Supplementary Information for

Brain endothelial STING1 activation by *Plasmodium*-sequestered heme promotes cerebral malaria via type I IFN response.

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Extended Material and Methods

Mice and treatments

All procedures involving laboratory mice were in accordance with national (Portaria 1005/92) and European regulations (European Directive 86/609/CEE) on animal experimentation and were approved by the Instituto Gulbenkian de Ciência Ethics Committee and the Direcção-Geral de Alimentação e Veterinária, the national authority for animal welfare.

Mice were housed and bred in the facilities of Instituto Gulbenkian de Ciência. *Myd88*$^{-/-}$ (MyD88 KO) mice were originally a gift from Prof. Shizuo Akira (Osaka University). *Mda5*$^{-/-}$mice were obtained from Dr. Maria Mota (IMM, Portugal). Transgenic mice expressing Cre under the GFAP promoter (GFAPCre) in C57BL/6 background were obtained from Dr. Henrique Veiga Fernandes (Champalimaud Foundation, Portugal).

Ifnβ1$^{-/-}$ (IFNβ KO), *Ifnβ1*$^{+/\Delta\beta\text{-luc}}$ (IFNβ-reporter mice), *Ifnβ1*$^{\text{floxβ-luc/floxβ-luc}}$ (IFNβ $^{\text{floxβ-luc/floxβ-luc}}$), *Trif*$^{-/-}$ (TRIF KO), B6(Cg)-*Tmem173*$^{tm1.2Camb}$/J (*Tmem173*$^{-/-}$, STING1 KO), Tg(Slco1c1-icre/ERT2)1Mrks (Slco1c1CreERT2) and *Myd88*$^{-/-}$*Trif*$^{-/-}$*Mavs*$^{-/-}$*Sting1*$^{-/-}$ (MyTrMaSt KO) mice were obtained from the TWINCORE, Centre for Experimental and Clinical Infection Research, Hanover, Germany.

C57BL/6J, B6;129-*Mavs*^{tm1Zjc}/J (MAVS KO), B6;SJL-*Sting1*$^{tm1.1Camb}$/J (STING1$^{\text{flox/flox}}$), B6.Cg-Tg(Tek-cre)1Ywa/J (Tie2Cre), B6J.B6N(Cg)-*Cx3cr1*$^{tm1.1(cre)Jung}$/J (CX3CR1Cre), B6.129P2(Cg)-*Cx3cr1*$^{tm2.1(cre/ERT2)Litt}$/*WganJ* (CX3CR1CreERT2) and B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/*J* mice (LysMCre) were originally purchased from The Jackson Laboratory (Bar Harbor, ME) (Table S1). MAVS KO mice were backcrossed to C57BL/6J mice up to 96.82% of recipient genome. IFNβ$^{\text{floxβ-luc/floxβ-luc}}$ and STING1$^{\text{flox/flox}}$ were crossed with the different Cre lines to generate cell lineage conditional IFNβ-reporter, IFNβ KO mice or STING1 KO mice. Experimental animals carrying one loxP-flanked and one KO allele

of *Sting1* were generated by crossing Slco1c1Cre^{ERT2}STING1$^{flox/flox}$ or CX3CR1Cre^{ERT2} STING1$^{flox/flox}$ mice with STING1 KO mice. The efficiency of the conditional *Sting1* deletion was expected to be improved by having just one floxed allele. Crossing of STING1$^{flox/flox}$ mice with CX3CR1CreSTING1$^{+/+}$ or Tie2Cre STING1$^{+/+}$ mice generated a deleted loxP-flanking Sting1 allele in the germ line. These mice were then crossed with STING1$^{flox/flox}$ and originated Cre^{+} mice with one deleted *Sting1* allele while another allele remained loxP-flanked and was conditionally deleted. Mice with either a deletion of the floxed allele or a KO allele but also expressing a floxed *Sting1* allele were designated STING1$^{flox/-}$.

Wild type, deleted and floxed alleles of *Ifnβ1* and *Sting1* were identified in experimental animals by genotyping using a 3-primer strategy (Table S2). For induction of *Sting1* deletion, 4-6 week old male and female mice were treated with tamoxifen (2 mg/10 g body weight) in corn oil administered by gavage every other day, totaling five doses. Mice were allowed to recover from tamoxifen treatment for 4 weeks.

For *in vivo* labelling of proliferating cells, mice received four intraperitoneal injections of BrdU (50 mg/kg,) every two hours. Brains were collected for FACS analysis two hours after the last injection and intracardiac perfusion of the mice with PBS.

