PNAS
www.pnas.org

Supplementary Information for

Dysregulation of TLR9 in neonates leads to fatal inflammatory disease driven by IFN-γ

Alison G. Stanbery, Zachary R. Newman, and Gregory M. Barton

Gregory M. Barton
barton@berkeley.edu

This PDF file includes:

Materials and Methods:

Mice

Mice were housed under specific pathogen-free conditions at the University of California, Berkeley. All mouse experiments were performed in accordance with the guidelines of the Animal Care and Use Committee at UC Berkeley. Unless noted, mice were analyzed as neonates (e18.5 to 1-3hrs post-birth) or as adults (6-12 weeks of age). Timed pregnancies were estimated considering the day of vaginal plug formation as 0.5day post coitus. Mouse lines Tmem163$^{Tg(ACTB-cre)2Mrt}$ (β-actin-cre), Commd10$^{Tg(Vav1-icre)A2Kio}$ (Vav-cre), Tg(Itgax-cre)1-1Reiz/J (Cd11c-cre), Lyz2$^{tm1(cre)Ifo}$ (Lyz2-cre), Cd19$^{tm1(cre)Cgn}$ (Cd19-cre), Ifngr1$^{-/-}$ (Ifngr1^{tm1Agt}), Ifnar1$^{-/-}$ (Ifnar1^{tm1Agt}) and FLPe mice (Tg(ACTFLPe)9205Dym/J) were obtained from The Jackson Laboratory. TLR9^{GFP} (B6(FVB)-*Tlr9$^{tm1.1Gbrt}$*/J) and TLR7^{TOMATO} (B6(FVB)-*Tlr7$^{tm1.1Gbrt}$*/J**)** mice have been described (17) and are available at The Jackson Laboratory.

TLR9^{fsTM} and TLR9^{fsWT} knock-in mice were generated by the UC Davis Mouse Biology Program. Briefly, JM8.N4 embryonic stem cells (C57BL/6 background) were electroporated with a targeting construct containing the Floxed-Stop cassette, relevant TLR9 allele, an internal FRT flanked neomycin resistance cassette, flanking homology arms (5' 3.7kb, 3' 1.5kb), and a DTA cassette external to the homology arms. ES clones were grown under selection with G418 and genomic DNA screened by Southern blot analysis for proper targeting then expanded for microinjection into Balb/c recipients. Pups chimeric for coat color were bred to FLPe mice to remove the neomycin resistance cassette. Progeny were screened for germline transmission by PCR and confirmed by Southern blot analysis.

Tissue harvest and stimulation

Blood was collected into tubes containing heparin sulfate (24 U/ml; Sigma-Aldrich), and red blood cells were lysed using ACK lysing buffer (Gibco). Spleens, lymph nodes, and neonatal livers were minced with scissors and digested with collagenase VIII (Sigma #C2139) with DNase I (Sigma #D4513) for 30min (15min for livers) in RPMI. Tissue was dissociated through a 70-micron filter, treated with ACK Lysis Buffer to eliminate red blood cells, and then resuspended in PBS containing 2% FBS (v/v) and 2mM EDTA (flow cytometry buffer). Bone marrow was crushed with a mortar and pestle, dissociated through a 40-micron filter, treated with ACK Lysis Buffer, and then resuspended in flow cytometry buffer. Cell counts were obtained using Count Bright absolute counting beads (Life technologies #C36950).

Splenocytes were harvested and stimulated with CpG-B (ODN1668: TCCATGACGTTCCTGATGCT, all phosphorothioate linkages, synthesized by Integrated DNA Technologies) or LPS (InvivoGen) for 6h and then harvested for flow cytometry.

For intracellular staining of IFN-γ, TNF, and IL-12p40, neonatal liver cells were harvested as described above, cultured for 6h in the presence of GolgiStop (BD 554724), and then stained for flow cytometry.

Flow cytometry and antibodies

Dead cells were excluded using 4',6-diamidino-2-phenylindole (DAPI, Thermo-Fisher) and all stains were carried out in flow cytometry buffer including anti-CD16/32 Fc blocking antibody (2.4G2, UCSF Antibody Core) and normal mouse serum (Sigma).

