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Supplementary Information for

Revisiting the role of IRF3 in inflammation and immunity by conditional and specifically targeted gene ablation in mice

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Supplementary Information Text

SI Materials and Methods

Construction of the targeting vector and generation of *Irf3* floxed mice. The preparation of the targeting vector, isolation of homologous recombinant embryonic stem cells, and derivation of *Irf3*-floxed mice (accession number CDB1028K: http://www2.clst.riken.jp/arg/mutant%20mice%20list.html) were performed as described previously (http://www2.clst.riken.jp/arg/Methods.html). The neomycin resistance gene was deleted from the mouse genome by crossing *Irf3*-floxed mice with *FLPe* transgenic mice. To obtain *Irf3*$^{-/-}$ mice, *Irf3*-floxed mice were crossed with *CAG-Cre* transgenic mice. Then, mice in the next generation were crossed with WT mice to delete the *CAG-Cre* transgene.

Southern blot analysis. Southern blot analysis was performed according to standard protocol. Briefly, genomic DNA from tail was isolated with the DNeasy Tissue kit (Qiagen) and digested with *Sca*I or *Nde*I (TaKaRa). The digested DNA (10 μg) was separated on a 0.8% agarose gel, hydrolyzed in 0.25 M HCl (Wako Chemical) for 20 min, neutralized in 0.4 M NaOH (Nacalai Tesque) for 20 min and transferred onto a nylon membrane Hybond N+ (GE Healthcare Life Sciences) using top to bottom blotting overnight (0.4 M NaOH). The DNA probe was generated by polymerase chain reaction (PCR) with primer set. The following primers were used: 5′-gtagctggtagctgcaggtctttgc -3′ (sense) and 5′- cagatgggaagactgagccttgtag -3′ (anti-sense). The amplified DNA fragments were labeled with [^{32}P]-dCTP (10 mCi/mL; PerkinElmer, USA) using BcaBEST labeling kit (TaKaRa, Japan) and purified with Probe Quant G-50 Micro Columns (GE Healthcare Life Sciences). The DNA membrane was pre-hybridized in ULTRAhyb ultrasensitive hybridization buffer (Thermo Fisher Scientific) at 42 ºC for one hour. The membrane was then hybridized with radioactive probe in ULTRAhyb buffer at 42ºC for 24 hours. After hybridization,

membranes were washed twice in 2 × SSC/0.1% SDS at 42ºC for 5 min, washed three times in 0.1 × SSC/0.1% SDS at 42ºC for 15 min, and then exposed to an X-ray film RX-U (Fuji Film).

Immunoblot analysis. Cell extracts were prepared with lysis buffer (50 mM Tris-HCl (pH 7.4), 500 mM NaCl, 20 mM β-glycerophosphate, 2 mM EDTA, 1.5% NP-40, 1 mM Na_3VO_4, 200 μM dithiothreitol, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 1 mM p-amidinophenylmethanesulfonyl fluoride). Samples were separated by 12.5% acrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane. Anti-IRF3 antibody (Thermo Fisher Scientific) and anti-β-actin antibody (AC-15; Sigma-Aldrich) were used for immunoblotting.

RNA analysis. Total RNA extraction and cDNA synthesis were performed as described previously (1-3). Quantitative real-time PCR analysis was performed using LightCycler480 and the SYBR Green system (Roche). The primer sequences for *Gapdh*, *Ifnb*, *Il6*, *Il12b*, *iNOS*, and *Arg1* mRNA have been described (1-3). The primers for *Bcl2l12* were as follows: 5′- ggttctgacggccttcctta -3′ (sense) and 5′- gcaaccagggcatagaactc -3′ (anti-sense). All experiments were performed more than three times and data are presented as relative expression units after normalization to *Gapdh* mRNA expression level.

Cell death assay. MEFs were washed with phosphate buffered saline, irradiated with UV (254 nm, 200 J/m^2) using UV cross-linker CL-100 (UVP), and then cultured for 18 hours. All cells were harvested, stained with Annexin V-APC apoptosis detection kit (BD Pharmingen), and then analyzed by flow cytometry.

