Immunity, Volume *45*

Supplemental Information

Cross-Regulation of Two Type I Interferon Signaling Pathways in Plasmacytoid Dendritic Cells Controls Anti-malaria Immunity and Host Mortality

Xiao Yu, Baowei Cai, Mingjun Wang, Peng Tan, Xilai Ding, Jian Wu, Jian Li, Qingtian Li, Pinghua Liu, Changsheng Xing, Helen Y. Wang, Xin-zhuan Su, and Rong-Fu Wang

Supplemental online information

Cross-regulation of two type I interferon signaling pathways in plasmacytoid dendritic cells controls anti-malaria immunity and host mortality

Xiao Yu, Baowei Cai, Mingjun Wang, Peng Tan, Xilai Ding, Jian Wu, Jian Li, Qingtian Li, Pinghua Liu, Changsheng Xing, Helen Y. Wang, Xin-zhuan Su, and Rong-Fu Wang

Supplemental Figures: Figure S1-S7.

Supplemental Experiment Procedures

Figure S1

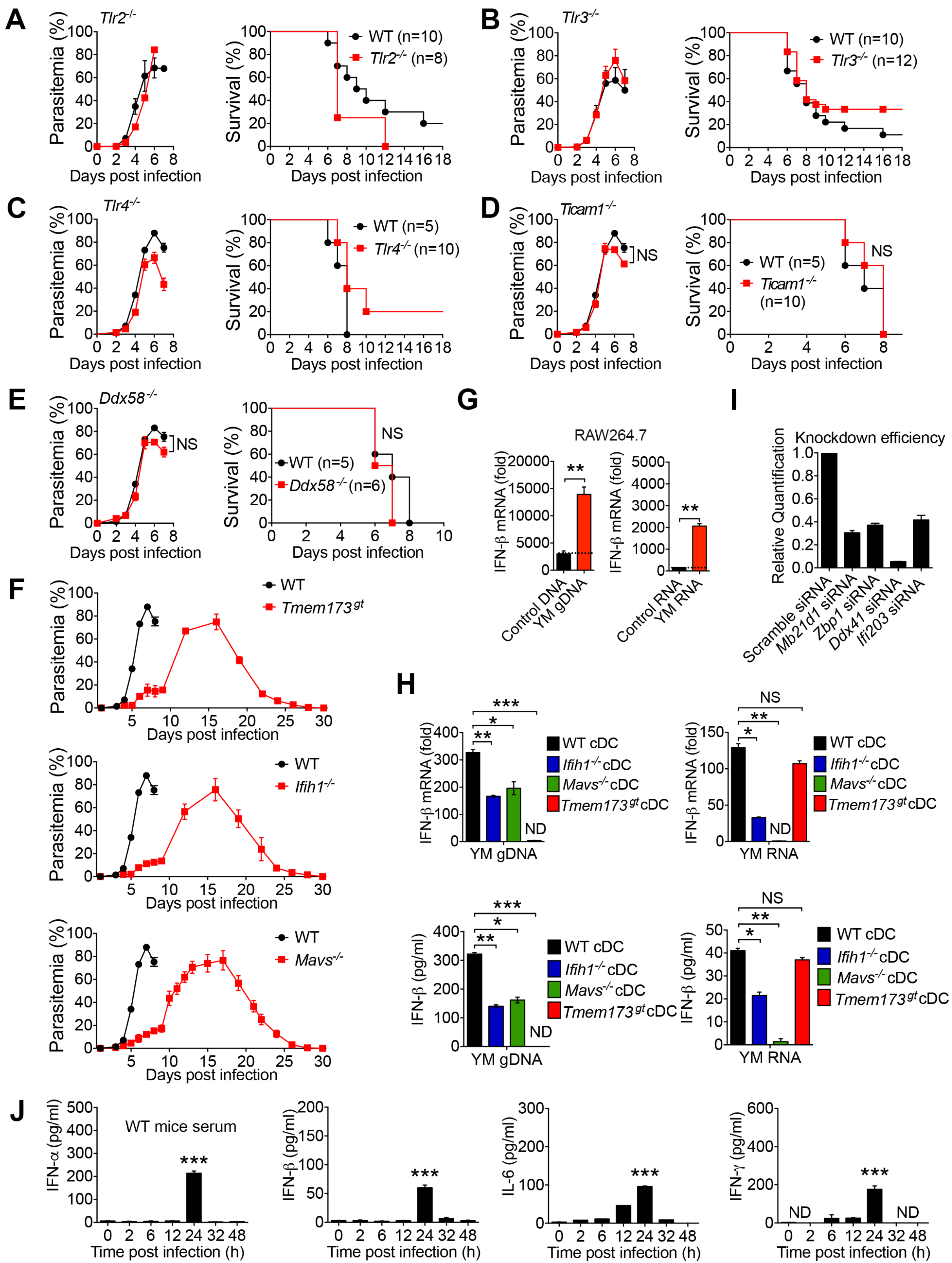

Figure S1. *Tlr2*$^{-/-}$, *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, *Ticam1*$^{-/-}$, and *Ddx58*$^{-/-}$ mice are susceptible to *Plasmodium yoelii* YM infection and Type I IFN gene expression after *P. yoelii* YM gDNA or RNA stimulation or serum cytokine production after *P. yoelii* YM infection. Related to Figure 1

