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RESEARCH ARTICLE

Normal B cell development and Pax5 expression in *Thy28/ThyN1*-deficient mice

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Abstract

Thy28, also known as ThyN1, is a highly conserved nuclear protein. We previously showed that in a chicken mature B cell line, Thy28 binds to the promoter of the gene encoding *Pax5*, a transcription factor essential for B cell development, and positively regulates its expression. Here, we generated a *Thy28*-deficient mouse line to analyze its potential role in B cell development in mice. *Thy28*-deficient mice showed normal development of B cells, and the expression of Pax5 was comparable between wild-type and *Thy28*-deficient primary B cells. Thus, species-specific mechanisms regulate Pax5 expression and B cell development.

Introduction

B cell development is a complex process regulated by the concerted actions of many gene products. Pax5 is an essential transcription factor in the process of B cell development [1]. Expression of the mouse *Pax5* gene is regulated by many transcription factors and DNA-binding proteins. Examples of such regulators include PU.1, IRF4, IRF8, NF-κB, and EBF1 [2, 3]. We previously used a locus-specific chromatin immunoprecipitation (ChIP) approach to analyze the mechanisms regulating the expression of *Pax5* in a chicken mature B cell line, DT40 [4][5]. We found that Thy28, which is also known as ThyN1, binds to the promoter region of the *Pax5* gene in a B cell-specific manner and positively regulates its expression [6].

Thy28 is an evolutionarily-conserved protein [7, 8] that is highly expressed in the bursa of Fabricius and in other lymphoid tissues in the chicken [7]. It is also expressed in the liver, heart, and brain in chickens [7]. In contrast to its relatively limited tissue distribution in the chicken, Thy28 is more broadly expressed in the mouse [8].

In the present study, we generated a mutant mouse strain lacking expression of Thy28 to examine its *in vivo* function in mice. The *Thy28*-deficient ($Thy28^{-/-}$) mice were viable and

collection and analysis, decision to publish, or preparation of the manuscript.

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showed normal development. Interestingly, B cell development in Thy28$^{-/-}$ mice was normal, suggesting that Thy28 is dispensable for B cell development in mice. Expression of Pax5 was comparable between wild-type and Thy28$^{-/-}$ primary B cells. These results suggest a species-specific role of Thy28 in B cell development and function.

Materials and methods

Mice

The targeting vector for the mouse *Thy28* gene (PG00147_X_4_A07) was obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM). The linearized plasmid was transfected into an embryonic stem (ES) cell line, EGR-G101, which was previously established from C57BL/6-Tg(CAG/Acr-EGFP)C3-N01-FJ002Osb mice, as described previously [9]. After G418 selection, surviving colonies were subjected to screening by PCR. ES cells retaining the transgene in the *Thy28* locus were injected into blastocysts derived from ICR mice (Japan SLC) to generate chimeras. The chimeric mice were crossed with C57BL/6 mice to generate heterozygous Thy28$^{KI/+}$ mice (strain name: C57BL/6-Thyn1$^{tm1a(EUCOMM)Osb/Osb}$) (RIKEN BioResource Center RBRC09564). The Thy28$^{KI/+}$ mice were then crossed with CAG-FLPe mice [10] to generate Thy28$^{flox/+}$ mice (strain name: B6.Cg-Thyn1$^{tm1c(EUCOMM)Osb/Osb}$) (RIKEN BioResource Center RBRC09563), and the Thy28$^{flox/+}$ mice were crossed with CAG-Cre mice [11] to generate Thy28$^{+/-}$ mice (strain name: B6.Cg-Thyn1$^{tm1d(EUCOMM)Osb/Osb}$) (RIKEN BioResource Center RBRC09565). Finally, the Thy28$^{+/-}$ mice were crossed with each other to generate Thy28$^{+/}$, Thy28$^{+/-}$, and Thy28$^{-/-}$ mice.

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Research Institute for Microbial Diseases, Osaka University.

Genotyping

For genotyping, genomic DNA was extracted and subjected to PCR with KOD FX (Toyobo). PCR conditions were as follows. Thy28$^{KI/+}$ mice: heating at 94˚C for 2 min, followed by 35 cycles of 98˚C for 10 s, 68˚C for 10 min, and 68˚C for 2 min. Thy28$^{flox/+}$ mice: heating at 94˚C for 2 min, followed by 37 cycles of 94˚C for 20 s, 64˚C for 20 sec, 72˚C for 30 sec, and 72˚C for 10 min. Tny28$^{+/-}$ mice: heating at 94˚C for 2 min; followed by 35 cycles of 98˚C for 10 s, 62˚C for 30 sec, 68˚C for 6 min, and 68˚C for 2 min. Primers used for genotyping PCR are shown in Table 1.

Immunoblot analysis

Nuclear extracts (NE) were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Aliquots of NE (10 μg) were subjected to immunoblot analysis with an anti-Thy28 Ab (kindly gifted by Dr. Compton) [7], as described previously [12].