Flow cytometric analysis. Flow cytometric analysis was performed using an LSRII Fortessa flow cytometer (BD Biosciences) as described previously (1, 3). The following antibodies were purchased and used: FITC, PE, Pacific Blue, APC, or PE-Cy7 anti-B220 mAb (Biolegend, RA3-6B2); PE anti-BP-1 mAb (Biolegend, 6C3); APC anti-CD43 mAb (Biolegend, S11); eFluor 450 anti-CD24 mAb (eBioscience, M1/69); FITC or APC anti-IgM mAb (Biolegend, R6-60.2); PE anti-CD25 mAb (BD Pharmingen, PC61); APC anti-c-kit mAb (Biolegend, 2B8); Pacific Blue anti-CD19 mAb (Biolegend, 6D5); FITC or PE anti-CD3 mAb (BD Biosciences, 145-2C11); PE anti-AA4.1 mAb (Biolegend, AA4.1); FITC, PE or APC anti-CD11b mAb (BD Pharmingen, M1/70); PE-Cy7 anti-Gr-1 mAb (Biolegend, RB6-8C5); PE anti-Fas mAb (eBioscience, 15A7); APC anti-GL7 mAb (eBioscience, GL-7); Alexa Fluor 647 anti-CD23 mAb (Biolegend, B3B4); eFluor 450 anti-IgD mAb (eBioscience, 11-26c); FITC anti-CD23 mAb (BD Pharmingen, B3B4); APC anti-NKp46 mAb (Biolegend, 29A1.4); FITC anti-CD11c mAb (Biolegend, N418); PE anti-CD21 mAb (Biolegend, 7E9); FITC anti-Ig light chain κ mAb (Biolegend, RMK-45); PE anti-Ig light chain λ mAb (Biolegend, RML-42); PerCP-Cy5.5 anti-F4/80 mAb (Biolegend, BM8); APC anti-CD138 mAb (Biolegend, 281-2); Alexa Fluor 488 or PE anti-CD45 mAb (Biolegend, IM7); FITC or PerCP anti-CD8 mAb (Biolegend, 53-6.7); FITC anti-IFN-γ mAb (Biolegend, XMG1.2); Pacific Blue or APC anti-TCRβ mAb (Biolegend, H57-597); PerCP-Cy5.5 anti-CD62L mAb (Biolegend, MEL-14); PE-Cy7 anti-CD4 mAb (Biolegend, GK1.5); APC anti-FoxP3 mAb (eBioscience, FJK-16s).

Viral and bacterial infections. VSV, HSV-1, EMCV and *L. monocytogenes* were prepared as described previously (3-6). Mice were intravenously infected with 10^5 pfu of EMCV and blood then collected at 6 hours after infection. For *in vitro* infection, cultured cells were infected with viruses or bacteria at a multiplicity of infection (MOI) of 1 for the indicated time periods.

Enzyme-linked immunosorbent assay (ELISA). Mouse IFN-β was measured using ELISA kit from PBL Assay Science, according to the manufacturer's protocol. Anti-OVA and anti-TNP ELISA were performed using standard procedures using OVA (Sigma Aldrich) or TNP-BSA (Biosearch Technologies) with SBA clonotyping system-C57BL/6-HRP (SouthernBiotech) (7).

LPS-induced lethal shock. Mice were injected intraperitoneally with LPS (8 mg/kg weight) and monitored every 12 hours. Whole blood samples were taken sequentially from tails, incubated at room temperature for 30 min, and centrifuged for 5 min (4 ºC at 8,000 rpm) to prepare sera.

Macrophage differentiation study. BMMs were seeded at 2.5×10^5 cells/well in 48-well plates for overnight. Cells were treated as described previously (3, 8). Briefly, for M1 macrophage differentiation, LPS (50 ng/ml) and IFN-γ (100 ng/ml; R&D systems); for M2a differentiation, IL-4 (20 ng/ml; Peprotech). Seven hours after treatment, cells were harvested and total RNA extracted.

Immunization and antibody production assay. For immunizations, mice were intraperitoneally injected with 100 μg of OVA emulsified at 1:1 (vol/vol) ratio with complete Freund's adjuvant (CFA; BD Difco), 50 μg of TNP-AECM-Ficoll (Biosearch Technologies) in PBS, or TNP-LPS (Biosearch Technologies) in PBS. Sera were collected at 3 weeks post injections.

