Supporting Information

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SI Materials and Methods

Mice. Toll-like receptor (TLR)$8^{-/-}$ and $TLR9^{-/-}$ mice were generated as described previously (1, 2). $TLR9^{-/-}$ mice were obtained from S. Akira (Osaka University, Osaka, Japan). Double $TLR8/9^{-/-}$ mice were generated by intercrossing $TLR8^{-/-}$ with $TLR9^{-/-}$ mice. All three TLR-deficient mouse genotypes were backcrossed on the C57BL/6 background for more than 10 generations. C57BL/6 mice were purchased from the Charles River Laboratories. Unless otherwise specified, age- and sex-matched mice were used for each experiment. Mice were housed under specific pathogen-free conditions at the Centre d'Immunologie de Marseille-Luminy and experiments were conducted in accordance with institutional guidelines for animal care and use and European directives.

Reagents. R848, LPS from *Escherichia coli* 0111-B4, Pam_3CSK_4, CpG ODN 1826, and poly I:C were purchased from Invivogen. Recombinant mouse IFN-α was from Calbiochem.

Preparation of Cells and Quantification of Cytokines. Bone marrow cells from mice were extracted from femur and tibias and cultured with GM-CSF or M-CSF for the production of bone marrow dendritic cells (BM-DCs) or BM-macrophages, respectively as previously described (1). For the production of bone marrow-plasmacytoid DCs (pDC), bone marrow cells depleted of erythrocytes were cultured in RPMI, 10% (vol/vol) FBS, 2 mM GlutaMAX (Gibco), 1 mM sodium pyruvate, 1% (vol/vol) MEM Non-Essential Amino Acids (Gibco), 50 μM β-mercaptoethanol, and 2.5% (vol/vol) B16-Flt3L cell line supernatant (source of Flt3 ligand) at 2×10^6 cells/mL in six-well plates for 9 d (3). B cells were purified from spleen by negative selection using the B-cell isolation kit (Miltenyi). Purity was assessed by FACS and above 96%. Cytokines levels in culture supernatants were measured by IL-6 and TNF (eBioscience) ELISA kits. For intracellular cytokine staining splenocytes were treated with the indicated stimuli in the presence of Brefeldin A (e-Biosciences) for 4 h.

RNA Isolation and Quantitative PCR. Total RNA was isolated with TRIzol reagent (Gibco, Invitrogen) or RNA easy kit (Qiagen). RNA was reversed-transcribed with Supercsript II reverse transcriptase (Invivogen) and quantitative PCR for TLR7, IFN-β, and β-actin was performed as described previously (1).

Serological Analysis. Evaluation of IgM and IgG2a, and IgG autoantibodies against DNA, RNA, ribonucleoprotein (RNP) and Smith ribonucleoprotein (smRNP) on serum samples were performed as described previously (1).

Flow Cytometric Analysis. Cell suspensions were incubated with 24G2 hybridoma supernatant and then stained using immunofluorescence-labeled antibodies against the following antigens: B220, CD3, CD4, CD8, NK1.1, CD19, CD11b, CD11c, Ly6G, Ly6C, CD21, CD23, CD5, CD44, CD86, CD38, CD138, CD24, and CD172a (SIRPα) from BD Biosciences, IA/IE (MHC class II), GL7 from eBioscience, and SiglecH from Biolegend. For intracellular cytokine staining cells were fixed with Cytofix/Cytoperm (BD Biosciences) for 20 min on ice and stained using immunofluorescence-labeled antibodies for TNF and IL12p40/p70 (BD Biosciences). Flow cytometry was conducted using an LSR2 (BD Biosciences) and data were analyzed with FlowJo (Tree Star).