Cell staining and flow cytometry

Cells were stained for 30 min at 4˚C with fluorochrome-conjugated antibodies (Abs). Abs used for surface staining were fluorescein isothiocyanate (FITC)-conjugated mouse CD19 (130-102-494, Miltenyi), phycoerythrin (PE)-Cy7-conjugated mouse CD3 (552774, BD Bioscience), allophycocyanin (APC)-conjugated mouse IgD (405713, BioLegend), APC-Cy7-conjugated mouse MHC class II (107628, BioLegend), BV510-conjugated mouse CD19 (562956, BD Pharmingen), BV421-conjugated CD5 (562739, BD Pharmingen), and PE-conjugated CD21/35 (552957, BD Pharmingen).

For detection of Pax5 protein, splenocytes from 7-week-old mice were stained with FITC-labeled anti-CD19 in autoMACS Running Buffer—MACS Separation Buffer (130-091-221,

Table 1. Oligodeoxyribonucleotides used in this study.

Number	Name	Sequence (5' → 3')	Experiments
27379	5'Gene-Specific (GF3)	gcaagtgtcaggccagtctgaggcaacatg	Genotyping of $Thy28^{KI/+}$ mice
27246	LAR3+2	cctacatagttggcagtgtttggggcaagtg	Genotyping of $Thy28^{KI/+}$ mice
26859	pNT1.1-Neo-R4	atggcgatgcctgcttgccgaatatcatgg	Genotyping of $Thy28^{KI/+}$ mice
27250	3'Gene-Specific (GR4)	cgagaacgacacaatagcgaagtatgag	Genotyping of $Thy28^{KI/+}$ mice
27701	LAR3+1_F	caacaagtttgtacaaaaaagcaggctggc	Genotyping of $Thy28^{floxed/+}$ and $Thy28^{KI/+}$ mice
27702	R2R_R	Ccgcctactgcgactataga	Genotyping of $Thy28^{floxed/+}$ and $Thy28^{KI/+}$ mice
27667	Thy28 Fow2	tatgtatccagccccaagaacagt	Genotyping of $Thy28^{+/-}$ mice
27668	Thy28 Rev2	agggtgagactgaggtgtttatcg	Genotyping of $Thy28^{+/-}$ mice

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Miltenyi), followed by staining with a PE-conjugated anti-Pax5 Ab (12–9918, eBioscience/Thermo Fisher Scientific) according to the manufacture's protocol. Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences) and data was analyzed with FlowJo software (TreeStar).

Statistics

Prism 8 software (GraphPad) was used for statistical analyses. One-way analysis of variance (ANOVA) or Student t-tests were used to calculate p-values.