***In vitro* T cell differentiation.** For *in vitro* T cell differentiation, naïve $CD4^+$ T cells were isolated from spleen using naive $CD4^+$ T cell isolation kit (Miltenyi Biotec). The cells (1×10^5 cells) were cultured on plates pre-coated with anti-CD3ε (2 μg/ml; 145-2C11, Biolegend) and anti-CD28 (2

μg/ml; 37.51, Biolegend) mAbs in the presence of either IL-2 (20 ng/ml; Peprotech), anti-IL-4 mAb (10 μg/ml; 11B11, Biolegend), and anti-IFN-γ mAb (10 μg/ml; Biolegend, XMG1.2) (Th0); IL-2 (20 ng/ml), anti-IL-4 mAb (10 μg/ml), and IL-12 (10 ng/ml; Peprotech) (Th1); IL-2 (20 ng/ml), anti-IFN-γ mAb (10 μg/ml), IL-4 (10 ng/ml; Peprotech) and anti-CD28 mAb (2 μg/ml) (Th2); IL-2 (20 ng/ml), anti-IL-4 mAb (10 μg/ml), anti-IFN-γ mAb (10 μg/ml), IL-6 (20 ng/ml; R&D systems, USA), TGF-β (1 ng/ml; Peprotech), IL1-β (50 ng/ml; Peprotech) and IL-12 (5 ng/ml) (Th17); and IL-2 (20 ng/ml), TGF-β (15 ng/ml), anti-IL-4 mAb (10 μg/ml), anti-IFN-γ mAb (10 μg/ml) (Treg) for 5 days. After the culture period, cells were treated with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (1 μg/ml; Sigma-Aldrich) and golgi stop (BD Biosciences) for 4 hours. Then, cells were harvested, stained with monoclonal antibodies using intracellular fixation & permeabilization buffer set (eBioscience) with PE-Cy7 anti-CD4 mAb, FITC anti-IFN-γ mAb (Biolegend, XMG1.2), PerCp-Cy5.5 anti-IL-17 mAb (Biolegend, TC11-18H10.1) or Alexa Fluor 700 anti-FoxP3 (eBioscience, FJK-16s), and then analyzed by flow cytometry.

***In vivo* tumor growth.** B16F10 cells (1×10^5 cells) were inoculated subcutaneously into mice as described previously (9). Tumor volume was calculated as $ab^2/2$ by measuring longer axes (a) and shorter axes (b) of the tumor every 3 or 4 days. Immune cells in tumor were analyzed by flow cytometry on day 17 after tumor inoculation.

Statistical analysis. Differences between control and experimental groups were evaluated with Student's *t*-test or F-test.

SI Figure legends

Fig. S1. Generation of *Irf3* gene conditional knock-out mice and UV-induced apoptosis in WT and *Irf3*$^{-/-}$ MEFs. (*A*) Genomic structure of the mouse *Irf3* gene and the targeting vector are shown. Exons 2 through 4 were flanked by two loxP sequences. Two frt sequences were introduced to flank the *neo* gene. (*B*) Tail genomic DNA was isolated from WT and *Irf3*$^{WT/f}$ mice, digested with *Sca*I restriction enzyme, electrophoresed, transferred to nylon membrane, and hybridized with the probes indicated in *A*. (*C*) Genomic DNA extracted from tails of WT or *Irf3*$^{f/f}$-*CAG*-*Cre*$^{+}$ mice was digested with *Nde*I restriction enzyme, and subjected to Southern blot analysis. (*D* and *E*) WT and littermate *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ (*D*) or *Irf3*$^{-/-}$ mouse embryonic fibroblasts (MEFs) (*E*) were left untreated or irradiated with UV (254 nm, 200 J/m^2). Eighteen hours after the irradiation, cells were stained with APC-conjugated Annexin-V and were subjected to flow cytometer analysis. Percentage of APC-Annexin-V-positive cells (gray) is shown. More than three independent experiments were performed with representative data shown.

Fig. S2. Induction of *Il6*, *Rantes* and *Il12b* mRNA in WT and *Irf3*$^{-/-}$ cells in response to PRR ligands. (*A–F*) BM-DCs (*A*, *C*, *E*), or BMMs (*B*, *D*, *F*) from WT and littermate *Irf3*$^{-/-}$ mice were stimulated with poly (I:C) (10 μg/ml), B-DNA (10 μg/ml), LPS (200 ng/ml), or CpG-B ODN (1 μM) for the indicated time periods. *Il6* (*A*, *B*), *Rantes* (*C*, *D*), and *Il12b* (*E*, *F*) mRNA expression levels were examined by qRT-PCR analysis. **P* < 0.05. Results shown are means ± s.d. of three independent experiments. (*G*) Bone marrow cells were prepared from WT and *Irf3*$^{-/-}$ mice and differentiated into macrophages (M0, M1, M2a) as described previously (3, 8). *Il12b* (left panel), *iNOS* (middle panel), and *Arg1* (right panel) mRNA expression levels were measured by qRT-PCR analysis. **P* < 0.05. Data are shown as means ± s.d. of triplicate determinants.

