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# Cytoskeleton protein 4.1R regulates B-cell fate by modulating the canonical NF-κB pathway

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## Summary

**During the immune response, B cells can enter the memory pathway, which is characterized by class switch recombination (CSR), or they may undergo plasma cell differentiation (PCD) to secrete immunoglobulin. Both of these processes occur in activated B cells, which are reported to relate to membrane-association proteins and adaptors. Protein 4.1R acts as an adaptor, linking membrane proteins to the cytoskeleton, and is involved in many cell events such as cell activation and differentiation, and cytokine secretion. However, the effect of 4.1R on regulating B-cell fate is unclear. Here, we show an important association between B-cell fate and 4.1R. *In vitro*, primary B cells were stimulated with lipopolysaccharide combined with interleukin-4; results showed that 4.1R-deficient ($4.1R^{-/-}$) cells compared with wild-type ($4.1R^{+/+}$) B cells augmented expression of activation-induced cytidine deaminase and germline, resulting in increased $IgG1^{+}$ B cells, whereas the secretion of IgG1 and IgM was reduced, and $CD138^{+}$ B cells were also decreased. Throughout the process, 4.1R regulated canonical nuclear factor (NF-κB) rather than non-canonical NF-κB to promote the expression of CSR complex components, leading to up-regulation of B-cell CSR. In contrast, 4.1R-deficient B cells showed reduced expression of Blimp-1, which caused B cells to down-regulate PCD. Furthermore, over-activation of canonical NF-κB may induce apoptosis signaling to cause PCD apoptosis to reduce PCD number. In summary, our results suggest that 4.1R acts as a B-cell fate regulator by inhibiting the canonical NF-κB signaling pathway.**

**Keywords:** class switch recombination; plasma cell differentiation; protein 4.1R; nuclear factor-κB pathway.

## Introduction

A functionally distinct subset of B cells, including class switching recombination (CSR) and plasma cell differentiation (PCD), is generated by a stochastic process during the immune response.[1–5]

CSR is a molecular rearrangement process, replacing the immunoglobulin heavy chain (IgH) constant (CH) region, for example *Cμ*, with a downstream *Cγ*, *Cα* and *Cε* region resulting in IgG, IgA and IgE antibodies with diverse biological effector functions.[6–8] CSR requires a complex – activation-induced cytidine deaminase (AID) is the member of the CSR complex that deaminates deoxycytidine, leading to the emergence of deoxyuracil.[9] Its expression in B cells is induced by T-cell-dependent or T-cell-independent signaling.[7] In T-cell-independent signaling, Toll-like receptor (TLR) induces AID through the canonical nuclear factor-κB (NF-κB) pathway, whereas B-cell receptor (BCR) initiates the non-canonical NF-κB pathway.[10] PCD is a crucial process for B cells to produce antibodies, providing immediate protection against current infections and long-term immunity against re-exposure to the same pathogen, which is guided by several transcription factors, such as *Blimp-1* and *IRF4*.[11–17] In B

Abbreviations: AID, activation-induced cytidine deaminase; CSR, class switch recombination; IL-4, interleukin-4; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; PCD, plasma cell differentiation


 

cells, *Blimp-1* is the major gene of PCD and is inhibited by *Bcl6* and *Pax5* (*Pax5* is also known as a member of the CSR complex), whereas *Pax5* and *Bcl6* are required for CSR.[15,18] Like *Pax5* and *Aicda* (which encodes the AID), *IRF4*, another member of the CSR complex, plays a key regulatory role in these processes as it is necessary for affinity maturation, CSR and PCD.[16,19] Although many essential factors involved in these processes have been identified, the interrelations in the regulatory network that determines cell fate after B-cell activation remain elusive.[20–23]

As a member of the protein 4.1 family, protein 4.1R functions as an adaptor linking cell membrane proteins with skeleton proteins, serving as a bridge between transmembrane proteins and the actin cytoskeleton.[24] 4.1R is associated with maintaining a large number of cellular events, including cell activation,[25–27] proliferation[25,28] and cytokine secretion,[25] and plays an essential role in cell signaling, such as extracellular-signal regulated kinase[25] and calcium flow.[29–32] Our previous research has found that protein 4.1R plays a vital role in the activation of immune cells and the regulation of immune cell function. In T cells, 4.1R is inversely related to T-cell activation, proliferation and cytokine production;[25] in addition to this, mice deficient in 4.1R display an elevated humoral response to immunization with the TD antigen NP-KLH (4-Hydroxy-3-nitrophenylacetyl-Keyhole Limpet Hemocyanin), serum IgM secretion in $4.1R^{-/-}$ mice was higher than in $4.1R^{+/+}$ mice, and the IgG1 level of $4.1R^{-/-}$ mice was also significantly increased.[25] These results suggest that 4.1R plays an indispensable role in the secretion and production of antibodies. We infer that protein 4.1R may play a crucial role in determining B-cell fate.

We speculated that protein 4.1R could play an important role in regulating the fate of activated B cells based on recent reports about the regulation effects of membrane cytoskeleton in B-cell function.[33–38] In this study, we explored how cytoskeleton protein 4.1R regulates the choice for the B-cell fate of PCD or CSR *in vitro*.

## Materials and method

### Mice

Wild-type mice ($4.1R^{+/+}$) were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China); $4.1R^{-/-}$ mice on the C57BL/6J genetic background were provided by the New York Blood Center (New York, NY). Mice were housed under specific pathogen-free conditions at a constant room temperature of 22–24°C with a 12-hr light/dark cycle. The experimental protocols were approved by the Zhengzhou University Animal Ethics and Experimentation Committee.

### B-cell preparation and culture

Splenic B cells were purified from 6- to 8-week-old $4.1R^{+/+}$ and $4.1R^{-/-}$ mice (after isotonic erythrocyte lysis) by positive selection of cells expressing CD19 using the B-cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's protocol, resulting in the preparation of more than 95% $B220^+$ B cells. B cells were cultured in 5% $CO_2$ at 37°C with complete RPMI-1640 medium containing 10% fetal bovine serum (FBS), 1% HEPES, 1% L-glutamate, 1% penicillin/streptomycin and 0·1% 2-mercaptoethanol.

### Flow cytometry

Cells were washed in phosphate-buffered saline containing 2% FBS and incubated at 4°C for 30 min with combinations of the following antibodies: fluorescein isothiocyanate-conjugated (FITC) anti-CD86, anti-B220, anti-Annexin V; phycoerythrin-conjugated (PE) anti-CD138; PE/Cy7-conjugated anti-IgG1; allophycocyanin-conjugated anti-CD69. The fluorescence channel of 7-aminoactinomycin D (7-AAD) is PE/Cy5·5. After two washes in phosphate-buffered saline containing 2% FBS, cells were analyzed by flow cytometry with a BD LSRFortessa, and data were processed with FlowJo X 10·0.7 software (Tree Star, Ashland, OR, USA. Live cells were gated based on forward and side scatter.

### Protein extraction and Western blot analysis

Proteins were extracted on ice for 30 min with lysis buffer RIPA (R0020; Solarbio, Beijing, China), and the amounts of protein were quantified using a Micro BCA Protein Assay Reagent (PC0020; Solarbio). Whole-cell lysates (40 μg per lane) were resolved on sodium dodecyl sulfate–polyacrylamide gels, electrotransferred onto polyvinylidene difluoride membranes, and examined by immunoblot analysis. The primary antibodies used were anti-4.1R (13014-1-AP; Proteintech, Chicago, USA), anti-NF-κB P65 (8242; Cell Signaling, Danvers, MA, USA), anti-Phospho-NF-κB p65 (3033; Cell Signaling), anti-IκB (4814; Cell Signaling), anti-Phospho-IκB (2859; Cell Signaling), anti-NF-κB P50/P100 (sc-1190; Santa Cruz), anti-Bcl2 (3498; Cell Signaling), anti-BAK (12105, Cell Signaling), anti-caspase3 (9662, Cell Signaling), anti-caspase8 (8592, Cell Signaling), anti-TLR4 (sc-293072, Santa Cruz) and anti-GAPDH (10494-1-AP; Proteintech).