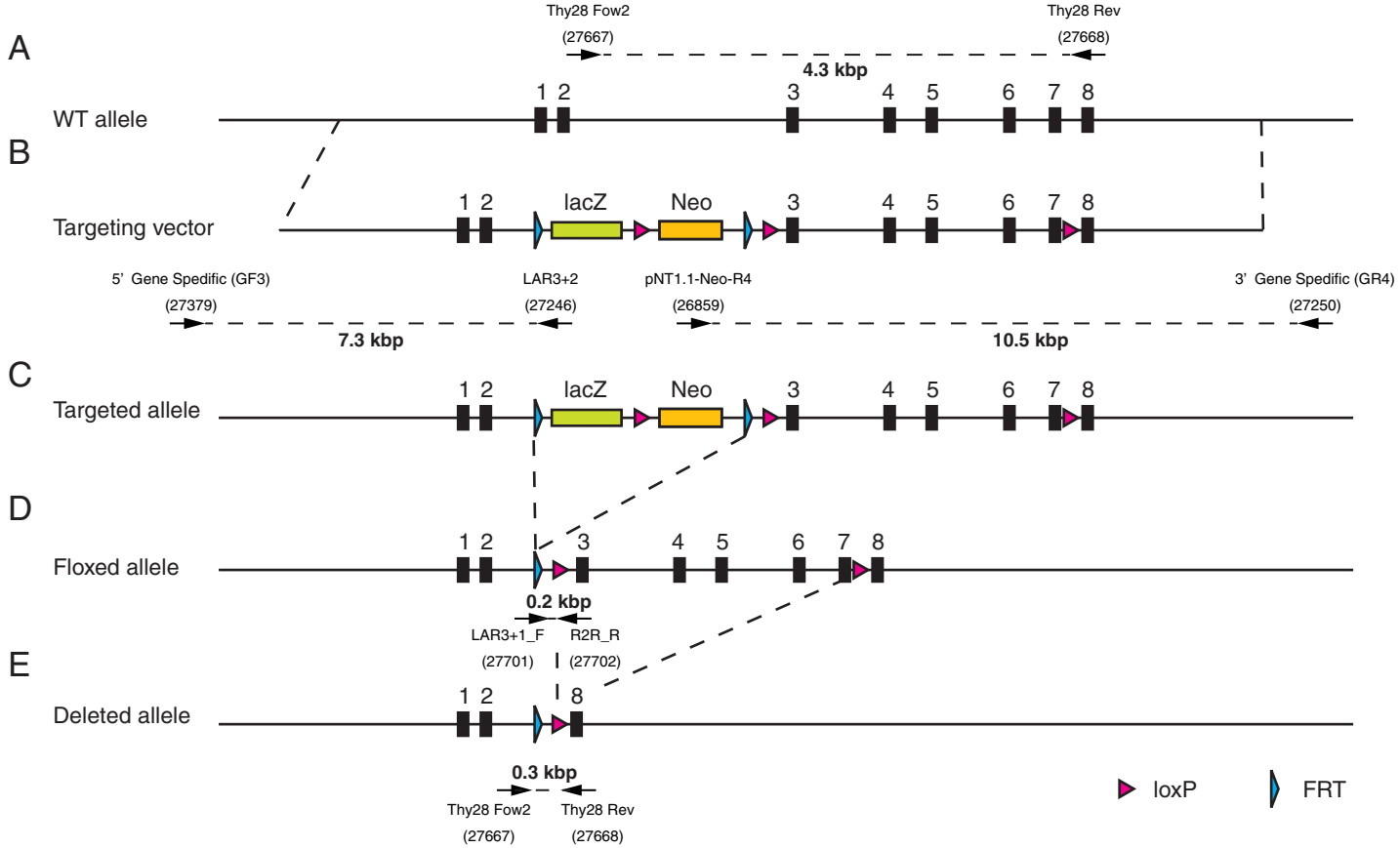

Fig 1. Generation of the *Thy28*-deficient mice. Schematic diagrams of the *Thy28* locus (**A**), the targeting vector (**B**), the targeted allele (**C**), the floxed allele (**D**), and the deleted allele (**E**).

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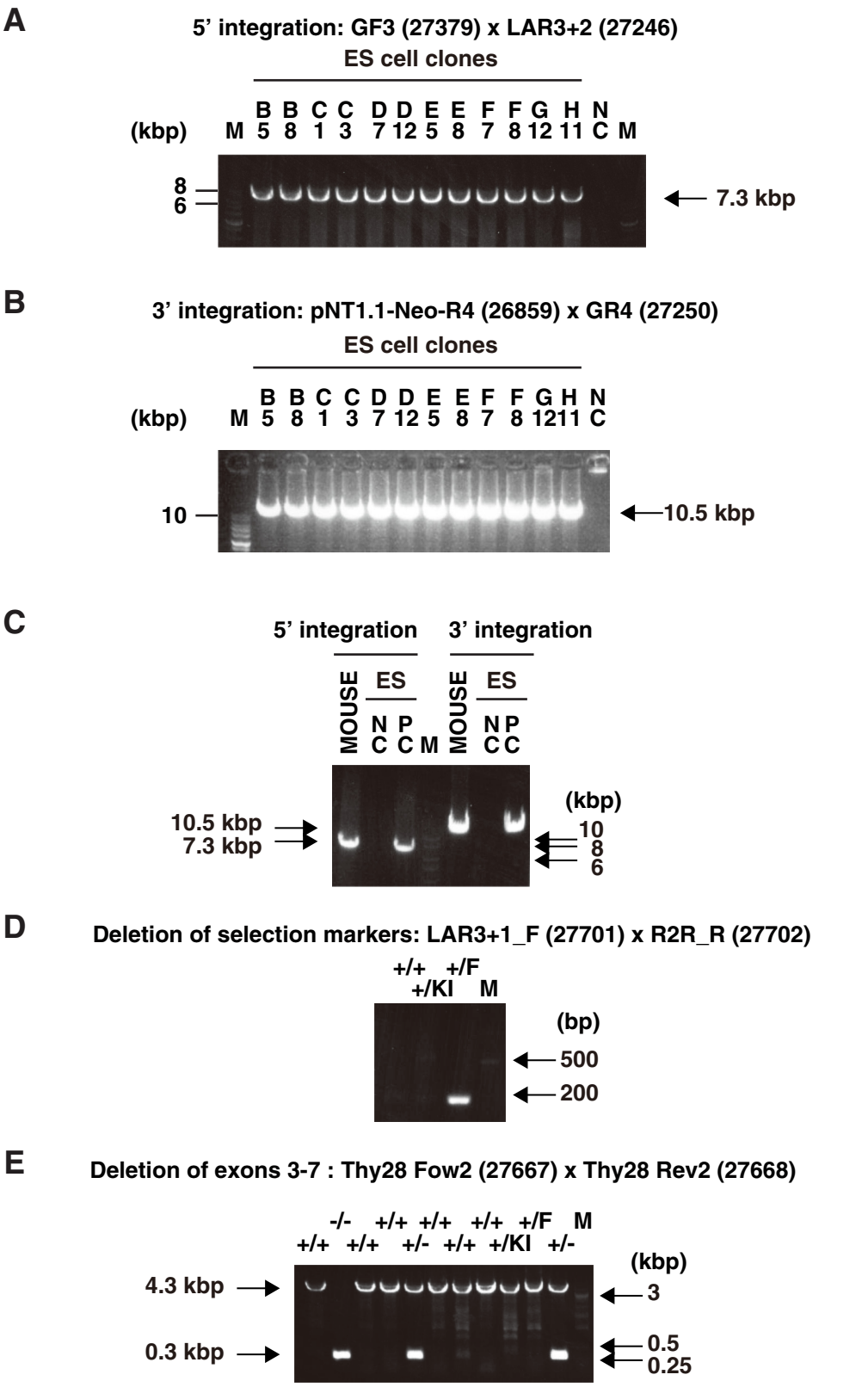