As a positive control for luciferase activity in IFNβ-reporter mice in GFAP^{+} cells, mice received an intraperitoneal injection (i.p.) of 0.4 mg of Poly I:C.

Parasites and infection

Mice were infected with 10^{6} IE with the following parasite strains: *Plasmodium berghei* ANKA (*Pba*) or *Pba*-GFP (*44*) to induce CM or with *Pb* NK65, a strain that proliferates in mice but does not induce neurological signs of CM.

Frozen IE stocks were expanded in C57BL/6 mice prior to infection. Parasitemia in mice infected with non-GFP parasites (*Pba* and *Pb* NK65) and GFP-*Pba* was determined by flow cytometry analysis as the percentage of $DRAQ5^+$ erythrocytes (blood samples incubated with DRAQ5™, 3 uM) and GFP^+ erythrocytes, respectively (LSRFortessa™ X-20 cell analyser, BD Biosciences and FACSDiVa software version 6.2).

Evaluation of CM

Neurological score

Wild-type mice developed severe signs of CM such as head deviation, paralysis, and convulsions at day 6–7 post infection (PI) and died within 4–6 h of severe disease development. In different genetic backgrounds death by CM occurred up to 11 days PI ("CM time window"). Mice resistant to CM died around day 20 with hyperparasitemia (~60%) without signs of severe neurological dysfunction.

Disease severity was evaluated in mice before and after infection following an adapted health score for CM (*45*). From day 4 PI mice were scored based on eleven parameters: gait/body posture, grooming, motor performance, balance, hindlimb clasping reflex, limb strength, toe pinch, aggressiveness, head deviation, paralysis and convulsions. For each parameter a maximum score of two was given to good response or health condition (normal gait, full body extension, clean and sheen hair, exploring of 3-4 corners in 30 sec, extending front feet on the wall of the cage, normal plantar reaction, active pull away, toe pinch reaction, normal aggressiveness, no convulsions, no paralysis or no head deviation). Clear signs of CM (convulsions, paralysis and head deviation) with no strength, no balance and no toe pinch reaction scored zero. Healthy mice scored 22 and mice dying of CM could score between 3-16 points at day 6 PI.

Blood–brain barrier integrity assay

At day 6 PI, when C57BL/6J WT mice show signs of CM, the different mouse groups were killed 1 hour after retro-orbital injection of 100 µl of 2% Evans blue (EB) dye (Sigma) and were perfused intracardially with 15 ml of PBS. Brains were dissected, weighed, and pictured. EB retained in the brain tissue was extracted by immersion in 2 ml formamide (Merck) at 37°C in the dark for 48 h and measured by spectrophotometry at 620 and 740 nm (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer). Amounts of EB were calculated using an EB standard and expressed as mg EB/g of brain tissue.

Immune cells analysis

Spleen (day 5 PI) and brain (day 6 PI) single cell suspensions of infected and non-infected C57BL/6J and KO mice were immuno-stained for T cell and monocyte/macrophage activation cell surface markers. Brain single-cell suspensions were prepared as described before (*25*). Briefly, mice were perfused with 20 ml of PBS via the left ventricle of the heart. Brains were removed and homogenized in HBSS containing collagenase VIII (0.2 mg/ml) (Sigma). After incubation for 45 min at 37 °C, the digested tissue was minced, forced through a nylon cell strainer of 100 µm (BD Falcon™) and centrifuged. The pellet was resuspended in a gradient of 30% Percoll (Amersham Biosciences) in PBS and centrifuged at 520 g for 30 min at RT to obtain a pellet with brain mononuclear cells.

Spleen and brain mononuclear cells were incubated with anti-mouse Fc block in FACS buffer (2% FCS and 0.01% NaN3 in PBS) for 30 min at 4 °C to block irrelevant binding of the antibodies to the Fc receptor of myeloid cells. After washing, cells were stained with defined cocktails of fluorochrome-conjugated antibodies (Table S3). For intracellular staining, cells were incubated for 5h in RPMI complete medium containing

eBioscience™ Cell Stimulation Cocktail (Invitrogen). Cells were then washed and fixed and permeablized with BD Cytofix/Cytoperm™ (BD) before staining with anti-mouse Granzyme B-efluor 450 and anti-mouse IFNγ-PE (BioLegend). Brain cells isolated from *Pba*-infected mice injected with BrdU were stained using the FITC BD BrdU Flow Kit (BD Pharmingen™) according to the manufacturer's instructions. Stained cells were resuspended in FACS buffer with 5,000 of 10-µm counting beads (SureCount™ Standards, Bang Laboratories) and analyzed by flow cytometry (LSR Fortessa X20™, BD). Data were analyzed with FlowJo v10 software and cell types identified according to the gate strategy (Fig. S6)