(A-E) Daily YM parasitemias and mortality rates of WT (black lines) and deficient mice (red lines). C57BL/6 (WT), *Tlr2*$^{-/-}$, *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, *Ticam1*$^{-/-}$, and *Ddx58*$^{-/-}$ mice were intraperitoneally infected with *P. yoelii* YM (0.2-0.5×10^6 iRBCs). *Tlr2*$^{-/-}$ **(A)**, *Tlr3*$^{-/-}$ **(B)**, *Tlr4*$^{-/-}$ **(C)**, *Ticam1*$^{-/-}$ **(D)**, and *Ddx58*$^{-/-}$ **(E)**. **(F)** Daily YM parasitemias of WT (black lines), *Ifih1*$^{-/-}$, *Mavs*$^{-/-}$, and *Tmem173*^{gt} mice (red lines) after *P. yoelii* YM (1×10^6 iRBCs). **(G)** Quantitative analysis of IFN-β mRNA in RAW264.7 cells stimulated with purified *P. yoelii* YM genomic DNA (gDNA) or RNA from YM-infected RBCs. gDNA or RNA purified from uninfected RBCs served as a control. **(H)** Quantitative analysis and ELISA analysis of IFN-β mRNA and protein amount in cDCs derived from WT and deficient (*Ifih1*$^{-/-}$, *Mavs*$^{-/-}$, and *Tmem173*^{gt}) mice after *P. yoelii* YM gDNA or RNA stimulation. **(I)** Silencing efficiency of *Mb21d1*, *Zbp1*, *Ddx41*, and *Ifi203* in RAW264.7 cells transfected with specific siRNAs for *Mb21d1*, *Zbp1*, *Ddx41*, *Ifi203* or scrambled siRNAs for 48 h. **(J)** Serum amounts of IFN-α, IFN-β, IL-6 and IFN-γ from WT mice at the indicated times after *P. yoelii* YM infection, assessed by ELISA. Data are plotted as the mean ± s.d. and are representative of three independent experiments. **P* <0.05, ***P* <0.01, ****P* <0.001 vs. corresponding control. ND, not detected; NS, not significant.

Figure S2

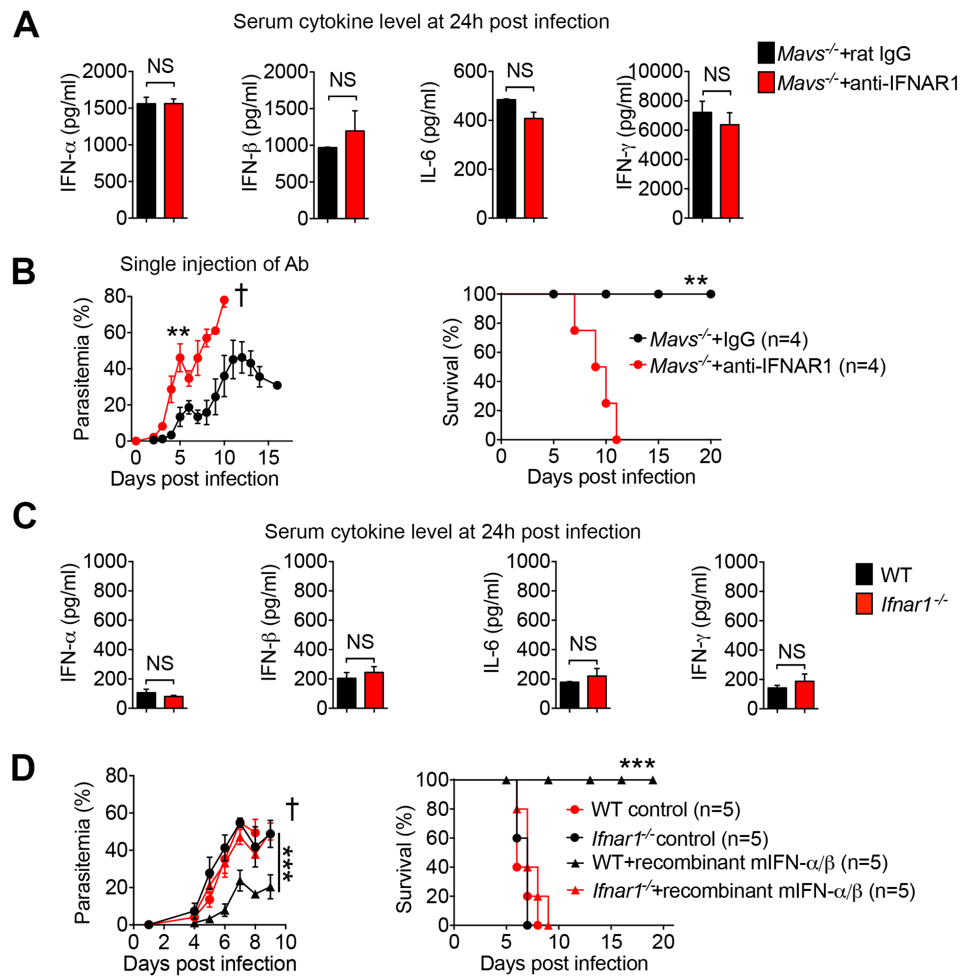

Figure S2. Blockage of type I IFN receptor determines the fate of *P. yoelii* YM infected mice. Related to Figure 2

(A) Serum amounts of IFN-α, IFN-β, IL-6, and IFN-γ collected at 24h after *P. yoelii* YM infection in *Mavs*$^{-/-}$ mice untreated or treated with anti-IFNAR1 blocking antibody (500 μg) single injection at 12 h before *P. yoelii* YM infection. **(B)** Parasitemias and mortality rates of *Mavs*$^{-/-}$ mice treated with anti-IFNAR1 blocking antibody (500 μg, single injection at 12h before infection), followed by infection with *P. yoelii* YM (2×10^6 iRBCs). **(C)** Serum amounts of IFN-α, IFN-β, IL-6, and IFN-γ in WT and *Ifnar1*$^{-/-}$ mice at 24 h after *P. yoelii* YM infection. **(D)** Parasitemias and mortality rates of WT and *Ifnar1*$^{-/-}$ mice infected with *P. yoelii* YM, followed by intravenous administration of recombinant mouse IFN-α and IFN-β at 18h post infection. Data are plotted as the mean ± s.d. and representative of three independent experiments. ***P* <0.01, ****P* <0.001 vs. corresponding control. NS, not significant. † denotes mouse death.