Fig 2. Genotyping of the *Thy28*-deficient mice. Detection of the 5' integration site (**A**) and the 3' integration site (**B**) in the ES cell clones. (**C**) Detection of 5' and 3' integration in an individual mouse. (**D**) Confirmation of deletion of selection markers by FLPe-mediated FRT recombination. (**E**) Confirmation of deletion of exons 3–7 of the *Thy28* gene by Cre-mediated loxP recombination.

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Table 2. Birth ratios of *Thy28*-deficient mice.

Number (%)	+/+	+/-	-/-
Male	35 (26.5%)	61 (46.2%)	36 (27.3%)
Female	30 (23.1%)	68 (52.3%)	32 (24.6%)
Total	65 (24.8%)	129 (49.2%)	68 (26.0%)

https://doi.org/10.1371/journal.pone.0220199.t002

Results and discussion

Generation of Thy28$^{-/-}$ mice

To examine the potential role of Thy28 in B cell development in mice, we generated mutant mice in which the *Thy28* gene was inactivated by deletion of its exons 3–7 (Thy28$^{-/-}$ mice) (Figs 1 and 2, Table 1). The linearized targeting vector for the mouse *Thy28* gene was transfected into an ES cell line, EGR-G101 [9]. After G418 selection, surviving colonies were subjected to screening by PCR. ES cells retaining the transgene in the *Thy28* locus were injected into blastocysts derived from ICR mice to generate chimeras. The chimeric mice were crossed with C57BL/6 mice to generate heterozygous Thy28$^{KI/+}$ mice. The Thy28$^{KI/+}$ mice were crossed with CAG-FLPe mice [10] to generate Thy28$^{flox/+}$ mice, and the Thy28$^{flox/+}$ mice were crossed with CAG-Cre mice [11] to generate Thy28$^{+/-}$ mice. Finally, the Thy28$^{+/-}$ mice were crossed each other to generate Thy28$^{+/}$, Thy28$^{+/-}$, and Thy28$^{-/-}$ mice. The Thy28$^{-/-}$ mice were viable and born in the expected Mendelian ratios (Table 2), suggesting that the *Thy28* gene is dispensable for normal development. As expected, the expression of Thy28 protein was lost in Thy28$^{-/-}$ mice, and reduced in heterozygous Thy28$^{+/-}$ mice (Fig 3). These results indicated that our targeting strategy effectively knocked out the *Thy28* gene in these mice.

Normal development of B cells in Thy28$^{-/-}$ mice

To examine the potential role of Thy28 in the development of mouse B cells and other lymphocytes, we analyzed the B cell population in Thy28$^{-/-}$ mice. As shown in Figs 4 and 5, no

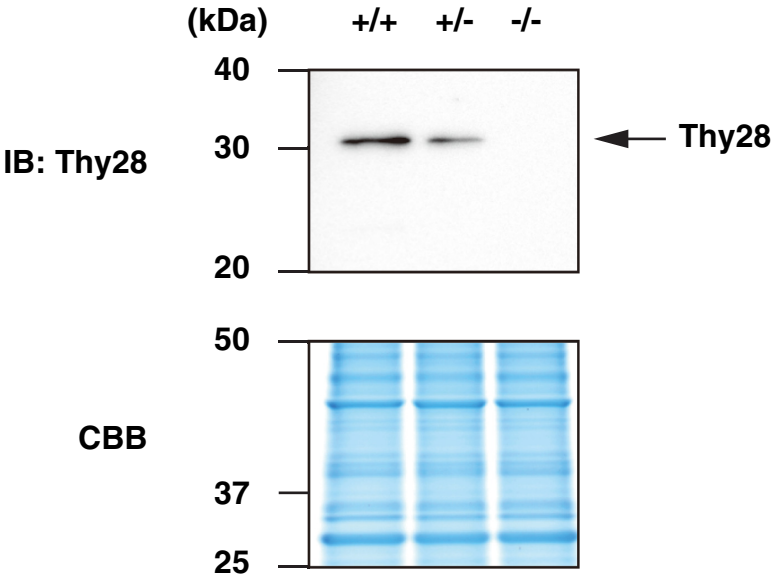

Fig 3. Expression of Thy28 in Thy28-mutant mice. Expression of Thy28 in murine spleocytes was detected by immunoblot analysis with an anti-Thy28 Ab. Coomassie Brilliant Blue (CBB) staining is shown as a protein loading control.

