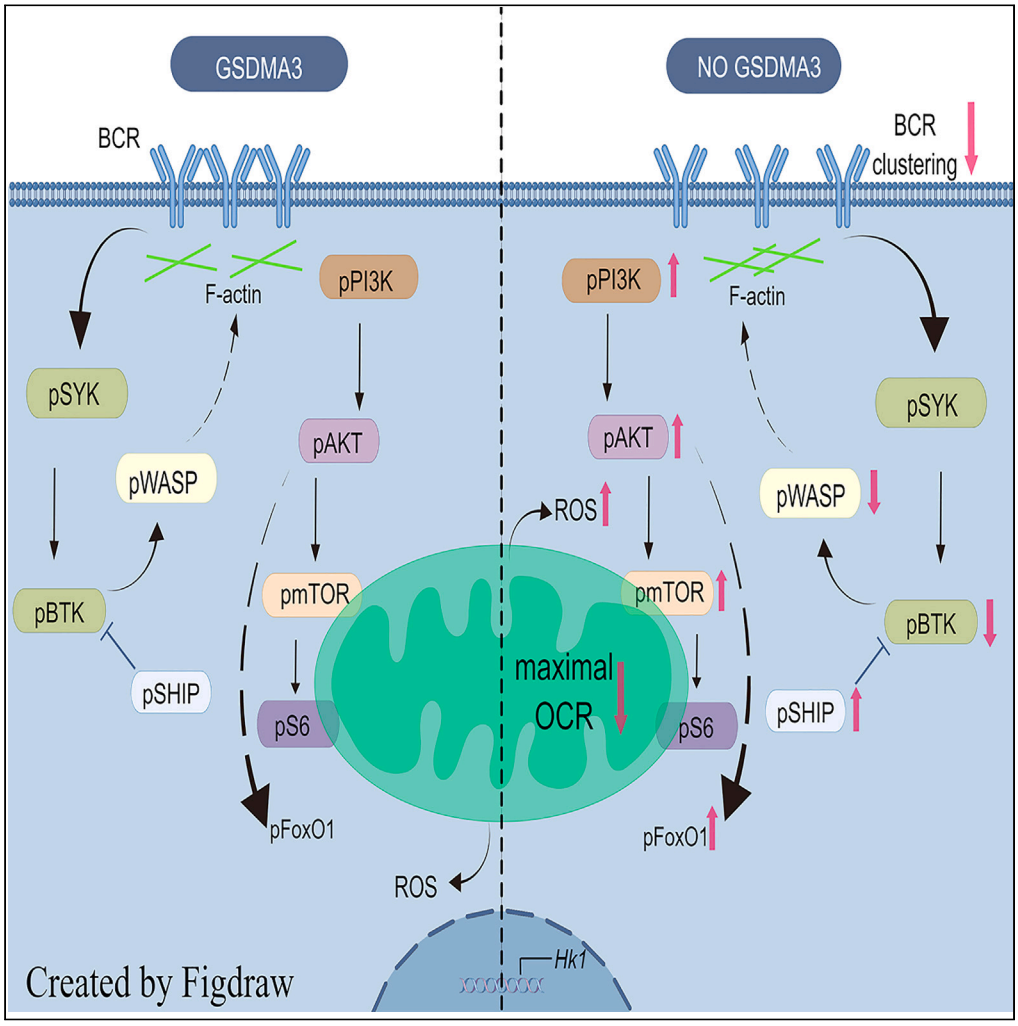

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Highlights

GSDMA3 deficiency modulates mitochondrial metabolism

GSDMA3 deficiency upregulates PI3K-Akt-mTOR signaling

GSDMA3 deficiency impairs BCR signaling and inhibits the activation of WASP

There is a compensation of upregulated GSDMA2 expression in the KO B cells

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GSDMA3 deficiency reprograms cellular metabolism and modulates BCR signaling in murine B cells

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SUMMARY

Metabolism plays a crucial role in B cell differentiation and function. GSDMA3 is related to mitochondrial metabolism and is involved in immune responses. Here, we used *Gsdma3* KO mice to examine the effect of GSDMA3 on B cells. The results demonstrated that GSDMA3 deficiency reprogrammed B cell metabolism, evidenced by upregulating PI3K-Akt-mTOR signaling, along with elevated ROS reproduction and reduced maximal oxygen consumption rate in mitochondria. Moreover, the BCR signaling in the KO B cells was impaired. The reduced BCR signaling was associated with decreased BCR clustering, caused by inhibited activation of WASP. However, GSDMA3 deficiency had no effects on B cell development and functions in humoral immunity, which might be associated with the compensation of upregulated GSDMA2 expression and the fine balance between PI3K signaling and BCR signals interaction. Our observations reveal a previously unknown influence of GSDMA3 on B cells under physiological and immunized states.

INTRODUCTION

The global COVID-19 pandemic has refreshed and expanded our understanding of the crucial role of antibodies in human immune defenses against pathogens.[1,2] B lymphocytes are responsible for protective antibody production during humoral immune responses. Emerging immunometabolism has tremendous effects on the development and function of immune cells.[3,4] Metabolic pathways not only offer energy and substrates for lymphocyte growth and survival, but also conduct its effector functions, gene expression, and differentiation.[5] Of note, mitochondria are important in B cell differentiation and activation to accommodate phenotypic and environmental changes.[6,7] Interestingly, the mitochondrion is proven to regulate cell differentiation by producing reactive oxygen species (ROS), and the PI3K signaling can affect ROS production and mitochondrial function through multiple pathways.[8] What is more, mitochondrial ROS can assist B cell receptor (BCR)-mediated B cell proliferation and regulate B cell differentiation in physiological states.[9] Recently, Urbanczyk et al. report that mitochondrial respiration in B cells is vital for humoral immune responses.[10] However, contrary to T cell metabolism, the detailed effects of immunometabolism on B cell is not deeply explored.[3] Therefore, more work is necessary to understand the impact of metabolic reprogramming on B cells.

The family Gasdermins (GSDM) is a new group of characterized proteins implicated in immune responses.[11,12] Up to now, six members have been classified as GSDM in humans. Humans carry genes encoding GSDMA, GSDMB, GSDMC, GSDMD, GSDME, and GSDMF (DFNB59 or PJVK).[11] However, mice have three homologs of GSDMA (GSDMA1-3), four homologs of GSDMC (GSDMC1-4), and one homolog each of GSDMD, GSDME, and GSDMF, while lacking a homolog of GSDMB.[12] Generally, GSDMs comprise a highly conserved N-terminal domain and a C-terminal inhibitory domain. The N-terminal fragments can be liberated via proteolytic cleavage and assemble in the cell membrane to form pores. GSDM pores can interrupt the integrity of cell membranes to promote pyroptosis, in which alarmins and pro-inflammatory cytokines are secreted into the extracellular space.[11,13–15]

GSDMA3, an ortholog of the murine GSDMA subfamily, is primarily expressed in the skin and gastrointestinal tract.[12,16] Recently, GSDMA3 has been reported to be associated with mitochondrial metabolism, which is involved in cell differentiation and death.[17–19] Mitochondrial ROS are regarded to be produced by the leakage of electrons in the mitochondrial respiratory chain, therefore changes in ROS usually indicate disorders of mitochondrial metabolism. Both the Y344H mutant protein and the unmasked N-terminal

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domain of GSDMA3 can cause increased production of mitochondrial ROS, promoting cell differentiation or autophagy.[17,18] Although GSDMA was the first identified member of the GSDMs family, it remains one of the least studied members of the family.[20] And the physiological function of GSDMA3 on cell metabolism including mitochondrial metabolism is still unclear.

Previous studies have indicated that GSDMA polymorphisms have been linked to immune-related diseases, including asthma, diabetes, systemic sclerosis, and inflammatory bowel diseases.[17,21–24] Furthermore, GSDMA expression can affect the level of interleukin (IL)-17 secreted by blood mononuclear cells and thus influence immune responses to allergens in the body.[25] All these above-mentioned data indicate that GSDMA is an important regulatory factor in immune responses.

Given that metabolism plays a crucial role in B cell differentiation and function, and that GSDMA3 is related to mitochondrial metabolism and is involved in immune responses, this study was to investigate whether GSDMA3 could influence B cell development and function in humoral immunity by regulating metabolism with a *Gsdma3* knockout (KO) mice model. Furthermore, BCR signaling and actin reorganization were explored, too.

RESULTS

GSDMA3 deficiency results in the metabolic reprogramming of B cells

As GSDMA3 has been reported to be associated with mitochondrial metabolism,[17,18] we investigated whether GSDMA3 affected the metabolism of B cells including the content of ROS, oxygen consumption rate (OCR) of mitochondria, and expression of genes related to glycolysis. The results showed that the ROS levels of the KO splenic B cells were increased significantly after being stimulated with LPS, biotin-F(ab')$_2$, or ODN 1826 (Figure 1A). We also detected the mitochondrial activity of splenic B cells and the results showed that the KO B cells had higher mean fluorescence intensity (MFI) of mitochondrial membrane than the wild-type (WT) B cells under LPS stimulation (Figure 1B). Furthermore, extracellular flux analysis of splenic B lymphocytes with biotin-F(ab')$_2$ and CD40 cultures in normal conditions revealed reduced maximal OCR in the KO mice (Figure 1C). In addition, GSDMA3 deficiency led to increased mRNA expression of hexokinase 1 (*Hk1*), while there was no effect on expression levels of pyruvate kinase (*Pkm2*) and hexokinase 3 (*Hk3*) (Figure 1D). Given that the PI3K pathway is crucial for the metabolism homeostasis of B cells, next we investigated effect of GSDMA3 defect on the PI3K signaling axis in the KO B cells. As Figure 1E indicated, the protein levels of pPI3K, pAKT, pmTOR, and pFoxO1 were upregulated in the KO B cells, except pS6. Taken together, GSDMA3 absence results in the metabolic reprogramming in murine B cells.

