The endoplasmic reticulum-resident E3 ubiquitin ligase Hrd1 controls B cell immunity through degradation of the death receptor CD95/Fas

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Supplemental files include:

1. **Supplemental Materials & Methods**
2. **Supplemental Figures 1-6 & legends**
3. **Supplemental Table 1**

Supplemental Materials and Methods

Animals. Hrd1 floxed mice at pure C57/B6 genetic background were used as described previously(1). CD19-Cre transgenic mice in the C57BL/6 genetic background were purchased from The Jackson Laboratory (Bar Harbor, ME). B cell-specific Hrd1-null mice were generated by breeding Hrd1 floxed mice with CD19-Cre transgenic mice. The IRE1α floxed mice were generated as previously described and had been backcrossed with C57BL/6J mice for more than 10 generations to maintain the C57BL strain background. Fas mutant mice (*Fas*^{lpr}) at the MRL/Lpr genetic background (000485) were purchased from The Jackson Laboratory. CHOP floxed mice were used as reported (2). Double knockout mice were generated by mating CD19-Cre Hrd1 floxed mice with IRE1α or Chop floxed mice or with Fas mutant mice. Hrd1/Fas double knockout at the mixed genetic background were used. All mice used in this study were maintained and used at the Northwestern University Mouse Facility under pathogen-free conditions according to institutional guidelines and animal study proposals approved by the Institutional Animal Care and Use Committee.

Cell lines. Mature B cell line A20 (TIB-208) were purchased from ATCC (www.atcc.org) and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 0.05 mM 2-Mercaptoethanol (2ME). Human embryonic kidney (HEK) 293 cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS, 1% penicillin/streptomycin.

Plasmids and antibodies. Hrd1 and the ubiquitin expression plasmids were constructed as before (Gao et al., 2008). Flag-Fas expression plasmids were purchased from Sino Biological, Inc. (North Wales, PA). The truncation mutants of Hrd1 plasmids were generated by PCR and subcloned into

pCMV-Flag (Sigma-Aldrich, St. Louis, MO) or pCMV-Myc vectors (Invitrogen). Antibodies used for immunoblotting, co-immunoprecipitation and immunofluorescence staining were from Santa Cruz Biotechnology (Santa Cruz, CA): anti-epitope tags (Flag, HA, and Myc); Abcam (Cambridge, MA): anti-Hrd1 (EP7459) and anti-Fas (ab82419); Calbiochem (Pasadena, CA): anti-Tubulin (DM1A); Sigma-Aldrich: anti-Hrd1 (HRD1-5); Cell Signaling (Cambridge, MA): anti-caspase3, anti-cleaved caspase 3 and anti-caspase 8; GeneTex (Irvine, CA): anti-p84 (5E10). Fluorescence-labeled antibodies used for flow cytometry were purchased from eBioscience (San Diego, CA): anti-CD16/32, anti-CD43 (eBioR2/60), anti-CD79a (HM47), anti-IgM (II/41), anti-Mouse CD268 (BAFF Receptor; eBio7H22-E16) and anti-CD267 (TACI; ebio8F10-3); Biolegend (San Diego, CA): anti-CD45R (B220; RA3-6B2), anti-IgM (RMM-1), anti-CD2 (RM2-5), anti-CD21/CD35 (7E9), anti-CD23 (B3B4), anti-CD5 (53-7.3), anti-CD3 (17A2), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD11c (N418), anti-NK1.1 (PK136); BD Biosciences (San Jose, CA): anti-CD138 (281-2), anti-CD127 (SB199) and anti-CD95 (Jo2); and Novus (Littleton, CO): anti-BCMA/TNFRSF17 (Vicky-2).

Primary B cell isolation and culture. Primary B cells were negatively or positively isolated from 8- to 12-week-old mice using Dynabeads Mouse CD43 Dynabeads® or Mouse Pan B (B220; Life Technologies, Grand Island, NY) per the manufacturer's instructions. Negatively purified B cells were cultured in RPMI 1640 supplemented with 10% FBS, 50 mM 2-ME, 1 mM sodium pyruvate, 10 mM HEPES buffer, and 1% penicillin/streptomycin at 10^6/ml. Purified B cells were stimulated with goat F(ab)2 anti-mouse IgM (10 mg/ml; Jackson Immunoresearch, West Grove, PA), anti-CD40 (1 mg/ml; eBioscience), LPS (500 ng/ml) and tunicamycin as indicated. For cell proliferation assays, purified B cells were stained with Cell Trace (5 mm; Life Technologies) and maintained at 10^6 cells/ml for 5 days with the indicated treatment. For the activation-induced cell

death (AICD) assay, after activation for 24 hours, agonistic anti-Fas antibody (500ng/ml, BD, Jo2) was added for additional 4 hours and then cell apoptosis was detected by AnnexinV and PI staining (Biolegend).

Flow cytometry and immunofluorescence staining. BM cells were obtained by flushing femur and tibia with PBS containing 3% FBS. Single-cell suspensions from bone marrow and spleen were blocked with antibody against Fc receptor and then stained with the appropriate fluorophore-conjugated antibodies. For intracellular staining, cells were fixed and permeabilized using the CytoFix/Perm Kit (eBioscience) per the manufacturer's instructions followed by staining with the appropriate fluorophore-conjugated antibodies. Cells were collected in an Accuri C6 Flow Cytometer or FACS Canto (BD Biosciences). The proximity ligation assay was performed using Duolink® In Situ Red Starter Kit Mouse/Rabbit purchased from Sigma (DUO92101). Cells were cyto-spun onto slides, fixed with 4% formaldehyde at room temperature (RT) for 15 minutes. After washing three times with PBS, cells were permeabilized with 0.1% Triton-X100 at RT for 5 minutes, and then incubated with primary antibodies diluted with 1-4% BSA for 1 hour at RT. After washing three times with PBS, cells were incubated with secondary antibodies for 1 hour at RT. Cells were processed and visualized with the Nikon Eclipse Ti fluorescence microscope at original magnification ×40 after staining with DAPI.