Isolation of extracellular particles (EPs)

All the solutions were filtered (0.22 µm) before use. Blood EPs were obtained from mouse blood collected by cardiac puncture with a syringe containing 100 µl of EDTA, 0.5 M. Plasma was prepared by two successive centrifugations at 1500 g for 15 minutes at 4ºC. Harvested plasma was further centrifuged at 20 000g for 25 minutes and the pellet containing EPs was ressuspended in 0.2% BSA in PBS (*46*).

IE-derived EPs were prepared from the supernatant of IE cultures. Whole blood of *Pba* infected mice (day 5-6 PI, 10-20% parasitaemia) was collected by cardiac puncture with a syringe containing heparin and suspended in 5 ml of RPMI 1640 medium (Biowest), followed by centrifugation at 450*g* for 10 min. The pellet was resuspended in RPMI 1640 medium containing Glutamax and Neomycin (0.05 mg/ml), supplemented with 20% FBS (Biowest) without or with chloroquine, 8 µM, and then incubated in culture flasks overnight in an orbital shaker at 36.5ºC and 50 rpm. EPs were isolated adapting a previously described procedure (*34*) (Fig. 5a). Briefly, the cell suspension was first centrifuged at 600g to remove IE and non-IE and then centrifuged at 1600g for 15min to

remove cell debris. The supernatant of this centrifugation was then sequentially centrifuged at 5630g and 20 000g for 20 min (total EPs). The pellet resulting from centrifugation at 5630g was designated fraction 1 (Fr1) and the supernatant was further centrifuged at 20 000g producing a pellet, "–Fr1". Fr1 was resuspended in PBS and passed through a LS magnetic exclusion column according to the manufacturer's instructions (MACS Milteny Biotec). Particles retained in this column were designated Fr2. The flow through (Fr3) was centrifuged at 10 000g for 10 min and the obtained pellet incubated with biotinylted anti-mouse TER119 antibody (1 μg/ml) (Invitrogen). The pellet was then washed twice and resuspended in 80 μl of labeling buffer (PBS, 2 mM EDTA) containing 20 μl of Anti-Biotin MicroBeads (Milteny Biotec). After incubation for 15 min at 4ºC and washing, the pellet was resuspended in separation buffer (0.5% BSA, 2 mM EDTA in PBS) followed by magnetic separation through a second LS column. Particles retained in this column and flow through were designated Fr4 and Fr5, respectively.

Characterization of EPs

NanoSight

Blood-derived EPs were analyzed for particle concentration and size distribution by the NS300 Nanoparticle Tracking Analysis (NTA) system with a red laser (638 nm) (NanoSight –Malvern Panalytical, United Kingdom). Samples were pre-diluted in PBS to a concentration range of $5x10^7$-$1x10^9$ particles/ml and 10-50 particles/frame for optimal NTA analysis. Video acquisitions were performed using camera level 16 and threshold 5. Five videos of 30s each were captured per sample. Analysis of particle concentration per milliliter and size distribution were performed using the NTA software v3.4.

Flow cytometry

Blood MPs or IE-derived EPs were pre-incubated with Fc-block and then stained with PE-conjugated anti-mouse Ter119, CD41, CD45 and CD105 antibodies (1:100) (Life Technologies) during 20 min. After staining, the samples were washed in PBS (10 000*g*, 10 min, 4ºC) and the pellet was resuspended in PBS and acquired with flow cytometer LSRFortessa™ (BD Biosciences, run with FACSDiVa software version 6.2) using side scatter of blue violet laser (SSC-BV) and FSC-PMT tuned to detect small particles. PBS was used to set background signal and unstained samples used to define PE-negative particles. Total EPs numbers were calculated using 5000 counting beads (10 µm) in each sample.

Transmission electron microscopy

For negative staining, 5 µl of the pellet of serum MPs in HEPES buffer (10 mM) was adhered to glow discharged Mesh100 grids coated with 2% formvar in chloroform and carbon for 2 min. Following attachment, samples were washed with distilled H_2O and stained with 2% uranyl acetate for 2 min.