GSDMA3 deficiency has no effect on the development of B cells in the bone marrow and the periphery

Growing evidence indicates that cellular metabolism plays a crucial role in B cell development.[26] Given that GSDMA3 deficiency leads to reprogramming B cell metabolism and the metabolic reprogramming of B cells is integral for their development and function, we further explored the effects of GSDMA3 deficiency on B cell development. The murine bone marrow (BM) B cells were stained with BP-1 (CD249) and CD24 to differentiate pre-pro, pro, and early-pre B cells, while B220 and IgM were to differentiate late-pre, immature, and recirculating B cells by flow cytometry, respectively. As Figures 2A–2C showed, there was no difference in the percentages of BM subsets between the WT and KO mice. In addition, the KO mice showed no difference in CD127 expression among different subsets of BM B cells relative to the WT (Figure 2D). Next, we further investigated whether GSDMA3 affected B cell differentiation in the periphery. Splenic lymphocytes were stained with IgD and IgM to define the follicular (FO) B cells, transitional B cells of type 1 (T1), and T2 cells, and CD21 and CD23 to define the marginal zone (MZ) B cells, and CD95 and GL7 to define the germinal center (GC) B cells, respectively. The results indicated that no significant differences were found in both the frequency and number of splenic FO, T1, T2, or MZ B cells between the WT and KO mice (Figures 2E–2H). Furthermore, the percentage and number of splenic GC B cells had no change in the KO mice, compared with the WT mice (Figures 2I and 2J). Altogether, GSDMA3 deficiency does not impair the B cell development in both the BM and the periphery.

GSDMA3 deficiency impairs BCR signaling

Since we found that GSDMA3 is involved in cellular metabolism in B cells, which is closely associated with BCR signaling,[27] we next examined the influence of GSDMA3 in BCR activation using confocal microscopy

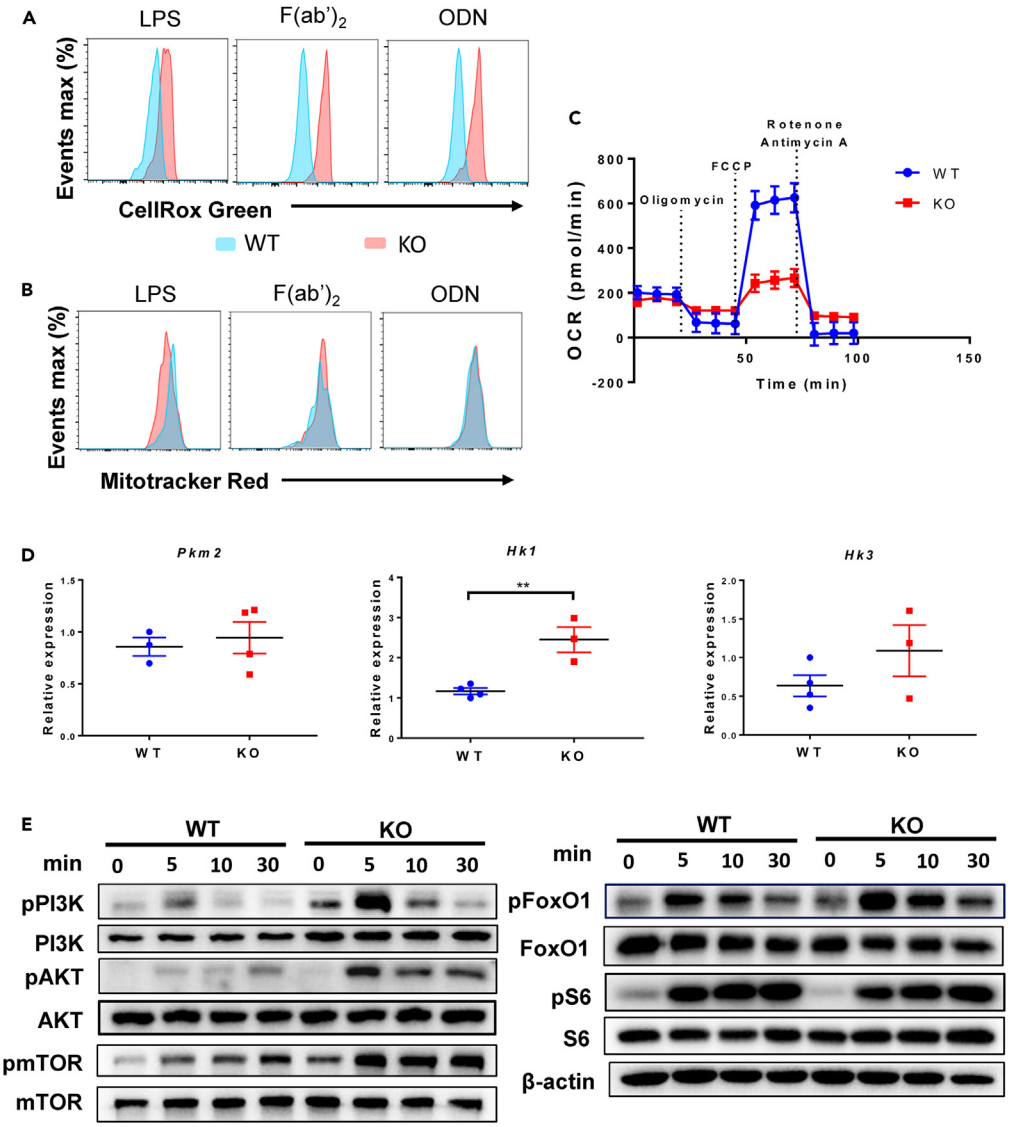


Figure 1. Metabolic changes in splenic B cells from *Gsdma3* KO mice

(A and B) Purified splenic B cells from WT and *Gsdma3* KO mice were activated with LPS, biotin-F(ab')$_2$ or ODN 1826 for 24 h, ROS (A) and mitochondrial activity (B) were detected by flow cytometry.

(C) Splenic B cell oxygen consumption rate was analyzed with a seahorse analyzer.

(D) The mRNA expression of *Pkm2*, *Hk1* and *Hk3* was detected by real-time PCR.

(E) Purified splenic B cells were stimulated with sAg at indicated times, and the expression of pPI3K, pAKT, pmTOR, pFoxO1, pS6, PI3K, AKT, mTOR, FoxO1, and S6 in cell lysates were analyzed using Western blot with β-actin as controls. The data were expressed as mean ± SEM (n ≥ 3), **p < 0.01 (Student's *t* test). Data are representative of three independent experiments.

(CFm). All B cells from the murine spleens were stimulated with soluble antigens (sAg) in 37°C for 5, 10, and 30 min, respectively. As Figure 3A revealed, the KO mice had a lower level of BCRs clustered on B cell surface than the WT group. The total levels of BCR signaling in the KO B cells, evidenced by anti-phosphorylated phosphotyrosine (pY) and pBTK, were decreased at 10 min and 30 min post stimulation. Next the co-localization was examined by the Pearson's correlation coefficient. As shown in Figure 3B, the colocation between pY and BCR in B cells from the KO mice was decreased significantly at 10 and 30 min post sAg stimulation. The expression of pBTK and the coefficient between pBTK and BCR were reduced in the KO B cells, too (Figure 3C), whereas, no differences were found in pSYK expression and the coefficient between pSYK and BCR in B cells between the KO and WT groups (Figures 3D and 3E). In addition, acting as

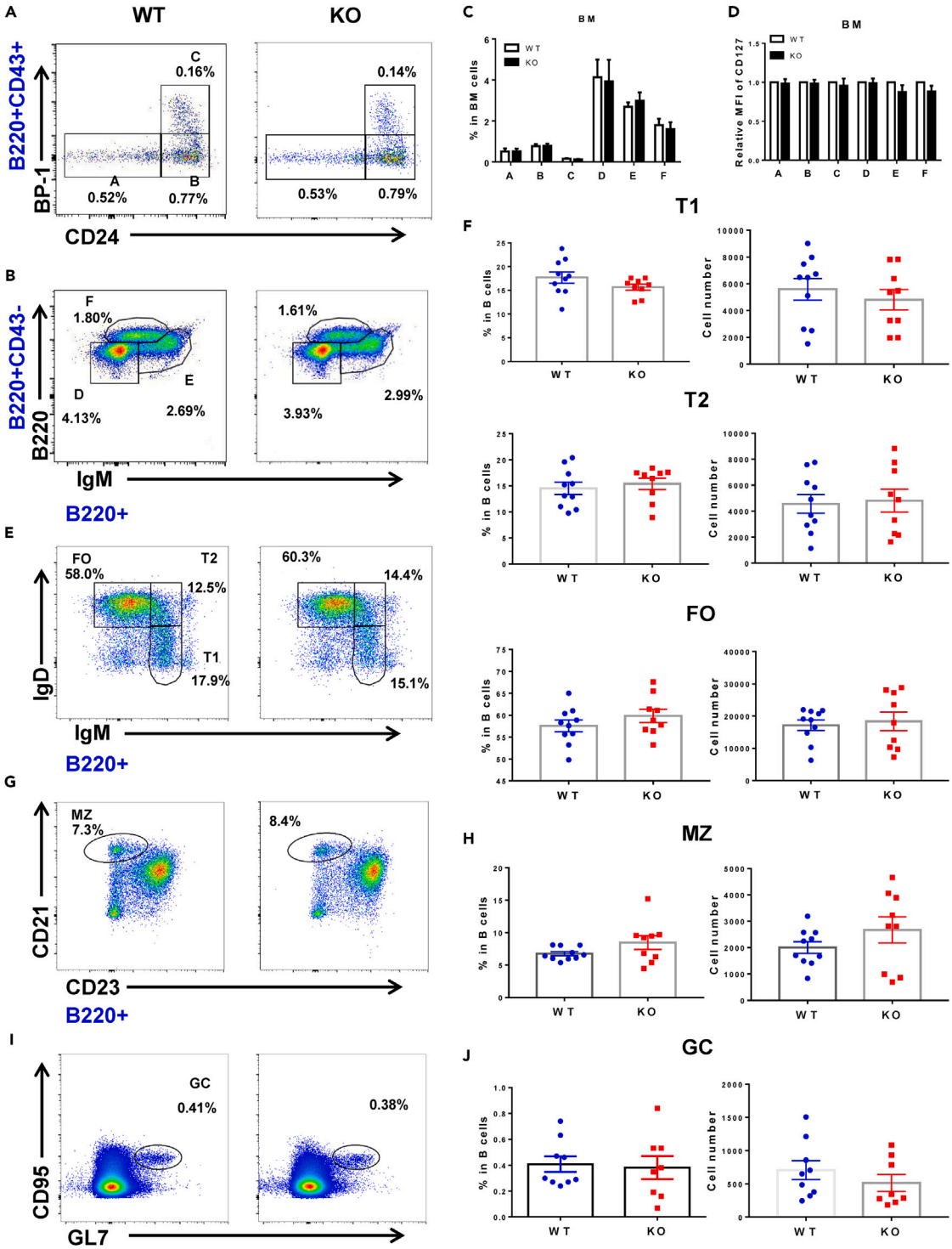


Figure 2. Absence of GSDMA3 does not impact the development and differentiation of B cells in BM and periphery

(A–J) BM cells from WT and *Gsdma3* KO mice were labeled with antibodies specific for surface markers of pre-pro (A group), pro (B group), early-pre (C group), late-pre (D group), immature (E group), and recirculating mature B cells (F group) and analyzed by flow cytometry. Shown were representative dot plots (A, B), the average percentages (C) of total cells extracted from BM, and the ratio MFI of CD127 in B cell subset (D). Splenic cells were stained with antibodies specific for FO, T1, T2, MZ, and GC ($n \geq 9$) B cells. Shown were representative dot plots (E, G, I) and the average percentage and number of splenic FO, T1, T2 (F), MZ (H), and GC (J) B cells. The data were mean ± SEM ($n \geq 9$) from three independent experiments.