Fig. S3. Normal tumor-inflitrating immune cells in tumor-bearing *Irf3*$^{-/-}$ mice. WT (n = 6) and *Irf3*$^{-/-}$ (n = 7) were subcutaneously inoculated with B16F10 cells (1×10^5 cells). Tumor-infiltrating immune cells in *Irf3*$^{-/-}$ mice were analyzed by flow cytometry on day 17 after tumor inoculation. Data are shown as a percentage of $CD45^+$ cells. Data are shown as means ± s.d.

Fig. S4. B cell populations in tissues from WT and *Irf3*$^{f/f}$-*Mb1-Cre*$^{+}$ mice. (*A*) CD19^{+}CD3^{-} B cells in the spleen (upper left panel), blood (upper right panel), mesenteric lymph nodes (mLNs; lower left panel), and Peyer's patches (PPs; lower right panel) from WT and *Irf3*$^{f/f}$-*Mb1-Cre*$^{+}$ mice were analyzed by flow cytometry. Percentages of cells in the total population are shown. (*B*) Splenocytes were prepared from WT and *Irf3*$^{f/f}$-*Mb1-Cre*$^{+}$ mice and analyzed by flow cytometry. AA4.1^{+}B220^{+} cells (upper left panel), B220^{+} cells (upper right panel), and CD19^{+} cells (lower right panel) were gated, respectively. Percentages of cells in the total or gated population are shown. MZ, marginal zone B cells. Fo, follicular B cells. P, plasma B cells. GC, germinal center B cells. (*C*) Splenocytes were prepared from WT and *Irf3*$^{f/f}$-*Mb1-Cre*$^{+}$ mice and subjected to flow cytomery analysis. Percentages of cells in the total population are shown. (*D*) Peritoneal exudate cells from WT and *Irf3*$^{f/f}$-*Mb1-Cre*$^{+}$ mice were analyzed by flow cytometry (upper panel). Percentages of B1 and B2 B cells in the total population are shown. CD19^{+}GL7^{+}Fas^{+} germinal center B cells in mLNs (lower left panel) or PPs (lower right panel) from WT and *Irf3*$^{f/f}$-*Mb1-Cre*$^{+}$ mice were analyzed by flow cytometry. Percentages of cells in the gated population are shown. All results shown are representative of three independent experiments.

Figure S5. Abnormal T cell populations in the thymus and spleen from *Irf3*$^{f/f}$-*Lck-Cre*$^{+}$ mice. (*A*) Naïve CD4^{+} T cells in spleen of WT and *Irf3*$^{f/f}$-*Lck-Cre*$^{+}$ mice were purified, differentiated into T helper (Th) 1 (upper left panel), Th2 (lower left panel), Th17 (upper right panel) or *in vitro* induced Treg (iTreg; lower right panel) cells, and analyzed by flow cytometry. Percentages of differentiated T cells in the total CD4^{+} T cell population are shown. (*B*) Thymocytes from WT and *Irf3*$^{f/f}$-*Lck-Cre*$^{+}$ mice were analyzed by flow cytometry. Percentages of CD4^{+}CD8^{+} and CD4^{-}CD8^{-} cells in the total cell population are shown. (*C*) Splenocytes from WT and *Irf3*$^{f/f}$-*Lck-Cre*$^{+}$ mice were analyzed by flow cytometry. CD4^{+}TCRβ^{+} cells (left panel) and CD8^{+}TCRβ^{+} cells (right panel) were gated, respectively. Percentages of CD44^{-}CD62L^{+} naïve T cells and effector/memory T cells including CD44$^{int\text{-}hi}$CD62L^{-} and CD44^{+}CD62L^{+} cells in the gated population are shown. (*D*) Naïve T cells and effector/memory T cells in peripheral blood from WT and *Irf3*$^{f/f}$-*Lck-Cre*$^{+}$ mice were analyzed by flow cytometry as in *C*. All results shown are representative of three independent experiments.