Cells were stained for 30min at 4°C with antibodies. For intracellular TNF or IFN-γ, splenocytes or neonatal liver cells were resuspended in live/dead stain for 15min and then Fcblock and mouse serum were added for 30min. Surface stain was added for additional 30min at 4C. Cells were then washed and resuspended in eBiosciences fix/perm for 30min, stained for TNF or IFN-γ, and then resuspended for flow cytometry. All cells were analyzed on an X-20 or LSR Fortessa (BD Biosciences), and data was analyzed with FlowJo (TreeStar). The following antibodies were used: B220 (clone: RA3-6B2; vendor: eBioscience), CD11c (clone: N418; vendor: BioLegend), CD19 (clone: 6D5; vendor: BioLegend), CD3ε (clone: 145-2C11; vendor: BioLegend), CD11b (clone: M1/70; vendor: BioLegend), F4/80 (clone: BM8; vendor: Tonbo), CD45 (clone: 30-F11; vendor: eBioscience), CCR2 (clone:475301 vendor: R&D systems), CD115 (clone: AFS98, vendor: eBioscience), Ly6C (clone: HK1.4; vendor: BioLegend), Ly6G (clone: 1A8; vendor: BioLegend), TNF (clone: MP6-XT22; vendor: eBioscience), IL-12p40 (clone: C17.8; vendor: eBioscience), Sca-1 (Ly-6A/E) (clone: D7; vendor: eBioscience), NK1.1 (clone: PK136; vendor: eBioscience), IFN-γ (vendor: BD Biosciences), NKG2D (clone: CX5; vendor: BioLegend), NKG2A/C/E (clone: 20d5; vendor: eBioscience), DNAM-1 (clone: 10E5; vendor: BioLegend), NKp46 (clone: 29A1.4; vendor: BioLegend), Mertk (clone: 2B10C.42 vendor: BioLegend), live/dead (near-IR fluorescent reactive dye; vendor: Life Technologies).

RNA isolation and qPCR gene analysis

RNA from neonatal livers was stored at -80C in RNAzol until purification. Total RNA was isolated using a DirectZol RNA purification kit (Zymo Research). RNA was treated with Turbo DNase (Thermo Fisher) for 30 min and purified using RNA Clean and

Concentrator kit (Zymo Research). cDNA was generated using iScript Reverse Transcription Supermix (Life Sciences). For quantitative RT-PCR, cDNA was mixed with appropriate primers and SYBR Green master mix (Thermo Fisher) and run on a QuantStudio 5 (Applied Biosystems). The following primers were used: *Actb*-5'-GGCTGTATTCCCCTCCATCG–3' (forward) and 5'-CCAGTTGGTAACAATGCCATGT-3' (reverse); *Ifit1*-5'-CTGAGATGTCACTTCACATGGAA-3' (forward) and 5'-GTGCATCCCCAATGGGTTCT-3' (reverse); *Ifit2*-5'-AGTACAACGAGTAAGGAGTCACT-3' (forward) and 5'-AGGCCAGTATGTTGCACATGG-3' (reverse); *Ifi44*-5'-AACTGACTGCTCGCAATAATGT-3' (forward) and 5'-GTAACACAGCAATGCCTCTTGT-3' (reverse); *Ifnb*-5'-ATAAGCAGCTCCAGCTCCAA-3' (forward) and 5'-CTGTCTGCTGGTGGAGTTCA-3' (reverse); *Ifng*-5'-TGGCTGTTTCTGGCTGTTACTG-3' (forward) and 5'-AGGTGTGATTCAATGACGCTTATG-3' (reverse); *Irf7*-5'-GAGACTGGCTATTGGGGGAG-3' (forward) and 5'-GACCGAAATGCTTCCAGGG-3' (reverse); *Oas2*-5'-TTGAAGAGGAATACATGCGGAAG-3' (forward) and 5'-GGGTCTGCATTACTGGCACTT-3' (reverse); *Oas3*-5'-TCTGGGGTCGCTAAACATCAC-3' (forward) and 5'-GATGACGAGTTCGACATCGGT-3' (reverse); *Mx2*-5'-GAGGCTCTTCAGAATGAGCAAA-3' (forward) and 5'-CTCTGCGGTCAGTCTCTCT-3' (reverse).

ELISpot

For ELISpot analysis, multiscreen plates (Millipore) were coated with anti-mouse IFN-γ capture antibody 1:200 in PBS (BD Biosciences purified anti-mouse IFN-γ #51-2525KZ) overnight at 4C. Plates were blocked with complete RPMI with 10% FCS. Plates were washed with PBS and cells from processed neonatal livers were serially diluted in complete RPMI and incubated at 37°C overnight. Following several washes in PBS + 0.05% Tween-20, biotinylated secondary anti-mouse IFN-γ antibodies (BD #51-1818KA) were used at 1:50 in PBS + 10% FCS to detect IFN-γ secreting cells. Following several washes, SA-alkaline phosphatase was diluted 1:1000 in PBS + 10% FCS and incubated for 30 minutes. Plates were developed with AEC developing reagent (Vector Laboratories) according to manufacturer's instructions. Plates were read on an ImmunoSpot C.T.L. Analyzer (v3.2) and quantitated using ImmunoSpot 5.1 Pro.