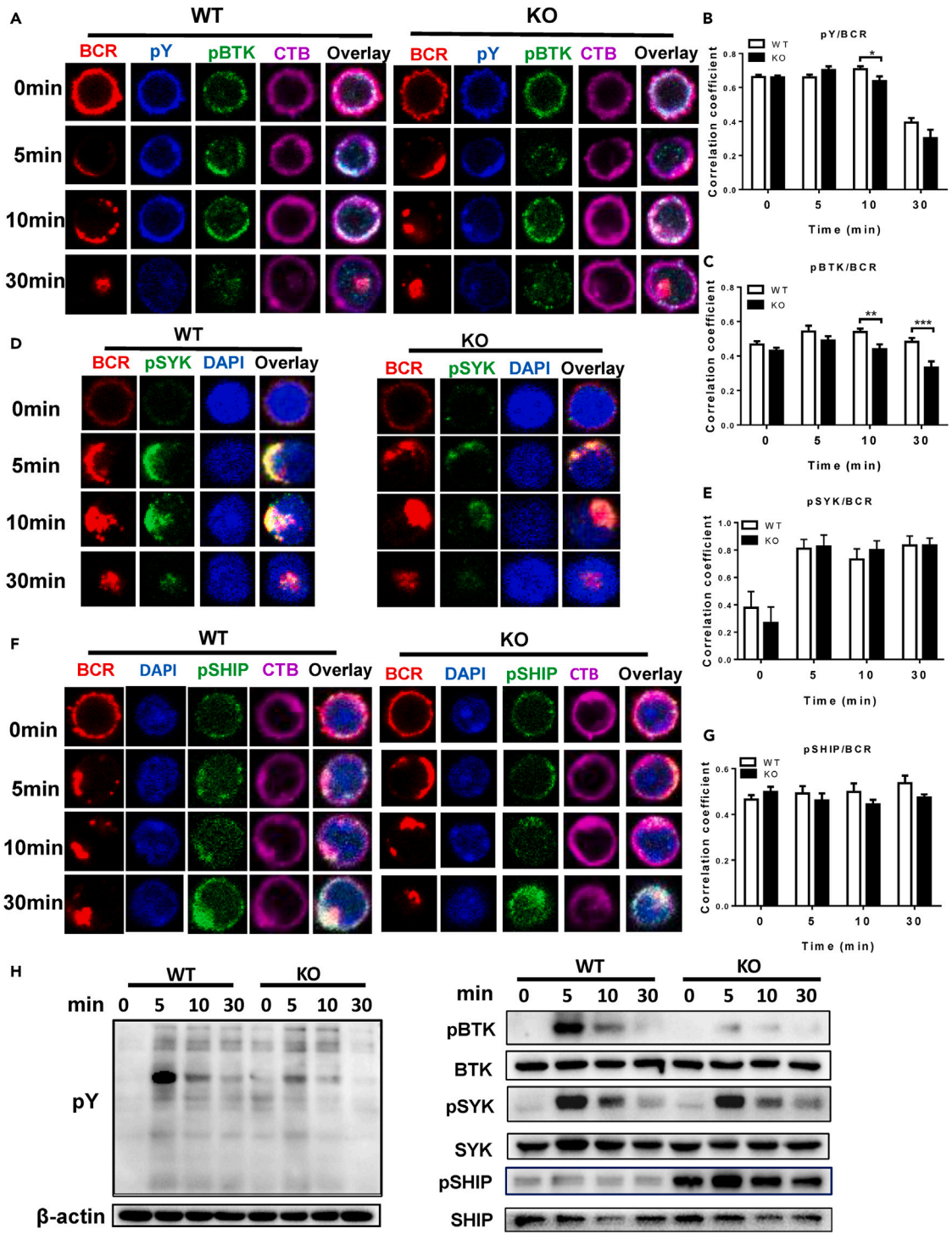


Figure 3. BCR signaling is decreased in *Gsdma3* KO mice

(A–G) Splenic B cells were incubated with anti-mouse antibodies and the correspondence secondary antibodies conjugated with fluorescence, the BCR signal molecules of pY (A), pBTK (A), pSYK (D) and pSHIP (F) were detected by CFm. The Pearson's correlation coefficients between BCR and pY (B), pBTK (C), pSYK (E), pSHIP (G) in stimulated cells were determined by NIS-Elements AR 3.2 software.

(H) The expression of pY, pBTK, BTK, pSYK, SYK, pSHIP, SHIP, and β-actin of cell lysates were analyzed by Western blot. The data were expressed as mean ± SEM (n = 30 cells), *p < 0.05, **p < 0.01, ***p < 0.001 (Student's *t* test). Data are representative of three independent experiments.

a proximal negative regulator of BCR signaling, pSHIP was investigated too. The results suggested that the MFI of pSHIP and the coefficient between pSHIP and BCR had no difference between the KO and WT B cells upon the varying time of sAg stimulation (Figures 3F and 3G). Furthermore, expressions of the aforementioned molecules regulating the BCR signaling were examined with Western blot. The results showed that the expression levels of pY and pBTK were decreased in the KO B cells during the BCR activation (Figure 3H), although there were no changes in the level of pSYK, consistent with the results obtained from the CFm. However, the KO mice had increased expression of pSHIP in B cells at different times post sAg stimulation, relative to the WT mice (Figure 3H). Collectively, the absence of GSDMA3 results in the impairment of BCR signals, which may be related to the downregulation of positive regulating signal pBTK and the upregulation of negative regulating signal pSHIP.

GSDMA3 deficiency weakens the formation of BCR cluster and BCR signalosome

Our previous work has shown that the cytoskeleton is closely related to BCR signaling and BCR clustering.[28] As GSDMA3 was associated with the activation of BCR signaling, we further investigated its effect on the early activation of B cells including BCR clustering and signalosome accumulation. Total internal reflection fluorescent microscopy (TIRFm) was used to detect splenic B cells stimulated with membrane antigens (mAgs) tethered to lipid bilayers. The surface area of B cells' connection with mAgs (B cell contact zone) was detected. As Figure 4I shown, all B cells spread rapidly, and the contact area of B cells was enhanced to the peak at 5 min and subsequently reduced concurrent with the cells contracted. However, GSDMA3 defect has no impact on B cell spreading and contraction. MFI, which refers to the BCR accumulation, was significantly inhibited in the KO B cells at 3, 5, and 7 min post mAgs treatment, indicated by the impaired BCR aggregation (Figure 4I). To explore the formation of BCR signalosome in the KO B cells, we detected the MFIs of pY, pBTK, pSYK, which present a crucial role in the transmission of activating signals of B cells. Accumulation of pY and pBTK in the contact zone was significantly reduced in the KO B cells relative to those of the WT B cells (Figures 4A–4H), although that of pSYK showed no difference (Figure 4F). Moreover, we found that the MFI of pSHIP, which negatively controls the BCR signaling through inhibiting the phosphorylation of BTK, was not different in the KO B cells from the WT B cells at 3, 5, or 7 min after stimulation. Overall, these data suggest that GSDMA3 deficiency results in decreased BCR clustering and signalosome recruitment.

GSDMA3 deficiency weakens the WASP activation during BCR activation

It has been proved that depolymerization and repolymerization of actin are mainly controlled by BCR signaling and Wiskott-Aldrich syndrome protein (WASP), which is involved closely in B cell spreading and BCR cluster formation.[29] Next, we investigated whether GSDMA3 affected cytoskeletal proteins in B cells. B cells from the WT and KO groups were treated with sAg for varying time points with CFm. The results suggested that the correlation coefficient between BCR and F-actin had no difference in the WT and KO B cells upon activation with sAg (Figures 5A and 5B). Then, we investigated the expression of F-actin on B cells during the B cells activation by mAg with TIRFm. The results indicated that compared with that in the WT mice, the MFI of F-actin in the contact area of early activated B cells had no difference in the KO mice (Figures 5C and 5D), while the MFI of pWASP was reduced in the KO B cells (Figure 5D), consistent with the immunoblot results (Figure 5E). Moreover, the Pearson's correlation coefficient between pWASP and BCR was reduced in B cells absent of GSDMA3 at 30 min post sAg stimulation (Figure 5B). Taken together, these results indicate GSDMA3 deficiency inhibits the expression of pWASP in B cells after sAg activation.

GSDMA3 deficiency does not alter the humoral immunity in the T-dependent immune response

Since we found that GSDMA3 was involved in the cellular metabolism and BCR signaling in B cells, next we asked whether GSDMA3 defect had an influence on humoral immunity in the immunized state. To explore T-cell-dependent immunity, mice were immunized with NP-KLH intraperitoneally twice. As Figure 6A-L displayed, the frequencies of PC, PBC, and MBC in the KO mice had no significant differences compared with those in the WT, as well as the frequencies of FO, MZ, T1, T2, and GC cells. However, the KO mice had higher frequency of switched B cells than the WT mice, along with a decreased frequency of unswitched B cells. Interestingly, the antibody titers of NP-specific IgM and IgG1 after NP-KLH immunization, showed no difference in the splenic B cells between the WT and KO mice (Figures 6M–6N). Overall, B cells are not affected by GSDMA3 in primary and recall antibody responses in the T-dependent immunity.