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Figure S1

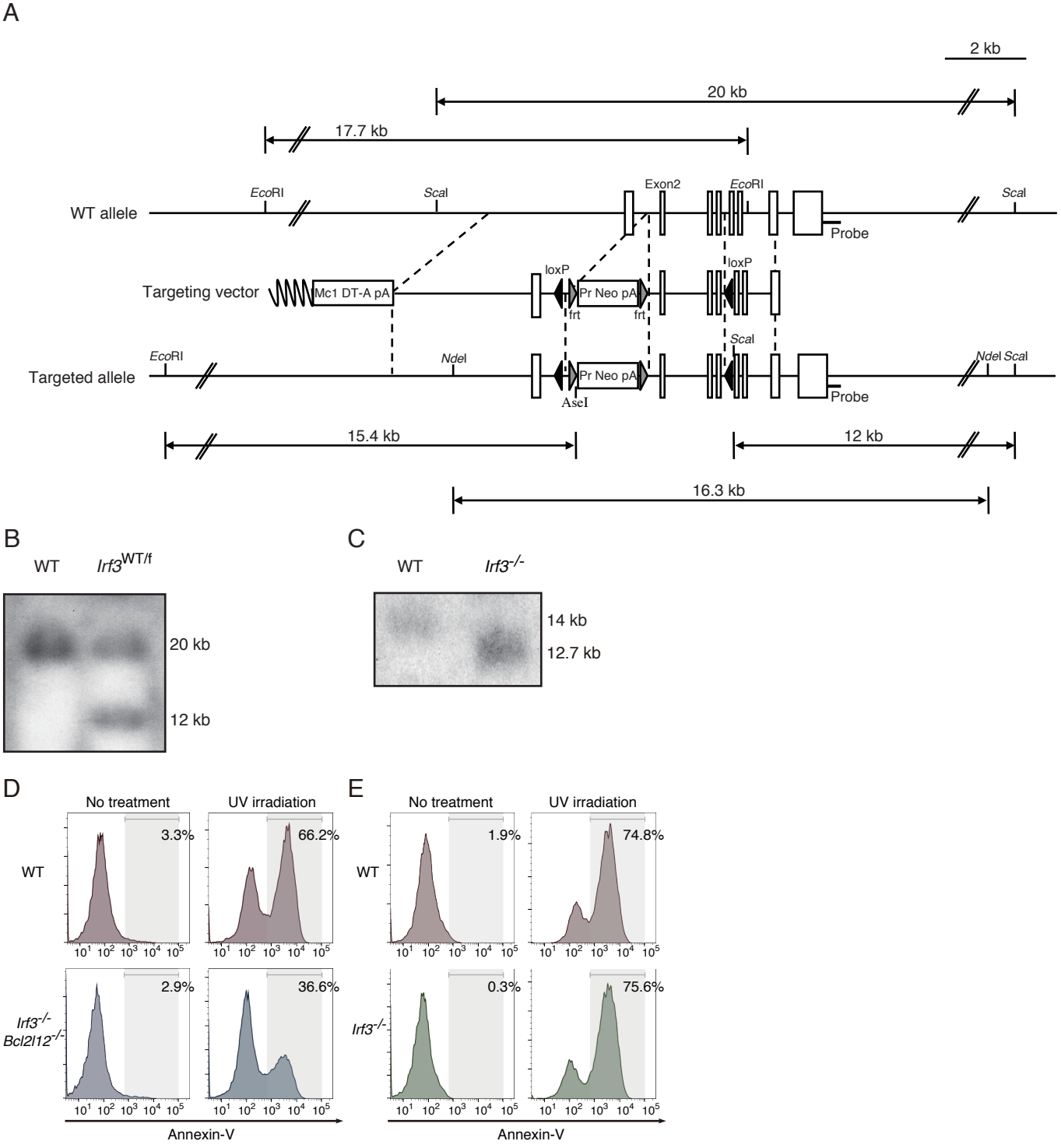
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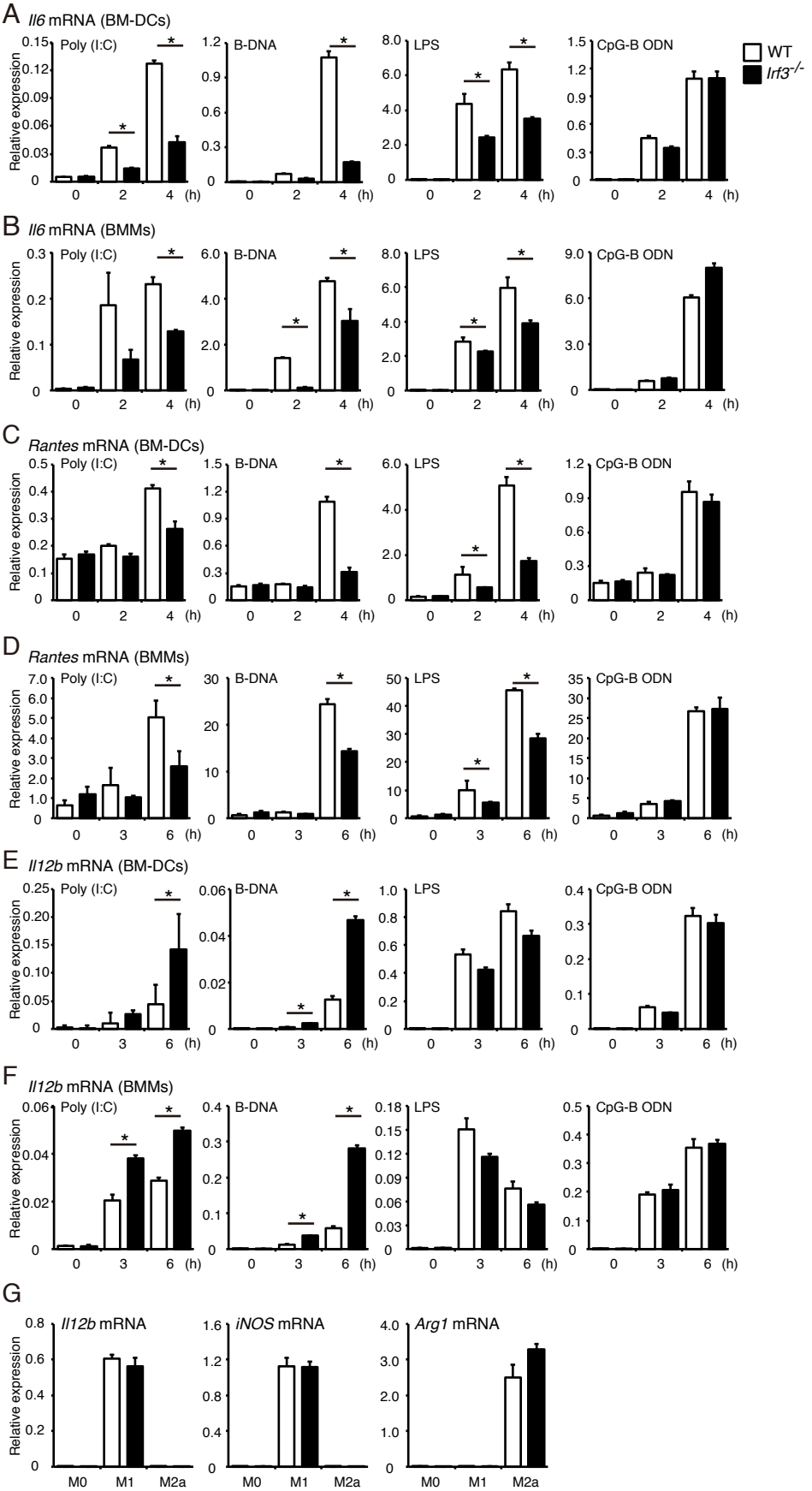
Figure S2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B Il6 mRNA (BMMs)
C Rantes mRNA (BM-DCs)