EPs samples were fixed with 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) overnight at 4ºC. After washing three times, samples were embedded for support in 2% low melting point agarose. After solidifying, samples were post-fixed with 1% osmium tetroxide in 0.1 M PB for 30 min at 4ºC. Samples were then washed in PB and distilled water and pre-stained with 1% tannic acid for 20 min at 4ºC and with 0.5% uranyl acetate for 1h at RT in the dark followed by dehydration using increasing concentrations of ethanol. After dehydration, samples were infiltrated and embedded using a graded series of Embed-812 epoxy resin polymerized at 60ºC overnight. Ultrathin sections (70 nm) were obtained with a Leica UC7 Ultramicrotome

and collected in 2% formvar in chloroform-coated slot grids that were post-stained with 1% uranyl acetate and Reynold's Lead Citrate for 5 min each.

Electron microscopy images were acquired on a FEI Tecnai G2 Spirit BioTWIN transmission electron microscope operating at 120 keV and equipped with an Olympus-SIS Veleta CCD Camera.

Brain endothelial cell cultures

Mouse brains were removed from the skull and kept in a petri dish with HBSS medium (Biowest) on ice. The brains were transferred to a freezer block covered with gauze and the meninges removed by rolling a cotton swab over each brain. Brain blood vessels were then isolated adapting a previously described procedure (*47*). Briefly, brains were minced with a blade in 1 ml of HBSS medium and aspirated with a syringe. The tissue was then homogenized by pushing it five times through a 23G needle. This homogenate was mixed with equal volume of cold 30% (wt/vol) dextran (MW ~60 kDa) (Sigma) in PBS and then centrifuged at 10 000g for 15 min. After centrifugation the myelin top layer was removed and the pellet containing the vessels was suspended in DMEM and passed through a 40-μm cell strainer. Five milliliters of medium was added to wash away other cells and myelin debris. The strainer was back-flushed with 5 ml of MCDB 131 to collect the brain vessels. These were digested by supplementing the medium with 1mg/ml of collagenase type IV (Millipore), 10 μg/ml DNAse (Roche) and 2% FBS, followed by incubation for 2-3 hours at 36.5 ºC in an orbital shaker. The digested vessels were then centrifuged and suspended in EGM™-2 Endothelial Cell Growth Medium-2 BulletKit™ (LONZA) supplemented with GlutaMax (ThermoFisher), 10% of FBS and 4 μg/ml puromycin, and cultured in 24 well-plates. Medium was changed for fresh medium without puromycin every four days.

When cultures reached 50-80% confluence (~14 days, 95% of the cells are $CD31^+$ by flow cytometry analysis), cells were exposed for 18-20h to different amounts ($2x10^5$-$2x10^6$) of IE and NIE, EPs fractions (Total EPs, -Fr1 and Fr1 to Fr5), MPs (present in 600 µl of serum), or different activators of IFNβ signaling (IFNβ, 1000 U/ml; LPS, 1µg/ml; Poly I:C, 1-5 µg/ml). To inhibit endosome acidification, BECs were pre-incubated for 20 min with 20 mM of NH4Cl before adding the stimuli.

Heme was added as ferric chloride heme (hemin, SIGMA) to BECs at a final concentration range of 10-30 µM. A freshly prepared stock solution was obtained by dissolving hemin in NaOH 0.1 M for 1h at 37ºC and then adding 1M Tris, pH 7.5 to a final concentration of 0.1 M. HCl was added to the solution to bring the pH back to 7.5.

Molecular docking

STING1-c-di-GMP crystal structure (PDB ID: 6S26) was retrieved from Protein Data Bank (PDB). The crystal structure was then prepared using the Molecular Operating Environment (MOE v2019.01) structure preparation wizard by stripping out the water molecules. Hydrogen atoms were added, and protonation states were assigned using Protonate-3D tool at pH=7.4 of the MOE software package. The co-crystallized ligand (c-di-GMP) was also prepared using the MOE software package. The EHT10 force field was used in all minimization steps. Again, hydrogen atoms were added using the Protonate-3D tool at pH=7.4.

C-di-GMP was then removed and re-docked into the corresponding STING1 crystal structure. Self-docking was performed in GOLD v5.7 software using ChemPLP as scoring function. The docking binding site was centered at threonine T263 (Fig. S5G), a crucial residue for c-di-GMP and STING1 interaction, and a search radius of 15 Å was set. Upon self-docking successfully validated the developed protocol, molecular docking

of heme was performed, 500 GA runs. Heme followed the same ligand preparation procedure as c-di-GMP.