Hemoglobin

For blood hemoglobin analysis, 10uL of neonatal blood was collected immediately post-sacrifice. Hemoglobin blood levels were measured using an AimStrip Hemoglobin (Hb) testing system from Ermaine Laboratories (catalog: 78200).

Luminex

For secreted cytokine analysis, fetal liver cells were collected and processed as previously stated and incubated in tissue-culture treated wells for 4h. Suspension cells were removed and adherent cells were incubated for an additional 14h. Supernatants were collected and analyzed using the 36-plex Luminex Mouse Inflammation kit (ProcartaPlex, catalog: EPX360-26092-901).

Tamoxifen-containing diet

For induction of Cre activity, mice carrying the ER^{T2}-*cre*$^{+}$ transgene and their littermates were fed a tamoxifen-containing diet (400 mg/kg tamoxifen citrate, 5% sucrose, 95% Teklad Global, 16% Rodent Diet) from Harlan Teklad. Mice were placed on diet starting at three weeks of age and weights were recorded every three days.

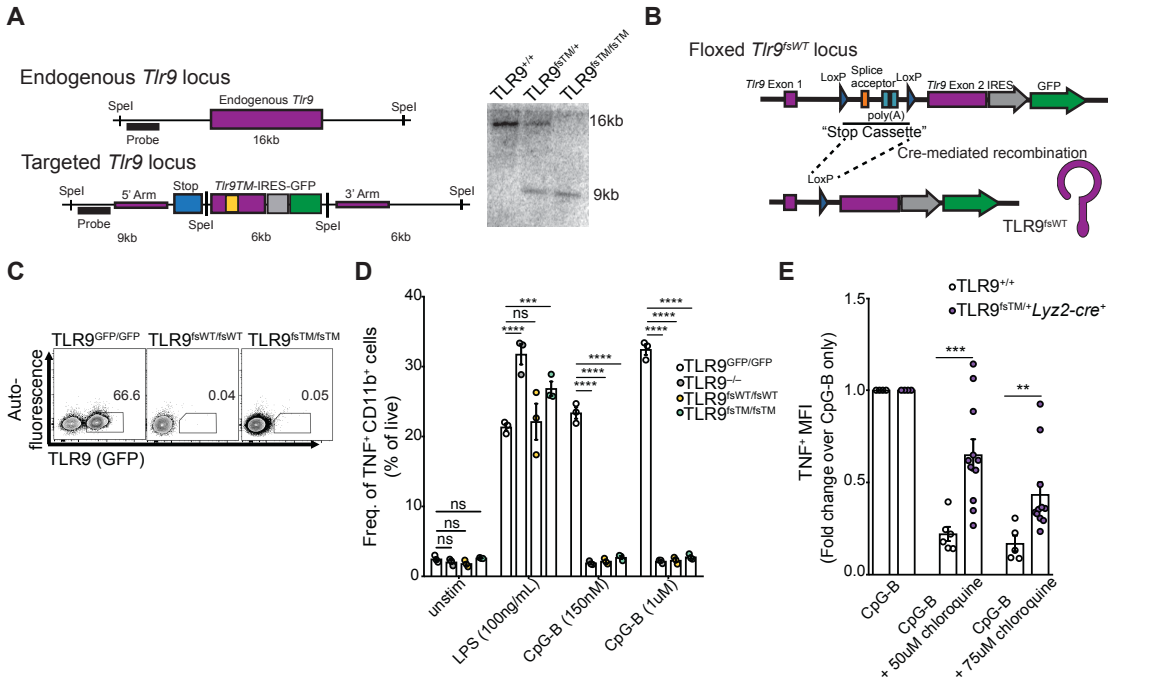
Histology

Neonates were euthanized immediately following birth and fixed in 10% Neutral Buffered Formalin. Embedding, slicing, and imaging was performed by HistoWiz Inc. (histowiz.com). Samples were processed, embedded in paraffin, and sectioned at 5μm. Hematoxylin and eosin staining was performed. After staining, sections were dehydrated and film coverslipped using a TissueTek-Prisma and Coverslipper (Sakura). Whole slide scanning (40x) was performed on an Aperio AT2 (Leica Biosystems). Histology scoring was performed at the Comparative Pathology Lab at UC-Davis.