### Canonical NF-κB inhibition

Neochlorogenic acid (NCA) as an inhibitor of canonical NF-κB was investigated as previously described.[39]Briefly, $4.1R^{+/+}$ and $4.1R^{-/-}$ B cells were pre-treated with NCA

(100 μM) (HY-N0722, MedChemExpress, Monmouth Junction, NJ, USA) for 1 hr, then stimulated with LPS + IL-4 for 48 hr. Flow cytometry, CCK-8 assay and ELISA were used to measured B-cell proliferation, CSR and PCD, respectively.

## Co-immunoprecipitation assay

B cells were purified as described previously and lyzed at 4°C for 30 min in ice-cold RIPA buffer. The supernatant was separated by centrifugation (12 000 g 10 min at 4°C) and incubated at 4°C overnight with the mouse anti-4.1R (sc-166759, Santa Cruz Biotechnology. Dallas, TX, USA) or mouse anti-TLR4 (sc-293072, Santa Cruz Biotechnology) and the control IgG (normal mouse IgG sc-2025, Santa Cruz Biotechnology), then incubated with ProteinA/G PLUS-Agarose (Santa Cruz Biotechnology) at 4°C for 3 hr. Immunoprecipitation proteins were collected by centrifugation at 3000 rpm at 4°C for 5 min and pellets were washed five times with 1 ml RIPA buffer. Samples were boiled for 10 min and then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by transfer onto a polyvinylidene difluoride membrane. The membrane was probed with rabbit anti-4.1R antibody (13014-1-AP; Proteintech) and rabbit anti-TLR4 antibody, respectively.

## Immunofluorescence microscopy

Cells were double-stained with mouse polyclonal anti-4.1R antibody and rabbit anti-mouse TLR4 antibody (19811-1-AP; Proteintech) and stained with Alexa fluor 594 anti-mouse secondary antibody and Alexa fluor 488 anti-rabbit antibody. Samples were mounted in Vectashield hard-set mounting media (Vector Labs, Burlingame, CA, USA), and fluorescence images were obtained with an LSM 510 META confocal microscope (Carl Zeiss, Thornwood, NY) equipped with a Plan Neofluar 100×/1·3 oil-immersion objective. The software used for taking images was LSM 510 META 3·2. Images were captured using the Zeiss ZEN software. Fluorescence integrated density measurements were taken in ZEN software. Pearson correlation coefficient was generated using the PRISM7 software.

## Analyses of mRNA expression

Total RNA was prepared from $1 \times 10^7$ mouse B cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand complementary DNA was synthesized from equal amounts of total RNA (4 μg) using Hifair® III 1st Strand cDNA Synthesis SuperMix for quantitative PCR (11141ES10, Takara, Kyoto, Japan) and analyzed by Lightcycler 480 SYBR green I master supermix (CW0659S, CWBIO) incorporation in PCR involving specific primers (see Supplementary material, Table S1) and performed in a real-time quantitative PCR system (Rotor-Gene3000). The levels of transcripts were quantified by the ΔΔCT (cycle threshold) method.

## Detection of immunoglobulin

Supernatant from lipopolysaccharide plus interleukin-4 (LPS + IL-4) -stimulated $4.1R^{+/+}$ and $4.1R^{-/-}$ B cells was taken at different times using a Mouse IgM ELISA Kit (ab133047, AbCam, Cambridge, UK) and Mouse uncoated IgG1 ELISA Kit (88-50410-22, Thermo Fisher Scientific, Waltham, MA, USA). In brief, 96-well plates were coated with goat anti-mouse immunoglobulin capture antibody overnight at 4°C, followed by incubation with diluted samples and developed with horseradish peroxidase-conjugated goat anti-mouse isotype-specific antibodies and tetramethylbenzidine substrate solution. Reactions were read at 450 nm after substrate addition using an absorbance reader (Molecular Devices CMax Plus, Molecular Devices, San Jose, CA, USA).

## Assays for cell proliferation and apoptosis

For *in vitro* B-cell proliferation assays, purified splenic naive B cells ($5 \times 10^6$ cells) were stained with 500 μl of 5 μM CFSE solution (C34554; Thermo Fisher) following the manufacturer's protocol before culture. B cells were seeded at $2 \times 10^5$ cells/well and were cultured in 96-well flat-bottom plates with LPS (10 μg/ml; Sigma, St Louis, MO, USA), recombinant mouse IL-4 (3 ng/ml; Invivogen, San Diego, CA, USA), for 24, 48 and 72 hr was measured by flow cytometry. Proliferation was also assessed by Cell Counting Kit-8 (CK04, Dojindo Laboratories, Rockville, MD, Japan) according to the manual of the manufacturer. Briefly, $2 \times 10^5$ B cells were seeded into each well of a 96-well plate and treated with LPS + IL-4 and examined at 48 hr. CCK-8 (10 μl) was added to each well and incubated for 4 hr at 37°C; absorbance was measured at 450 nm with a Microplate Reader (Bio-Rad, Hercules, CA, USA).

For analyses of apoptosis, purified B cells seeded at $2 \times 10^5$ cells/well in 96-well flat-bottom plates were stimulated with LPS + IL-4 for 24, 48 and 72 hr. Cells were then stained with FITC-coupled Annexin V (BD Biosciences, Franklin Lakes, NJ, USA) and 7-AAD (eBioscience, San Diego, CA, USA) for FACS analysis.

### Statistical analysis

All statistical analyses were conducted using PRISM7 (GraphPad Software, San Diego, CA, USA). Differences between $4.1R^{+/+}$ and $4.1R^{-/-}$ B cells were calculated using Student's unpaired *t*-test. All bar graphs are presented as SE and $P$ values $<0{\cdot}05$ were considered significant.


 

Statistical details for each experiment are indicated in the figure legends.

## Results

### Expression and location of 4.1R in B cells

Isoforms of 4.1R have previously been shown to be present in human MOLT4 T-cell lines.[40] Because there is great disparity in which 4.1R isoforms are expressed in non-erythroid cells, we first cloned 4.1R from B cells by RT-PCR with primers initiating from ATG-1 and ATG-2, and clones were all sequenced. As shown in Fig. 1(a) 4.1R isoforms isolated from B cells and all clones lacked exon 14, exon 15 and exon 16, compared with MOLT4. Western blotting showed two bands with molecular weights of approximately 135 kDa and 80 kDa, corresponding to 4.1R transcripts from ATG1 and ATG2 initiation sites, respectively. The expression of 4.1R was not detected in $4.1R^{-/-}$ B cells, (Fig. 1b). Immunofluorescence staining of 4.1R showed that in resting B cells 4.1R was evenly distributed in a punctate pattern on the membrane (Fig. 1c).

### Increased activation and proliferation of $4.1R^{-/-}$ B cells

To investigate the effect of 4.1R deficiency on B-cell activation, we examined the expression of CD69 and CD86, which is an inducible cell surface glycoprotein acquired during B-cell activation by flow cytometry. The results revealed that the expression of CD69 and CD86 in $4.1R^{+/+}$ and $4.1R^{-/-}$ B cells were significantly increased after stimulation with LPS (10 μg/ml) for 24 hr, and the percentages of $CD86^+$ and $CD69^+$ B cells in $4.1R^{-/-}$ B cells are higher than in $4.1R^{+/+}$ B cells (Fig. 2a). It was suggested that the deficiency of 4.1R increased the activation of B cells.

B-cell proliferation is an important event after its activation, the difference in the proliferation response between $4.1R^{+/+}$ and $4.1R^{-/-}$ B cells was explored in CFSE-labeling experiments after stimulation with LPS (10 μg/ml) for 24, 48 and 72 hr. Figure 2(b) displays the representative CFSE of both $4.1R^{+/+}$ and $4.1R^{-/-}$ B cells, and it clearly shows the increased division rate of $4.1R^{-/-}$ B cells, especially at 48 hr. The statistical analysis results of three CFSE experiments are shown in Figure 2(c), $4.1R^{-/-}$ B cells divide significantly faster than $4.1R^{+/+}$ B cells in 48 hr, and there are fewer cells in stages D0 (undivided) and D1 (one division) and more cells in D2 (two divisions), D3 (three divisions) and D4 (four divisions) within the same experimental time-frame. Data at 24 and 72 hr showed no significant difference between $4.1R^{+/+}$ and $4.1R^{-/-}$ B cells, but decreased cells in D1 and a moderate increase in cells in the D4 stage were observed at 72 hr.