Histology, Immunofluorescence, and Antinuclear Antibody Staining. For histopathology studies, kidneys were fixed in formalin and embedded in paraffin. For light microscopy, 3- to 4-μm-thick tissue sections were stained with H&E and periodic acid-Schiff. To determine the extent of renal damage, all renal biopsies were analyzed in a blinded fashion by a pathologist. Typical glomerular active lesions of lupus nephritis were evaluated: glomerular cellularity (mesangial cell proliferation, endocapillary proliferation, and inflammatory cells), glomerular deposits, extracapillary proliferation, as well as, tubulointerstitial chronic lesions: tubular atrophy, interstitial fibrosis, and interstitial infiltrates. Lesions were graded semiquantitatively using a scoring system from 0 to 4 (0, no changes; 1, mild; 2, moderate; 3, high; 4, severe). The total histological score for each specimen was derived from the sum of all of the described parameters. Immunofluorescence IgG and IgM staining on kidney sections was performed as described previously (1). Antinuclear antibodies (ANA) in mouse sera were tested with Hep-2 cells fixed on slides (Biomedical Diagnostics). Sera were diluted in PBS 1:60 and an Alexa Fluor 488 anti-mouse IgG antibody (Invitrogen) was used to detect mouse antibodies.

Statistical Analysis. Statistics were calculated using Prism 5 (GraphPad Software) by Mann–Whitney U test with P values indicated throughout as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

1. Demaria O, et al. (2010) TLR8 deficiency leads to autoimmunity in mice. *J Clin Invest* 120(10):3651–3662.
2. Hemmi H, et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* 408(6813): 740–745.
3. Gilliet M, et al. (2002) The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J Exp Med* 195(7):953–958.

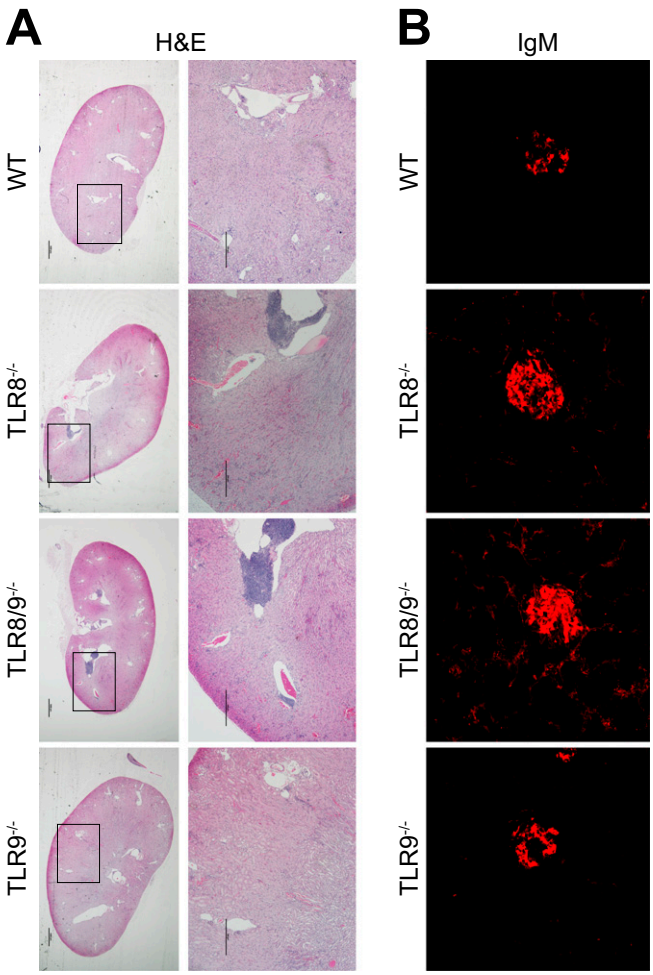

Fig. S1. Increased renal pathology in TLR8/9$^{-/-}$ mice compared with TLR8$^{-/-}$ or TLR9$^{-/-}$ mice. (*A*) H&E-stained kidney sections from 6 mo old female WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice (n = 3 per group). (*Left*) (Scale bars, 1 mm.) (*Right*) Magnified details of black bordered areas. (Scale bars, 0.5 mm.) (*B*) Kidney sections from 5-mo-old female WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, or TLR9$^{-/-}$ mice (n = 3 per group) were stained with immunofluorescence anti-IgM (original magnification × 100).