Peroxidase activity and gel migration shift assay

Recombinant human STING1 protein (Gentaur) (0.3 μg, 1μM) in 20 mM Tris-HCl, 0.15 M NaCl, pH=8.0 (Tris buffer) was incubated for 15 min at room temperature (RT) with 3000, 600, 300, 150, 60 and 6 μM of heme. After adding Tris sample buffer (pH=6.8) for native PAGE (no SDS or reducing agents), the samples were loaded on 12% Criterion™ TGX™ Stain-Free™ precast gels for PAGE (Bio-Rad). Heme-STING1 complexes were separated by gel electrophoresis in non-denaturing running buffer. Peroxidase-like activity of heme bound to STING1 was detected on the gel after incubation with TMB Substrate Reagent Set (BD Biosciences). After developing a blue-colored band, the gel was washed with 0.5 M sodium acetate (pH=5) and isopropanol (30%). Migration shift of protein-heme complexes (*37*) was examined after washing the gel in Milli-Q water and staining with Commassie Brilliant Blue R250 (0.05% in 50% of methanol and 10% of acetic acid in H_2O) for 2 hours at RT, followed by incubation with destaining solution (10% etanol and 7.5% acetic acid in H_2O). STING1 without incubation with heme and heme alone were used as negative controls for peroxidase-like activity. Serum bovine albumin (3.3 μg, 5μM) incubated with 600 μM of heme was used as a positive control (*48*).

Gene expression

RNA was prepared from total mouse brains of mice and from BECs using Trizol and Cells-to-CT kit (Thermo Fisher Scientific), respectively. One microgram of total brain RNA was converted to cDNA (Transcriptor High Fidelity cDNA Synthesis Kit; Roche).

A final volume of 20 µl of the cDNA reaction was diluted 1:3 in RNase-free water to be used in qRT-PCR. *Ifnβ1*, *Cxcl10*, *Irf7*, *Irf1* and *Ifit1* mRNA were quantified using Mm00439552_s1, Mm00445235_m1, Mm00516788_m1, Mm01288580_m1 and Mm00515153_m1 TaqMan Gene Expression Assays from ABI, respectively.

IFNβ-luciferase reporter assay

Tissues were lysed in Glo Lysis Buffer (Promega) followed by centrifugation at 20,000g for 20 min. BECs were gently washed with PBS before lysis. 50 µl of tissue or cell lysates were mixed with 50µl of Bright-Glo™ Luciferase Assay System (Promega) in a 96-well luminometer plate. The luminescence was immediately measured with GloMax® Explorer (Promega) (excitation, 405nm; emission, 500-550nm; integration time, 5). Protein was quantified by Bradford Assay (BioRad) and luciferase activity expressed as RFU per mg of protein.

Satistical Analysis

Statistical analysis was performed using Prism v6.01 software (GraphPad Software, Inc.). Comparisons between treated and non-treated conditions used two-tailed unpaired t-test, comparisons between multiple groups used one-way ANOVA, comparisons between treated cells of different genotypes used two-way ANOVA as indicated in figure legends. Bars in figures represent the mean (±SD) and each symbol represents one mouse or well as indicated in the figures. Sample size and number of the independent experiments performed can be found in the figures and figure legends.