Statistical analysis

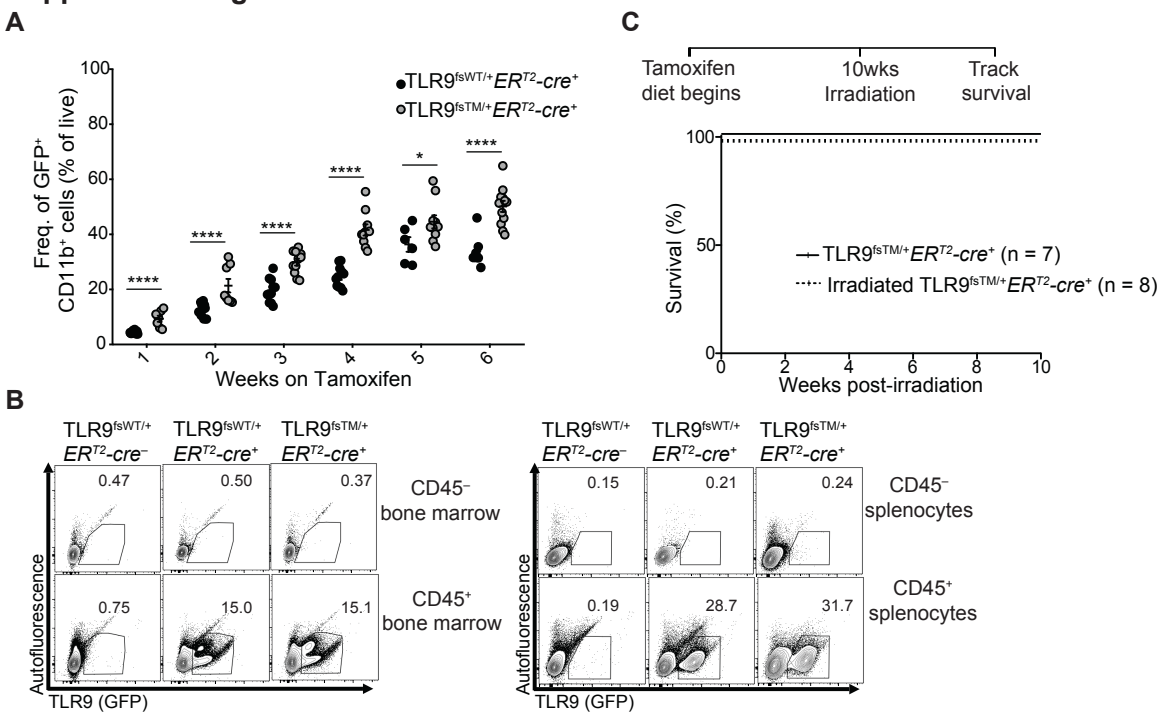
Statistical analysis was performed with the Prism software (GraphPad software). P-values were determined using ordinary unpaired ANOVA with Tukey's multiple comparisons or two-tailed Student's t test as indicated. Histology was scored using Kruskal-Wallis analysis of variance and Dunn's multiple comparisons test. Statistical analysis of survival curves was performed with using Log-rank (Mantel-Cox) test.

Supplemental Figure 1



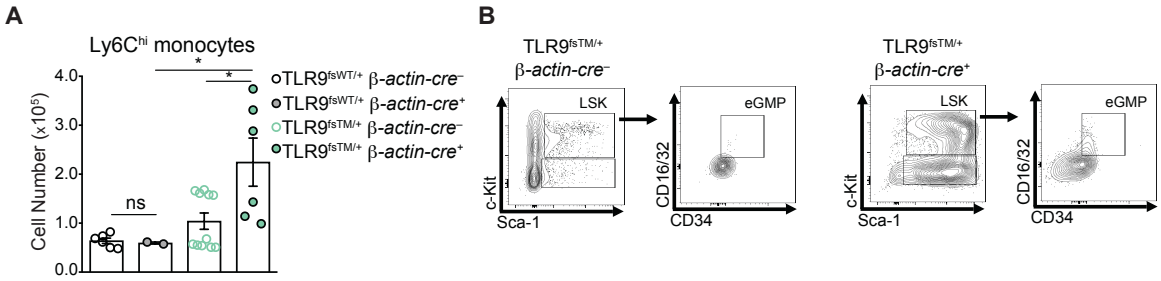
Supplementary Figure 1. Targeting and validation of TLR9^{fsTM} and TLR9^{fsWT} knock-in mice. (**A**) Targeting of endogenous *Tlr9* locus for TLR9^{fsTM} mice. The endogenous locus generates a 16kb fragment, while correct targeting introduced a new SpeI site that allowed for the detection of a 9kb and 6kb band. Image of genomic DNA probed by Southern with a probe that binds proximal to the 5'arm of the targeting construct. (**B**) Schematic for targeting of endogenous *Tlr9* locus for TLR9^{fsWT} mice. (**C**) In the absence of Cre, TLR9$^{fsTM/fsTM}$ and TLR9$^{fsWT/fsWT}$ mice do not have GFP^{+} splenocytes. Representative flow cytometry plots of live, single CD45^{+} splenocytes from TLR9$^{GFP/GFP}$, TLR9$^{fsWT/fsWT}$, and TLR9$^{fsM/fsTM}$ mice. (**D**) TLR9$^{fsTM/fsTM}$ and TLR9$^{fsWT/fsWT}$ mice do not respond to TLR9 ligand stimulation. Quantification of live, single CD45^{+}CD11b^{+}TNF^{+} splenocytes from TLR9$^{GFP/GFP}$, TLR9$^{-/-}$, TLR9$^{fsWT/fsWT}$, and TLR9$^{fsTM/fsTM}$ mice stimulated with TLR4 (LPS) or TLR9 (CpG-B) ligands. Data are representative of two independent experiments with 3 mice per genotype and analyzed using one-way ANOVA with Tukey's multiple comparisons post-test. (**E**) TLR9$^{fsTM/+}$ *Lyz2-cre*$^{+}$ bone-marrow macrophages respond to DNA ligand in the presence of chloroquine. Shown is the fold change of TNF MFI of macrophages stimulated with CpG-B with or without chloroquine pretreatment. Data are combined from four independent experiments with 3 mice or more per genotype and analyzed using two-tailed Student's t test. *P*-values in all panels are defined as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplemental Figure 2