Figure S3

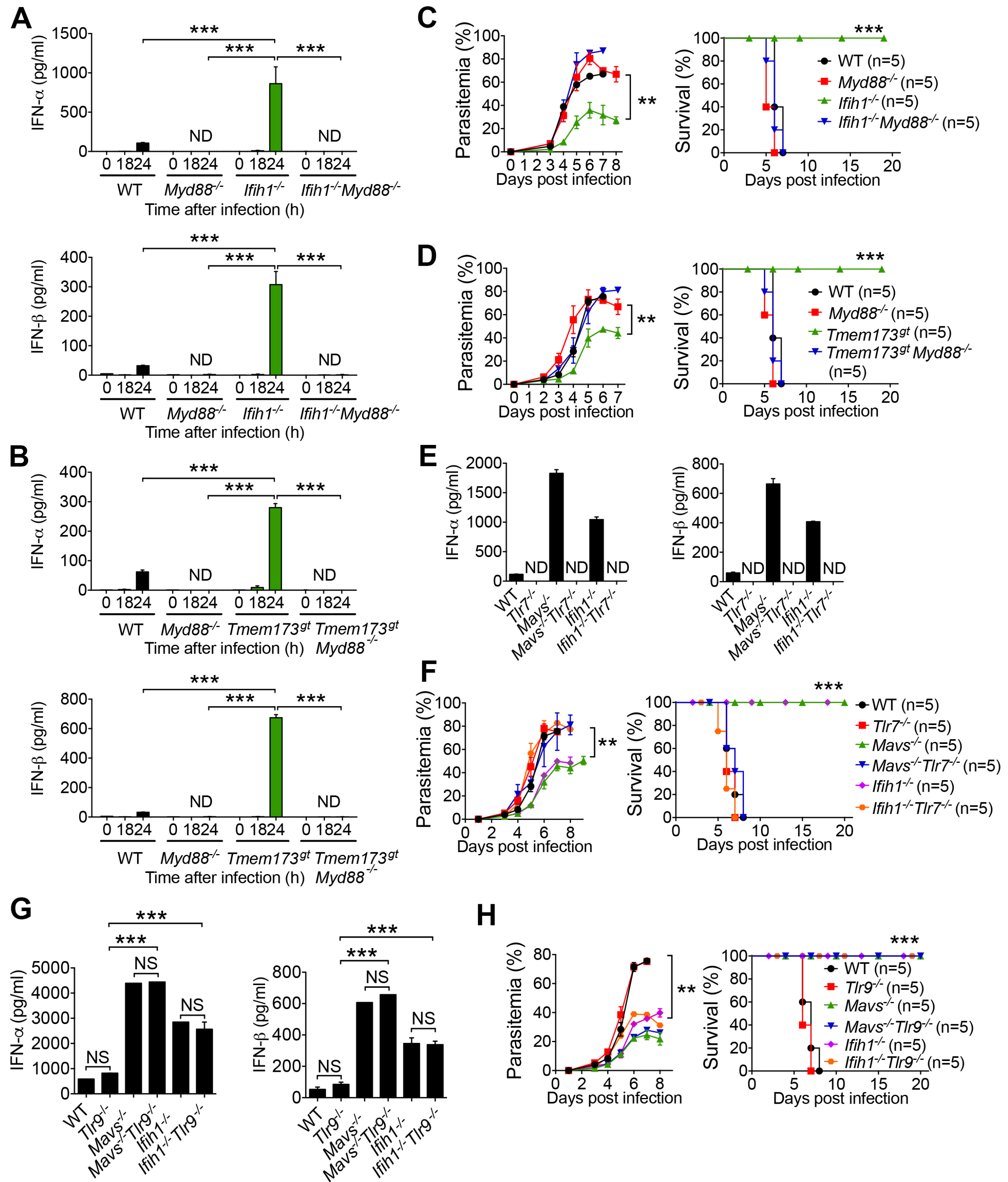

Figure S3. Requirement and regulation of TLR7-MyD88-IRF7 in type I IFN mediated protection of mice from *P. yoelii* YM infection. Related to Figure 3

(A-B) Serum amounts of IFN-α and IFN-β in WT and deficient (*Myd88*$^{-/-}$, *Ifih1*$^{-/-}$, *Ifih1*$^{-/-}$:*Myd88*$^{-/-}$, *Tmem173*^{gt} and *Tmem173*^{gt}:*Myd88*$^{-/-}$) mice at 0, 18, and 24 h after *P. yoelii* YM infection. **(C-D)** Daily YM parasitemias and mortality rates of WT and deficient (*Myd88*$^{-/-}$, *Ifih1*$^{-/-}$, *Ifih1*$^{-/-}$:*Myd88*$^{-/-}$, *Tmem173*^{gt} and *Tmem173*^{gt}:*Myd88*$^{-/-}$) mice after *P. yoelii* YM (1×10^6 iRBCs) infection. **(E)** Serum amounts of IFN-α and IFN-β in WT and deficient (*Tlr7*$^{-/-}$, *Mavs*$^{-/-}$, *Mavs*$^{-/-}$:*Tlr7*$^{-/-}$, *Ifih1*$^{-/-}$ and *Ifih1*$^{-/-}$:*Tlr7*$^{-/-}$) mice at 24 h after *P. yoelii* YM infection. **(F)** Daily YM parasitemias and mortality rates of WT and deficient (*Tlr7*$^{-/-}$, *Mavs*$^{-/-}$, *Mavs*$^{-/-}$:*Tlr7*$^{-/-}$, *Ifih1*$^{-/-}$ and *Ifih1*$^{-/-}$:*Tlr7*$^{-/-}$) mice after *P. yoelii* YM (1×10^6 iRBCs) infection. **(G)** Serum amounts of IFN-α and IFN-β in WT, *Tlr9*$^{-/-}$, *Mavs*$^{-/-}$, *Mavs*$^{-/-}$:*Tlr9*$^{-/-}$, *Ifih1*$^{-/-}$ and *Ifih1*$^{-/-}$:*Tlr9*$^{-/-}$ mice at 24 h after *P. yoelii* YM infection. **(H)** Daily YM parasitemias and mortality rates of WT and deficient (*Tlr9*$^{-/-}$, *Mavs*$^{-/-}$, *Mavs*$^{-/-}$:*Tlr9*$^{-/-}$, *Ifih1*$^{-/-}$ and *Ifih1*$^{-/-}$:*Tlr9*$^{-/-}$ mice) mice after *P. yoelii* YM (1×10^6 iRBCs) infection. Data are plotted as the mean ± s.d. and are representative of three independent experiments. ***P* <0.01, ****P* <0.001 vs. corresponding control. ND, not detected. NS, not significant.