### 4.1R inhibits B-cell CSR

We next examined the effect of 4.1R on B-cell CSR, which entails *AID*, IgH locus germline $I_H$-$C_H$

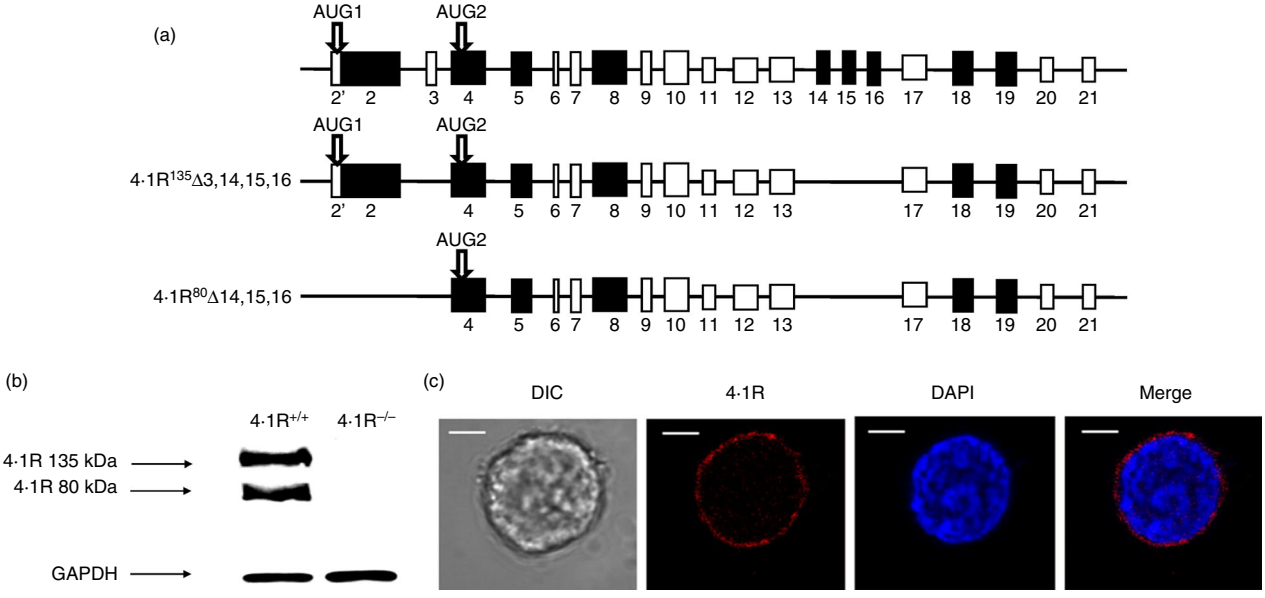


**Figure 1.** Expression and location of 4.1R in B cells. (a) Exon composition of 4.1R isoforms. Schematic representation of the exon map of 4.1R is displayed. Two translation initiation sites are indicated. Alternatively, spliced exons are shown in black, constitutive exon in white, and non-coding exons in open boxes. Exon compositions of 4.1R 135 kDa and 80 kDa are shown in the middle and bottom panels, respectively. (b) Western blot analysis of protein 4.1R. B cells ($10^7$ cells) were subjected to immunoblot analysis with polyclonal rabbit antibodies against 4.1R. The positions of approximately 135 kDa and approximately 80 kDa 4.1R are indicated. (c) Localization of 4.1R in B cells. Unstimulated primary mouse B cells were stained with anti-4.1R antibodies and analyzed by confocal microscopy. Scale bar, 2 μm.

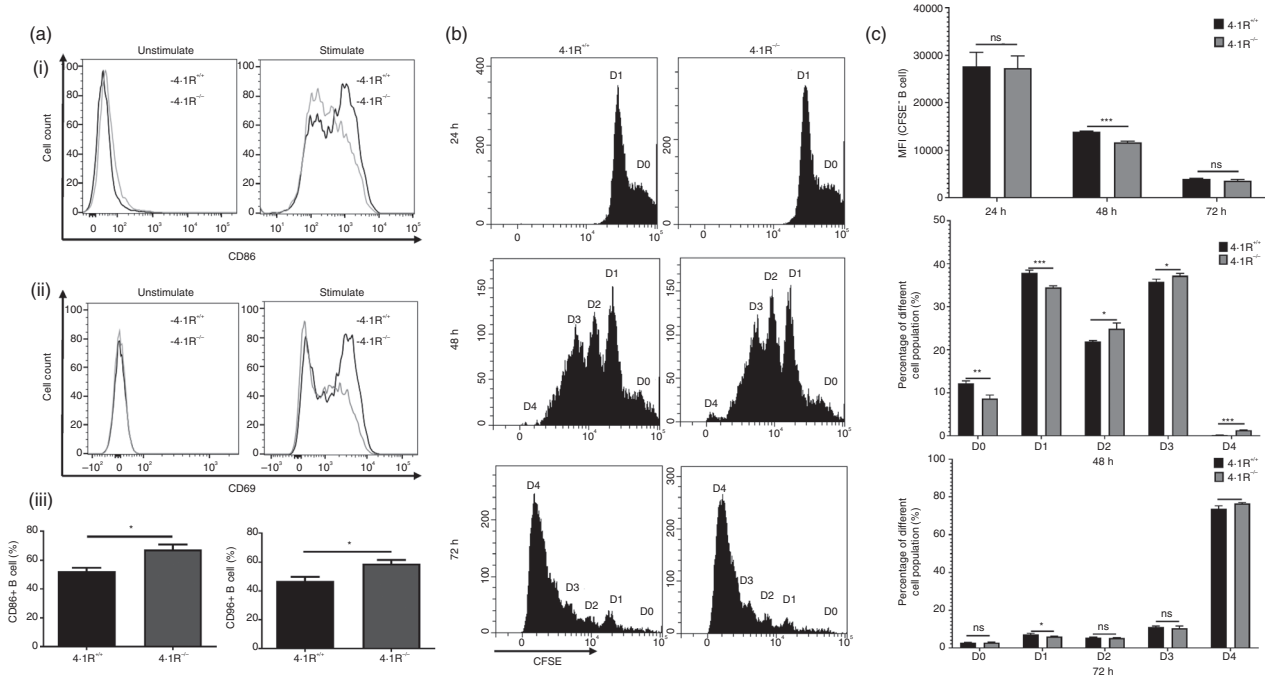

**Figure 2.** Overactivation and hyperproliferation of $4.1R^{-/-}$ B cells. (a) Purified $4.1R^{+/+}$ or $4.1R^{-/-}$ B cells were stimulated with lipopolysaccharide (LPS) (10 μg/ml) and unstimulated for 24 hr, the surface expression of CD86 and CD69 was assessed by flow cytometry. The representative profiles of CD86 and CD69 expression of $4.1R^{+/+}$ or $4.1R^{-/-}$ B cells were shown (i) (ii) and the statistical analysis of three independent experiments was shown (iii). (b) B-cell expansion. $4.1R^{+/+}$ or $4.1R^{-/-}$ B cells were stimulated with LPS (10 μg/ml) and unstimulated for 24, 48 and 72 hr, B-cell expansion *in vitro* was assessed by CFSE dilution as described in the Materials and methods section. (c) Statistical analysis of the effects of protein 4.1R on B-cell proliferation. The experiments were performed three times, $4.1R^{-/-}$ B cells showing faster division compared with $4.1R^{+/+}$ B cells. D0 (undivided), D1 (one division), D2 (two divisions), D3 (three divisions), D4 (four divisions); Error bars indicate SE; *P* values: Student's *t*-test. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$.