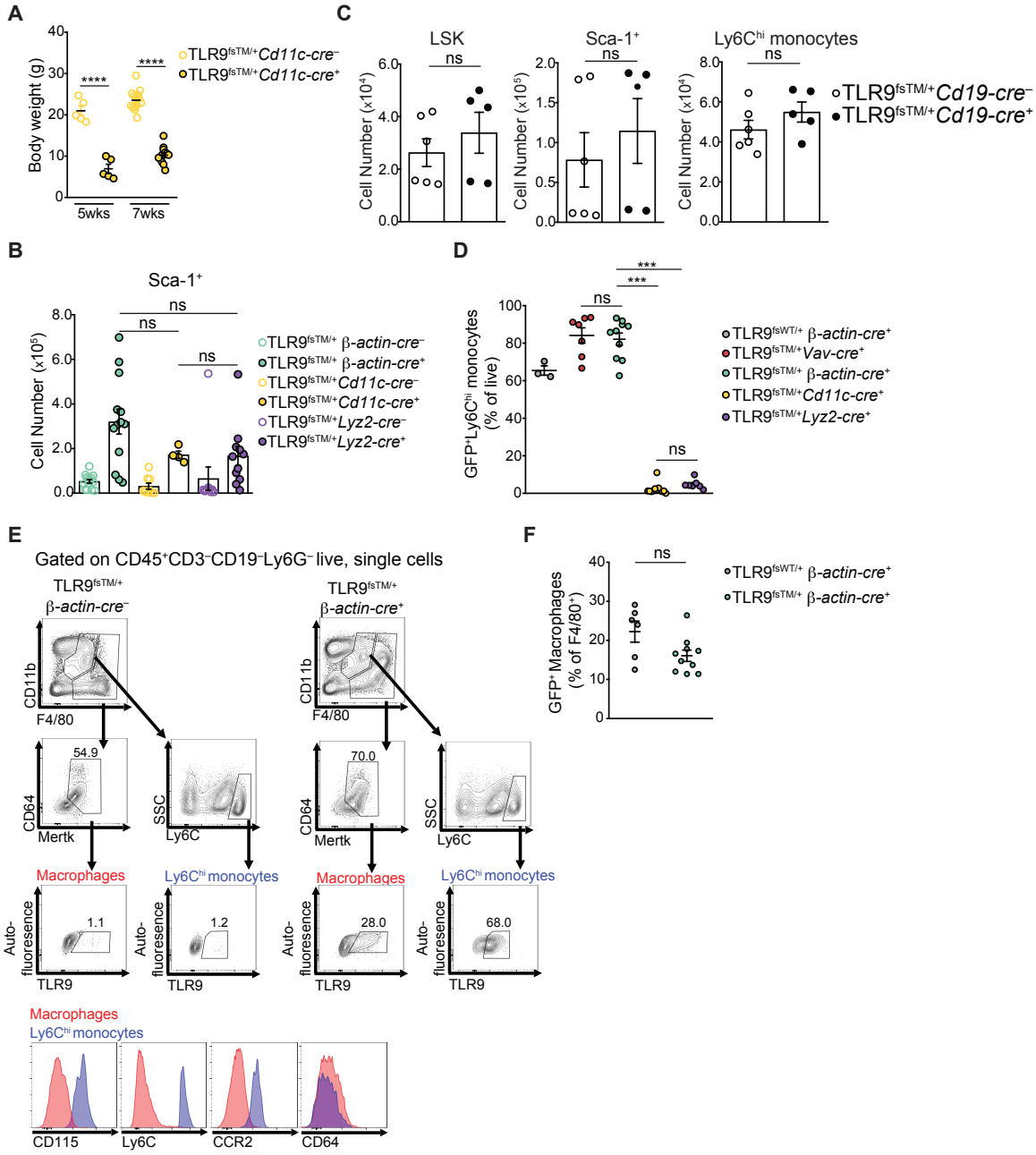
Supplementary Figure 2. Excess nucleic acids do not exacerbate disease in adult mice expressing TLR9^{fsTM}. (**A**) TLR9$^{TM/+}$*ER^{T2}-cre*$^{+}$ and TLR9$^{fsWT/+}$*ER^{T2}-cre*$^{+}$ mice were fed tamoxifen diet beginning at weaning and bled every week to monitor GFP expression. Frequency of circulating live, single CD11b^{+}GFP^{+} cells each week on tamoxifen diet. Data combined from independent experiments examining 3 or more mice per genotype per timepoint and analyzed using two-tailed Student's t test. (**B**) Representative flow cytometry plots showing the frequency of live, single CD45^{+}GFP^{+} and CD45^{-}GFP^{+} cells in the bone marrow and spleen from TLR9$^{fsWT/+}$*ER^{T2}-cre*$^{+}$ and TLR9$^{TM/+}$*ER^{T2}-cre*$^{+}$ mice after tamoxifen diet for six weeks. Data are representative of three independent experiments. (**C**) Irradiation of TLR9$^{fsTM/+}$*ER^{T2}-cre*$^{+}$ mice does not exacerbate disease. Shown is the survival curve of indicated genotypes. Data are representative of two independent experiments. P-values in all panels are defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Supplemental Figure 3