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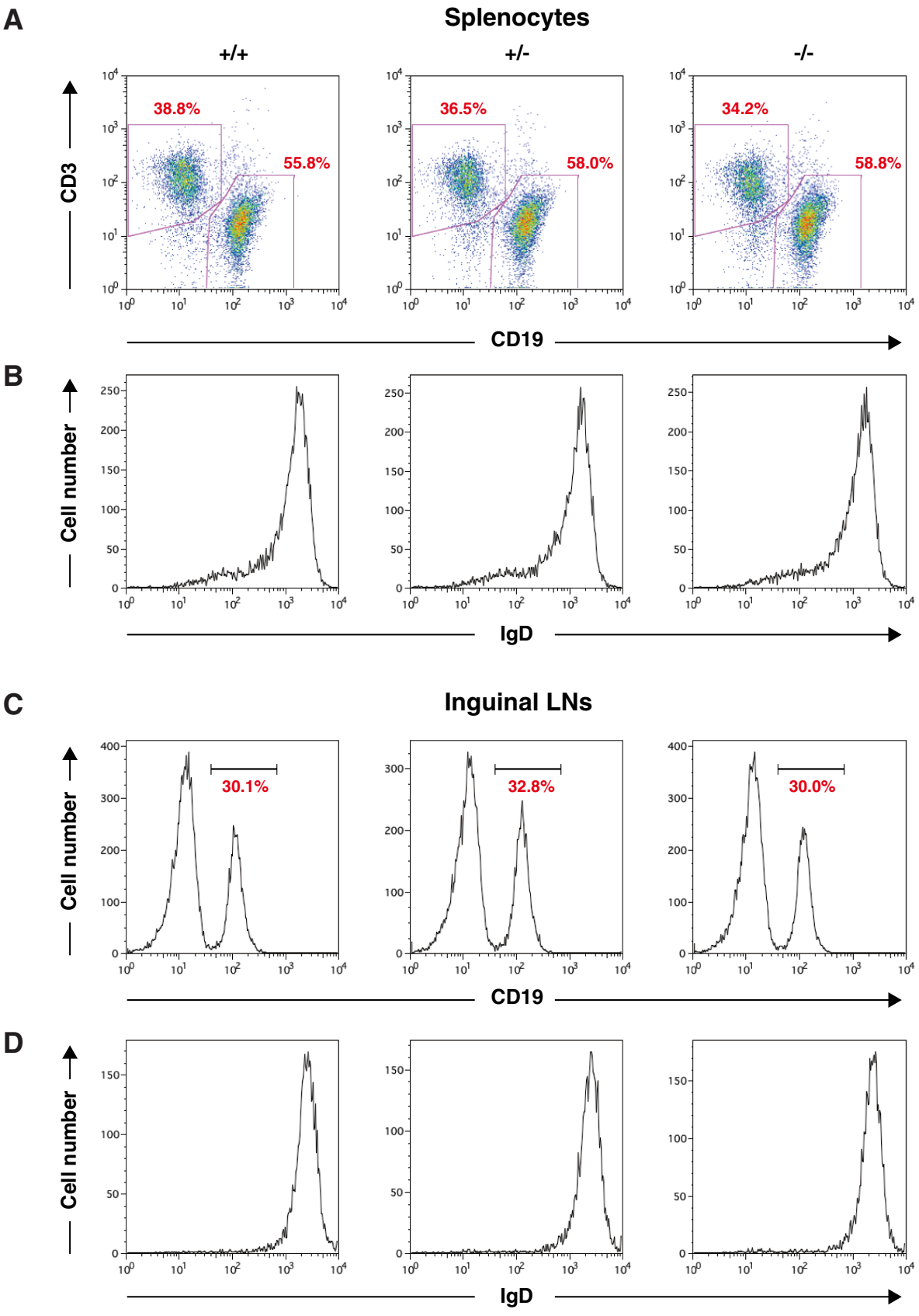
A
Splenocytes
+/+
+/-
-/-
38.8%
55.8%
36.5%
58.0%
34.2%
58.8%
CD3
CD19
B
Cell number
IgD
C
Inguinal LNs
30.1%
32.8%
30.0%
Cell number
CD19
D
Cell number
IgD

Fig 4. B cell profiles. (**A**) Percentages of B cells and T cells in the spleen. (**B**) Expression of IgD on splenic B cells. (**C**) Percentages of $CD19^+$ B cells in the inguinal lymph nodes (LNs). (**D**) Expression of IgD on B cells in the inguinal LNs.

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abnormalities were detected in cellularity in the B cell population. B cell numbers in $Thy28^{-/-}$ mice were normal, as determined by the percentages of $CD19^+$ cells in the spleen and lymph node (LN) (Figs 4 and 5). The percentages of total B cells, B1B cells, B2B cells, follicular B cells, marginal zone B (MZB) cells, and pre-B cells in spleens from $Thy28^{-/-}$ mice were normal (Fig 6). These data suggest that Thy28 is dispensable for B cell development in mice.