Cell transfection, immunoblotting and co-immunoprecipitation assay. HEK293 cells in 60-mm or 12-well dishes were transfected with appropriate plasmids using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. 24 or 48 hours after transfection, cells were lysed in 1× Nonidet P-40 lysis buffer, which was freshly added with protease inhibitor cocktail. After adding pre-washed protein A or G magnetic beads to the cell lysates, antibodies (1 μg) were added and the lysates were rotated in a rotating wheel at 4°C for 2 hours followed by the addition of 30

μl of protein G-Sepharose beads (GE Healthcare, Arlington Heights, IL) for an additional 2 hours at 4°C or overnight. Immunoprecipitates were washed four times with Nonidet P-40 lysis buffer, and then were boiled at 95°C in 20 μl of 2× Laemmli's buffer. Samples were analyzed on an 8–12% SDS-PAGE gel and electro-transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking with 4% milk dissolved in TBST, membranes were probed with the appropriate primary antibodies. After washing twice with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. After washing twice with TBST, membranes were treated with enhanced chemiluminescence detection system (ECL; GE Healthcare) and exposed. If necessary, membranes were stripped with stripping buffer (Bio-Rad Laboratories, Hercules, CA), washed, blocked and then reprobed with other antibodies.

Immunizations. For the NP-KLH immunization study, 8- to 12-week-old WT and Hrd1 KO mice were given an intraperitoneal injection of 100 μg NP-KLH (Biosearch Technologies, Petaluma, CA) emulsified 1:1 in CFA (Santa Cruz) on day 0 and 100 μg NP-KLH emulsified 1:1 in IFA (Santa Cruz) on day 8. Blood was harvested from the femoral vein on days 0, 7, and 12. Mice were sacrificed on day 12. For NP-LPS immunization, on day 0, 8- to 12-week-old WT and Hrd1 KO mice were immunized with 100 μg NP-LPS (Biosearch Technologies). Blood was harvested from the femoral vein on days 0 and 7. Mice were sacrificed on day 7. Serum Ig level was detected by ELISA. Splenocytes were stained with antibodies and then analyzed by flow cytometry.

ELISA. 96-well flat-bottom plates were coated overnight at 4˚C with capture antibodies diluted in 100 μl coating buffer (bicarbonate, pH 9). For detection of total Ig levels, 50 ng purified anti-mouse antibodies for the specific isotypes were used. Wells were then blocked with 1% BSA at RT for 2 hours. After washing twice with PBST, wells were incubated with mouse sera (diluted 1:200–1:10,000) for 2 hours at RT or overnight at 4˚C. Wells were washed three times, then biotin-

coated secondary antibodies against specific Ig were added and incubated for 1 hour at RT. After washing the wells 4 times, avidin-HRP was added and incubated for 30 minutes in dark. Wells were washed again and then TMB substrate (Thermo Scientific) was added and developed in the dark. Absorbance at 450 nm was detected using a FilterMax F5 microplate reader (Molecular Devices, Sunnyvale, CA). Mouse ANA total Ig were measured with an ELISA kit (Alpha Diagnostic, San Antonio, TX) per the manufacturer's instructions.

Real-time quantitative PCR. Total RNA was extracted from cells with TRIzol reagent per the manufacturer's instructions (Ambion, Grand Island, NY). First-strand cDNA was synthesized using qScript cDNA Synthesis kit (Quanta Biosciences, Gaithersburg, MD). Real-time qPCR was performed using iQ5 and the SYBRGreen Detection system (Bio-Rad Laboratories). Data were normalized to the expression of β-actin in each sample. Primers are shown in Table S1.

Statistical analysis. All experiments used the Student's t-test for statistical analysis. P values less than 0.05 were considered statistically significant.

References:

1. Yang H, *et al.* (2014) Hrd1-mediated BLIMP-1 ubiquitination promotes dendritic cell MHCII expression for CD4 T cell priming during inflammation. *The Journal of experimental medicine* 211(12):2467-2479.
2. Zhou AX, *et al.* (2015) C/EBP-Homologous Protein (CHOP) in Vascular Smooth Muscle Cells Regulates Their Proliferation in Aortic Explants and Atherosclerotic Lesions. *Circulation research* 116(11):1736-1743.