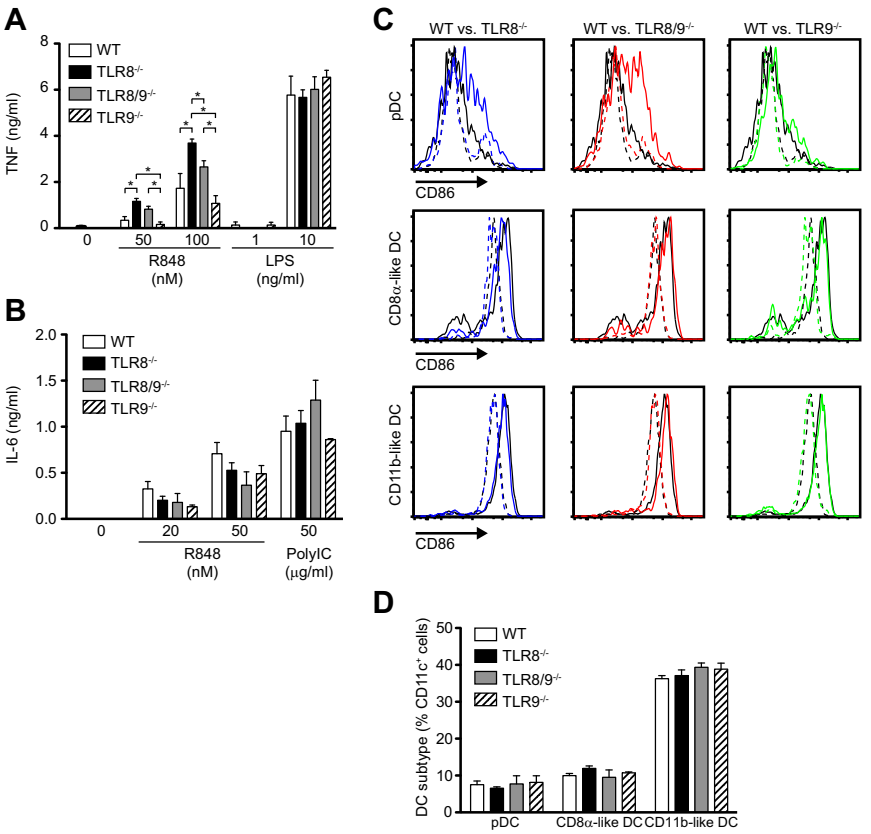

Fig. S2. Cytokine production and CD86 expression by WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ DCs or macrophages. (*A*) BM-DCs or (*B*) BM-macrophages from WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice left untreated or stimulated with the indicated amounts R848, LPS, or poly I:C. After 16 h, the concentration of TNF (*A*) or IL-6 (*B*) in culture supernatants was assessed by ELISA. Data are representative of two to three independent experiments ($n = 3$ per group). (*C*) Total splenocytes were left untreated or stimulated with 30 nM R848. After 16 h, the surface expression of CD86 was analyzed by FACS analysis on splenic pDCs, CD8α-like DCs, or CD11b-like DCs from WT (black line), TLR8$^{-/-}$ (blue line), TLR8/9$^{-/-}$ (red line), or TLR9$^{-/-}$ (green line) mice ($n = 3$ mice per group). Untreated cells (dotted lines), R848 treated cells (full lines). $n = 3$ mice per group. (*D*) Frequencies of splenic pDCs, CD8α-like DCs, or CD11b-like DCs in the four genotypes. Data are average percentages ± SD ($n = 3$ mice per group). *$P < 0.05$.