Figure S4

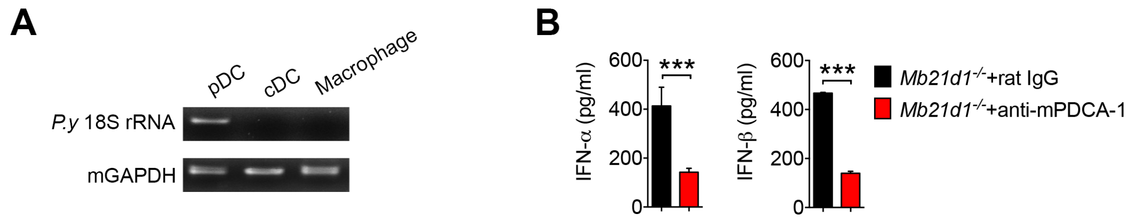

Figure S4. Detection of malaria 18S rRNA in pDCs, cDCs and macrophages and serum amount of type I IFN in cGAS-deficient mice after pDC depletion. Related to Figure 4

(A) The cell populations of pDCs, cDCs and macrophages were isolated from WT mice splenocytes at 18h post YM infection by cell isolation kits, and then analyzed for cell-specific expression of *P. yoelii* 18S rRNA by PCR. **(B)** Depletion of pDCs cell population in *Mb21d1*$^{-/-}$ mice by administration of anti-mPDCA-1 antibody at 12 h before and 12 h after YM infection, rat IgG treatment served as a control. Serum amounts of IFN-α and IFN-β collected at 24 h after infection in *Mb21d1*$^{-/-}$ mice untreated or treated with anti-mPDCA-1 are shown. Data are plotted as the mean ± s.d. and are representative of three independent experiments. ***P <0.001 vs. corresponding control.

Figure S5

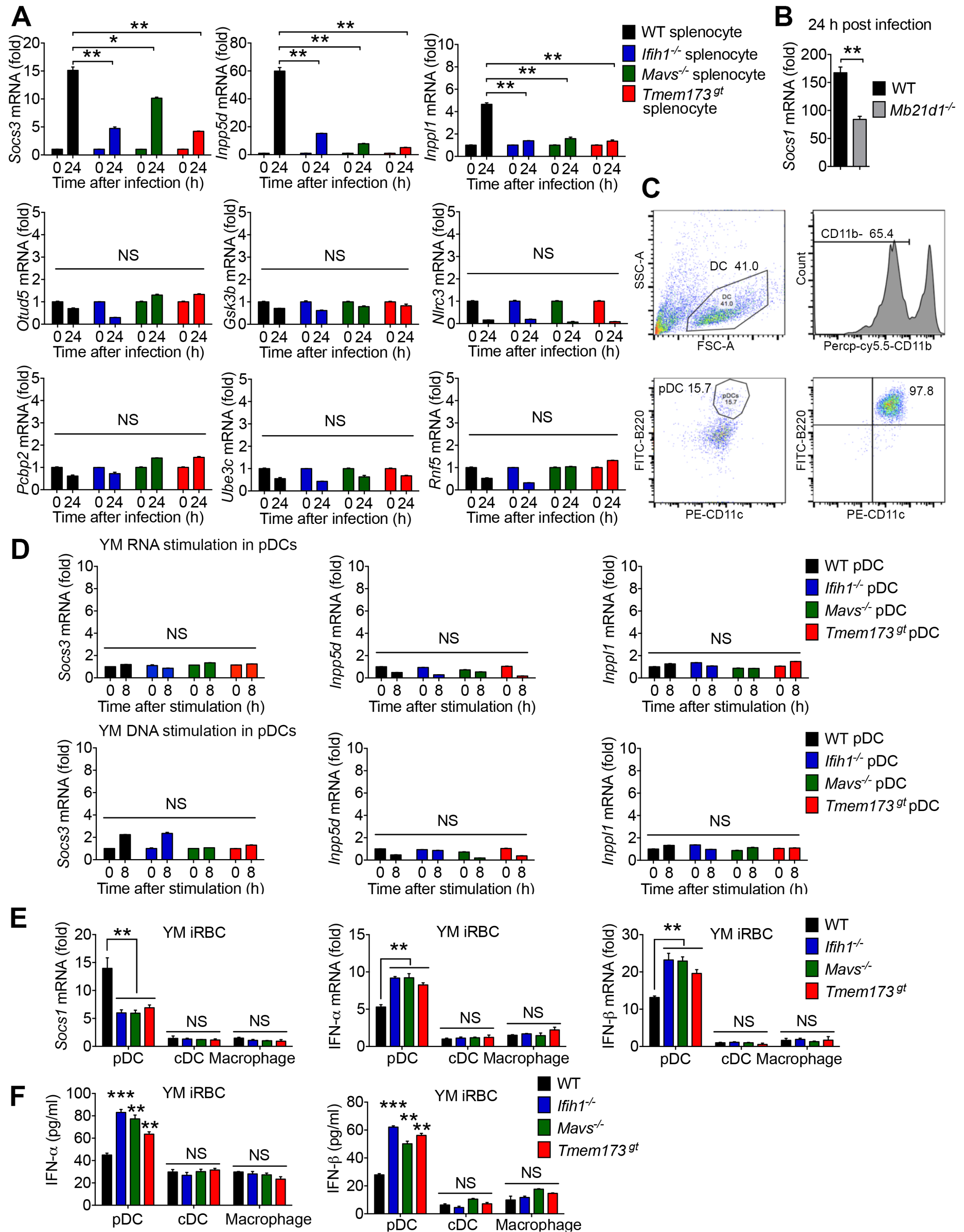

Figure S5. Identify putative negative regulators of type I interferon signaling after *P. yoelii* YM infection. Related to Figure 5