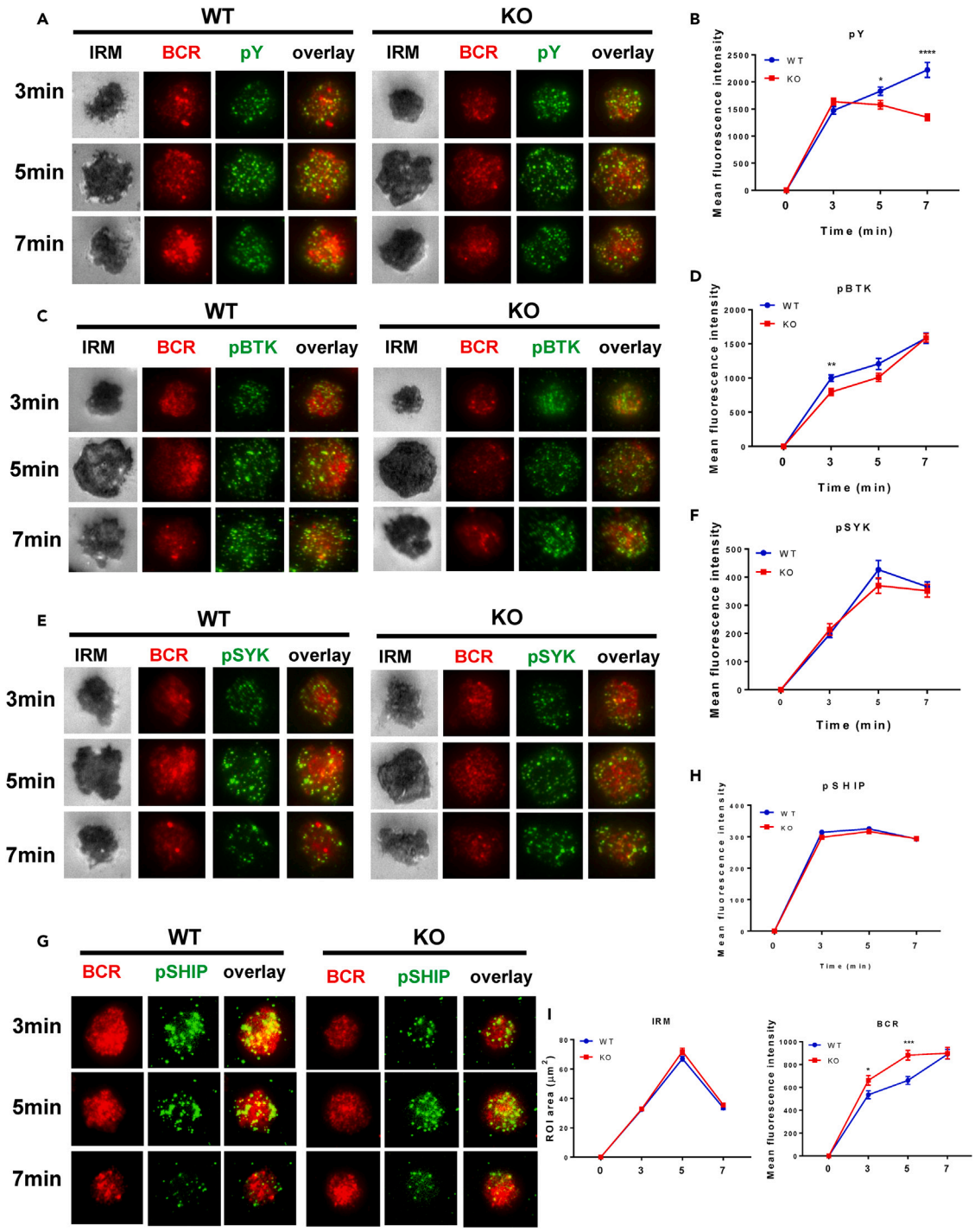


Figure 4. GSDMA3 deficiency impairs BCR formation and BCR signalosome during early activation of B cells

(A–I) Splenic B cells from WT and *Gsdma3* KO mice were used for detection of pY (A), pBTK (C), pSYK (E) and pSHIP (G) and analyzed with TIRFm. MFI of the pY (B), pBTK (D), pSYK (F), pSHIP (H) and BCR (I) in the contact zone and the average values of the B cell contact area (I) were analyzed. The data were presented as mean ± SEM (n = 30 cells), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (Student's *t* test). Data are representative of three independent experiments.

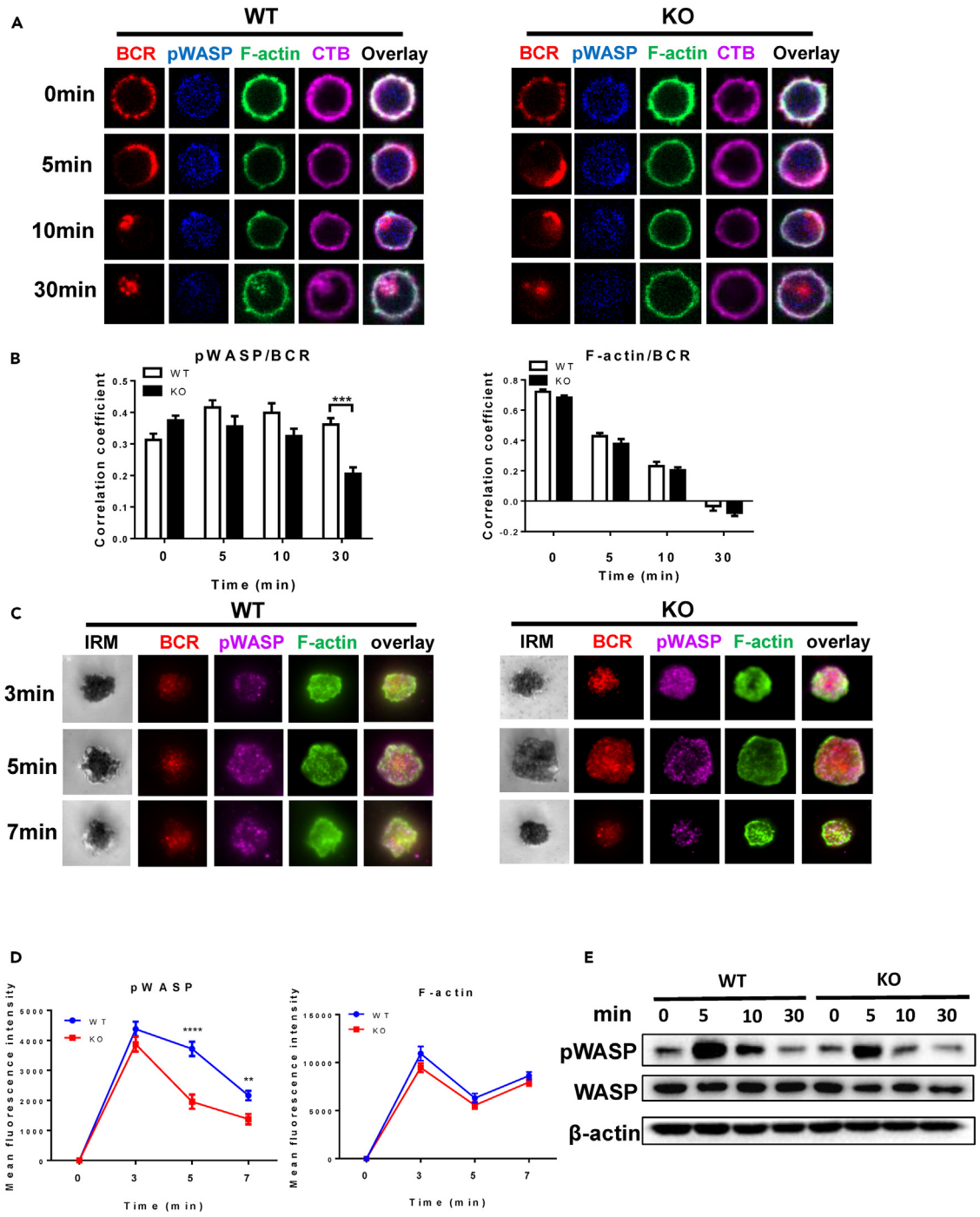
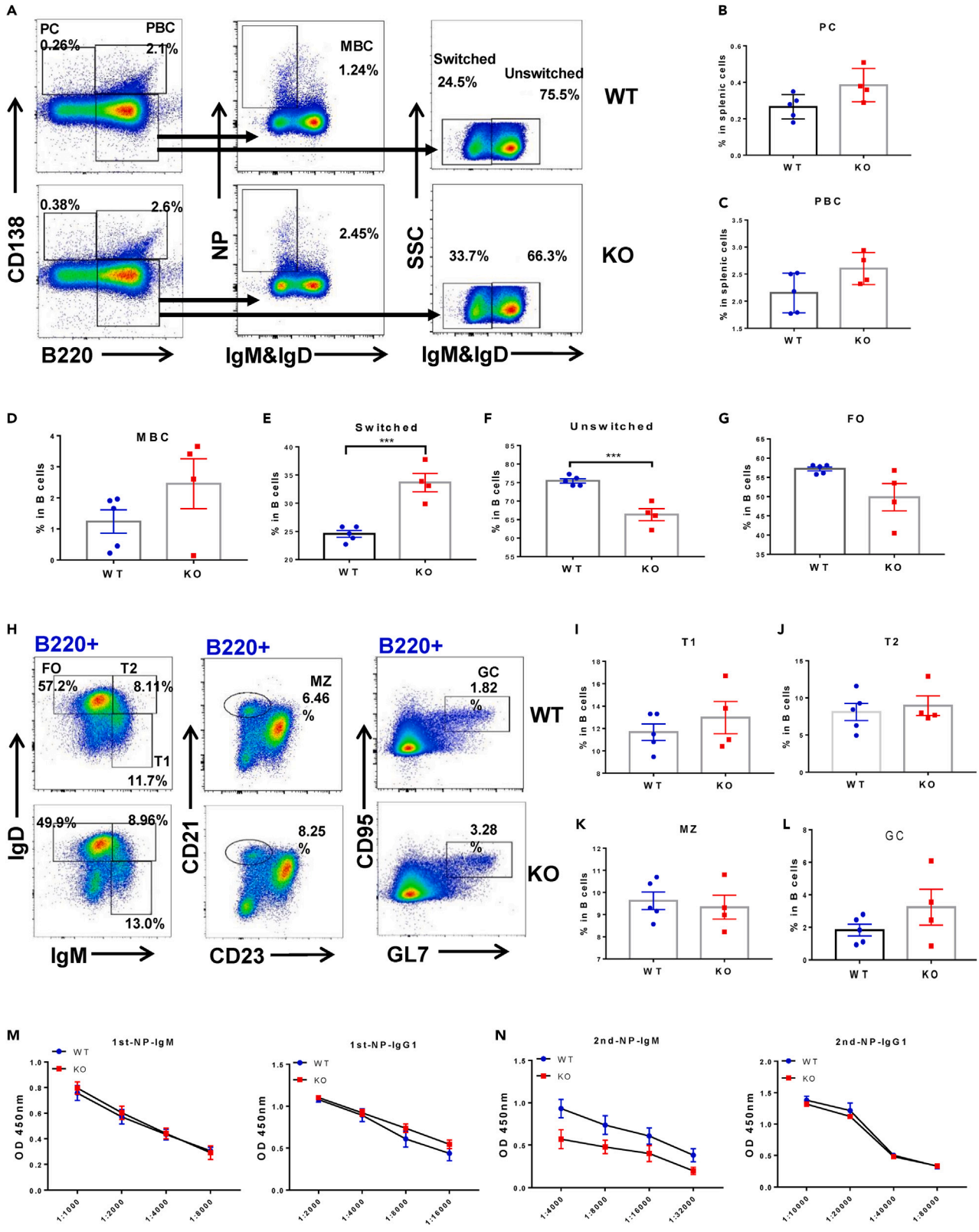