Supplemental Figures 1-6

Fig. s1A-G

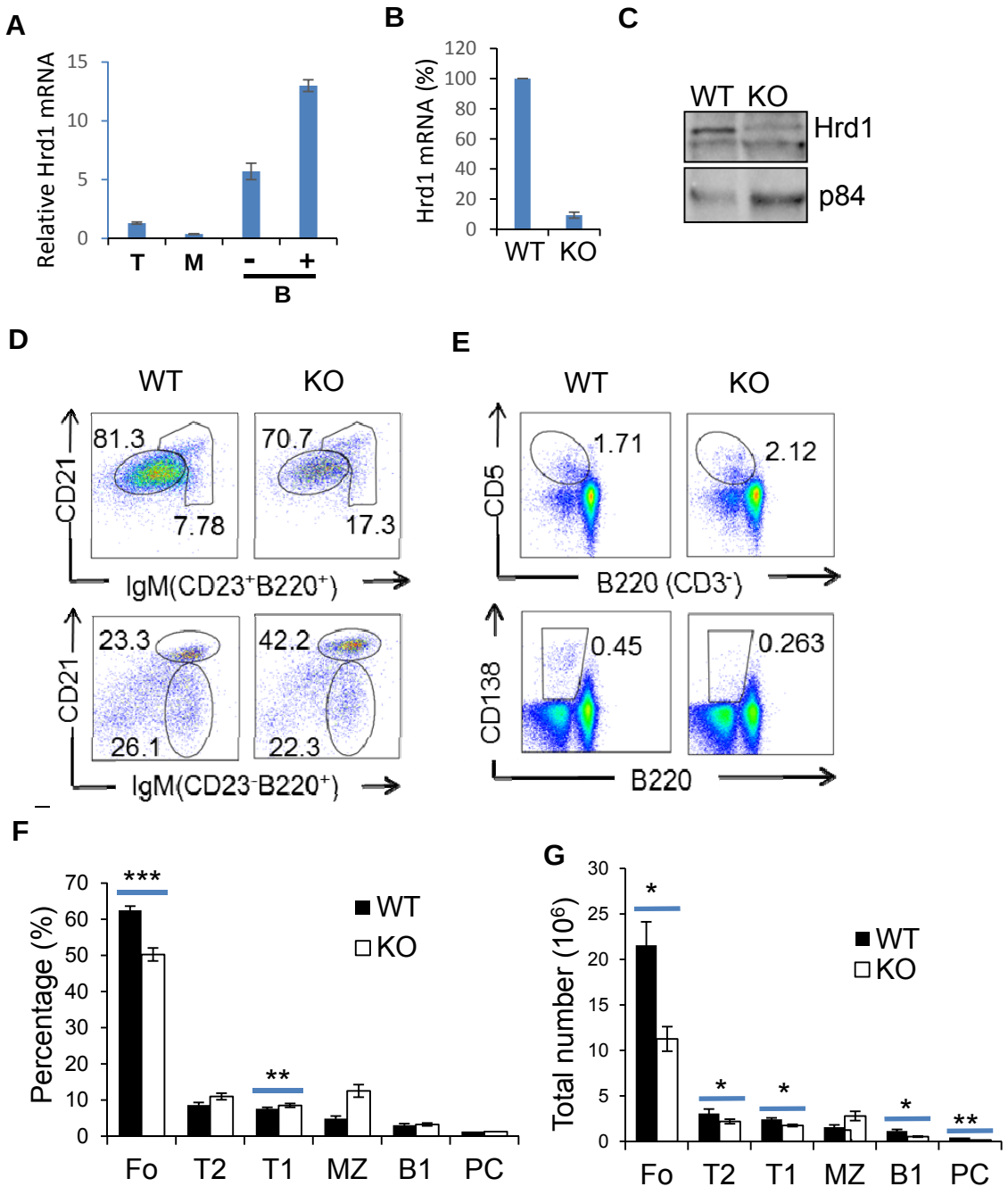

A
Relative Hrd1 mRNA
15
10
5
0
T
M
-
+
B
B
Hrd1 mRNA (%)
120
100
80
60
40
20
0
WT
KO
C
WT KO
Hrd1
p84
D
WT
KO
CD21
81.3
7.78
70.7
17.3
IgM(CD23+B220+)
CD21
23.3
26.1
42.2
22.3
IgM(CD23-B220+)
E
WT
KO
CD5
1.71
2.12
B220 (CD3-)
CD138
0.45
0.263
B220
F
Percentage (%)
70
60
50
40
30
20
10
0
***
**
WT
KO
Fo
T2
T1
MZ
B1
PC
G
Total number (10^6)
30
25
20
15
10
5
0
*
*
*
*
**
WT
KO
Fo
T2
T1
MZ
B1
PC

Fig. s1H-K

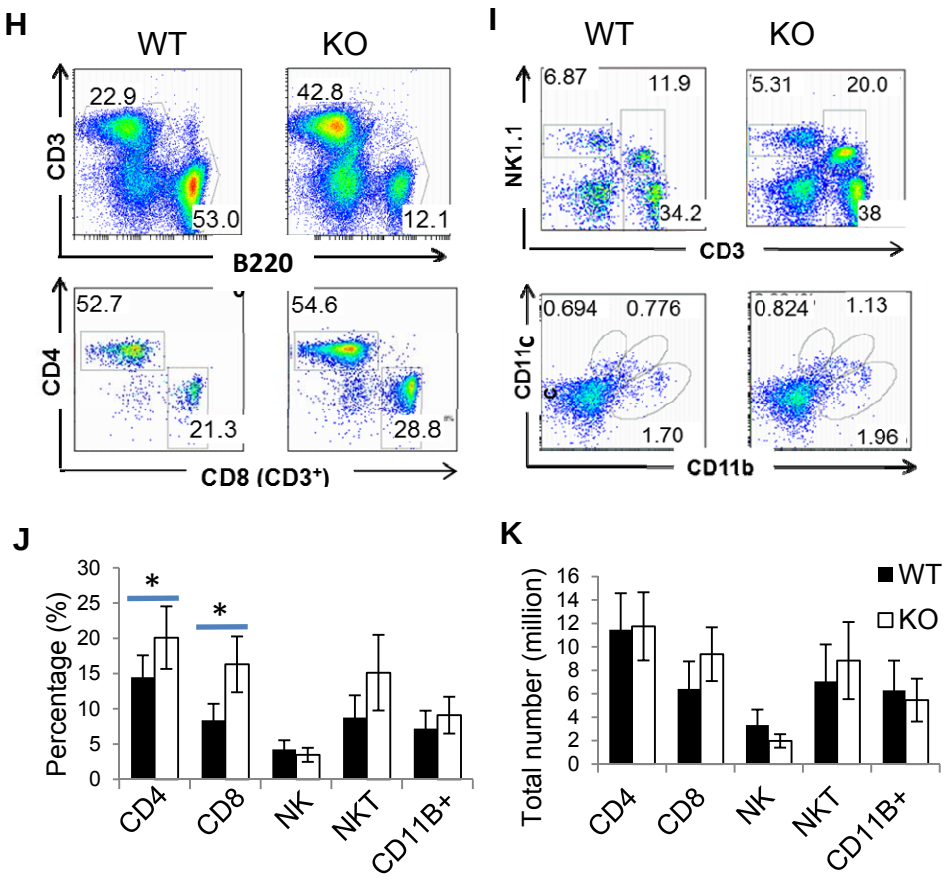

H
WT
KO
22.9
53.0
42.8
12.1
CD3
B220
52.7
21.3
54.6
28.8
CD4
CD8 (CD3+)
I
WT
KO
6.87
11.9
34.2
5.31
20.0
38
NK1.1
CD3
0.694
0.776
1.70
0.824
1.13
1.96
CD11c
CD11b
J
Percentage (%)
CD4
CD8
NK
NKT
CD11B+
K
Total number (million)
WT
KO
CD4
CD8
NK
NKT
CD11B+