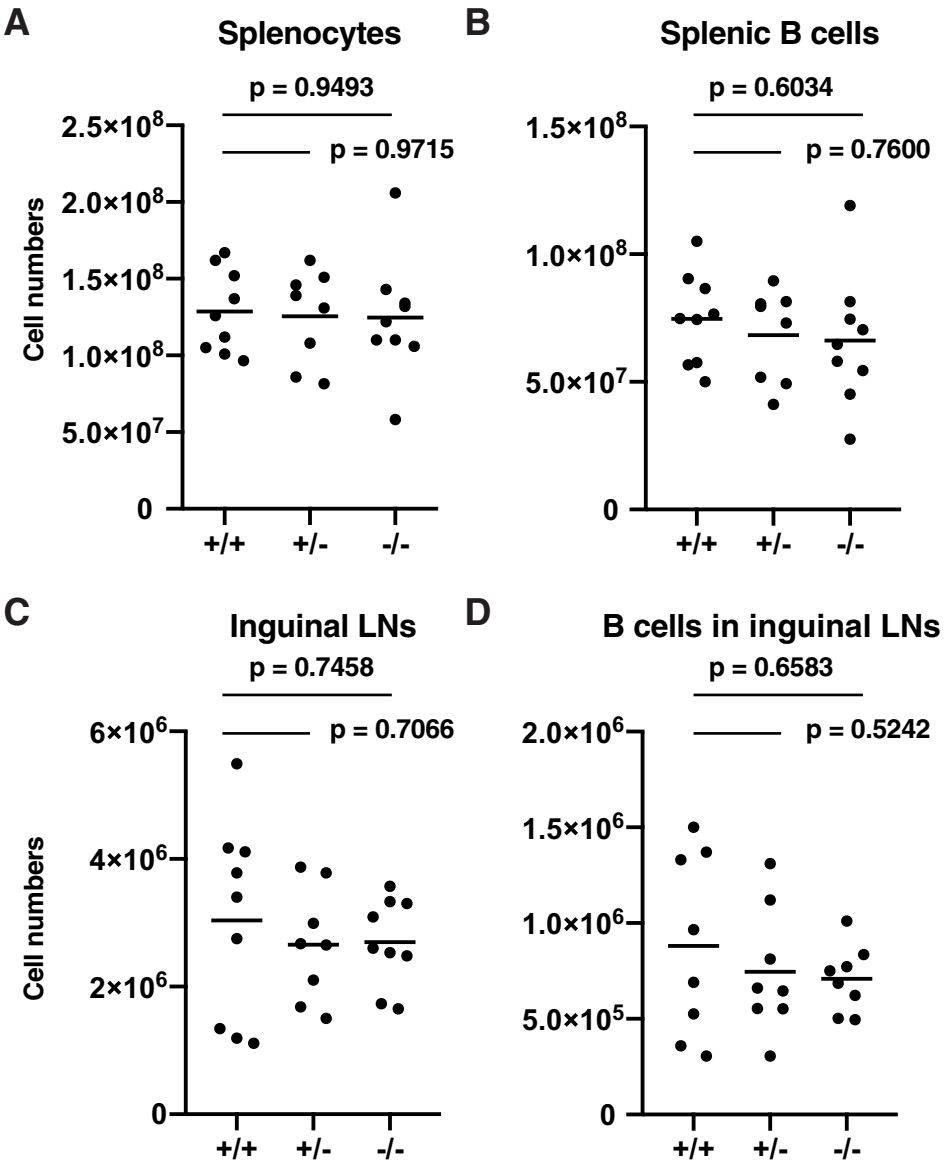

Fig 5. Numbers of B cells in spleens and inguinal LNs. Numbers of (**A**) splenocytes, (**B**) splenic $CD19^+$ B cells, (**C**) cells in inguinal LNs, and (**D**) $CD19^+$ B cells in inguinal LNs are shown. The age range of the mice was 8.1–38.7 weeks. One-way ANOVA was used to calculate p-values.