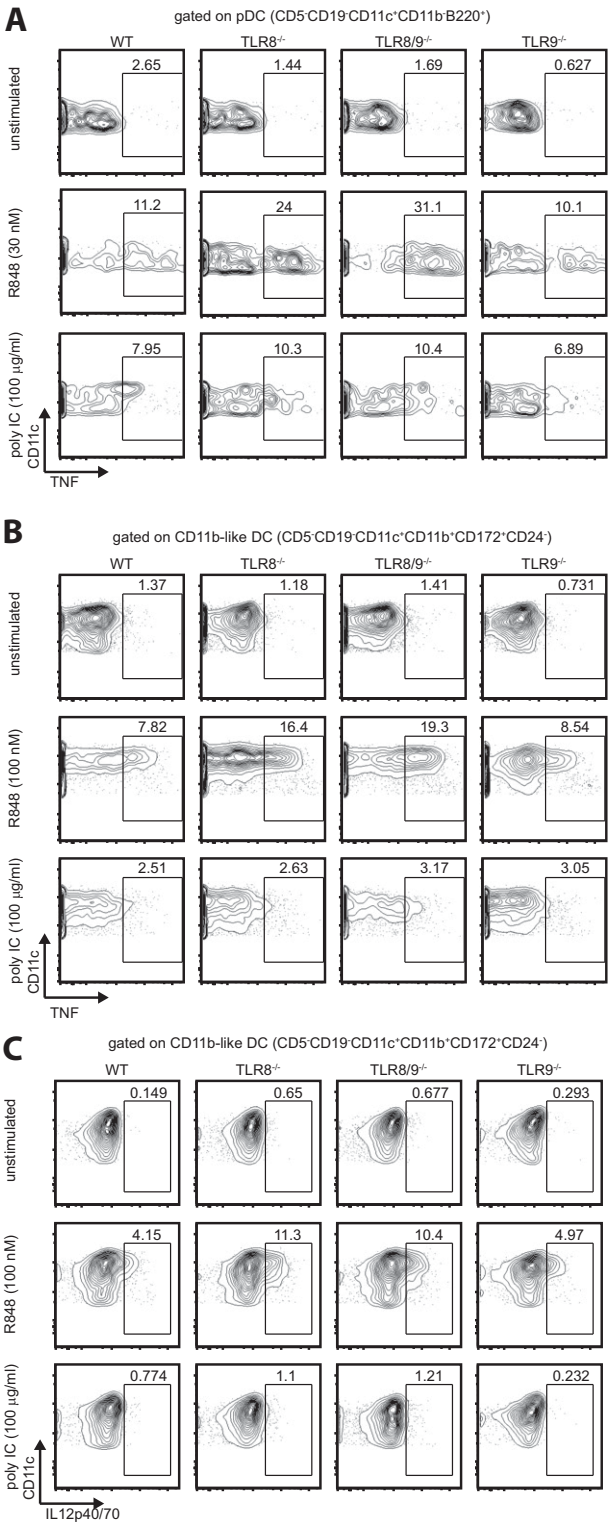

Fig. S3. Analysis of TNF and IL-12p40/70 cytokine production by splenic pDCs and CD11b-like DCs. Total splenocytes were left untreated or stimulated with R848 or poly I:C. After 4 h, intracellular staining for TNF and IL-12p40/p70 was performed. Expression of TNF was evaluated after gating on (*A*) pDCs and (*B*) CD11b-like DCs, and expression of IL-12P40/70 was evaluated after gating on (*C*) CD11b-like DCs from WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, or TLR9$^{-/-}$ mice (n = 3 per genotype). Numbers in dot plots represent the percentage of cells that produce TNF or IL-12. Plots are representative of the data shown in Table S4.