Fig. s2

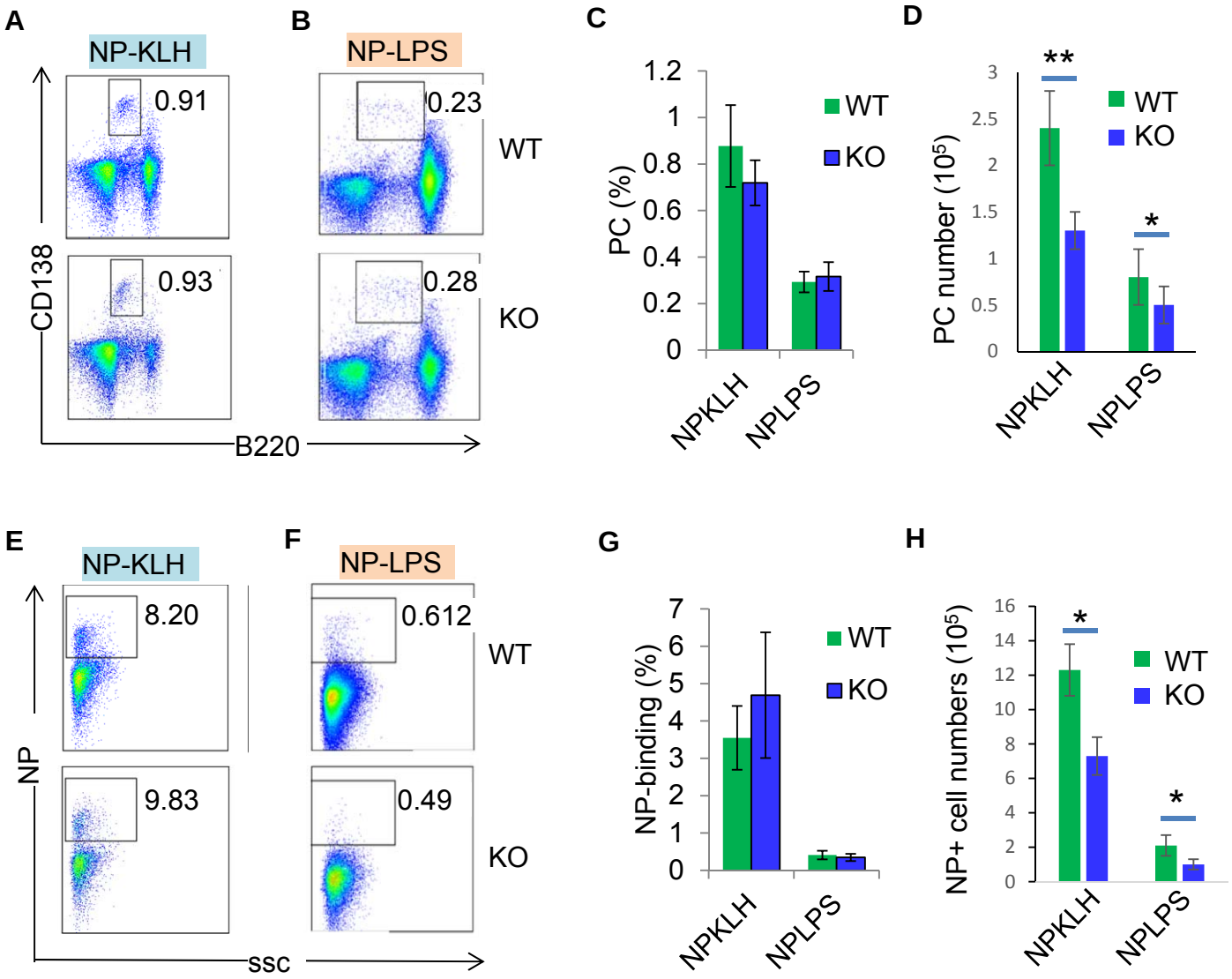

A
NP-KLH
0.91
0.93
CD138
B220
B
NP-LPS
0.23
0.28
WT
KO
C
PC (%)
1.2
1
0.8
0.6
0.4
0.2
0
WT
KO
NPKLH
NPLPS
D
PC number (10⁵)
3
2.5
2
1.5
1
0.5
0
**
*
WT
KO
NPKLH
NPLPS
E
NP-KLH
8.20
9.83
NP
ssc
F
NP-LPS
0.612
0.49
WT
KO
G
NP-binding (%)
7
6
5
4
3
2
1
0
WT
KO
NPKLH
NPLPS
H
NP+ cell numbers (10⁵)
16
14
12
10
8
6
4
2
0
*
*
WT
KO
NPKLH
NPLPS