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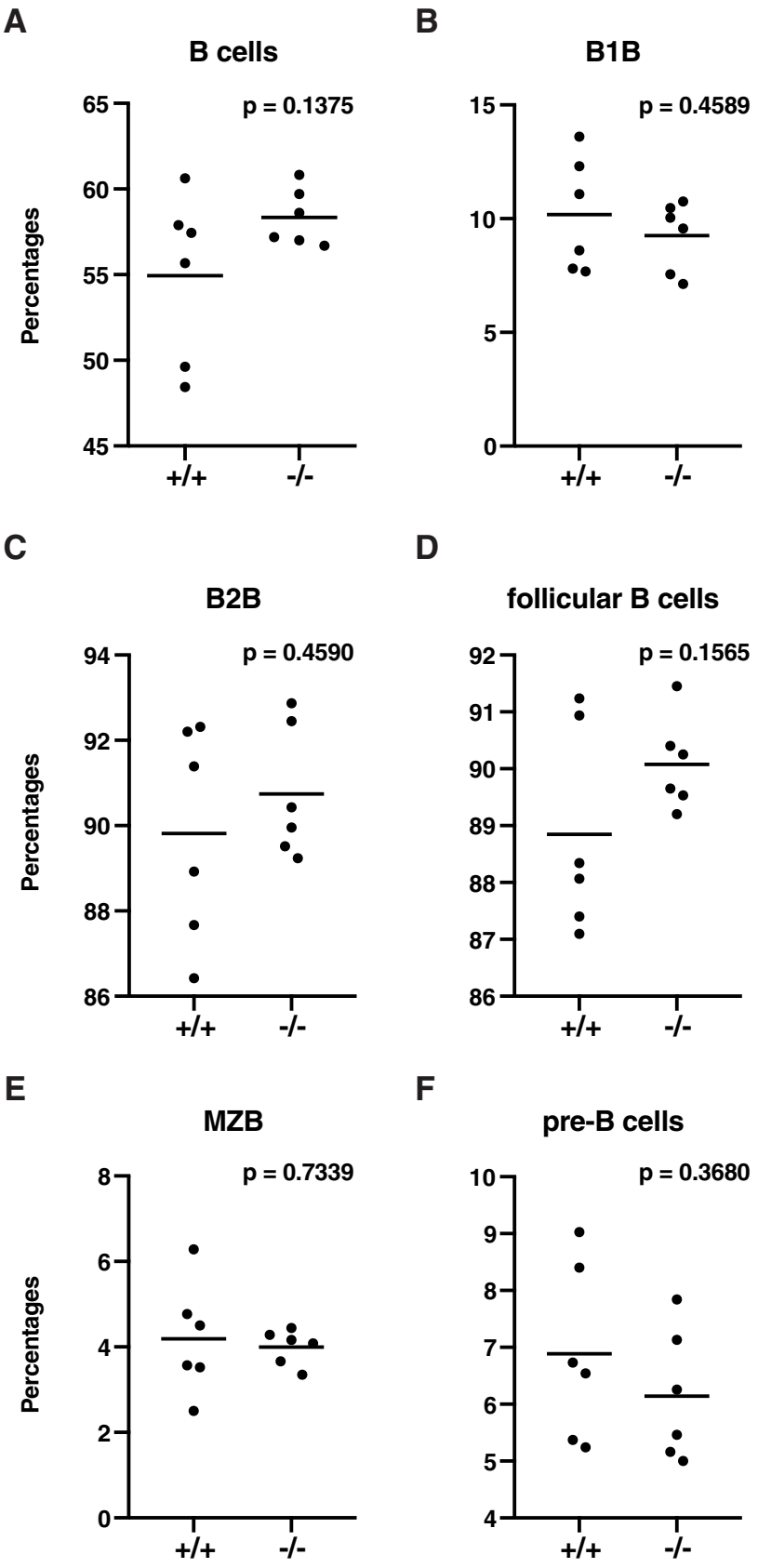
A
B cells
p = 0.1375
Percentages
65
60
55
50
45
+/+
-/-
B
B1B
p = 0.4589
15
10
5
0
+/+
-/-
C
B2B
p = 0.4590
Percentages
94
92
90
88
86
+/+
-/-
D
follicular B cells
p = 0.1565
92
91
90
89
88
87
86
+/+
-/-
E
MZB
p = 0.7339
Percentages
8
6
4
2
0
+/+
-/-
F
pre-B cells
p = 0.3680
10
9
8
7
6
5
4
+/+
-/-

Fig 6. Cellularity of B cells in the spleen. (**A**) B cells, (**B**) B1B cells, (**C**) B2B cells, (**D**) follicular B cells, (**E**) marginal zone B (MZB) cells, and (**F**) pre-B cells. Gating steps are as follows: B cells: MHC class II^+ $CD19^+$; B1B cells: MHC class II^+ $CD19^+$ $CD5^+$; B2B cells: MHC class II^+ $CD19^+$ $CD5^-$; follicular B cells: MHC class II^+ $CD19^+$ $CD5^-$ $CD21/35^+$; MZB cells: MHC class II^+ $CD19^+$ $CD5^-$ $CD21/35^{high}$; and pre-B cells: MHC class II^+ $CD19^+$ $CD5^-$ $CD21/35^{low}$. Student's t-tests were used to calculate p-values.

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Normal expression of Pax5 in $Thy28^{-/-}$ B cells

Finally, we examined the effect of the loss of Thy28 on the expression of Pax5. Expression of Pax5 in $CD19^+$ splenic B cells was comparable between $Thy28^{+/}$, $Thy28^{+/-}$, and $Thy28^{-/-}$ mice (Fig 7). Expression of Pax5 in mature B cells in inguinal LNs was also comparable between $Thy28^{+/}$, $Thy28^{+/-}$, and $Thy28^{-/-}$ mice (S1 Fig). These data show that Thy28 is dispensable for Pax5 expression in mature B cells in the mouse. We previously showed that Thy28 binds to the promoter region of the *Pax5* gene in a B cell-specific manner in a chicken mature B cell line, DT40, and down-regulation of Thy28 resulted in a decrease in the expression of the *Pax5* gene [6]. These results in a chicken B cell line were in clear contrast with the present results in mice. We also knocked down Thy28 in the human B cell lines Nalm-6 and Raji. As shown in S2 Fig, down-regulation of Thy28 in these cell lines did not affect the expression of Pax5. These results demonstrate that Thy28 is dispensable for Pax5 expression in B cells from at least two mammals, mice and humans, and suggest a species-specific mechanism for the regulation of Pax5 expression.