Supplementary Figure 3. Quantification of Ly6C^{hi} monocytes and gating strategy for eGMP in neonatal livers. (**A**) TLR9$^{fsTM/+}$ *β-actin-cre*$^{+}$ neonates exhibit an increase in Ly6C^{hi} monocyte cells in the liver as compared to TLR9$^{fsWT/+}$ *β-actin-cre*$^{+}$ neonates. Data combined from multiple litters are shown as mean ± SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons post-test. Mouse numbers: TLR9$^{fsWT/+}$ *β-actin-cre*$^{–}$ n = 6, TLR9$^{fsWT/+}$ *β-actin-cre*$^{+}$ n = 2, TLR9$^{fsTM/+}$ *β-actin-cre*$^{–}$ n = 11, and TLR9$^{fsTM/+}$ *β-actin-cre*$^{+}$ n = 6. * indicates $p < 0.05$ (**B**) Representative flow cytometry plots from neonatal livers of TLR9$^{fsTM/+}$ *β-actin-cre*$^{–}$ and TLR9$^{fsTM/+}$ *β-actin-cre*$^{+}$ mice showing the gating strategy for identification of eGMP populations. Gating is shown on CD45^{+}Lin$^{–}$ live, single cells.

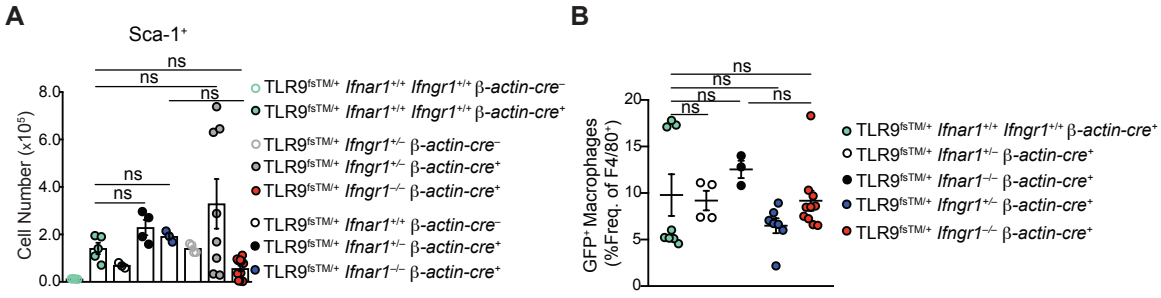
Supplemental Figure 4



Supplementary Figure 4. Characterization of TLR9^{TM} expression by CD11c^{+} cells, B cells, monocytes, and macrophages. (**A**) TLR9$^{fsTM/+}$ *Cd11c-cre*$^{+}$ mice are runted compared to littermate controls. Weight data are combined from multiple experiments at 5- and 7-weeks of age, shown as mean ± SEM, and analyzed using two-tailed Student's t test. Mouse numbers: TLR9$^{fsTM/+}$ *Cd11c-cre*$^{-}$ n = 5 and n = 15, TLR9$^{fsTM/+}$ *Cd11c-cre*$^{+}$ n = 5 and n = 10. (**B**) Quantification of Sca-1^{+} cell population in neonatal livers of the indicated mice. Data combined from multiple litters are shown as mean ± SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons post-test. Mouse numbers: TLR9$^{fsTM/+}$ *β-actin-cre*$^{-}$ n = 15, TLR9$^{fsTM/+}$ *β-actin-cre*$^{+}$ n = 13, TLR9$^{fsTM/+}$ *Cd11c-cre*$^{-}$ n = 9, TLR9$^{fsTM/+}$ *Cd11c-cre*$^{+}$ n = 4, TLR9$^{fsTM/+}$ *Lyz2-cre*$^{-}$ n = 10, and