Fig. s3

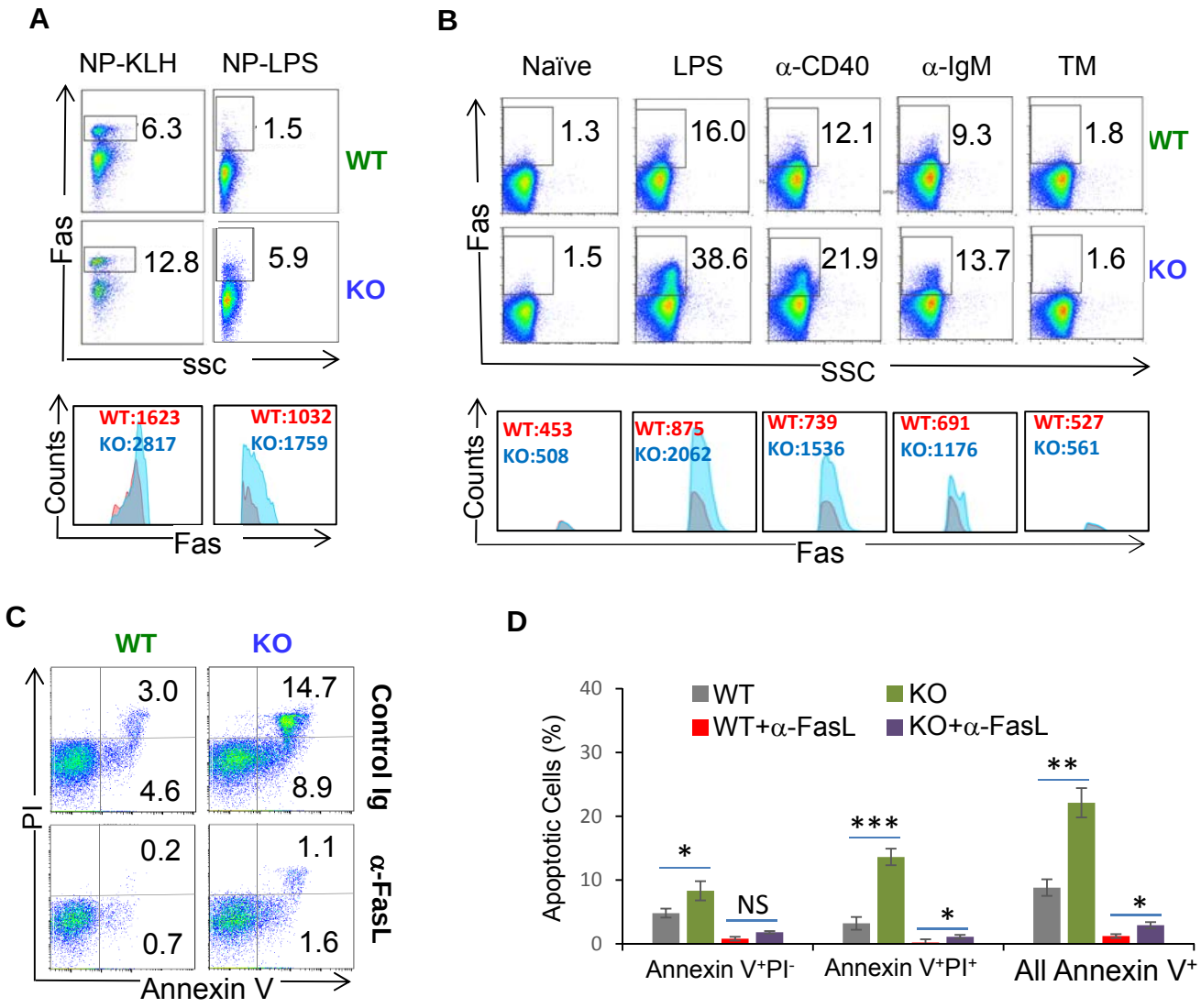

A
NP-KLH
NP-LPS
6.3
1.5
WT
12.8
5.9
KO
Fas
ssc
WT:1623
KO:2817
WT:1032
KO:1759
Counts
Fas
B
Naïve
LPS
α-CD40
α-IgM
TM
1.3
16.0
12.1
9.3
1.8
WT
1.5
38.6
21.9
13.7
1.6
KO
Fas
SSC
WT:453
KO:508
WT:875
KO:2062
WT:739
KO:1536
WT:691
KO:1176
WT:527
KO:561
Counts
Fas
C
WT
KO
3.0
14.7
Control Ig
4.6
8.9
0.2
1.1
α-FasL
0.7
1.6
PI
Annexin V
D
WT
KO
WT+α-FasL
KO+α-FasL
Apoptotic Cells (%)
40
30
20
10
0
*
NS
***
*
**
*
Annexin V+PI-
Annexin V+PI+
All Annexin V+