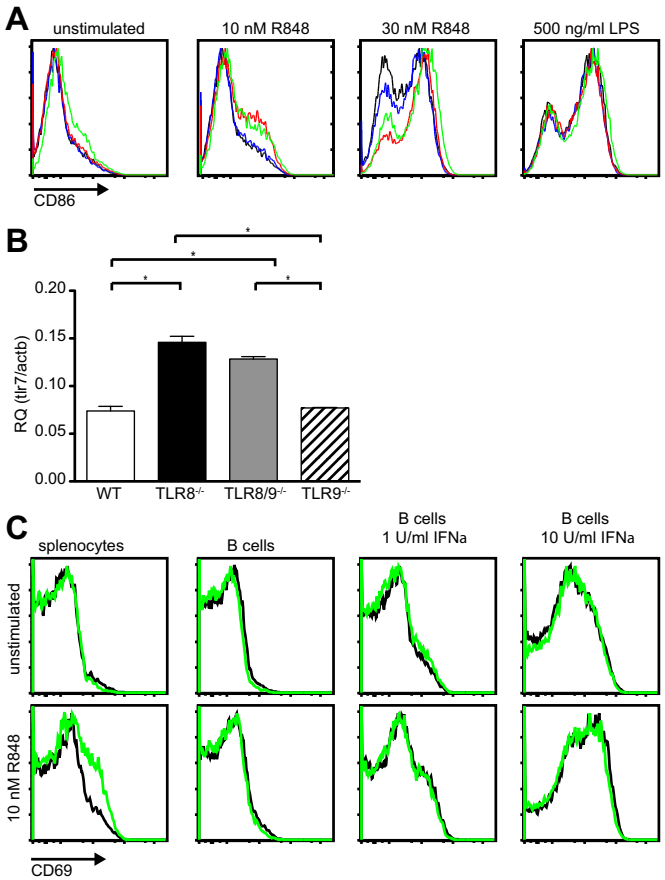

Fig. S4. CD86 expression by splenocytes and TLR7 expression and response of isolated B cells from WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice. (*A*) Total splenocytes were left untreated or stimulated with R848 or LPS for 16 h. Representative flow cytometry plots of CD86 on WT (black line), TLR8$^{-/-}$ (blue line), TLR8/9$^{-/-}$ (red line), and TLR9$^{-/-}$ (green line) CD119^{+}B220^{+} splenocytes. (*B*) Total RNA was extracted from WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, or TLR9$^{-/-}$ splenic isolated B cells and the expression of TLR7 and β-actin were evaluated by quantitative PCR. *$P < 0.05$. (*C*) Splenocytes or isolated B cells from WT (black line) or TLR9$^{-/-}$ (green line) mice were left untreated or stimulated with the indicated amount of R848 in the presence or absence of IFN-α. The surface expression of CD69 was analyzed by flow cytometry on CD19^{+} cells. Data are representative of two independent experiments ($n = 3$ per group).

Table S1. Spleen weight, total cell count, and major cell populations of WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice