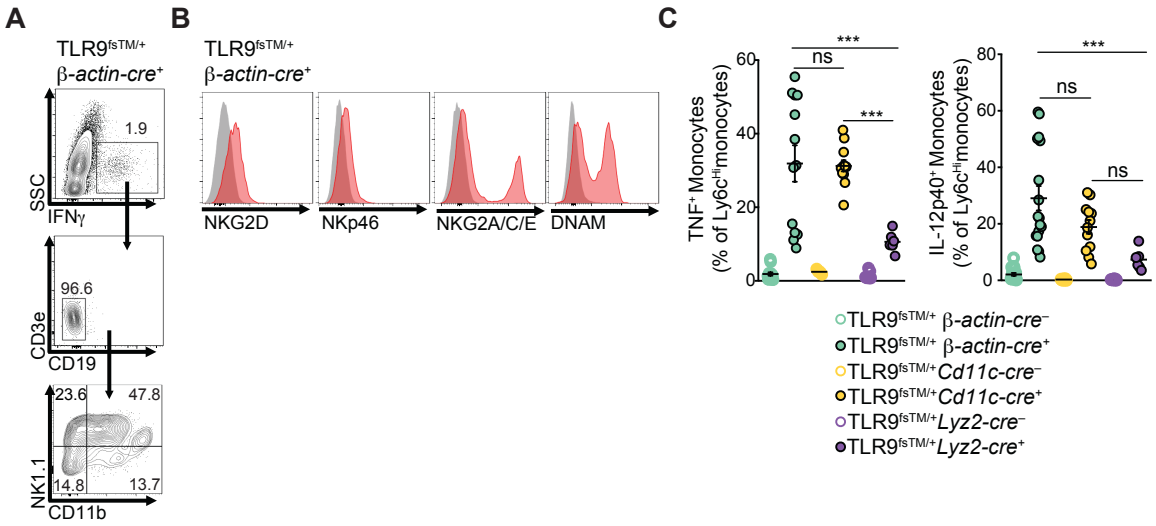
TLR9$^{fsTM/+}$ *Lyz2-cre*$^{+}$ n = 11. (**C**) TLR9$^{fsTM/+}$ *Cd19-cre*$^{+}$ neonates do not exhibit an expansion of progenitor cells or Ly6C^{hi} monocytes in the liver when compared to TLR9$^{fsTM/+}$ *Cd19-cre*$^{-}$ neonates. Shown are quantification of combined results from independent experiments, shown as mean ± SEM, and analyzed using two-tailed Student's t test. (**D**) The frequency of TLR9^{TM}-expressing Ly6C^{hi} monocytes does not correlate with disease severity. Quantification of the frequency of GFP+ Ly6C^{hi} monocytes from indicated genotypes. Data combined from multiple litters are shown as mean ± SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons post-test. Mouse numbers: TLR9$^{fsWT/+}$ *β-actin-cre*$^{+}$ n = 3, TLR9$^{fsTM/+}$ *Vav-cre*$^{+}$ n = 7, TLR9$^{fsTM/+}$ *β-actin-cre*$^{+}$ n = 12, TLR9$^{fsTM/+}$ *Cd11c-cre*$^{+}$ n = 9, and TLR9$^{fsTM/+}$ *Lyz2-cre*$^{+}$ n = 7. (**E**) Representative flow cytometry plots from neonatal livers of TLR9$^{fsWT/+}$ *β-actin-cre*$^{+}$ neonates demonstrating gating strategy for identification of neonatal macrophages. Gating is shown on CD45^{+}CD3^{-}CD19^{-}Ly6G^{-} live, single cells. (**F**) The frequency of TLR9-expressing (GFP+) macrophages is similar between TLR9$^{fsWT/+}$ *β-actin-cre*$^{+}$ and TLR9$^{fsTM/+}$ *β-actin-cre*$^{+}$ neonates. Data combined from multiple litters are shown as mean ± SEM and analyzed using two-tailed Student's t test. Mouse numbers: TLR9$^{fsWT/+}$ *β-actin-cre*$^{+}$ n = 6 and TLR9$^{fsTM/+}$ *β-actin-cre*$^{+}$ n = 10. *P*-values in all panels are defined as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplemental Figure 5