Fig. s4

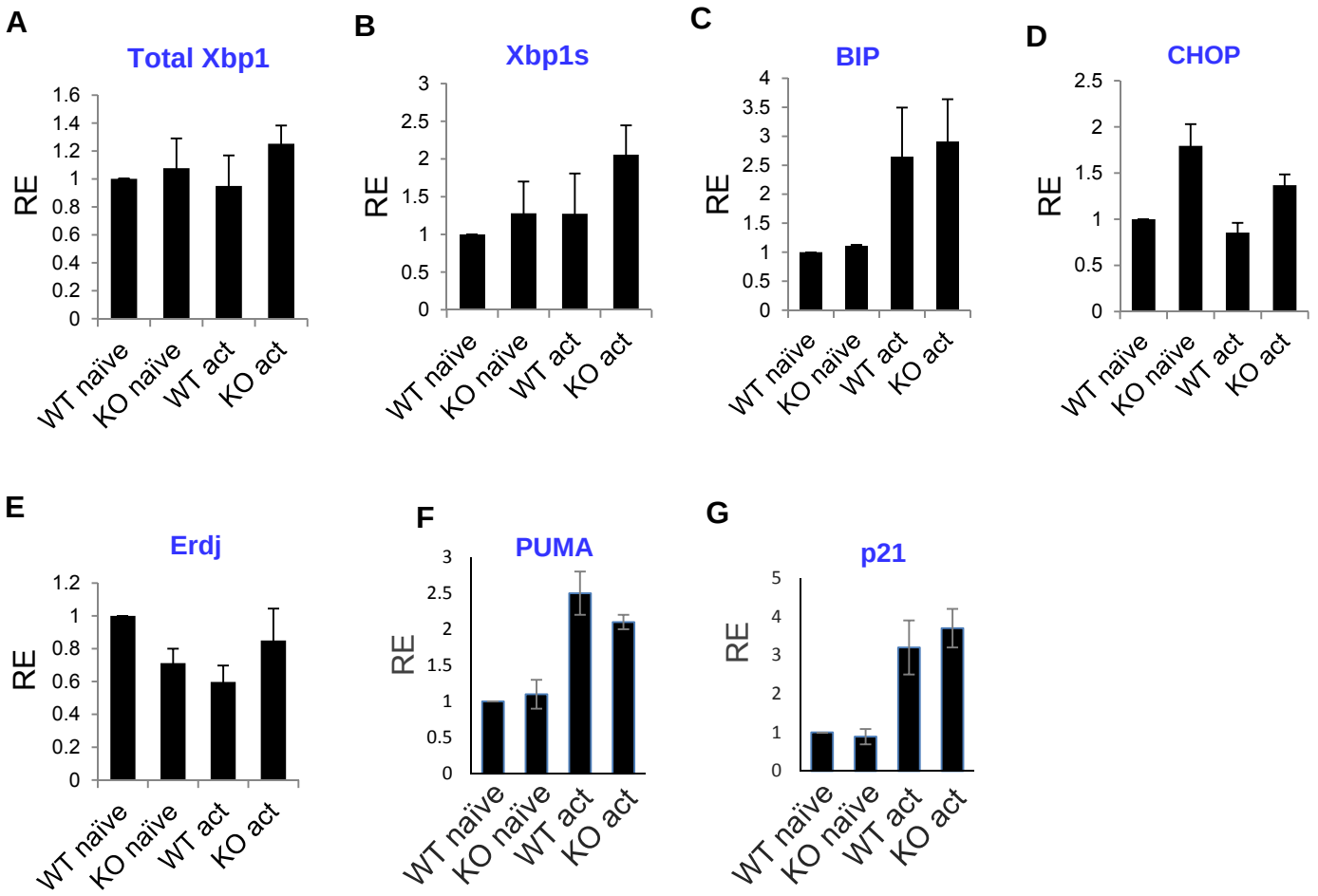

A
Total Xbp1
RE
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT naïve
KO naïve
WT act
KO act
B
Xbp1s
RE
3
2.5
2
1.5
1
0.5
0
WT naïve
KO naïve
WT act
KO act
C
BIP
RE
4
3.5
3
2.5
2
1.5
1
0.5
0
WT naïve
KO naïve
WT act
KO act
D
CHOP
RE
2.5
2
1.5
1
0.5
0
WT naïve
KO naïve
WT act
KO act
E
Erdj
RE
1.2
1
0.8
0.6
0.4
0.2
0
WT naïve
KO naïve
WT act
KO act
F
PUMA
RE
3
2.5
2
1.5
1
0.5
0
WT naïve
KO naïve
WT act
KO act
G
p21
RE
5
4
3
2
1
0
WT naïve
KO naïve
WT act
KO act