D Rantes mRNA (BMMs)
E Il12b mRNA (BM-DCs)
F Il12b mRNA (BMMs)
G
Poly (I:C)
B-DNA
LPS
CpG-B ODN
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Relative expression
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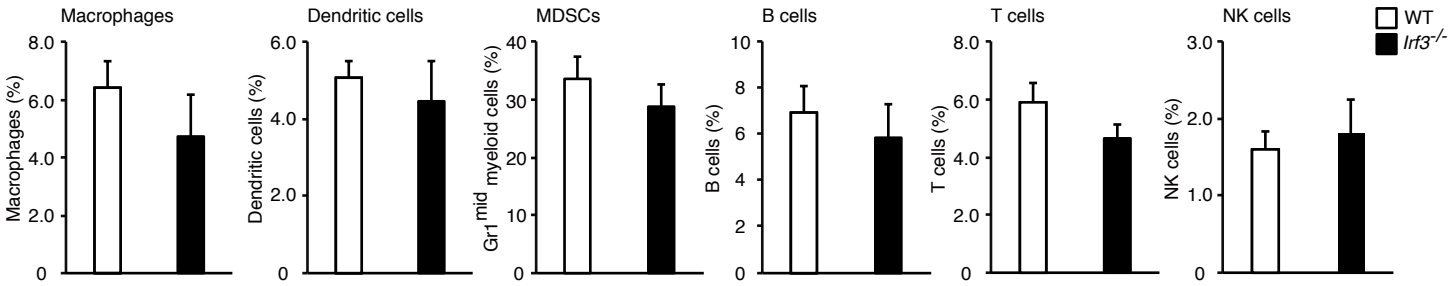
iNOS mRNA
Arg1 mRNA
M0
M1
M2a