(A) Expression of putative negative regulators *Socs3*, *Inpp5d*, *Inppl1*, *Otud5*, *Gsk3b*, *Nlrc3*, *Pcbp2*, *Ube3c,* and *Rnf5* in the spleens of WT and deficient (*Ifih1*$^{-/-}$, *Mavs*$^{-/-}$, and *Tmem173*^{gt}) mice at the indicated times after *P. yoelii* YM infection. RNA from splenocytes was isolated and used for expression analysis using qPCR. **(B)** Expression of *Socs1* in the spleens of WT and *Mb21d1*$^{-/-}$ mice at 24 h after *P. yoelii* YM infection. RNA from splenocytes was isolated and used for expression analysis using qPCR. **(C)** Purification of pDCs (CD11b^{-}B220^{+}CD11c^{+}) by flow cytometry analysis. The last panel shows the percentage of pDCs after sorting. **(D)** Expression of *Socs3*, *Inpp5d* and *Inppl1* in pDCs of WT and deficient (*Ifih1*$^{-/-}$, *Mavs*$^{-/-}$, and *Tmem173*^{gt}) mice after *P. yoelii* YM RNA or gDNA stimulation. **(E)** Quantitative analysis of *Socs1* mRNA, IFN-α and IFN-β mRNA in pDCs, cDCs, and macrophages from WT and deficient (*Ifih1*$^{-/-}$, *Mavs*$^{-/-}$, and *Tmem173*^{gt}) mice after co-culture with *P. yoelii* YM iRBCs for 9 h. **(F)** ELISA analysis of IFN-α and IFN-β protein in supernatants of pDCs, cDCs, and macrophages from WT and deficient (*Ifih1*$^{-/-}$, *Mavs*$^{-/-}$, and *Tmem173*^{gt}) mice after co-culture with *P. yoelii* YM iRBCs for 24 h. Data are plotted as the mean ± s.d. and are representative of three independent experiments. **P* <0.05, ***P* <0.01, ****P* <0.001 vs. corresponding control. NS, not significant.

Figure S6

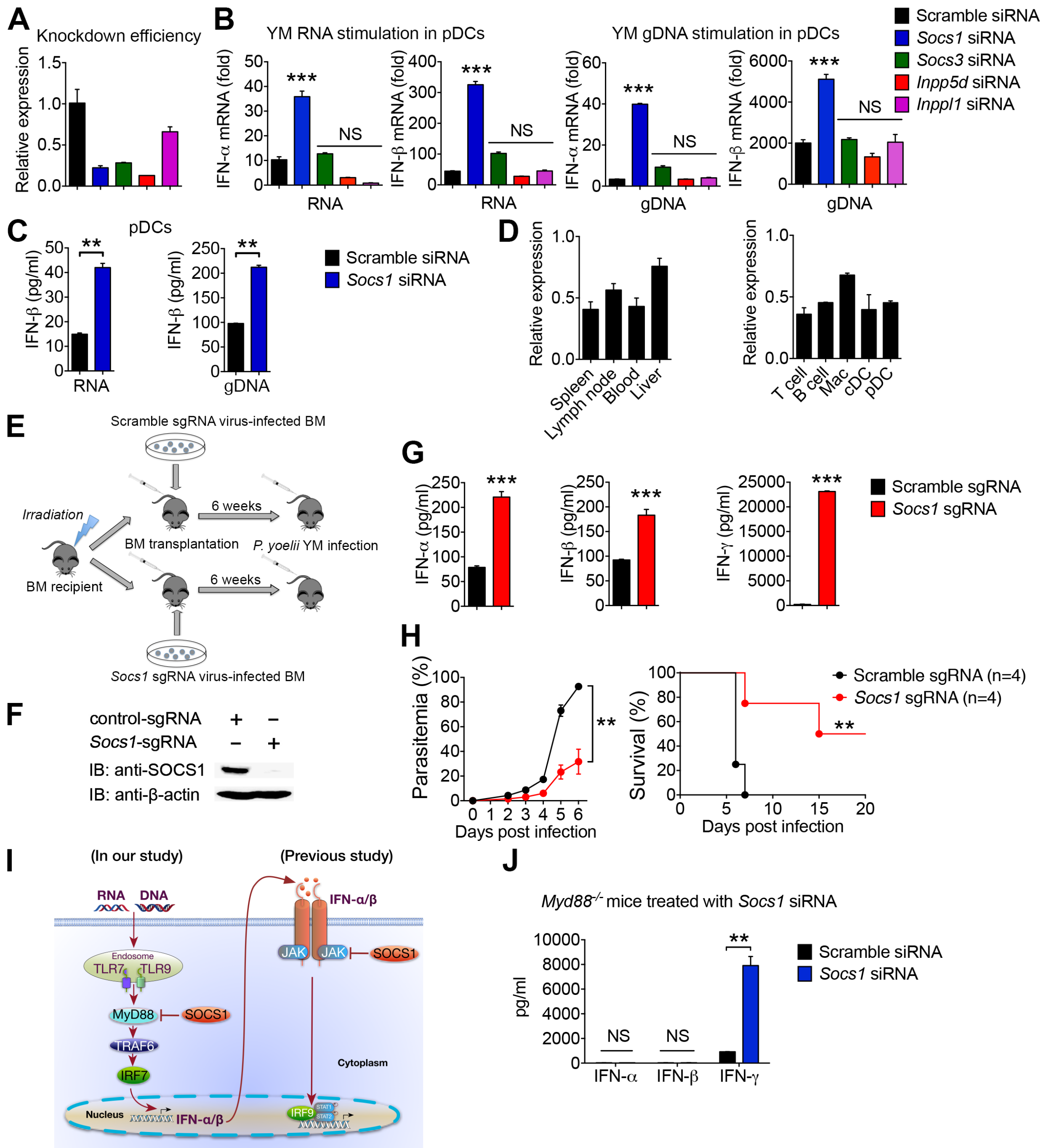

Figure S6. SOCS1 is responsible for inhibition of Myd88-dependent type I IFN in pDCs. Related to Figures 5 and 6