Fig. s5

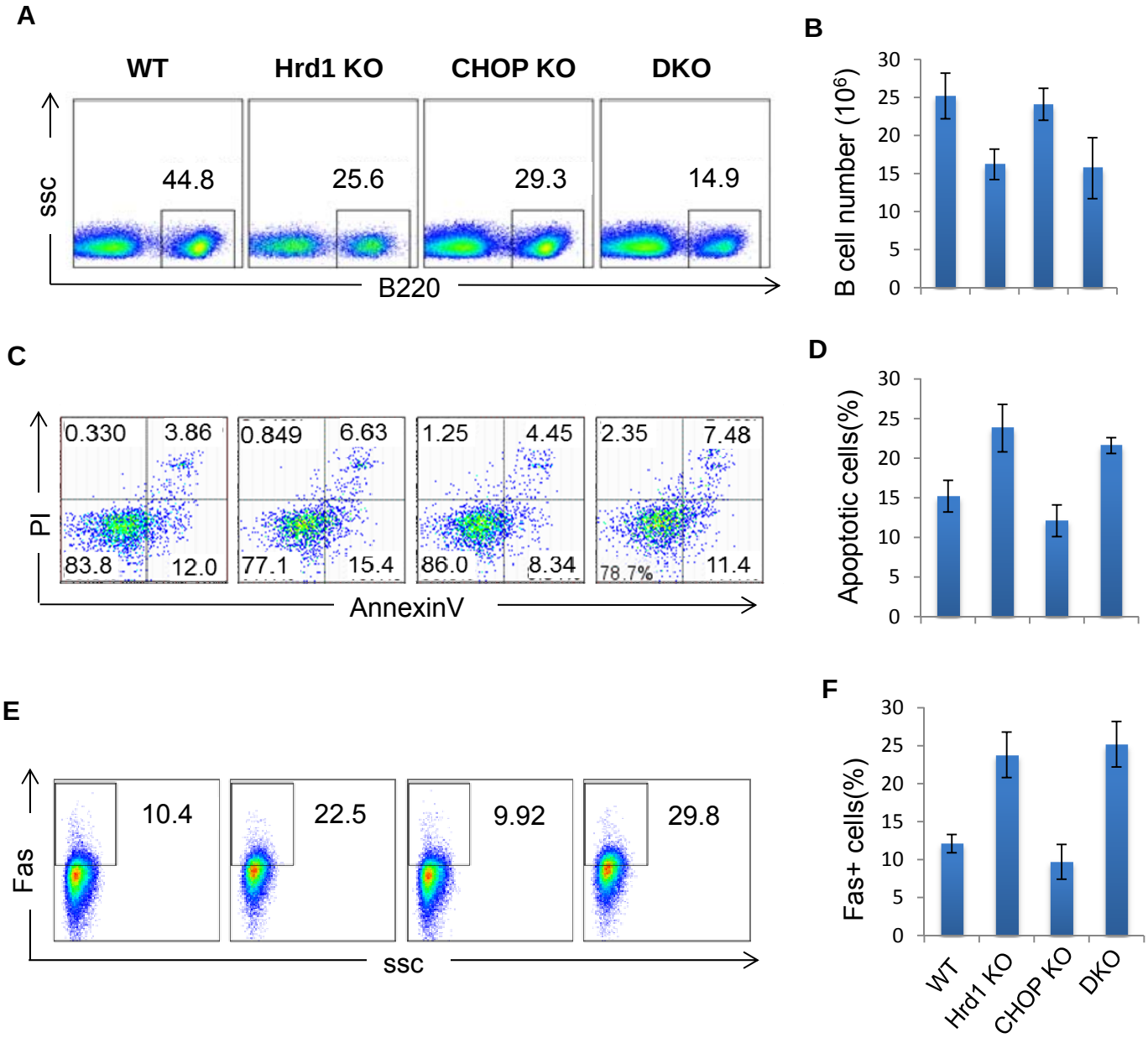
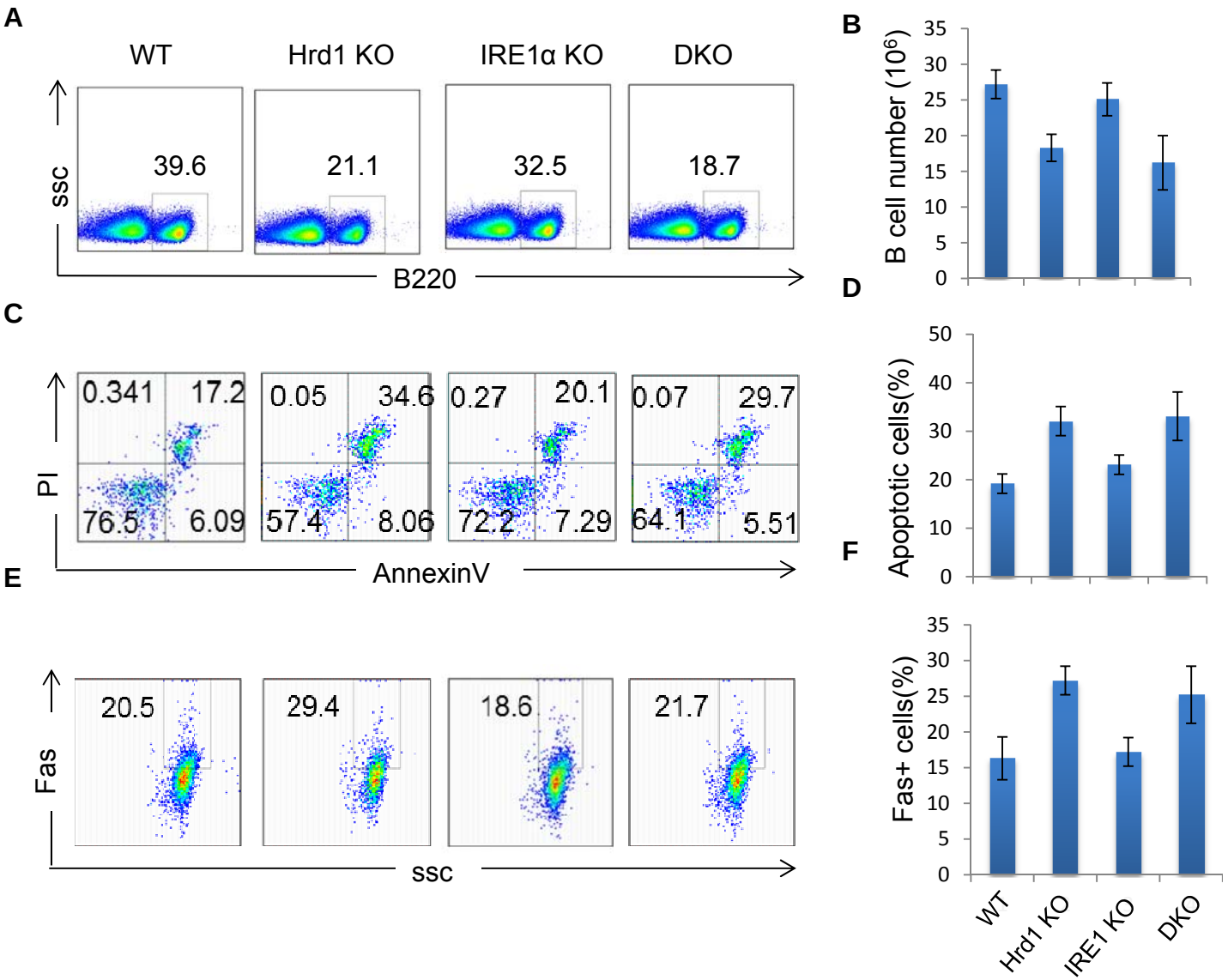

A
WT
Hrd1 KO
CHOP KO
DKO
ssc
44.8
25.6
29.3
14.9
B220
B
B cell number (10^6)
30
25
20
15
10
5
0
C
PI
0.330
3.86
83.8
12.0
0.849
6.63
77.1
15.4
1.25
4.45
86.0
8.34
2.35
7.48
78.7%
11.4
AnnexinV
D
Apoptotic cells(%)
30
25
20
15
10
5
0
E
Fas
10.4
22.5
9.92
29.8
ssc
F
Fas+ cells(%)
30
25
20
15
10
5
0
WT
Hrd1 KO
CHOP KO
DKO

Fig. s6


A
WT
Hrd1 KO
IRE1α KO
DKO
ssc
39.6
21.1
32.5
18.7
B220
B
B cell number (10^6)
35
30
25
20
15
10
5
0
C
PI
0.341
17.2
76.5
6.09
0.05
34.6
57.4
8.06
0.27
20.1
72.2
7.29
0.07
29.7
64.1
5.51
AnnexinV
D
Apoptotic cells(%)
50
40
30
20
10
0
E
Fas
20.5
29.4
18.6
21.7
ssc
F
Fas+ cells(%)
35
30
25
20
15
10
5
0
WT
Hrd1 KO
IRE1 KO
DKO