Figure S3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B cells
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B cells (%)
T cells (%)
NK cells (%)
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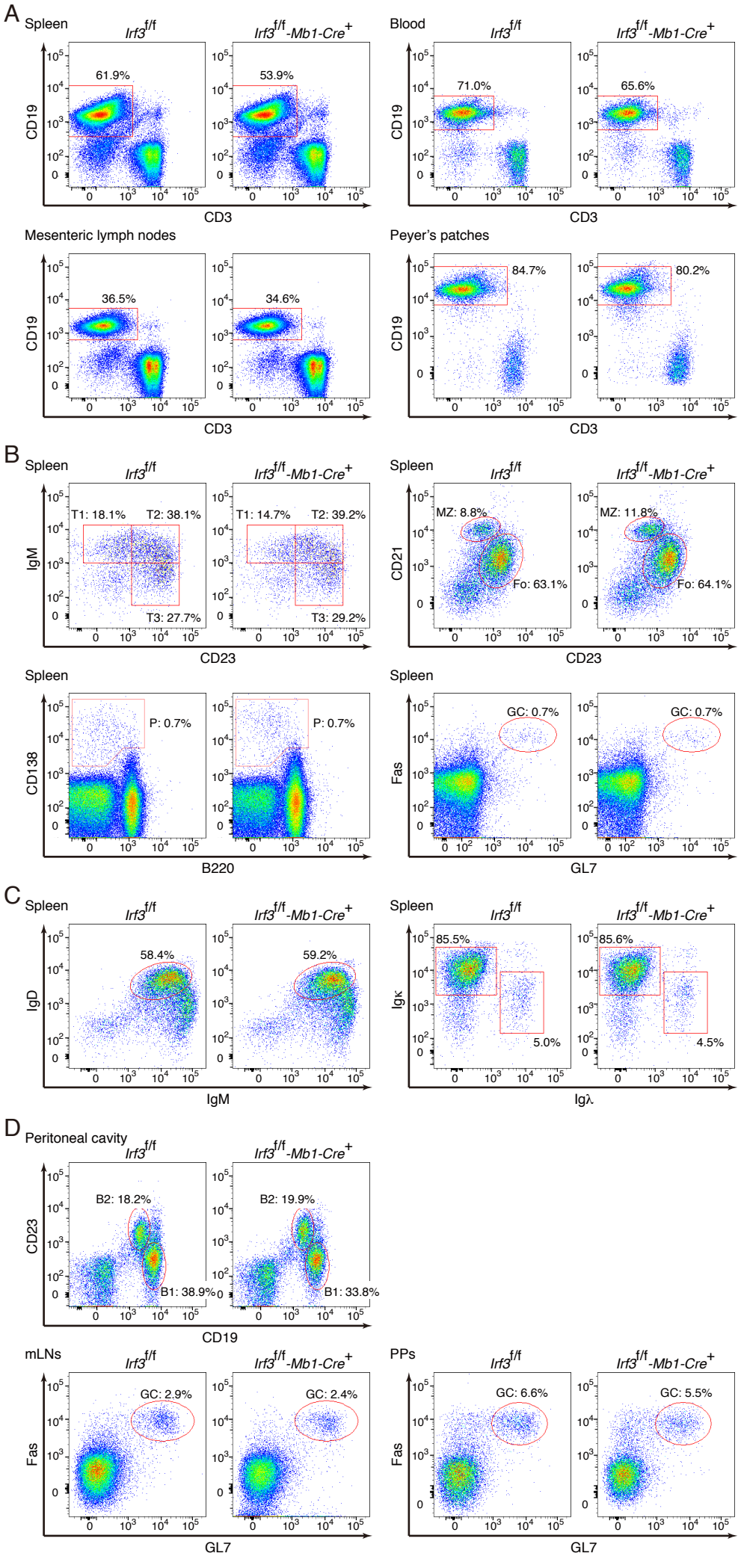
Figure S4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Irf3f/f-Mb1-Cre+
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53.9%
CD19
CD3
Blood
71.0%
65.6%
Mesenteric lymph nodes
36.5%
34.6%
Peyer's patches
84.7%
80.2%
B
IgM
CD23
T1: 18.1%
T2: 38.1%
T3: 27.7%
T1: 14.7%
T2: 39.2%
T3: 29.2%
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GC: 0.7%
C