(A) Silencing efficiency of *Socs1*, *Socs3*, *Inpp5d* and *Inppl1* in pDCs transfected with specific siRNAs for *Socs1*, *Socs3*, *Inpp5d*, *Inppl1*, or scrambled siRNAs for 48 h. **(B)** Expression of IFN-α and IFN-β mRNA in WT pDCs transfected with *Socs1*-specific, *Socs3*-specific, *Inpp5d*-specific, *Inppl1*-specific, or scrambled siRNAs for 48h, followed by *P. yoelii* YM RNA or gDNA stimulation for 6 h. **(C)** IFN-β protein of pDCs transfected with *Socs1*-specific or scrambled siRNAs, followed by *P. yoelii* YM RNA or gDNA stimulation for 24 h. **(D)** Silencing efficiency of *Socs1* at 48 h after *Socs1*-specific siRNA delivery. C57BL/6 mice were injected intravenously with invivofectamine 3.0 (Invitrogen) mixed *Socs1*-specific siRNA or scrambled siRNA at dosage of 5mg/kg. Spleen, lymph node, liver and peripheral blood were collect at 48 h after siRNA injection and subjected to QPCR analysis of SOCS1 expression. Meanwhile, pDCs were isolated from spleen with anti-mPDCA-1 beads (Miltenyi); T cells, B cells, macrophages, and cDCs were isolated from spleen with PE-staining (CD3 for T cells, CD19 for B cells, F4/80 for macrophages and CD11c for cDCs, respectively.), followed by PE positive selection kit (Stemcell). RNA were isolated and subjected to QPCR analysis of SOCS1 expression. **(E)** Experimental procedure of genetically deletion of *Socs1* using CRISPR/CAS9 system in bone marrow for generating chimera mice. **(F)** Western blot analysis of *Socs1* deficiency in bone marrow cells after transduction with *Socs1*-specific or scrambled sgRNA lentiviral supernatant for 48 h. **(G)** Serum amounts of IFN-α, IFN-β, and IFN-γ in *Socs1*-specific or scramble sgRNA-treated mice at 24 h after *P. yoelii* YM infection. **(H)** Daily YM parasitemias and mortality rates of *Socs1*-specific or scrambled sgRNA-treated mice after *P. yoelii* YM infection. **(I)** A working model to explain SOCS1 inhibits Myd88 dependent type I IFN signaling and Jak1 dependent downstream signaling of type I IFN. (**J**) Serum amounts of IFN-α, IFN-β, and IFN-γ in *Socs1*-specific or scramble siRNA-treated *Myd88*$^{-/-}$ mice at 24 h after *P. yoelii* YM infection. Data are plotted as the mean ± s.d. and are representative of three independent experiments. ***P* <0.01, ****P* <0.001 vs. corresponding control. NS, not significant.