transcription, and *PAX5*, *Bcl-6* and *IRF4*. We measured the level of B-cell CSR by flow cytometry and quantitative RT-PCR. Resting B cells from $4.1R^{-/-}$ and $4.1R^{+/+}$ mice were labeled with CFSE and activated *in vitro* with LPS (10 μg/ml) and IL-4 (3 ng/ml). At 24, 48 and 72 hr after stimulation, cells were harvested and counterstained for surface IgG1 expression. Dilution of intracellular CFSE staining allowed assessment of cell division, whereas surface immunoglobulin staining allowed the identification of IgG1-switched B cells. The $4.1R^{-/-}$ B cells showed increased cell division within 48 hr in response to LPS (10 μg/ml) and IL-4 (3 ng/ml), as reflected by a higher proportion of $IgG1^+$ B cells compared with $4.1R^{+/+}$ B cells, which had undergone fewer cell divisions. However, after 72 hr of stimulation, the proportion of $IgG1^+$ B cells in $4.1R^{-/-}$ was decreased (Fig. 3a). The quantitative RT-PCR result shows that the *AID*, *IgH* locus germline $I_H$-$C_H$ transcription, and *PAX5*, *Bcl-6*, *IRF4* expression of $4.1R^{-/-}$ B cells also increased (Fig. 3b). This indicates that 4.1R can regulate B-cell CSR by controlling the expression of *AID*. Interestingly, we found that the function of $4.1R^{-/-}$ B cells to secrete antibodies (IgM and IgG1) was reduced, which is consistent with flow cytometry results (Fig. 3c).

### 4.1R promotes B-cell PCD

It has been reported that PCD is essential for B cells to resist foreign antigens and secrete antibodies; plasma cells highly express CD138 and are regulated by the transcription factor *Blimp-1*.[13] We tested the percentage of $CD138^+$ B cells by flow cytometry after stimulating B cells with LPS (10 μg/ml) and IL-4 (3 ng/ml) for 72 hr. A modest decrease in $CD138^+$ cell numbers was observed in $4.1R^{-/-}$ B cells (Fig. 3d), and the transcriptional factor *Blimp*-1 measured by quantitative RT-PCR was reduced in $4.1R^{-/-}$ B cells. All of these results showed that activated $4.1R^{-/-}$ B cells moderately reduced PCD. The PCD results are contrary to our common knowledge that activation enhancement of B cells leads to increased plasma differentiation.

## CSR is up-regulated through the canonical NF-κB pathways in $4.1R^{-/-}$ B cells

Both BCR and TLR can each activate only one NF-κB pathway, non-canonical and canonical, respectively. When LPS activates B cells, it triggers both BCR-


 

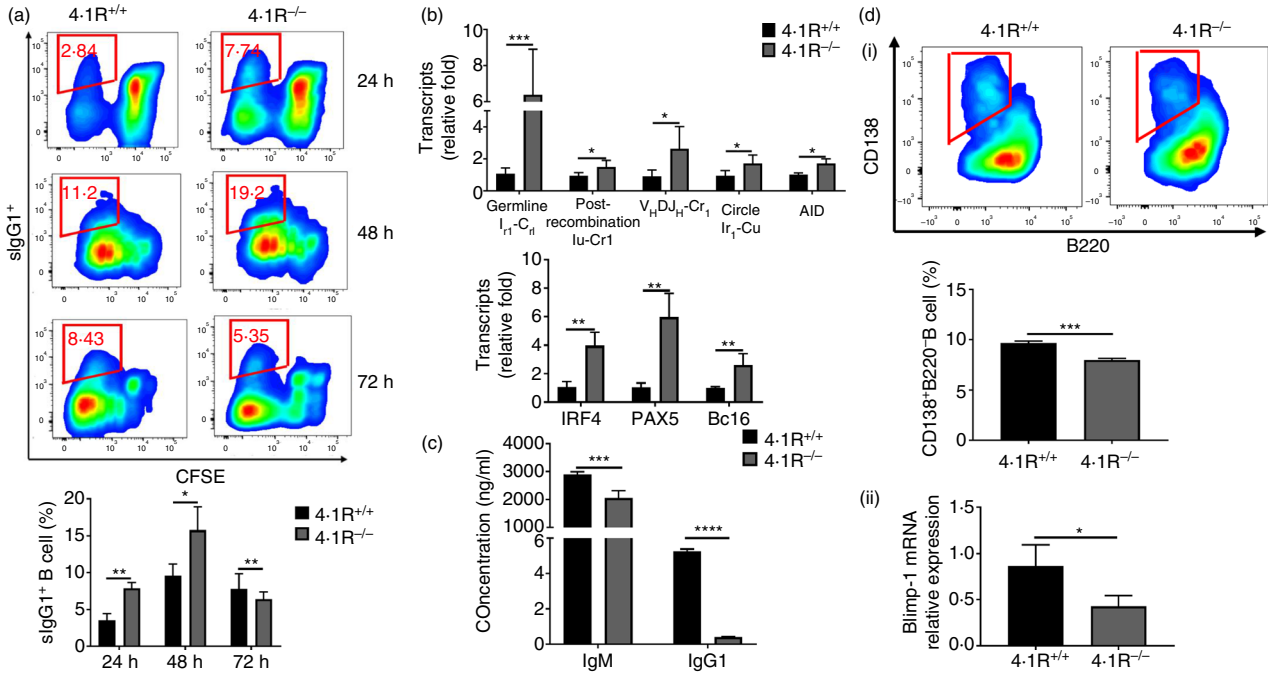


**Figure 3.** 4.1R modulates class switch recombination (CSR) and plasma cell differentiation (PCD) fate of B cells. (a) Proportions of surface IgG1$^+$ (sIgG1$^+$) B cells. CFSE-labeled 4.1R$^{+/+}$ or 4.1R$^{-/-}$ B cells were measured after stimulate with lipopolysaccharide (LPS; 10 μg/ml) plus interleukin -4 (IL-4; 3 ng/ml) 24, 48 and 72 hr. (b) Levels of germline *Iγ1-Cγ1*, post-recombination *Iμ-Cγ1*, $V_HDJ_H$-*Cγ1*, *circle Iγ1-Cμ* transcripts, and *Aicda* in B cells stimulated for 72 hr with LPS (10 μg/ml) plus IL-4 (3 ng/ml), and mRNA levels of *IRF4*, *PAX5* and *Bcl6* transcripts. (c) The concentration of IgM and IgG1 in B-cell culture supernatant after 72 hr measured by ELISA. (d) (i) Percentage and statistical analysis of plasma cell (CD138$^+$ B220$^-$) in 4.1R$^{+/+}$ or 4.1R$^{-/-}$ B cells. (ii) The levels of Blimp-1 in B cells after stimulation with LPS (10 μg/ml) plus IL-4 (3 ng/ml) for 72 hr. Error bars indicate SE; *P* values: Student's *t*-test. **P* < 0·05, ***P* < 0·01, ****P* < 0·001, *****P* < 0·0001.

signaling through its polysaccharidic moiety and TLR4-signaling through its lipid A moiety, thereby activating both the non-canonical (p52) and canonical (p65) NF-κB pathways to induce *AID* expression and enable CSR, but which one is the dominant pathway is still under study. To further discuss the mechanism by which 4.1R mediates the synergy of BCR and TLR in inducing B-cell CSR, Western blotting was performed to track IκBα phosphorylation at Ser32 and p65 phosphorylation at Ser536 (canonical) and p100 to p52 (non-canonical) treatment in B cells stimulated with TLR4 in the presence of IL-4. Phosphorylation events of the canonical NF-κB pathway were accelerated and enhanced in 4.1R$^{-/-}$ B cells (Fig. 4a). However, there was no significant difference in the levels of p52 and p100 within 3–48 hr (Fig. 4b).