Supplemental Figure legends

Fig. s1: Hrd1 deletion in B cells does not affect non-B cell populations. (A) Real-time PCR analysis of Hrd1 expression in immune cells. T: T cells; M: macrophages; B: B cells stimulated with (+) or without LPS (-) for 4 hours. **(B & C)** $B220^+$ cells were isolated from bone marrow of WT and Hrd1 KO (KO) mice. Hrd1 mRNA levels were determined by real-time RT-PCR (B) and its protein expression was determined by immunoblotting. p84 protein served as loading control (C). **(D)** $B220^+$ cells from spleens of WT and KO mice were analyzed for $CD23^+$ $CD21^{INT}$ IgM^{INT} mature B cells and $CD23^+$ $CD21^{HI}$ IgM^{HI} T2 B cells (top panel) and $CD23^-$ $CD21^{LO}$ IgM^+ T1 and $CD23^-CD21^{HI}$ IgM^+ marginal zone (MZ) B cells (bottom panel). **(E)** Splenocytes from WT and KO mice were analyzed for $CD3e^-$ $B220^{INT}$ $CD5^+$ B1 cells (top panel) and $B220^{INT}$ $CD138^+$ plasma cells. **(F)** Percentages of indicated B cell populations as analyzed in (D) and (E). **(G)** Total number of indicated B cell populations as analyzed in (D) and (E). **(H)** Splenocytes from WT and KO mice were analyzed for $CD3e^+$ T cells and $B220^+$ B cells (top panel) and $CD4^+$ and $CD8^+$ T cells (bottom panel). **(I)** Splenocytes were analyzed for $NK1.1^{HI}$ $CD3e^-$ NK cells and $NK1.1^{INT}$ $CD3e^+$ NKT cells (top panel) and $CD11c^+$ $CD11b^-$, $CD11c^+$ $CD11b^+$ and $CD11c^-$ $CD11b^+$ myocytes (bottom panel). **(J & k)** Percentages (J) and total numbers (K) of leukocytes populations as analyzed in (H) and (I). Error bars represent SEM. N=6. *$p<0.05$, ** $p<0.01$, ***$p<0.001$.

Fig. s2: NP-binding B cells and plasma cells are comparable in immunized WT and KO mice. (A-D) $B220^{INT}$ $CD138^+$ plasma cells of WT and Hrd1 KO (KO) mice immunized with NP-KLH (A) or NP-LPS (B) were detected by flow cytometry 14 and 7 days p.i., respectively. The average percentages from five pairs of mice are shown (C & D). **(E-H)** Percentages of plasma cells (PC) as measured in (A) and (B). **(D, E)** NP-specific splenic B cells in WT and KO mice immunized with NP-KLH (D) or NP-LPS (E) were analyzed by flow cytometry 14 and 7 days p.i.,

respectively. The average percentages from five pairs of mice are shown (C & D). Error bars represent SEM. N=5, **p<0.05, ** p<0.01*.

Fig. s3. Hrd1 inhibits Fas-induced B cell death. (A & B) The histogram analysis of gated B220+Fas+ cells in Fig 3A (A) and 3D (B), respectively. (C & D) Solenocytes from WT and Hrd1 KO mice were stimulated with LPS in the presence of 10 μg/ml anti-FasL neutralization antibodies. The apoptosis of B220+IgM+ B cells were accessed by PI and annexin V staining. Representative images are shown (C) and data from 4 independent experiments are indicated (D). NS: not significantly different; *p<0.05, ** p<0.01, ***p<0.001.

Fig. s4: Hrd1 deletion does not affect expression of UPR and p53 target genes. WT and Hrd1 KO splenic B cells were either freshly isolated (naïve) or stimulated with LPS for 4 hours. mRNA levels of **(A)** *Xbp1*, **(B)** *Xbp1s*, **(C)** *Bip*, **(D)** *Chop*, **(E)** *Erdj3*, **(F)** *Puma* and **(G)** *Noxa* were quantified by qPCR. Error bars represent SEM. N=5.

Fig. s5: B cell development defect in Hrd1 KO mice is independent of ER-stress associated pro-apoptotic protein CHOP. (A & B) Splenocytes from WT, Hrd1 KO, CHOP KO and double KO (DKO) littermates were analyzed for $B220^+$ mature B cells (A). The average B cells numbers from 5 mice each strain are shown (B). **(C-F)** Purified B cells from WT, Hrd1 KO, CHOP KO and double KO (DKO) were stimylated with LPS for 24 hours. Cell apoptosis (C & D) and Fas expression (E & F) were analyzed. Error bars represent SEM. N=5.

Fig. s6: B cell development defect in Hrd1 KO mice is independent of ER-stress sensor IRE1α. (A & B) Splenocytes from WT, Hrd1 KO, IRE1α KO and double KO (DKO) littermates were analyzed for $B220^+$ mature B cells (A). The average B cells numbers from 5 mice each strain are shown (B). **(C-F)** Purified B cells from WT, Hrd1 KO, IRE1α KO and double KO (DKO) were

stimylated with LPS for 24 hours. Cell apoptosis (C & D) and Fas expression (E & F) were analyzed. Error bars represent SEM. N=5. *$p<0.05$, ** $p<0.01$.

Supplemental Table 1. Primers used for qPCR analysis in this study.

Primer set	Sense primer 5'→3'	Antisense primer 5'→3'
Hrd1 (qPCR)	CTAGGACCTTGTCCTTTGGGG	CAGAGACCTGTGAACGCTAGG
Hrd1 (genotyping)	CTTCTGTGCAGCTGGTATTTGG	ACACGTCTCCTGGGTGATCTAC
Fas	TGCTGGCTCACAGTTAAGAGTT	TGTTCACACGAGGCGCAG
Xbp1	AAGAACACGCTTGGGAATGG	CTGCACCTGCTGCGGAC
Xbp1s	AGTTAAGAACACGCTTGGGAAT	AAGATGTTCTGGGGAGGTGAC
CHOP	GTCCCTAGCTTGGCTGACAGA	TGGAGAGCGAGGGCTTTG
BiP	TCATCGGACGCACTTGGA	CAACCACCTTGAATGGCAAGA
Erdj3	CCCCAGTGTCAAACTGTACCAG	AGCGTTTCCAATTTTCCATAAATT
puma	TGGGGTCTGCCCAGGCATGT	CGCTCCCTGGGGCCACAAAT
P21	AGTGTGCCGTTGTCTCTTCG	ACACCAGAGTGCAAGACAGC