		WT	TLR8$^{-/-}$	TLR8/9$^{-/-}$	TLR9$^{-/-}$
Spleen weight (mg)		77.0 ± 19.5	116.9 ± 29.1*,†	145.7 ± 50.8*,‡,§	103.7 ± 17.8*,†
Total cell count (×10^{6})		67.7 ± 26.4	108.6 ± 32.8*,†	143.0 ± 46.8*,‡	113.4 ± 34.1*
Cell type	Cell surface markers	Frequency (% of total)			
T cells	CD3^{+}	25.7 ± 3.7	20.7 ± 5.4§	21.3 ± 3.4§	26.1 ± 1.8†,‡
CD4 T cells	CD3^{+}/CD4^{+}	14.5 ± 2.7	12.3 ± 3.0§	12.8 ± 1.7§	15.9 ± 1.2†,‡
CD8 T cells	CD3^{+}/CD8^{+}	9.7 ± 1.3	6.7 ± 2.3*	6.5 ± 1.6*	8.4 ± 1.1
NKT cells	CD3^{+}/NK1.1^{+}	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	ND
NK cells	CD3^{-}/NK1.1^{+}	2.2 ± 0.1	1.8 ± 0.4	1.5 ± 0.2*	ND
B cells	CD19^{+}	58.8 ± 3.7	65.9 ± 5.1*	66.8 ± 5.5*	63.4 ± 6.0
CD11c^{+} cells	CD11c^{+}	3.9 ± 0.4	5.0 ± 0.5*	5.3 ± 0.6*	5.4 ± 0.9
DC	CD11c^{hi}/IA-IE^{+}	1.6 ± 0.1	1.8 ± 0.1	2.1 ± 0.5	2.0 ± 0.1
pDC	CD11c^{int}/SiglecH^{+}	0.7 ± 0.1	0.9 ± 0.3	0.8 ± 0.3	1.0 ± 0.2
Monocytes	CD11b^{+}/Ly6G^{-}/Ly6C^{+}	0.4 ± 0.3	1.1 ± 0.4*	0.8 ± 0.2§	1.1 ± 0.2*,†
Neutrophils	CD11b^{+}/Ly6G^{+}/Ly6C^{-}	0.4 ± 0.3	0.9 ± 0.2*,†	0.5 ± 0.3§	0.9 ± 0.2*

Data are from 4- to 7-mo-old male or female mice. No differences were observed between males and females. Values shown are average of at least four mice ± SD. ND, not determined.

*$P < 0.05$ vs. WT.

†$P < 0.05$ vs. TLR8/9$^{-/-}$.

‡$P < 0.05$ vs. TLR8$^{-/-}$.

§$P < 0.05$ vs. TLR9$^{-/-}$.

Table S2. Frequency of splenic marginal zone (MZ) B cells and peritoneal B1a and B1b B cells of WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice

Genotype	MZ B cells	B1a B cells	B1b B cells
WT	4.7 ± 0.2 (n = 8)	18.8 ± 1.2 (n = 9)	10.9 ± 0.4 (n = 5)
TLR8$^{-/-}$	2.1 ± 0.2*,† (n = 8)	9.1 ± 1.2*,†,‡ (n = 8)	5.1 ± 1.0† (n = 4)
TLR8/9$^{-/-}$	1.5 ± 0.3*,† (n = 8)	4.7 ± 0.6*,†,§ (n = 7)	7.2 ± 1.7*,† (n = 3)
TLR9$^{-/-}$	4.8 ± 0.5‡,§ (n = 3)	14.9 ± 1.5‡,§ (n = 7)	11.7 ± 0.8‡,§ (n = 3)

Data are from 4- to 7-mo-old female mice. Values shown are average ± SD. In parenthesis is the number of mice per group.
$^{*}P < 0.05$ vs. WT.
$^{\dagger}P < 0.05$ vs. TLR9$^{-/-}$.
$^{\ddagger}P < 0.05$ vs. TLR8/9$^{-/-}$.
$^{\S}P < 0.05$ vs. TLR8$^{-/-}$.

Table S3. Renal scores for WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ female 6-mo-old mice

	Glomerular lesions			Tubulo-interstitial lesions			
Genotype	Glomerular cellularity	Extracapillary proliferation	Glomerular depositis	Tubular atrophy	Fibrosis	Interstitial infiltration	Total score
WT	0	0	0	0	0	0	0
TLR8$^{-/-}$	1.33 ± 0.57	0	1.00 ± 0.00	0	0	0.66 ± 0.57	2.9
TLR8/9$^{-/-}$	1.66 ± 0.57	0.33 ± 0.57	1.66 ± 0.57	0	0	1.00 ± 0.00	4.5
TLR9$^{-/-}$	1.00 ± 0.00	0	1.00 ± 0.00	0	0	0	2

Pathologist scored H&E and periodic acid-Schiff stained paraffin embedded kidney sections displayed as mean ± SD, n = 3 mice per group shown. Pathologist scoring method and definitions can be found in *SI Materials and Methods*.