To confirm the inhibitory effects of 4.1R on LPS-stimulated activation of the canonical NF-κB pathway, B cells from 4.1R$^{+/+}$ and 4.1R$^{-/-}$ mice that had been pretreated with NCA for 1 hr and subsequently stimulated with LPS + IL-4 for 48 hr. Cells were collected to measure B-cell proliferation, CSR and PCD. Results showed that the proliferation, the IgG1$^+$ B cells, and the IgM secretion between 4.1R$^{+/+}$ and 4.1R$^{-/-}$ B cells pretreated with NCA were not significantly different (Fig. 4c,d,e). Hence, 4.1R acts as a regulator through the canonical NF-κB pathway.

## Over-activation of the canonical NF-κB pathways enhances B-cell apoptosis

Theoretically, excessive activation of NF-κB in B cells can result in either accelerated proliferation or preferably apoptosis.[41] To distinguish between these two possibilities, in addition to detecting B-cell proliferation by CFSE (Fig. 2b), we also used 7-AAD and Annexin V staining to monitor apoptosis of B cells after 24, 48 and 72 hr of LPS stimulation. Flow cytometry results showed that the percentage of Annexin-V$^+$ B cells in 4.1R$^{-/-}$ was statistically increased by nearly 10% compared with 4.1R$^{+/+}$ B cells at 72 hr (Fig. 5a,b). To further investigate the causes of B-cell apoptosis in 4.1R$^{-/-}$, we used Western blotting to detect the balance of anti-apoptosis (BAK, Bcl-2) and pro-apoptosis (caspase-3, caspase-8) proteins in this process. The results showed that 4.1R deficiency disrupted the balance of apoptosis-related proteins and induced apoptosis of B cells (Fig. 5c). In conjunction with Fig 3, these data support the view that the accumulation of IgG1$^+$ B cells within 48 hr is a result of accelerated B-cell

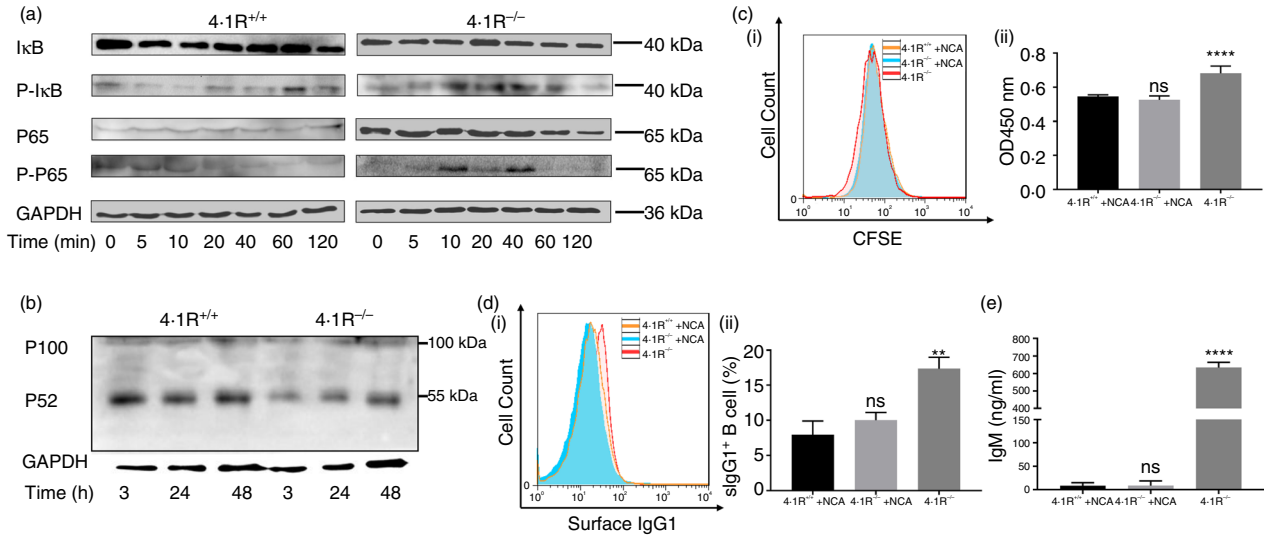


**Figure 4.** 4.1R$^{-/-}$ B cells up-regulate canonical nuclear factor-κB (NF-κB) pathways for activation-induced cytidine deaminase (AID) induction. (a) Level of phosphorylated IκBα, total IκBα, phosphorylated p65, total p65 and GAPDH in 4.1R$^{+/+}$ (left) and 4.1R$^{-/-}$ (right) B cells were measured by Western blot. (b) The protein level of P100 and P52 in 4.1R$^{+/+}$ (left) and 4.1R$^{-/-}$ (right) B cells. B cells pre-treated with or without the canonical NF-κB inhibitor NCA 1 hr, following lipopolysaccharide (LPS; 10 μg/ml) plus interleukin-4 (IL-4; 3 ng/ml) stimulation for 48 hr. Flow cytometry (c(i)) and CCK-8 assay (c(ii)) measure B-cell proliferation. (d) Proportion (i) and statistical analysis (ii) of surface IgG1$^{+}$ (sIgG1$^{+}$) B cells. (e) The concentration of IgM in B-cell culture supernatant measured by ELISA. Error bars indicate SE; *P* values: Student's *t*-test. $^{**}P < 0{\cdot}01$, $^{****}P < 0{\cdot}0001$.

CSR and enhanced proliferation. On the other hand, over-activation of the canonical NF-κB pathway is responsible for B-cell apoptosis, and in turn, reduces antibody levels in cell culture supernatants.

## Co-localization of 4.1R and TLR4 in B cells

We sought direct evidence for the association of 4.1R with TLR4 in B cells by performing immunofluorescence and co-immunoprecipitation studies. As shown in Fig. 6a, immunofluorescence staining of 4.1R shows that in resting B cells, 4.1R is evenly distributed in a punctate pattern on the membrane, where it was rarely co-localized with TLR4 (co-localization coefficient: $r = 0{\cdot}3750$). Interestingly, in B cells stimulated with LPS, 4.1R was recruited to one point on the membrane, seemingly like a B-cell immune synapse and obviously co-localized with TLR4 (co-localization coefficient: $r = 0{\cdot}7388$). We then further confirmed the immunofluorescence results by using immunoprecipitation. 4.1R was immunoprecipitated using a specific antibody in 4.1R$^{+/+}$ B cells, then followed by Western blotting with the anti-4.1R antibody and anti-TLR4 antibody, which revealed the presence of protein 4.1R and the presence of TLR4 (Fig. 6b(i)). On the other hand, we used TLR4-specific antibody, followed by Western blotting using anti-TLR4 and anti-4.1R antibodies and revealed the presence of TLR4 and protein 4.1R, respectively (Fig. 6b(ii)). The results of immunoprecipitation indicated that TLR4 and protein 4.1R have co-localization, especially with the 80 kDa 4.1R. No signal was detected when this experiment was performed with 4.1R$^{-/-}$ B cells. These results imply that 4.1R and TLR4 band *in situ*.

## Enhanced TRAM signaling levels in B cells from 4.1R$^{-/-}$ mice

Activation of TLR4-mediated signal transduction is required for B-cell activation, proliferation and antibody secretion. To explore whether 4.1R deficiency affects TLR4 signal transduction, the products from the real-time quantitative PCR were used to make a semi-quantitative measurement of the mRNA expression of adaptor proteins MyD88 and TRAM (TRIF-related adaptor molecule) in TLR4-mediated signal transduction in 4.1R$^{+/+}$ and 4.1R$^{-/-}$ B cells after stimulation with LPS (10 μg/ml). The results showed that there was no significant difference in MyD88 expression in 4.1R$^{+/+}$ and 4.1R$^{-/-}$ B cells, whereas the mRNA expression of TRAM was enhanced in 4.1R$^{-/-}$ B cells (Fig. 7a).