Figure 5. GSDMA3 deficiency down-regulates the activation of WASP during BCR activation

(A–E) The colocalization between BCR and pWASP, F-actin and the MFI of the F-actin, and pWASP in splenic B cells stimulated by Ag were detected with CFm (A-B) and TIRFm (C-D). (E) Cell lysates were analyzed by Western blot to detect the expression of pWASP. The data were expressed as mean ± SEM (n = 30 cells), from three independent experiments. **p < 0.01, ***p < 0.001, ****p < 0.0001 (Student's *t* test).

Given that Ig isotype switching, mainly occurring within GC B cells, is crucial for humoral immunity,[30] next we investigated the effect of GSDMA3 on the isotype class-switch in the murine B cells. The results indicated that no significant differences were found among all isotype switching (IgA, Ig2b, IgG3, IgG1, and IgE) between the WT and KO B cells (Figure S1).


A
PC
0.26%
PBC
2.1%
MBC
1.24%
Switched
24.5%
Unswitched
75.5%
WT
0.38%
2.6%
2.45%
33.7%
66.3%
KO
CD138
NP
SSC
B220
IgM&IgD
IgM&IgD
B
PC
% in splenic cells
WT
KO
C
PBC
% in splenic cells
WT
KO
D
MBC
% in B cells
WT
KO
E
Switched
***
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WT
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% in B cells
WT
KO
G
FO
% in B cells
WT
KO
H
B220+
FO
57.2%
T2
8.11%
T1
11.7%
49.9%
8.96%
13.0%
IgD
IgM
B220+
MZ
6.46
%
8.25
%
CD21
CD23
B220+
GC
1.82
%
3.28
%
CD95
GL7
WT
KO
I
T1
% in B cells
WT
KO
J
T2
% in B cells
WT
KO
K
MZ
% in B cells
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N
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1:8000
1:16000
1:32000
2nd-NP-IgG1
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KO
OD 450nm
1:10000
1:20000
1:40000
1:80000

Figure 6. GSDMA3 deficiency does not alter the humoral immunity of B cells

(A–N) WT and *Gsdma3* KO mice were immunized with NP-KLH twice. After the second immunization, the frequency of PC, PBC, MBC, FO, MZ, T1, T2 and GC cells were analyzed. Shown representative images and percentages of PC, PBC (A-C), MBC (A, D), Switched and unswitched (A, E-F), FO, T1, T2 (G-J), MZ (H, K), and GC (H, L) B cells. (M-N) The antibody levels of NP-specific IgM and IgG1 after NP-KLH immunization were detected by ELISA. The data were mean ± SEM ($n \geq 4$), ***$p < 0.001$ (Student's *t* test). Data are representative of three independent experiments.

All results indicate that GSDMA3 deficiency has no effect on the humoral immunity induced by T-cell-dependent antigens.

GSDMA3 deficiency has no effect on the expression of pyroptosis-associated molecules in B cells

GSDMs have been recently regarded as the mediators in pyroptosis and GSDMA is a typical member of the family.[11] Then we asked whether GSDMA3 deficiency affected pyroptosis in splenic B cells upon activation. The expression of *Nlrp3*, *Caspase 1*, *Gsdmd*, *IL1β*, and *IL18* in stimulated B cells with LPS and Nigericin were detected through real-time PCR (The relevant primers are shown in Table 1) and Western blot, respectively. Except for a slightly increased mRNA level of *IL1β*, there were no differences in the mRNA expression of *Nlrp3*, *Caspase 1*, *Gsdmd*, and *IL18* between the WT and KO B cells (Figure 7A). In addition, the protein expression of NLRP3, Caspase 1, GSDMD, and IL1β had no difference between WT and KO B cells, too (Figure 7B). Furthermore, no difference was found in LDH release between WT and KO B cells after stimulation (Figure 7C). Collectively, GSDMA3 deficiency has no effect on the expression of pyroptosis-associated molecules in the murine B cells.

GSDMA2 expression is upregulated in the GSDMA3 deficient B cells

As GSDMA3 affects the metabolism and BCR signaling of B cells without influencing B cell development and function, we further investigated possible underlying mechanisms with RNA sequencing analyses (RNA-seq). The result showed that 83 genes were upregulated among 151 differential expression genes, while the other 68 genes were downregulated in B cells from the KO mice. Most importantly, among these differential expression genes, the upregulated expression of *Gsdma2* was most significant (Figure 8A). To confirm the results of RNA-seq, we assessed the mRNA expression levels of *Gsdma2*, *Mid1* and *Slc15a2*, two genes involved in metabolic pathways, with real-time PCR (The relevant primers are shown in Table 1). As Figure 8B revealed, the expression levels of *Gsdma2* and *Mid1* were consistent with those of RNA-seq, respectively. However, the expression level of *Slc15a2* was higher in the KO B cells, but the difference was not statistically significant. Therefore, real-time PCR results tended to be consistent with RNA-seq results, showing that the RNA-seq results were reliable. Altogether, one possibility that GSDMA3 deficiency does not affect B cell development and function, is related to the compensatory up-regulation of GSDMA2 expression in the murine B cells.

DISCUSSION

This work explored the impacts of GSDMA3 deletion on B cell metabolism, development, BCR signaling, and functions in humoral immunity in a mouse model. The results showed that GSDMA3 deficiency resulted in the reprogramming of B cell metabolism, evidenced by upregulating PI3K-Akt-mTOR signaling, along with elevated ROS reproduction and reduced maximal OCR in mitochondria. The KO B cells had impaired BCR signaling relative to the WT ones. And during the early B cell activation, BCR signaling was downregulated by GSDMA3 deficiency, too. Furthermore, the reduced BCR signaling was associated with reduced BCR clustering in the KO B cells, which might be caused by decreased activation of WASP. However, GSDMA3 deficiency had no effects on B cell development and functions in humoral immunity.

There is increasing evidence that the GSDMA family is an essential regulatory factor in immune responses.[21–25] Mutation or deletion of the C-terminal domain and the unmasked N-terminal domain of GSDMA3 have been shown to result in abnormal protein aggregation in mitochondria, significantly decreased mitochondrial activity, and increased ROS levels, and thus alter cellular differentiation and function.[17,18] In addition, defects in GSDMD oligomerization are reported to be associated with ROS production in macrophages.[31] It has been identified that a lack of GSDMD is associated with increased B cell activation and differentiation of plasma cells in systemic autoimmunity.[15] In light of these results, we sought to assess the effect of the full-length GSDMA3 on B cell metabolism in a murine model. Our results showed that both mitochondrial activity and ROS were upregulated in the GSDMA3 KO B cells. In addition, the maximal OCR

Table 1. The sequences of primers used in real-time PCR

Genes	Sequences (5′-3′)
Gapdh	Forward: GGTGAAGGTCGGTGTGAACG Reverse: CTCGCTCCTGGAAGATGGTG
Gsdmd	Forward: CGATCTCATTCCGGTGGACA Reverse: CAAAACACTCCGGTTCTGGTT
Nlrp3	Forward: TCTGTCCCCACCGACCAAT Reverse: GGAAAGATGGAGTGGCAGACA
Caspase 1	Forward: AGAAACGCCATGGCTGACAA Reverse: CGTGCCTTGTCCATAGCAGTA
Il1β	Forward: TGCCACCTTTTGACAGTGATG Reverse: TGATGTGCTGCTGCGAGATT
Il18	Forward: ACTTTGGCCGACTTCACTGT Reverse: GTCTGGTCTGGGGTTCACTG
Pkm2	Forward: AAGTACGCCCGAGCTCTTC Reverse: GAATGAAGGCAGTCCCTGCT
Hk1	Forward: CCGCGCAACTACTGGCATA Reverse: TGAAATCCCCCTTTTCTGAGCC
Hk3	Forward: TGGGGAGCTGACCCAGAGTT Reverse: ATTGCTGCAAGCATTCCAGTTC
Slc15a2	Forward: AGAGAGTAAGGAGCCAGCCA Reverse: AGTGGGTAGCTAGAGCCGAA
Mid1	Forward: TGTGATGAGTGCCTGAAAGC Reverse: ACTCAAAGCTGCCACCTGAT
Gsdma2	Forward: TATGGACGCCATCCTCTACTT Reverse: GGACCTTGTTCTCCAAGGATTT

was downregulated in the KO B cells upon F(ab')$_2$ stimulation, reflecting the impaired mitochondrial respiration. The ectopic expression of the N-terminal domain of GSDMA3 is reported to upregulate mitochondrial ROS generation and promote autophagy in HEK293T cell lines, while wild-type GSDMA3 has no effect on cell morphology and function.[18] Another group also finds that the N-terminal GSDMA, associating with HSP90, is delivered to the mitochondria via mitochondrial importer receptor Tom70, and leads to increased production of mitochondrial ROS.[17] A recent study reports that GSDMA is preferentially localized in mitochondria and that N-terminal GSDMA leads to early mitochondrial dysfunction associated with plasma membrane penetration due to this subcellular localization.[20] These previous studies reveal the function of the GSDMA N-terminal domain. However, our results reveal that the full length of GSDMA3 deletion can promote ROS production by regulating mitochondrial activity and mitochondrial respiration in B cells, although the underlying molecular mechanism remains to be elucidated.