Table S4. Percentages of TNF- and IL-12p40/p75-expressing splenic pDCs, CD8α^{+}-like DCs, and CD11b^{+}-like DCs from WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice upon R848 or poly I:C stimulation

Genotype	Untreated	R848	Poly I:C	Untreated	R848	Poly I:C
	TNF by pDC			IL-12 by pDC		
WT	2.5 ± 0.7	10.9 ± 2.3	7.4 ± 1.3	0.3 ± 0.2	5.0 ± 2.8	0.4 ± 0.2
TLR8$^{-/-}$	1.4 ± 0.1*	44.1 ± 21.5*,†	10.2 ± 1.3*	0.5 ± 0.2	8.2 ± 1.6	0.9 ± 0.1*
TLR8/9$^{-/-}$	2.9 ± 1.4*	30.1 ± 4.0*,†	12.9 ± 4.5	0.2 ± 0.1	12.4 ± 2.1*	1.2 ± 0.4
TLR9$^{-/-}$	0.8 ± 0.1†,‡,§	11.5 ± 0.7‡,§	5.8 ± 0.6‡	0.2 ± 0.1	4.9 ± 1.0§	0.6 ± 0.2‡
	TNF by CD8α^{+}-like DC			IL-12 by CD8α^{+}-like DC		
WT	3.5 ± 2.3	2.7 ± 1.5	6.7 ± 0.8	2.1 ± 1.0	3.4 ± 1.4	10.9 ± 2.4
TLR8$^{-/-}$	1.0 ± 0.3	1.5 ± 0.3	9.7 ± 1.5	4.2 ± 0.8	5.4 ± 0.6	13.8 ± 0.4*
TLR8/9$^{-/-}$	1.3 ± 0.1*	2.1 ± 0.3*	7.2 ± 0.4	4.4 ± 0.1*,†	5.4 ± 0.8	13.2 ± 1.4
TLR9$^{-/-}$	0.9 ± 0.1†,§	1.2 ± 0.2§	8.2 ± 1.9	3.8 ± 0.2†,§	4.5 ± 0.8	9.9 ± 1.5‡
	TNF by CD11b^{+}-like DC			IL-12 by CD11b^{+}-like DC		
WT	2.1 ± 1.1	7.1 ± 1.9	2.1 ± 0.2	0.1 ± 0.0	3.9 ± 1.5	0.7 ± 0.1
TLR8$^{-/-}$	1.2 ± 0.2*	14.6 ± 3.7	2.2 ± 0.1§	0.6 ± 0.1*,†	10.7 ± 0.6*,†	1.0 ± 0.2
TLR8/9$^{-/-}$	1.6 ± 0.4*	16.5 ± 1.4*,†	3.0 ± 0.2†,‡	0.6 ± 0.1*,†	12.9 ± 1.9*,†	0.8 ± 0.3
TLR9$^{-/-}$	0.7 ± 0.1†,‡,§	8.1 ± 1.2§	2.4 ± 0.4	0.2 ± 0.1‡,§	5.3 ± 1.0‡,§	0.1 ± 0.1

Data represent the average percentage ± SD of TNF or IL-12p40/p70- expressing pDCs, CD8α^{+}-like DCs, or CD11b^{+}-like DCs after stimulation of splenocytes from three mice per group with 30 nM R848 for pDCs and CD8α^{+}-like DCs, 100 nM R848 for CD11b^{+}-like DCs, or 100 μg/mL poly I:C.
$^{*}P < 0.05$ vs. TLR9$^{-/-}$.
$^{\dagger}P < 0.05$ vs. WT.
$^{\ddagger}P < 0.05$ vs. TLR8/9$^{-/-}$.
$^{\S}P < 0.05$ vs. TLR8$^{-/-}$.