## Discussion

Protein 4.1R is a member of the 4·1 protein family (including 4.1R, 4.1B, 4.1G and 4.1N), and plays a key role in immune cell activation and differentiation. Several studies have shown that ezrin in the FERM domain of 4.1R is associated with B-cell biology. However, this study has not revealed the role of protein 4.1R in determining


 

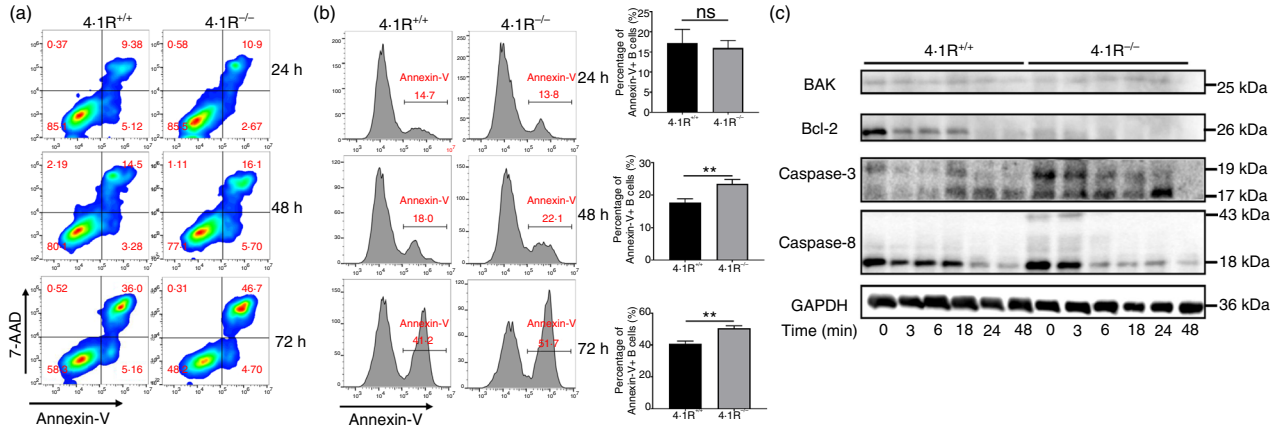


**Figure 5.** Over-activation of canonical nuclear factor-κB (NF-κB) pathways enhances apoptosis. (a) $4.1R^{+/+}$ and $4.1R^{-/-}$ B cells were stimulated with lipopolysaccharide (LPS; 10 μg/ml) plus interleukin-4 (IL-4; 3 ng/ml) for 24, 48 and 72 hr, then double-stained with Annexin V-FITC and 7-AAD and the percentage of apoptotic cells (Annexin $V^+$ and 7-AAD cells) was determined by flow cytometry. (b) The statistical analysis of Annexin $V^+$ B cells in 24, 48 and 72 hr, data are representative of three independent experiments. (c) The expression of pro-apoptosis protein cleaved caspase-3, caspase-8 and anti-apoptosis protein BAK, Bcl-2 was measured by Western blot. Equal protein loading in all lanes was assessed by probing the blots with GAPDH antibody. Error bars indicate SE; *P* values: Student's *t*-test. $^{**}P < 0{\cdot}01$.

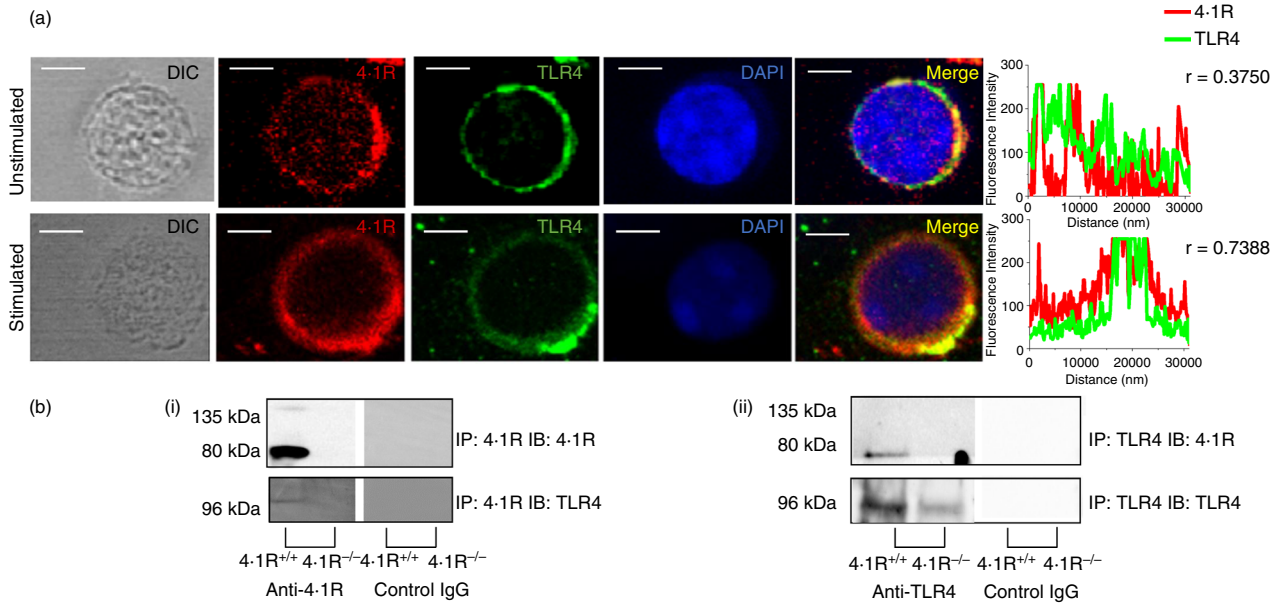


**Figure 6.** Localization of 4.1R and Toll-like receptor 4 (TLR4) in B cells. (a) Unstimulated and stimulated primary mouse B cells were double-stained with anti-4.1R and anti-TLR4 antibodies and analyzed by confocal microscopy. Pearson correlation coefficient was generated using the Prism7 software; Scale bar, 2 μm. (b) Co-immunoprecipitation of 4.1R and TLR4. $4.1R^{+/+}$ and $4.1R^{-/-}$ B cells were lyzed, and extracts were subjected to immunoprecipitation with anti-4.1R antibody or pre-immune IgG. The presence of protein 4.1R or TLR4 was determined by Western blot in immunoprecipitation eluates using anti-4.1R antibodies (i) or anti-TLR4 antibodies (ii), respectively.

activated B-cell fate and its molecular mechanism. In the present study, with the use of $4.1R^{-/-}$ mice, we have shown that 4.1R is crucial for maintaining normal B-cell activation and proliferation, and identified the mechanism for this novel function of 4.1R in B-cell fate.

In B cells, there are two 4.1R isoforms ($4.1R^{135}$ and $4.1R^{80}$; 135 kDa and 80 kDa, respectively), located in the B-cell membrane, which are consistent in T cells. Although both T and B cells belong to lymphocytes, there are several differences between T and B cells in function. An obvious event in this study is that the lack of 4.1R enhances the expression of B-cell activation markers CD69 and CD86, as well as that of B-cell proliferation. It was suggested that 4.1R may act as a negative regulator in B-cell activation, and that