Another major finding of our study was that GSDMA3 deficiency resulted in elevated activation of the PI3K-Akt-mTOR pathway in B cells. Both oxidative phosphorylation and glycolysis metabolism play critical roles in B cells.[3,10,32] The function of the PI3K signaling in the survival, development, and differentiation of B cells has been mentioned in numerous studies.[33–36] Earlier data show that the PI3K-AKT pathway promotes ROS production through decreasing glycogen synthase kinase 3 (GSK3), which induces metabolic quiescence and inhibits proliferation during B cell activation.[37] Therefore, the increased ROS production may be associated with the enhancement of the PI3K pathway in the KO B cells. Moreover, PI3K-Akt-mTOR signaling can promote the expression of glucose transporter GLUT1 and its membrane localization, thus upregulating the glucose uptake capacity of cancer cells.[38] It had been reported that in the control of mTORC1, HK1-dependent glycolysis is a vital metabolic pathway in macrophages[39] and PI3K-Akt-FoxO1 signaling is involved in GSDMD-mediated microglia pyroptosis.[40] Together with the increased mRNA expression of *Hk1*, our results suggest that GSDMA3 defect leads to enhanced glycolysis metabolism in the murine B cells.

BCR signaling is crucial for B cell survival, growth, and development,[27,41] as such we studied the effect of GSDMA3 deficiency on BCR signaling using the CFm, Western blot and TIRFm. The results showed that

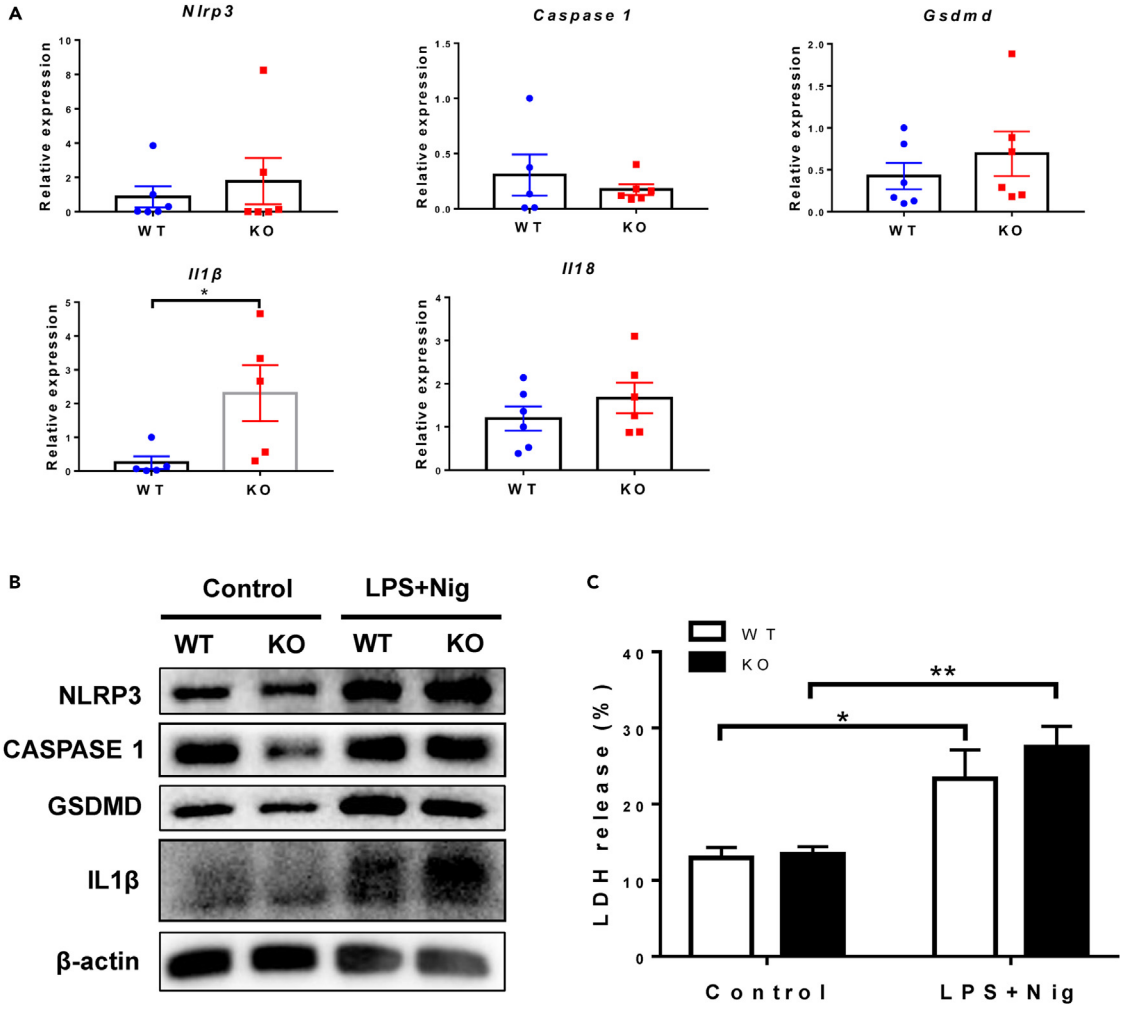

Figure 7. Absence of GSDMA3 does not affect the expression of pyroptosis associated molecules in B cells

(A) Real-time PCR was used to detect the expression of *Nlrp3*, *Caspase 1*, *Gsdmd*, *IL1β*, and *IL18* in B cells stimulated with LPS (500 ng/mL) for 4 h, followed with Nigericin (10 μM) for 2 h.

(B) The expression of NLRP3, GSDMD, CASPASE 1 and IL1β was detected with Western blot.

(C) LDH release was measured in B cells stimulated with LPS and Nigericin. The data were expressed as mean ± SEM ($n \geq 5$) from three independent experiments, *$p < 0.05$, **$p < 0.01$ (Student's *t* test).

GSDMA3 defect impaired BCR signals, evidenced by inhibited pBTK and increased pSHIP. Moreover, there were decreased BCR clustering and BCR signalosome recruitment in the KO B cells, accompanied by reduced pWASP. Our previous research has indicated that BCR clustering and BCR signaling are closely associated with the activation of WASP.[29] Thus, the reduced BCR signaling might be related to the inhibited expression of pWASP by GSDMA3 defect after sAg activation in B cells of the KO mice.

To our surprise, GSDMA3 expression was not indispensable for B cell development. What is more, deficiency of GSDMA3 had no impact on isotype switching of B cells, antibody production in immunized mice with T-cell-dependent antigen, or expressions of pyroptosis-associated molecules in B cells. It has proved that signal transduction and metabolic pathways interact with each other to cooperatively define cellular outcomes in B cells.[42] It is noteworthy that GSDMA3 deficiency upregulates B cell metabolism, while leading to inhibited BCR signaling, including BCR signaling in the early activation. Immune responses are not a standalone process, but are interconnected with cellular metabolism in health and disease. PI3K signaling is the BCR downstream, with the transcription factor FoxO1 playing a vital role.[35] PI3K signaling is able to inhibit Ig isotype switching through the Akt-dependent FoxO1 pathway in GC B cells.[43] Moreover, previous research has shown that constitutive activation of PI3K can rescue BCR-negative B cells.[33] This may be the reason why the development and function did not

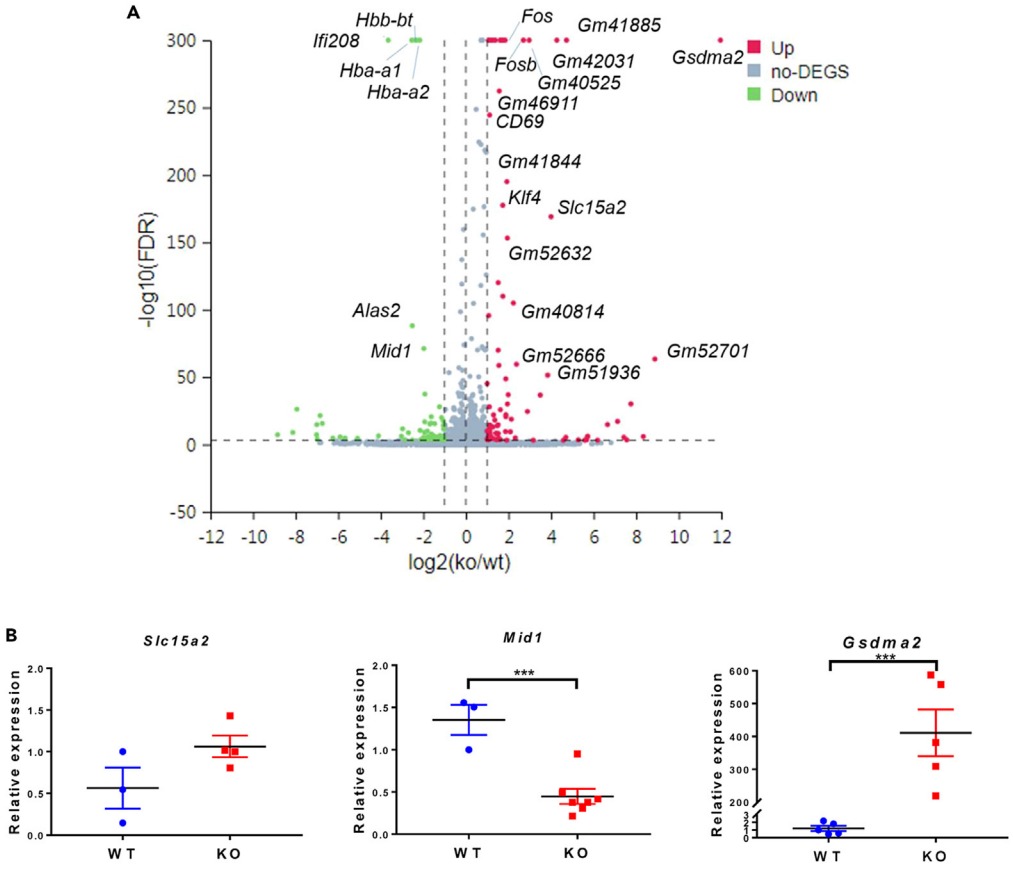

Figure 8. GSDMA3 deficiency results in changes of metabolic gene and *Gsdma2* in B cells

Purified splenic B cells from WT and KO mice were separated and resolved in TRIzol reagent, the RNA was isolated and used for RNA sequencing.