Figure S7

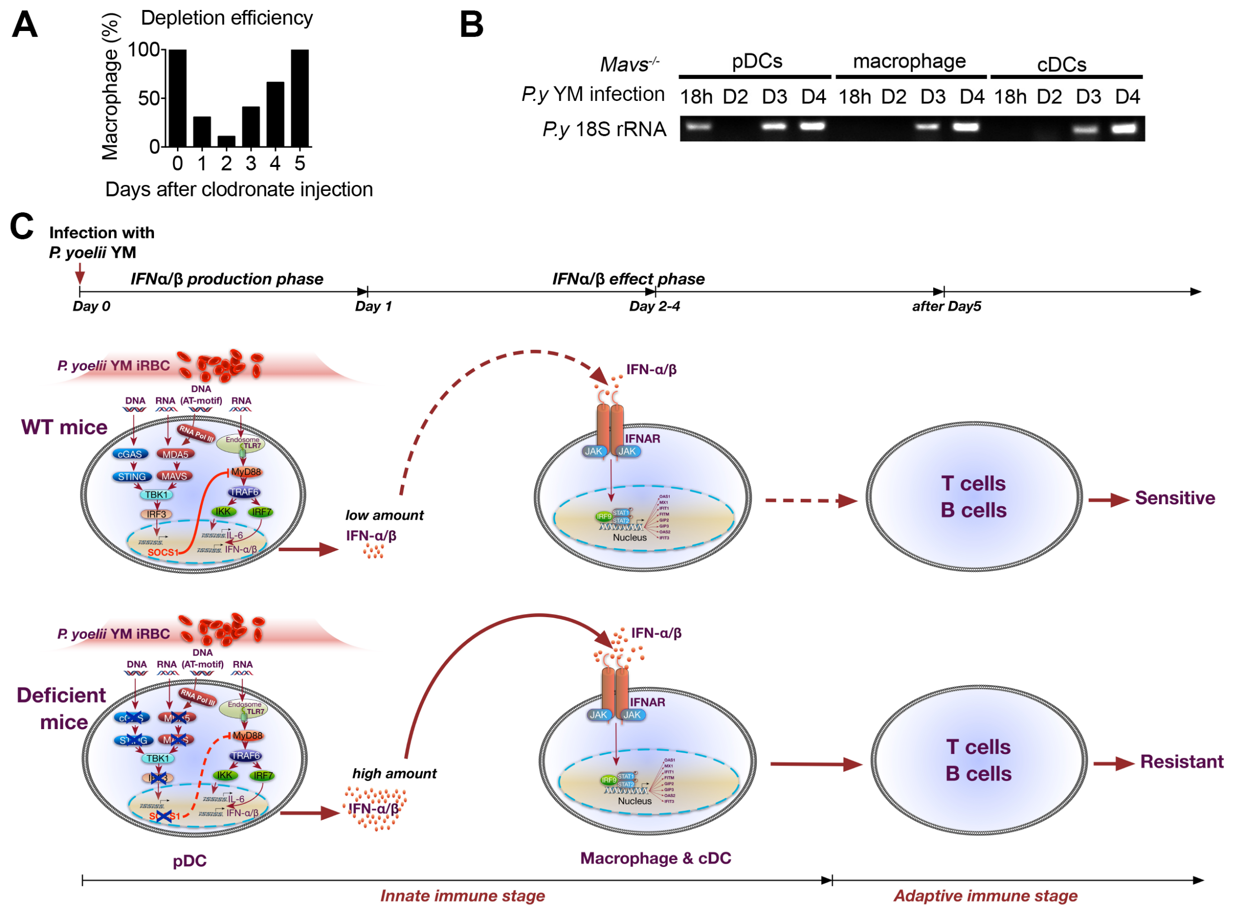

Figure S7. Stage-specific role of pDCs, cDCs, macrophages, T cells and B cells in generating IFN-α/β-induced immunity. Related to Figures 7

(A) *Tmem173*^{gt} mice were injected with clodronate (750 µg, intraperitoneally) and peripheral blood was collected at the indicated times and stained with CD11b, F4/80 for the flow cytometry analysis of macrophage percentage. Percentage of macrophage was compared to which in untreated mice. **(B)** The cell populations of pDC, cDC and macrophage were isolated from *Mavs*$^{-/-}$ mice splenocytes at indicated times post YM infection by cell isolation kits, and then analyzed for cell-specific expression of *P. yoelii* 18S rRNA by PCR. **(C)** A working model to illustrate how DNA/RNA sensors (cGAS, MDA5 and TLR7) detect malaria infection to activate two type I IFN signaling pathways in pDCs and the stage-specific roles of pDCs, macrophages, cDCs and T/B cells in generating IFN-α/β-induced immunity. Importantly, cGAS-STING and MDA5-MAVS-induced IRF3-dependent type I IFN signaling inhibits TLR7-MyD88-induced IRF7-dependent type IFN signaling pathway through upregulation of SOCS1 in WT pDCs in response to lethal *P. yoelii* YM infection. Deficiency in cGAS, STING, MDA5, MAVS or

IRF3 markedly increases IFN-α and IFN-β production at the early stage of infection (24 h) in deficient pDCs in response to lethal *P. yoelii* YM infection. Data are plotted as the mean ± s.d. and are representative of three independent experiments. **P <0.01, ***P <0.001 vs. corresponding control. NS, not significant. † denotes mouse death.

Supplemental Experiment Procedures

Malaria parasites and mice

The parasite *Plasmodium yoelii* YM has been previously described (Li et al., 2011). For *plasmodium* infection, 1×10^6 iRBCs (otherwise, indicated specifically in the figure legend) suspended in 200 µl PBS from the donor mice were intraperitoneally injected into experimental mice. Parasitemias were monitored daily by examination of Giemsa-stained thin tail blood smears. Female mice of C57BL/6 (WT), *BDCA2-DTR*, *Zbtb46-DTR, Ifnar1*$^{-/-}$, *Mavs*$^{-/-}$, *Ifih1*$^{-/-}$, *Myd88*$^{-/-}$, *Tmem173*^{gt}, *Ticam1*$^{-/-}$, *Tlr2*$^{-/-}$, *Tlr3*$^{-/-}$, and *Tlr4*$^{-/-}$ mice were purchased from The Jackson Laboratory. *Tlr9*$^{-/-}$ mice were a gift from Dr. Marco Colonna (Washington University School of Medicine, St. Louis, MO), *Tlr7*$^{-/-}$ mice from Dr. Richard A. Flavell (Yale University, New Haven, CT), *Ddx58*$^{-/-}$ mice from Dr. Wenxin Wu (University of Oklahoma Health Science Center), *Irf3*$^{-/-}$:*Irf7*$^{-/-}$ mice from Dr. Kate Fitzgerald (University of Massachusetts Medical School) and Dr. Tadatsugo Taniguchi (The University of Tokyo), and *Mb21d1*$^{-/-}$ mice from Dr. Skip Virgin (Washington University at St. Luis). All mouse-related procedures were performed according to experimental protocols approved by the Animal Care and Welfare Committee at Houston Methodist Research Institute and in accordance with NIH–approved animal study protocol LMVR-11E.

In vivo receptor blockade, cytokine treatment, and cell depletion

To block type I IFN receptor, anti-mouse interferon α/β receptor antibodies (Leinco Technologies), in the amount of 500 µg in PBS at day 0, 2, 4, and 250 µg at day 6 after infection (or indicated specifically in the figure legend), were intraperitoneally injected into WT and deficient mice. Mouse IgG antibody was used as control, and parasitemias were monitored by daily Giemsa-stained blood smears. To test the role of type I IFN cytokines, C57BL/6 mice were first infected with *P. yoelii* YM, and then intravenously injected with 800 U/g body weight mouse recombinant IFN-α and IFN-β, recombinant IL-6, or recombinant IFN-γ at the indicated time points. Bovine serum albumin (BSA, 0.1%) in PBS served as a control, and parasitemias were monitored by daily Giemsa-stained blood smears. To deplete pDCs, pDC-depleting functional-grade mAb (anti-mPDCA-1 IgG, clone JF05-1C2.4.1) was purchased from Miltenyi Biotec (Auburn, CA), and the corresponding isotype control IgG served as control. Two intraperitoneally injections of antibody (250 µg/mouse) per mouse were administered 12 h prior and after plasmodium YM infection or indicated specifically in the figure legend. To deplete macrophage, clodronate liposomes (from Dr. Nico. Van Rooijen) were injected intraperitoneally at 750 µg/injection at the indicated times, control liposomes served as control. T cells were depleted by intraperitoneally injection of anti-CD4 and anti-CD8 antibody every 3 days from 1 day before infection. B cells were depleted by intraperitoneally injection of anti-CD20 antibody every 4 days from 7 days before infection.