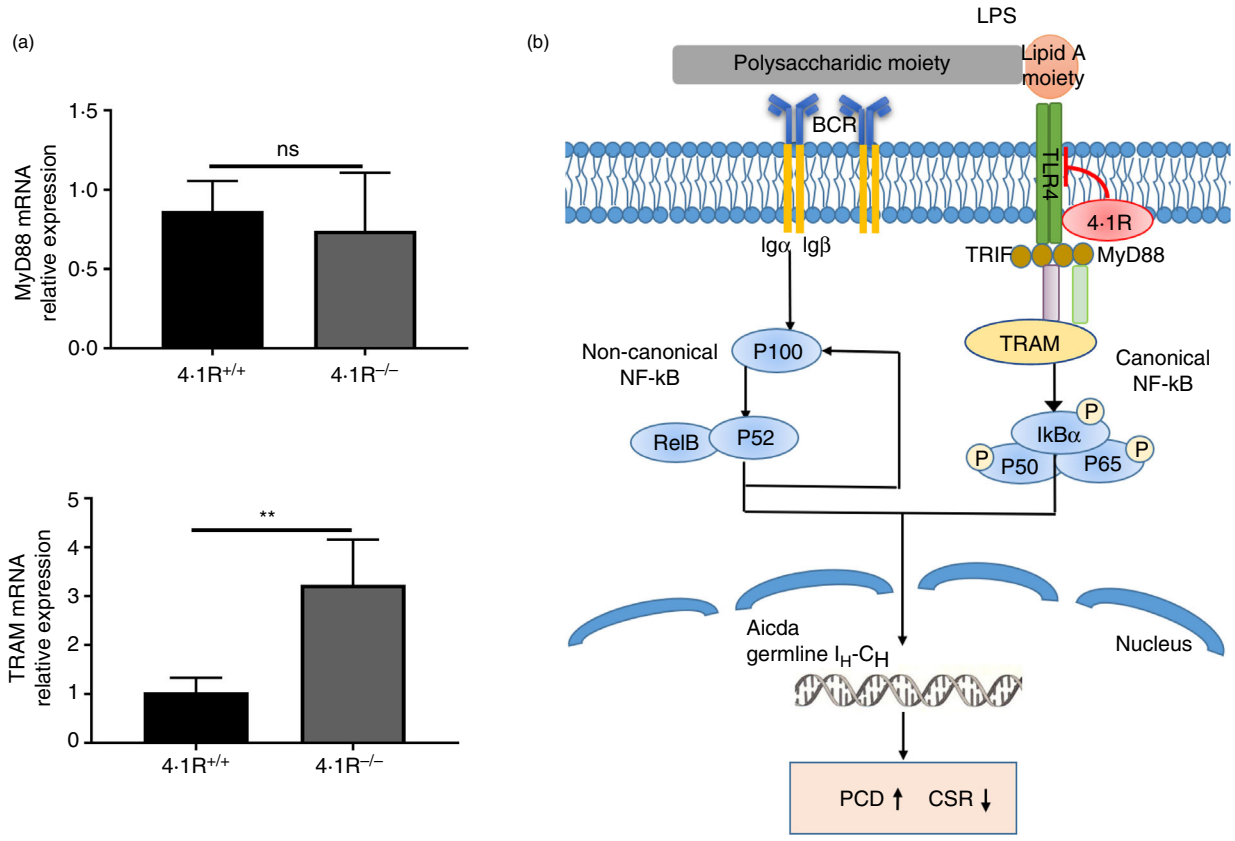


**Figure 7.** The effects of 4.1R mediate on B-cell fate. (a) mRNA expression of MyD88 and TRAM in B cells stimulated with lipopolysaccharide (LPS; 10 μg/ml) plus interleukin-4 (IL-4; 3 ng/ml) for 72 hr; (b) LPS triggers both BCR-signaling through its polysaccharidic moiety and TLR4-signaling through its lipid A moiety, thereby activating both the non-canonical and canonical nuclear factor-κB (NF-κB) pathways to induce activation-induced cytidine deaminase (AID) and class switch recombination (CSR). Protein 4.1R direct co-location with TLR4 and regulates the canonical NF-κB pathway of B cells, which subsequently regulates related transcription factors and then modulates the fate of CSR and plasma cell differentiation (PCD). Error bars indicate SE; *P* values: Student's *t*-test. ns $P > 0{\cdot}05$ $^{**}P < 0{\cdot}01$.

has also been validated in B-cell CSR. It is, however, worth noting that although 4.1R deficiency enhanced B-cell activation, the antibody secretion and PCD in $4.1R^{-/-}$ B cells showed a decreasing trend, which suggested that 4.1R impacts on B-cell fate involved in the regulation of intracellular signaling transduction and gene expression.

Most evidence has shown that the activation of canonical (P65) and non-canonical (P52/100) NF-κB is critical for signal transduction of B-cell CSR stimulated by LPS. 4.1R acts as an adaptor between the cell membrane and cytoskeleton proteins that are involved in many intercellular signal transductions, such as extracellular-regulated kinase and phosphoinositide 3-kinase signaling.[25,42] However, to our knowledge, whether 4.1R regulates the NF-κB signaling pathway is still unknown. Here, our results demonstrated that 4.1R impacts LPS-mediated B-cell fate by activating and regulating the canonical rather than the non-canonical NF-κB signaling pathway.

Studies have reported that excessive enhancement of the canonical NF-κB pathway might have been expected to reduce cell survival. To explain the reduction of antibody secretion and PCD during the activation of $4.1R^{-/-}$ B cells, we further explored whether excessive activation of the NF-κB pathway in $4.1R^{-/-}$ B cells can induce B-cell apoptosis. Our results confirmed the speculation and showed that $4.1R^{-/-}$ B cells stimulated by LPS had an increase in apoptosis up to 51·7%, and also the expression of pro-apoptotic protein caspase 3/8 increased, whereas the expression of anti-apoptotic protein Bcl-2 decreased. This is a possible explanation for why the lack of 4.1R leads to plasma apoptosis, which is achieved by disrupting the balance between pro-apoptotic proteins and anti-apoptotic proteins. Thereby reducing the number of the plasma cells ultimately results in reducing the level of immunoglobulin culture supernatant.

B cells recognize the antigen bound to antigen-presenting cells by forming immune synapses, which are initiated by the engagement of the BCR with antigens tethered at the surface of neighboring cells. Synapses are essential for immune cells and play an important role in antigen


 

capture, processing and presentation of B cells. It has long been reported that TLR4 can act as a co-receptor for BCR when stimulated by LPS. Although it is still unclear whether TLR4 is a synaptic protein member in the process, it is a possibility. TLR4 may be involved in synapse formation under LPS stimulation. On the other hand, there is increasing evidence that the 4.1 family plays an important role in cell synapse and signal transduction. However, the role of 4.1R in B-cell synapses and signals is completely unknown. Consequently, we used immunofluorescence assays and immunoprecipitation assays to identify the association of protein 4.1R with TLR4 and the accumulation of protein 4.1R in B-cell synapses after treatment with LPS. Regarding the immunological synapse, our previous study showed that protein 4.1R was recruited to the T-cell synapse on stimulation, whereas we show here that 4.1R may also recruit to the B-cell synapse on stimulation, then mediate the canonical NF-$\kappa$B pathway downstream of TLR4 signaling TRAM (Fig. 7B). The effect of 4.1R on B-cell synapses and its role in the BCR signaling pathway remain to be explored.

In summary, we have defined a hitherto unrecognized role for 4.1R in regulating activated B-cell fate by modulating the canonical NF-$\kappa$B pathway. More understanding of 4.1R in B-cell fate is still under exploration.

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## Conflict of interests

The authors declare no conflict of interests.

### Data availability statement

The data sets used and/or analyzed during the current study are available from the corresponding author upon request. All data generated or analyzed during this study are included in this published article.

## References

1 Chaudhuri J, Alt FW. Class-switch recombination: interplay of transcription, DNA deamination and DNA repair. *Nat Rev Immunol* 2004; **4**(7):541–52.

2 Ramiro AR, Nussenzweig MC. Immunology: aid for *AID*. *Nature* 2004; **430**(7003):980–1.

3 Gonda H, Sugai M, Nambu Y, Katakai T, Agata Y, Mori KJ, *et al*. The balance between *Pax5* and *Id2* activities is the key to *AID* gene expression. *J Exp Med* 2003; **198**(9):1427–37.

4 Muto A, Tashiro S, Nakajima O, Hoshino H, Takahashi S, Sakoda E, *et al*. The transcriptional programme of antibody class switching involves the repressor *Bach2*. *Nature* 2004; **429**(6991):566–71.

5 Nambu Y, Sugai M, Gonda H, Lee CG, Katakai T, Agata Y, *et al*. Transcription-coupled events associating with immunoglobulin switch region chromatin. *Science* 2003; **302**(5653):2137–40.

6 Stavnezer J, Schrader CE. IgH chain class switch recombination: mechanism and regulation. *J Immunol* 2014; **193**(11):5370–8.

7 Stavnezer J, Guikema JE, Schrader CE. Mechanism and regulation of class switch recombination. *Annu Rev Immunol* 2008; **26**:261–92.

8 Stavnezer J, Schrader CE. Response to comment on 'IgH chain class switch recombination: mechanism and regulation'. *J Immunol* 2015; **194**(5):2040.