Supplementary Figure 5. Quantification of Sca-1$^+$ cell population and TLR9^{TM}-expressing macrophages in TLR9$^{fsTM/+}$*β-actin-cre$^+$Ifngr1$^{-/-}$* and TLR9$^{fsTM/+}$*β-actin-cre$^+$Ifnar1$^{-/-}$* neonates. (**A**) The Sca-1$^+$ cell populations in TLR9$^{fsTM/+}$*β-actin-cre$^+$Ifngr1$^{-/-}$* neonates and TLR9$^{fsTM/+}$*β-actin-cre$^+$Ifnar1$^{-/-}$* neonates is similar to TLR9$^{fsTM/+}$*β-actin-cre$^+$* neonates. Quantification of Sca-1$^+$ cells from neonatal livers of indicated genotypes (combined from multiple litters) is shown as mean ± SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons post-test. (**B**) Frequency of TLR9^{TM}-expressing macrophages in the neonatal livers of indicated genotypes. Data from multiple litters are shown as mean ± SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons post-test; ns indicates not significant. Mouse numbers: TLR9$^{fsTM/+}$ *β-actin-cre$^-$* n = 4, TLR9$^{fsTM/+}$ *β-actin-cre$^+$* n = 5, TLR9$^{fsTM/+}$*β-actin-cre$^-$Ifnar1$^{+/+}$* n = 4, TLR9$^{fsTM/+}$*β-actin-cre$^+$Ifnar1$^{+/-}$* n = 4, TLR9$^{fsTM/+}$*β-actin-cre$^+$Ifnar1$^{-/-}$* n = 3, TLR9$^{fsTM/+}$*β-actin-cre$^-$Ifngr1$^{+/-}$* n = 4, TLR9$^{fsTM/+}$*β-actin-cre$^+$Ifngr1$^{+/-}$* n = 7, and TLR9$^{fsTM/+}$*β-actin-cre$^+$Ifngr1$^{-/-}$* n = 9.

Supplemental Figure 6



Supplementary Figure 6. Identification of IFN-γ producing cells in TLR9^{TM}-expressing neonates. (**A**) Shown is a representative flow cytometry plot to identify IFN-γ^{+}NK1.1^{+} cells in neonatal livers of TLR9$^{fsTM/+}$*β-actin-cre*$^{+}$ mice. (**B**) Expression of canonical NK cell markers in the neonatal livers of TLR9$^{fsTM/+}$*β-actin-cre*$^{+}$ mice. Representative histograms of Lin^{-}CD45^{+}NK1.1^{+} cells examining expression of NKG2D, NKp46, NKG2A/C/E, and CD226 (DNAM). (**C**) Ly6C^{hi} monocytes from TLR9$^{fsTM/+}$ *β-actin-cre*$^{+}$ and TLR9$^{fsTM/+}$ *Cd11c-cre*$^{+}$ neonates secrete more TNF and IL-12p40 as compared to TLR9$^{fsTM/+}$ *Lyz2-cre*$^{+}$ neonates. Results are combined from multiple experiments and analyzed using one-way ANOVA with Tukey's multiple comparisons post-test. Mouse numbers: TLR9$^{fsTM/+}$ *β-actin-cre*$^{-}$ n = 10, TLR9$^{fsTM/+}$ *β-actin-cre*$^{+}$ n = 8, TLR9$^{fsTM/+}$ *Cd11c-cre*$^{-}$ n = 7, TLR9$^{fsTM/+}$ *Cd11c-cre*$^{+}$ n = 12, TLR9$^{fsTM/+}$ *Lyz2-cre*$^{-}$ n = 8, and TLR9$^{fsTM/+}$ *Lyz2-cre*$^{+}$ n = 6. *P*-values are defined as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.