Conclusions

We generated *Thy28*-deficient mice to investigate the potential role of Thy28/ThyN1 in B cell development. *Thy28*-deficient mice were viable and showed a Mendelian birth ratio. *Thy28*-deficient mice had normal B cell numbers as well as normal percentages of subclasses of B cell lineages. Finally, the expression of Pax5 was normal in B cells from *Thy28*-deficient mice. These results indicate that Thy28/ThyN1 is dispensable for the regulation of Pax5 expression and the development of B cells in the mouse and suggest a species-specific role of Thy28/ThyN1 in Pax5 expression and B cell development.

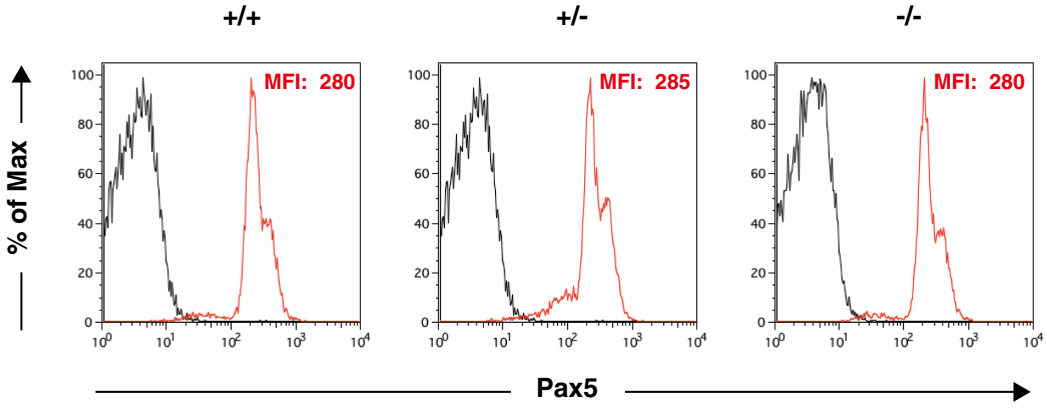

Fig 7. Expression of Pax5 in splenic B cells. Splenocytes from 7-week-old mice were stained with a FITC-conjugated anti-CD19 Ab and a PE-conjugated anti-Pax5 Ab. The expression level of Pax5 in $CD19^+$ B cells is shown. The mean fluorescence intensity (MFI) of Pax5 staining is shown. Black: unstained control; red: Pax5 staining. Percentages of $Pax5^+$ cells in $CD19^+$ splenic B cells from $Thy28^{+/}$, $Thy28^{+/-}$, and $Thy28^{-/-}$ mice were 95.6%, 94.7%, and 95.6%, respectively.

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Supporting information

S1 Fig. Expression of Pax5 in mature B cells in inguinal lymph nodes. Splenocytes from 9-week-old mice were stained with a FITC-conjugated anti-CD19 Ab, an APC-conjugated IgD Ab, and a PE-conjugated anti-Pax5 Ab. The expression of Pax5 in $CD19^{high}$ and IgD^{+} B cells is shown. The mean fluorescence intensity (MFI) of Pax5 staining is shown. Percentages of $Pax5^{+}$ cells in $CD19^{high}$ and IgD^{+} B cells from $Thy28^{+/}$, $Thy28^{+/-}$, and $Thy28^{-/-}$ mice were 99.6%, 99.7%, and 99.9%, respectively.
(PDF)

S2 Fig. Expression of Pax5 in human B cell lines. (**A**, **B**) shRNA-mediated knock-down of Thy28 in a human pre-B cell line, Nalm-6. Expression of Pax5 protein (A) and *Pax5* mRNA (B) was analyzed in Nalm-6 cells stably expressing an shRNA against GFP or human Thy28. The expression of *Pax5* mRNA was quantified by real-time RT-PCR and normalized to the expression of *GAPDH* mRNA (mean +/- SEM, n = 4). (**C**, **D**) Clustered regularly interspersed short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated knock-out of Thy28 in a human Burkitt's lymphoma cell line, Raji. (**C**) Nucleotide insertions or deletions generated by CRISPR/Cas9 in the human *Thy28* gene. The ATG codons in blue and the TGA codon in red indicate start codons and an inserted stop codon, respectively. The CRISPR/Cas9 target sequence is underlined. (**D**) Expression of Pax5 was analyzed in Thy28 mutant (KO) Raji cells.
(PDF)

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Author Contributions

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Supervision: Hodaka Fujii.

Writing – original draft: Hodaka Fujii.

Writing – review & editing: Toshitsugu Fujita, Hodaka Fujii.

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