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59.2%
Igκ
Igλ
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5.0%
85.6%
4.5%
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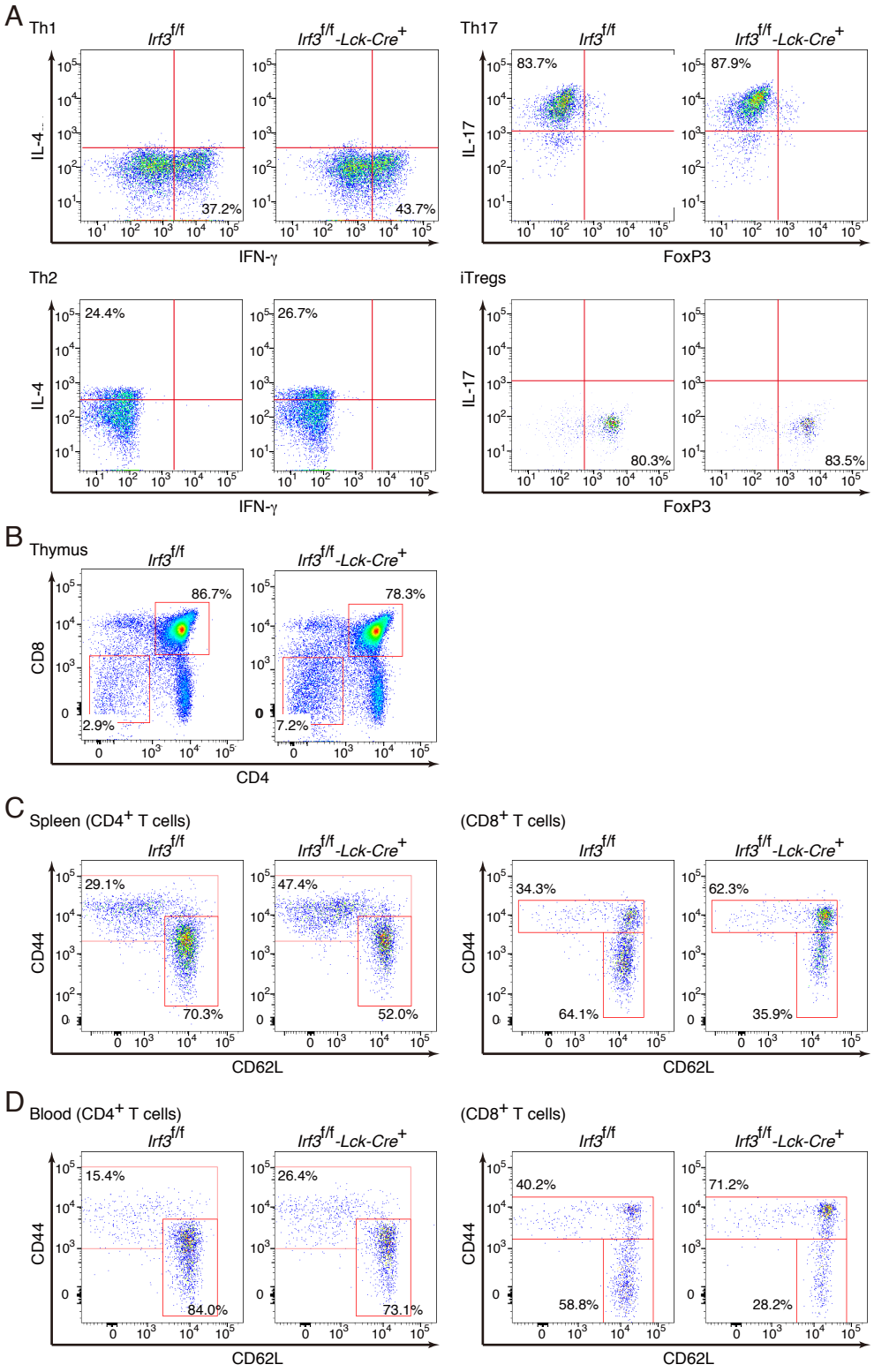
Figure S5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26.7%
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Thymus
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C
Spleen (CD4+ T cells)
(CD8+ T cells)
CD44
CD62L
29.1%
70.3%
47.4%
52.0%
34.3%
64.1%
62.3%
35.9%
D
Blood (CD4+ T cells)
(CD8+ T cells)
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26.4%
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