9 Kobayashi M, Takemoto M, Honjo T. The novel activation-induced deoxycytidine deaminase (*AID*) mutants, AIDv and AIDvDelta15 are defective in SHM and CSR. *DNA Repair (Amst)* 2017; **53**:1–3.

10 Pone EJ, Zhang J, Mai T, White CA, Li G, Sakakura JK, *et al*. BCR-signalling synergizes with TLR-signalling for induction of *AID* and immunoglobulin class-switching through the non-canonical NF-$\kappa$B pathway. *Nat Commun* 2012; **3**(1):767–79.

11 Nutt SL, Fairfax KA, Kallies A. *BLIMP1* guides the fate of effector B and T cells. *Nat Rev Immunol* 2007; **7**(12):923–7.

12 Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. *Blimp-1* is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* 2003; **19**(4):607–20.

13 Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A, *et al*. *Blimp-1* orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 2002; **17**(1):51–62.

14 Sciammas R, Shaffer AL, Schatz JH, Zhao H, Staudt LM, Singh H. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity* 2006; **25**(2):225–36.

15 Ochiai K, Muto A, Tanaka H, Takahashi S, Igarashi K. Regulation of the plasma cell transcription factor *Blimp-1* gene by *Bach2* and *Bcl6*. *Int Immunol* 2008; **20**(3):453–60.

16 Klein U, Casola S, Cattoretti G, Shen Q, Lia M, Mo T, *et al*. Transcription factor *IRF4* controls plasma cell differentiation and class-switch recombination. *Nat Immunol* 2006; **7**(7):773–82.

17 Ochiai K, Maienschein-Cline M, Simonetti G, Chen J, Rosenthal R, Brink R, *et al*. Transcriptional regulation of germinal center B and plasma cell fates by dynamical control of *IRF4*. *Immunity* 2013; **38**(5):918–29.

18 Lin KI, Angelin-Duclos C, Kuo TC, Calame K. *Blimp-1*-dependent repression of *Pax-5* is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. *MOL CELL BIOL* 2002; **22**(13):4771–80.

19 Ochiai K, Maienschein-Cline M, Simonetti G, Chen J, Rosenthal R, Brink R, *et al*. Transcriptional Regulation of Germinal Center B and Plasma Cell Fates by Dynamical Control of *IRF4*. *Immunity* 2013; **38**(5):918–29.

20 Thomas MJ, Klein U, Lygeros J, Rodriguez MM. A Probabilistic Model of the Germinal Center Reaction. *Front Immunol* 2019; **10**:689.

21 Duffy KR, Wellard CJ, Markham JF, Zhou JHS, Holmberg R, Hawkins ED, *et al*. Activation-induced B cell fates are selected by intracellular stochastic competition. *Science* 2012; **335**(6066):338–41.

22 Barnett BE, Ciocca ML, Goenka R, Barnett LG, Wu J, Laufer TM, *et al*. Asymmetric B cell division in the germinal center reaction. *Science* 2012; **335**(6066):342–4.

23 Tarlinton D. B-cell differentiation: instructive one day, stochastic the next. *Curr Biol* 2012; **22**(7):R235–R237.

24 Conboy J, Kan YW, Shohet SB, Mohandas N. Molecular cloning of protein 4.1, a major structural element of the human erythrocyte membrane skeleton. *Proc Natl Acad Sci USA* 1986; **83**(24):9512–6.

25 Kang Q, Yu Y, Pei X, Hughes R, Heck S, Zhang X, *et al*. Cytoskeletal protein 4.1R negatively regulates T-cell activation by inhibiting the phosphorylation of LAT. *Blood* 2009; **113**(24):6128–37.

26 Liu X, Zhou Q, Ji Z, Fu G, Li Y, Zhang X, *et al*. Protein 4.1R attenuates autoreactivity in experimental autoimmune encephalomyelitis by suppressing CD4$^+$ T cell activation. *Cell Immunol* 2014; **292**(1–2):19–24.

27 Fan D, Li J, Li Y, Guo Y, Zhang X, Wang W, *et al*. Protein 4.1R negatively regulates CD8$^+$ T-cell activation by modulating phosphorylation of linker for activation of T cells. *Immunology* 2019; **157**(4):312–21.

28 Men YL, Kang QZ, Ding C, Liu S-M, Jiang H, Wang X-D, *et al*. Expression of protein 4.1 family in melanoma cell lines and its effect on cell proliferation. *Nan Fang Yi Ke Da Xue Xue Bao* 2016; **36**(5):649–54.

29 Stagg MA, Carter E, Sohrabi N, Siedlecka U, Soppa GK, Mead F, *et al.* Cytoskeletal protein 4.1R affects repolarization and regulates calcium handling in the heart. *Circ Res* 2008; **103**(8):855–63.
30 Liu C, Weng H, Chen L, Yang S, Wang H, Debnath G, *et al.* Impaired intestinal calcium absorption in protein 4.1R-deficient mice due to altered expression of plasma membrane calcium ATPase 1b (PMCA1b). *J BIOL CHEM* 2013; **288**(16):11407–15.
31 Nunomura W, Sasakura D, Shiba K, Nakamura S, Kidokoro S, Takakuwa Y. Structural stabilization of protein 4.1R FERM domain upon binding to apo-calmodulin: novel insights into the biological significance of the calcium-independent binding of calmodulin to protein 4.1R. *BIOCHEM J* 2011; **440**(3):367–74.
32 Nunomura W, Isozumi N, Nakamura S, Jinbo Y, Ohki S, Kidokoro S, *et al.* Unique structural changes in calcium-bound calmodulin upon interaction with protein 4.1R FERM domain: novel insights into the calcium-dependent regulation of 4.1R FERM domain binding to membrane proteins by calmodulin. *CELL BIOCHEM BIOPHYS* 2014; **69**(1):7–19.
33 Tolar P. Cytoskeletal control of B cell responses to antigens. *Nat Rev Immunol* 2017; **17**(10):621–34.
34 Hoogeboom R, Natkanski EM, Nowosad CR, Malinova D, Menon RP, Casal A, *et al.* Myosin IIa Promotes Antibody Responses by Regulating B Cell Activation, Acquisition of Antigen, and Proliferation. *Cell Rep* 2018; **23**(8):2342–53.
35 Nowosad CR, Tolar P. Plasma Membrane Sheets for Studies of B Cell Antigen Internalization from Immune Synapses. *Methods Mol Biol* 2017; **1584**:77–88.
36 Li J, Yin W, Jing Y, Kang D, Yang L, Cheng J, *et al.* The coordination between b cell receptor signaling and the actin cytoskeleton during B cell activation. *Front Immunol* 2018; **9**:3096.
37 Pore D, Gupta N. The ezrin-radixin-moesin family of proteins in the regulation of B-cell immune response. *Crit Rev Immunol* 2015; **35**(1):15–31.
38 Sustar V, Vainio M, Mattila PK. Visualization and quantitative analysis of the actin cytoskeleton upon B cell activation. *Methods Mol Biol* 2018; **1707**:243–57.
39 Kim M, Choi SY, Lee P, Hur J. Neochlorogenic Acid Inhibits Lipopolysaccharide-Induced Activation and Pro-inflammatory Responses in BV2 Microglial Cells. *Neurochem Res* 2015; **40**(9):1792–8.
40 Luque CM, Lallena MJ, Perez-Ferreiro CM, *et al.* The N-terminal 209-aa domain of high molecularweight 4.1R isoforms abrogates 4.1R targeting to the nucleus. *Proc NatlAcad Sci USA.* 1999; **96**(26):14925–30.
41 Grimm T. EBV latent membrane protein-1 protects B cells from apoptosis by inhibition of BAX. *Blood* 2005; **105**(8):3263–9.
42 Chen L, Wang T, Ji X, Ding C, Liang T, Liu X, *et al.* Cytoskeleton protein 4.1R suppresses murine keratinocyte cell hyperproliferation via activating the Akt/ERK pathway in an EGFR-dependent manner. *Exp Cell Res* 2019; **384**(2):111648.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Real-time qPCR primers.


 