(A) The differential genes were analyzed from database.

(B) The mRNA expression of *Slc15a2*, *Mid1*, and *Gsdma2* were detected with real-time PCR. The data were presented as mean ± SEM ($n \geq 3$), ***$p < 0.001$ (Student's *t* test). Data are representative of three independent experiments.

change in the KO B cells during our experiment, that is, the increase of PI3K may compensate for the deficiency of BCR signaling in B cells. Further work will be needed to assess the underlying mechanism by which PI3K signaling cross-talks with BCR signaling in the KO B cells.

Of note, it has been proved that three homologs in the GSDMA family (GSDMA1-3) have similar functions.[18] Therefore, we wondered if there were compensatory increases in other homologs of GSDMA in the GSDMA3 KO B cells. The results from RNA-seq indicated that there was an increased expression of GSDMA2 in the KO mice. Growing evidence suggests that there is no obvious phenotype in GSDMA3 or GSDMA KO mice, demonstrating that *Gsdma* genes might be redundant in function, which is only manifest under challenge by pathogens or other danger signals.[11,18,44] Therefore, the compensation of upregulated GSDMA2 expression might be at least partly responsible for the aforementioned with no changes in the KO B cell differentiation, development, and function. In addition, given that it is a whole-gene KO mouse in our model, the loss of GSDMA3 in other immune cells may affect all aspects of B cells. Existing evidence suggests that in addition to intracellular metabolism, available fuel sources and environmental factors also have a general influence on cell fate and functional decisions of B cells.[3,32] Therefore, a B cell-specific KO model, especially upon pathogen challenge, is needed to determine whether this effect is cellular intrinsic.

Conclusion

In summary, to the best of our knowledge, it is the first time to detect the effects of GSDMA3 expression on B cells. Deletion of GSDMA3 leads to reprogramming B cell metabolism and reduced BCR signaling.

Mechanistically, the reduced BCR signaling was associated with inhibited BCR clustering in the KO B cells, which might be caused by decreased activation of WASP. However, GSDMA3 deficiency had no effects on B cell differentiation or development, and functions in humoral immunity, which might be associated with the compensation of upregulated GSDMA2 expression and the fine balance between PI3K signaling and BCR signals interaction.

Limitations of the study

Our study has some limitations. First, a whole-gene KO mouse was used, and thus the loss of GSDMA3 in other cells may affect all aspects of B cells. Therefore, a B cell-specific KO model, especially upon pathogen challenge, is needed to determine whether this effect of GSDMA3 is cellular intrinsic. Second, although this study revealed that the full length of GSDMA3 deletion can promote ROS production by regulating mitochondrial activity and mitochondrial respiration in B cells, the relevant mechanisms of how GSDMA3 affects mitochondrial metabolism remain to be elucidated. Finally, GSDMA3 deficiency had no effects on B cell differentiation or development, and functions in humoral immunity, which might be associated with the fine balance between PI3K signaling and BCR signals interaction. Further research will be needed to explore the detailed underlying mechanism.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

F.G., X.L., J.L., Y.M.H., Q.L., J.C., G.F.F., D.Q.K., H.G., and L.L. performed the experiments. F.G. and X.L. analyzed the data. F.G., L.Y., Z.Y.L., X.G., C.H.L., and J.H.L. designed and supervised the research. F.G. and J.H.L. wrote the manuscript.

DECLARATION OF INTERESTS

The authors have declared that no conflict of interest exists.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Purified anti-mouse CD90.2 Antibody	Biolegend	Cat # 105352; RRID: AB_2813937
FITC anti-mouse CD127	Biolegend	Cat # 135008; RRID: AB_1937232
PE anti-mouse BP-1	Biolegend	Cat # 108307; RRID: AB_313364
7AAD	BD	Cat # 6074701; RRID: AB_2869266
APC anti-mouse CD43	Biolegend	Cat # 143208; RRID: AB_11149685
PE/Cy7 anti-mouse CD24	Biolegend	Cat # 101822; RRID: AB_756048
Brilliant Violet 421 anti-mouse IgM	Biolegend	Cat # 406518; RRID: AB_2561444
Brilliant Violet 510 anti-mouse B220	Biolegend	Cat # 103247; RRID: AB_2561394
FITC anti-mouse CD19	Biolegend	Cat # 152404; RRID: AB_2629813
FITC anti-mouse CD95	Biolegend	Cat # 152606; RRID: AB_2632901
PE anti-mouse CD23	Biolegend	Cat # 101608; RRID: AB_312833
AF647 anti-mouse GL7	Biolegend	Cat # 144606; RRID: AB_2562185
Percp/Cy5.5 anti-mouse IgD	Biolegend	Cat # 405736; RRID: AB_2563346
APC anti-mouse CD21	Biolegend	Cat # 123412; RRID: AB_2085160
PE anti-mouse IgG1	BD	Cat # 550083; RRID: AB_393553
FITC anti-mouse IgA	BD	Cat # 559354; RRID: AB_397235
FITC anti-mouse IgG3	BD	Cat # 553403; RRID: AB_394840
FITC anti-mouse IgE	BD	Cat # 553415; RRID: AB_394848
FITC anti-mouse IgG2b	BD	Cat # 553395; RRID: AB_394833
Gasdermin D rabbit mAb	Cell signaling technology	Cat # 46451S; RRID: AB_2921367
IL1β rabbit pAb	Abclonal	Cat # A11369; RRID: AB_2758528
Caspase 1 rabbit pAb	Abclonal	Cat # A0964; RRID: AB_2757485
NLRP3 Rabbit pAb	Abclonal	Cat # A12694; RRID: AB_2759538
Mouse anti beta actin mAb	Proteintech	Cat # 60008-1-IG; RRID: AB_2289225
Goat anti-mouse IgG(H+L)	Proteintech	Cat # SA00001-1; RRID: AB_2722565
Goat anti-rabbit IgG(H+L)	Proteintech	Cat # SA00001-2; RRID: AB_2722564
HRP conjugated Goat anti-Mouse IgM	Bethyl	Cat # A90-101P; RRID: AB_67189
HRP conjugated Goat anti-Mouse IgG1	Bethyl	Cat # A90-105P; RRID: AB_67150
Anti-Phosphotyrosine	Merck-millipore	Cat # 05-321; RRID: AB_309678
pBTK (pY550) Rabbit anti-Hu/Ms	Abcam	Cat # Ab52192; RRID: AB_873715
Phospho-FoxO1 (Ser256) Antibody	Cell signaling technology	Cat # 9461S; RRID: AB_329831
Phospho-S6 Ribosomal Protein (Ser235/236) (2F9) Rabbit mAb	Cell signaling technology	Cat # 4856S; RRID: AB_2181037
Phospho-Akt (Ser473) (D9E) XP(R) Rabbit mAb	Cell signaling technology	Cat # 4060L; RRID: AB_2315049
Phospho-mTOR (Ser2448) (D9C2) XP® Rabbit mAb	Cell signaling technology	Cat # 5536S; RRID: AB_10691552
Phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) Antibody	Cell signaling technology	Cat # 4228S; RRID: AB_659940
Anti-WASP (phospho Y290) antibody	Abcam	Cat # Ab59278; RRID: AB_946348
pship1 (tyr1020)	Cell signaling technology	Cat # 3941S; RRID: AB_2296062
Phospho-Syk (Tyr525/526)	Cell signaling technology	Cat # 2710S; RRID: AB_2197222
PI3 Kinase p85 Antibody	Cell signaling technology	Cat # 4292S; RRID: AB_329869
FoxO1 (C29H4) Rabbit mAb	Cell signaling technology	Cat # 2880S; RRID: AB_2106495

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
S6 Ribosomal Protein (5G10) Rabbit mAb	Cell signaling technology	Cat # 2217S; RRID: AB_331355
Akt Antibody	Cell signaling technology	Cat # 9272S; RRID: AB_329827
SHIP1 (D1163) Antibody	Cell signaling technology	Cat # 2728S; RRID: AB_2126244
Btk (D3H5) Rabbit mAb	Cell signaling technology	Cat # 8547S; RRID: AB_10950506
Syk rabbit mAb	Cell signaling technology	Cat # 13198S; RRID: AB_2687924
WASP rabbit mAb	Abclonal	Cat # A5132; RRID: AB_2863460
Alexa Fluor® 594-AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG + IgM (H+L)	Jackson	Cat # 115-586-068; RRID: AB_2338895
Biotin-conjugated F(ab')2 anti-mouse Ig (M + G)	Jackson	Cat # 115-066-068; RRID: AB_2338581
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Thermo fisher	Cat # A-21245; RRID: AB_2535813
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo fisher	Cat # A-11008; RRID: AB_143165
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 405	Thermo fisher	Cat # A-31556; RRID: AB_221605
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 405	Thermo fisher	Cat # A-31553; RRID: AB_221604
Chemicals, peptides, and recombinant proteins		
Guinea pig complement	Rockland	Cat # C300-0500
NP-PE	Biosearch	Cat # N-5070-1
Anti mouse CD40	Bioxcell	Cat # BE0016-2-1MG
IL4	PeproTech	Cat # 214-14
Protease inhibitor cocktail	Servicebio	Cat # G2006
NP-KLH	Biosearch	Cat # N-5060-25
Adjuvant system	Sigma	Cat # S6322-1VL
ODN 1826	Invivogen	Cat # tlrl-1826-1
Red cell lysis buffer	TIANGEN	Cat # RT122-02
CellRox Green	Invitrogen	Cat # C10444
Mitotracker Red CMXRos	Invitrogen	Cat # M46752
Poly-D-lysine	Beyotime	Cat # C0132
Glucose	Sigma	Cat # G8769
L-glutamine	Sigma	Cat # G6392
Sodium pyruvate	Sigma	Cat # S8636
Oligomycin	Absin	Cat # abs42024304
Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP)	Sigma	Cat # C2920
Rotenone	Sigma	Cat # R8875
AF488-phalloidin	Thermo fisher	Cat # R37110
Alexa Fluor™ 647 Conjugate Cholera Toxin Subunit B	Thermo fisher	Cat # C34778
Critical commercial assays		
LDH cytotoxicity assay kit	Beyotime Biotechnology	Cat # C0017
Software and algorithms		
ImageLab	Bio-rad	N/A
NIS elements software	Nikon	N/A
GraphPad Prism	GraphPad	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Jiahui Lei (leijiahui@hotmail.com).