Isolation and preparation of *plasmodium* gDNA and RNA

Parasite-infected mice blood was collected in saline solution and filtered to deplete white blood cells. Parasites were spun down after RBC lysis buffer treatment, and lysate incubated with buffer A (150 mM NaCl, 25 mM EDTA, 10% SDS and protein kinase) overnight. gDNAs were isolated using phenol/chloroform, and RNAs were isolated using TRIzol reagent (Invitrogen).

Isolation and purification of immune cell populations

Bone marrow cells were isolated from the tibia and femur and cultured in RPMI1640 medium with 10% FBS, 1% penicillin-streptomycin, 55 µM β-mercaptoethanol, and 10% L929 conditioned media, containing macrophage-colony stimulating factor (M-CSF) for 5 days for BMDMs, 20 ng/ml murine GM-CSF and 10 ng/ml IL-4 for 6-8 days for cDCs. pDCs were generated from bone marrow in the culture medium containing 200 ng/ml Flt3L and further purified by flow cytometry analysis and were gated on the CD11b$^-$B220$^+$CD11c$^+$ cell population. For specific cell isolation from splenocytes, pDCs were isolated using anti-mPDCA-1 microbeads from Miltenyi Biotec (Auburn, CA). After pDC isolation, macrophages were isolated with CD11b microbeads from Miltenyi Biotec, cDCs were isolated with

mouse CD11cPE labeling and followed by PE selection cocktail from STEMCELL technologies, following the manufacturer's protocol.

Cell culture, transfection, immunoprecipitation and immunoblot analysis

HEK293T and RAW264.7 cells were maintained in DMEM (Hyclone) containing 10% FBS. STING stably expressing HEK293T cells (2×10^5) were plated in 24-well plates and transfected, through the use of Lipofectamine 2000 (Invitrogen), with 100 ng plasmids encoding Flag-tagged cGAS, DAI, DDX41, IFI16 or empty vector for 24 h, then stimulated with 1 ug YM gDNA or control DNA. Cells were collected at 18 h after DNA stimulation, and RNA were purified and subjected to QPCR analysis of IFN-β expression. For immunoprecipitation, 293T cells were transfected with 200 ng HA tagged SOCS1 and 300 ng Flag tagged IRAK1, IRAK4 or Myd88 for 48 h. Whole-cells extracts were prepared after transfection, followed by incubation overnight with the anti-Flag antibodies plus Protein A/G beads (Pierce). Beads were then washed five times with low-salt lysis buffer, and immunoprecipitates were eluted with 4×SDS loading buffer and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Bio-Rad) followed by further incubation with the appropriate antibodies. LumiGlo Chemiluminescent Substrate System was used for protein detection.

RNAi-mediated silencing in mice

In Vivo Ready siRNAs were mixed with Invivofectamine 3.0 liposomes (Invitrogen) following the manufacturer's instructions and injected intravenously in a volume of 100 µl at a dose of 5 mg/kg. Mice were infected with *P. yoelii* YM (0.5×10^6 iRBCs) at 48 h after siRNA treatment.

RNA preparation and qPCR

Total RNA was harvested from splenic tissue or stimulated cells using the TRIzol reagent (Invitrogen), and the complimentary cDNA was generated using reverse transcriptase III (Invitrogen). Real-time PCR was carried out using the ABI Prism 7000 analyzer (Applied Biosystems) using the SYBR GreenER qPCR Super Mix Universal (Invitrogen) and specific primers.

Isolation and purification of pDCs and Transfection with RNAi

Bone marrow cells were isolated and cultured with 200 ng/ml Flt3L for 9 days and isolated by flow cytometry analysis for pDCs ($CD11b^-B220^+CD11c^+$). siRNA oligonucleotides specific for *Mb21d1*, *Zbp1*, *Ddx41*, *Ifi203*, *Socs1*, *Socs3*, *Inpp5d*, *Inppl1*, and control (scrambled siRNA) were purchased from Invitrogen and nucleotransfected into RAW264.7 cells or pDCs cells for 48 h using Amaxa nucleofector kit, following the manufacturer's instructions (Lonza). Next, cells were stimulated with 1 µg *plasmodium* gDNA or RNA, using Lipofectamine 2000 reagent (Invitrogen), at the indicated time points. Supernatants were tested for cytokine production by ELISA, and RNAs were extracted for qPCR assay.

Mouse bone marrow transplant

Total bone marrow was isolated from the femurs and tibias from 8-week-old female C57BL/6 mice. Bone marrow was subjected to erythrocyte lysis, and then transduced with concentrated *Socs1*-specific or scrambled sgRNA lentiviral supernatant in the presence of 2 µg/ml polybrene. At 24 h post transduction, cells were collected and intravenously injected into lethally irradiated (950 cGy) 6-week-old female C57BL/6 mice.

Diphtheria Toxin (DT) treatment

Ifih1$^{-/-}$,*Mavs*$^{-/-}$ and *Tmem173*^{gt} mice were crossed with *BDCA2-DTR* transgenic mice to generate *Ifih1*$^{-/-}$: *BDCA2-DTR, Mavs*$^{-/-}$*:BDCA2-DTR* and *Tmem173*^{gt}*:BDCA2-DTR* mice, respectively, and then treated with diphtheria toxin (DT, Sigma-Aldrich) intraperitoneally (i.p.) at dose of 100-120 ng/mouse. pDCs were depleted by DT injection at 1 day before and 1, 3, and 5 days after *P. yoelii* YM infection. For cDC depletion, bone marrow chimeras were reconstituted for at least 6 to 8 weeks after lethal irradiation (950

cGy) and *i.v.* transferred with 10×10^6 bone marrow cells from *Mavs*$^{-/-}$:*Zbtb46-DTR* mice, then injected with DT at a dose of 2.5 ng per gram of body weight.

Statistical Analysis

All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Data are presented as means ± s.d., unless otherwise stated. Statistical significance of differences between two groups was assessed by unpaired Student *t* tests and a *p* value of <0.05 was considered significant.