Materials availability

All unique/stable reagents generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- All additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse studies

WT C57BL/6 mice and *Gsdma3* KO mice on C57BL/6 background were bought from Nanjing Biomedical Research Institute. All the mice were bred in animal center of Tongji Medical College of Huazhong University of Science and Technology, Wuhan. Eight to ten weeks old female and male mice were euthanized and tissues were collected. All animal studies were performed with the approval of the Institutional Animal Care and Ethics Committee of Animal Experimentation of Tongji Medical College.

NP-KLH immunization

Five female wild type and *Gsdma3* KO mice were chosen for NP-KLH immunization. Each mouse was intraperitoneally immunized with 40 μg of NP-KLH and adjuvant twice at an interval of four weeks. The sera were collected at two weeks post the 1st immunization from tail vein. Five days post the 2nd immunization, the sera and spleen were collected.

Study approvals

This study was approved by the Institutional Animal Care and Ethics Committee of Animal Experimentation of Tongji Medical College (Wuhan, China).

METHOD DETAILS

Preparation of BM and splenic single cells

For BM cell preparation, cell suspensions were lysed with red cell lysis buffer to remove the red blood cells. Splenic lymphocytes were isolated with Ficoll density centrifugation from splenic single cells. In order to obtain purified splenic B cells, T cells were removed by anti-mouse CD90.2 antibody and guinea pig complement, and adhesive mononuclear cells were deleted by incubating at 37°C for 1 h.

Flow cytometry

BM cells were incubated on ice with the following antibodies: B220 (BioLegend, Brilliant Violet (BV) 510 conjugated), CD43 (BioLegend, allophycocyanin (APC) conjugated), CD24 (BioLegend, PE/Cy7 conjugated), IgM (BioLegend, pacific blue (PB) conjugated), BP-1 (BioLegend, phycoerythrin (PE) conjugated), CD127 (BioLegend, fluorescein isothiocyanate (FITC) conjugated), and 7AAD (BD Pharmingen, PerCP conjugated) to analyze BM B lymphocyte subsets. Six subsets of BM B cells were gated, including pre-pro-B cells ($CD24^{-}BP1^{-}$), pro-B cells ($CD24^{+}BP1^{-}$), early pre-B cells ($CD24^{+}BP1^{+}$), late pre-B cells ($B220^{Int}IgM^{-}$), immature B cells ($B220^{Int}IgM^{+}$), and recirculating B cells ($B220^{hi}IgM^{+}$).

Splenic single cells were surface-stained with the following conjugate-labelled antibodies (from BioLegend): PerCP-B220, FITC-CD19, AF 647-GL7, FITC-CD95, PB-IgM, Percp/Cy5.5-IgD, APC-CD21, PE-CD23, PE-NP, BV510-CD138. The subsets of splenic B cells were gated, including FO B cells ($B220^{hi}IgM^{low}IgD^{hi}$), MZ B cells ($B220^{hi}CD21^{hi}CD23^{low}$), T1 ($B220^{+}IgM^{hi}IgD^{low}$), T2 B cells ($B220^{+}IgM^{hi}IgD^{hi}$), GC B cells ($B220^{+}GL7^{hi}CD95^{hi}$),

plasma cells (PC, $B220^{-}CD138^{+}$), plasmablast cells (PBC, $B220^{+}CD138^{+}$) and memory B cells (MBC, $NP^{+}IgM^{-}IgD^{-}$).

For mitochondrial analyses, the purified splenic B cells were cultured with LPS (5 μg/ml), biotin-$F(ab')_2$ (10 μg/ml) plus anti-CD40 (10 μg/ml) and IL-4 (10 ng/ml) or ODN 1826 (5 μg/ml) plus IL-4 (10 ng/ml) for 24 hours, then the cells were collected and then stained with BV510 conjugated B220 and CellRox Green or Mitotracker Red CMXRos.

All the data were performed by flow cytometer (AttuneNxt, ThermoFisher) and analyzed with FlowJo software.

Mito stress test

The 24 well cell plates were coated with 10 μg/ml Poly-D-Lysine overnight. At the same time, the purified splenic B cells were cultured with biotin- $F(ab')_2$ (10 μg/ml) and anti-CD40 (10 μg/ml). After 12 hours, the cells were seeded into the seahorse plates at a density of 2×10^6 cells/well. The cells were cultured in cultivator without CO_2 for 1 h and then put into a seahorse extracellular flux analyzer (XF^e 24) according to manufacturer's instructions.

Confocal microscopy (CFm)

About $2\text{-}5 \times 10^5$ splenic B cells were incubated with AF 594 conjugate affinipure $F(ab')_2$ fragment anti-mouse Ig (M+G) (AF594-$F(ab')_2$) at 4 °C for 30 min, and then incubated at 37°C for 5, 10, or 30 min. The cells were fixed with 4% paraformaldehyde, washed twice with PBS, permeabilized with 0.05% saponin (Sigma-Aldrich), and stained with anti-pY, anti-pBTK, anti-pSYK, anti-pSHIP, anti-pWASP, AF488-phalloidin at 4°C for 30 min, followed by AF405 conjugate goat anti-rabbit IgG, AF488 conjugate goat anti-rabbit IgG, and AF647 conjugate Cholera Toxin subunit B (CTB) at 4°C for 30 min. The images were obtained with Nikon confocal fluorescence microscope (C2+N-SIM, Nikon, Japan) and analyzed using NIS Elements software.

Total internal reflection fluorescent microscopy (TIRFm)

Mice splenic B cells from the KO and WT were incubated with 10 μg/ml AF594-$F(ab')_2$ on the ice for 30 min. After stimulated with a mAg at 37°C for 3, 5, or 7 min, the cells were fixed with paraformaldehyde, washed with PBS and permeabilized with solution containing saponin and then stained with anti-pY, anti-pBTK, anti-pSYK, anti-pSHIP, pWASP, AF488-phalloidin, and followed by AF488 goat anti-mouse IgG and AF488 goat anti-rabbit IgG.

Western blot

Purified splenic B cells were incubated with biotin- $F(ab')_2$ (10 μg/ml) at 4°C for 30 min, and plus streptavidin for 10 min. Then the cells were stimulated in water bath at 37°C for 5, 10, 30 min and lysed with RIPA buffer containing protease inhibitor cocktail. Cell lysates were centrifuged and supernatants were analyzed by SDS-PAGE and immunoblot with anti-pY, anti-pBTK, anti-pSYK, anti-pSHIP, anti-pWASP, anti-pPI3K p85/p55, anti-pAKT, anti-pmTOR, anti-pS6, anti-pFoxO1, anti-BTK, anti-SYK, anti-PI3K, anti-AKT, anti-mTOR, anti-S6, anti-FoxO1, anti-SHIP, anti-WASP and β-actin.

RNA isolation and real-time PCR

Total RNA was isolated from the purified splenic B cells using chloroform/isopropyl alcohol method. cDNA was synthetized using reverse transcription Kit (Abclonal, #RK20429) according to manufacturer's instructions. Then real-time PCR was performed on qTower 3 (Germamy) with SYBR green (Abclonal, #RK21203) and the following probes: *Gapdh*, *Gsdmd*, *Il1β*, *Il18*, *Caspase 1*, *Nlrp3*, *Pkm2*, *Hk1*, *Hk3*, solute carrier family member 2 (*Slc15a2*), Midline 1 (*Mid1*) *and Gsdma2*. The primer sequences were listed in Table 1.

LDH assay

The purified splenic B cells were stimulated with LPS (500 ng/ml) for 4 hours followed with Nigericin (10 μM) for 2 hours. The LDH release in cells was assessed by LDH cytotoxicity assay kit.

ELISA

Sera were isolated by centrifugation from the whole blood. ELISA plates were coated with NP-BSA overnight at 4°C, then blocked with 5% BSA in TBS for 1 h. The sera were diluted in series of dilution, and added into the plates, incubated at 37°C for 1 h. After three times of washing with TBST solution, the secondary antibodies of rabbit-anti mouse IgM or IgG1 were incubated at 37°C for 1 h. The substrate TMB was added and the absorbance level (OD value) was detected at 450 nm.

Isotype switching assay

Purified splenic B cells were seeded into 24-well plate with the density of 4-8 × 10^5 cells per well under the following conditions: anti-CD40 (10 μg/ml) plus IL-4 (10 μg/ml) to induce Immunoglobulin (Ig) G1 and IgE switch, LPS (10 μg/ml) to induce IgG2b and IgG3 switch, and LPS (10 μg/ml) plus TGF-beta (0.5 ng/ml) to induce IgA switch. After culturing in incubator at 37°C for 4-5 days, cells were collected and cells were fixed and permeabilized with a Fixation/Permeabilization Kit (Invitrogen, #00-5523-00) for intracellular staining of FITC-IgA, FITC-IgG2b, FITC-IgG3, PE-IgG1, FITC-IgE. Finally, flow cytometry analysis was implemented to evaluate the levels of IgA, IgG2b, IgG3, IgG1 and IgE isotypes of B cells.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were presented as mean ± SEM and analyzed with GraphPad Prism 7 software. Comparisons of two groups were analyzed by Student's unpaired *t*-test, while comparison among multiple groups was performed by multivariate analysis of variance (ANOVA). *P* value of less than 0.05 was considered significant.