Supplemental Figure 1

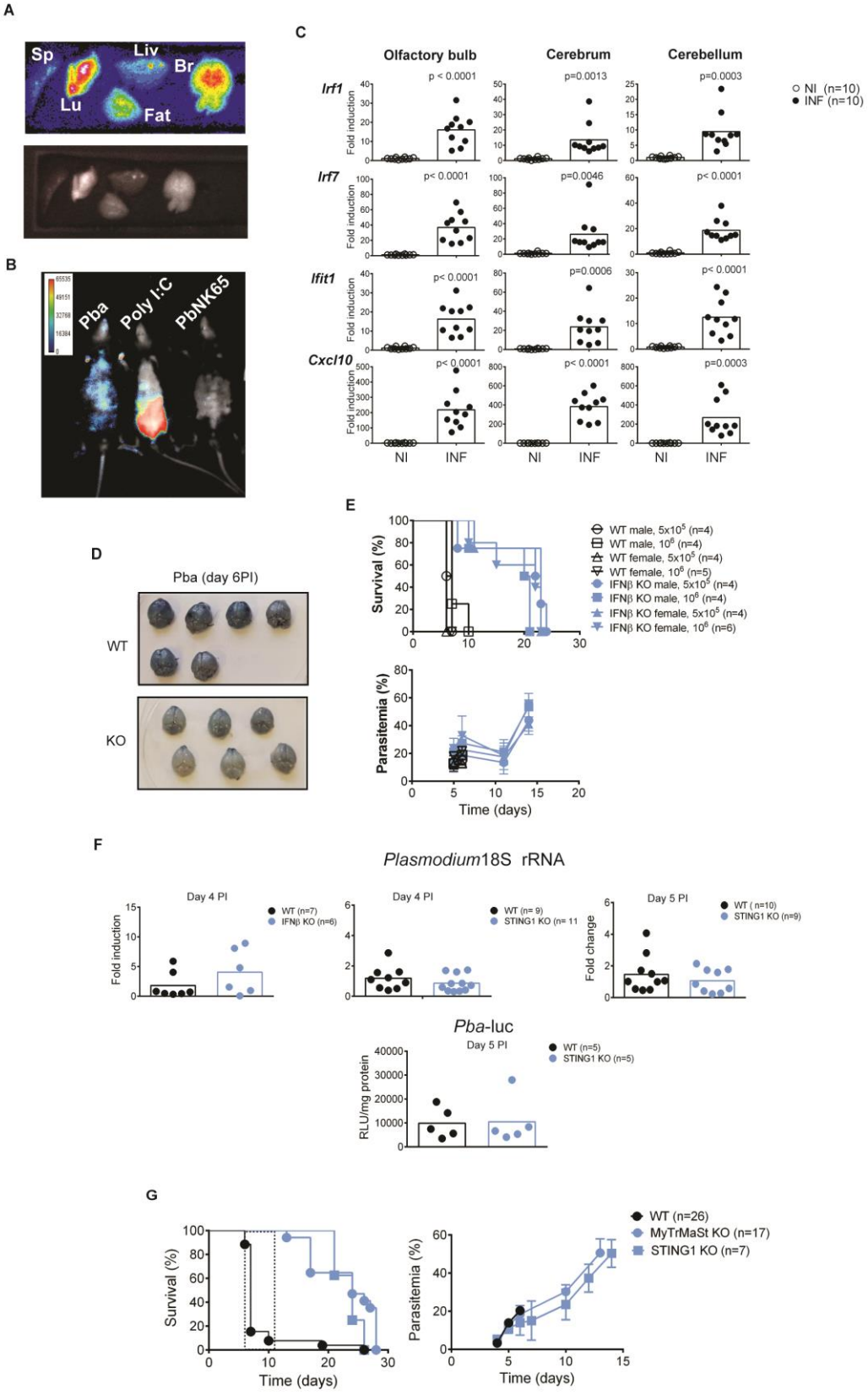

Fig. S1. **Role of IFNβ signaling in CM development.**

(A) Ex vivo luminescence (upper panel) and corresponding light imaging (lower panel) of spleen (Sp) lung (Lu), liver (Liv), abdominal fat (Fat) and brain (Br) of IFNβ-reporter mice infected with *Pba* (day 5, 20 % parasitaemia). **(B)** *In vivo* luminescence imaging of IFNβ-reporter mice infected with *Pba* (10-20% parasitaemia with CM), injected with a IFNβ inducer (Poly I:C) or with a strain of *P. berghei* that does not induce CM (PbNK65) (day 18, 13% parasitaemia). **(C)** Interferon response signature detected in the designated brain regions was evaluated by measuring interferon-stimulated gene expression by quantitative PCR (qPCR). Data points represent individual mice from two independent infection experiments. Symptomatic *Pba*-infected mice (day 6 PI) were compared to non-infected mice using unpaired, two-tailed *t*-test (p values on the figure). **(D)** Photograph of brains collected from WT and IFNβ KO mice infected with *Pba* (day 6 PI) after Evans blue i.v. and perfusion. **(E)** Survival and parasitemia curves of WT and IFNβ KO male and female mice, infected with the indicated amounts of *Pba*-IE. **(F)** Parasite load in the brain of WT and KO mice was detected by measuring the *Plasmodium 18S rRNA* gene expression by qPCR. Luciferase activity was measured in the brains of WT and STING1 KO mice infected with a transgenic *Pba* line expressing luciferase (Pba-luc).

(G) Survival and parasitemia curves of WT, STING1 KO and quadruple KO mice for IFNβ critical components of IFNβ induction (*MyD88*$^{-/-}$, *Trif*$^{-/-}$, *Mavs*$^{-/-}$ and *Sting1*$^{-/-}$) infected with *Pba*. Protection from CM development in quadruple KO mice, is recapitulated in the STING1 KO alone.

Supplemental Figure 2

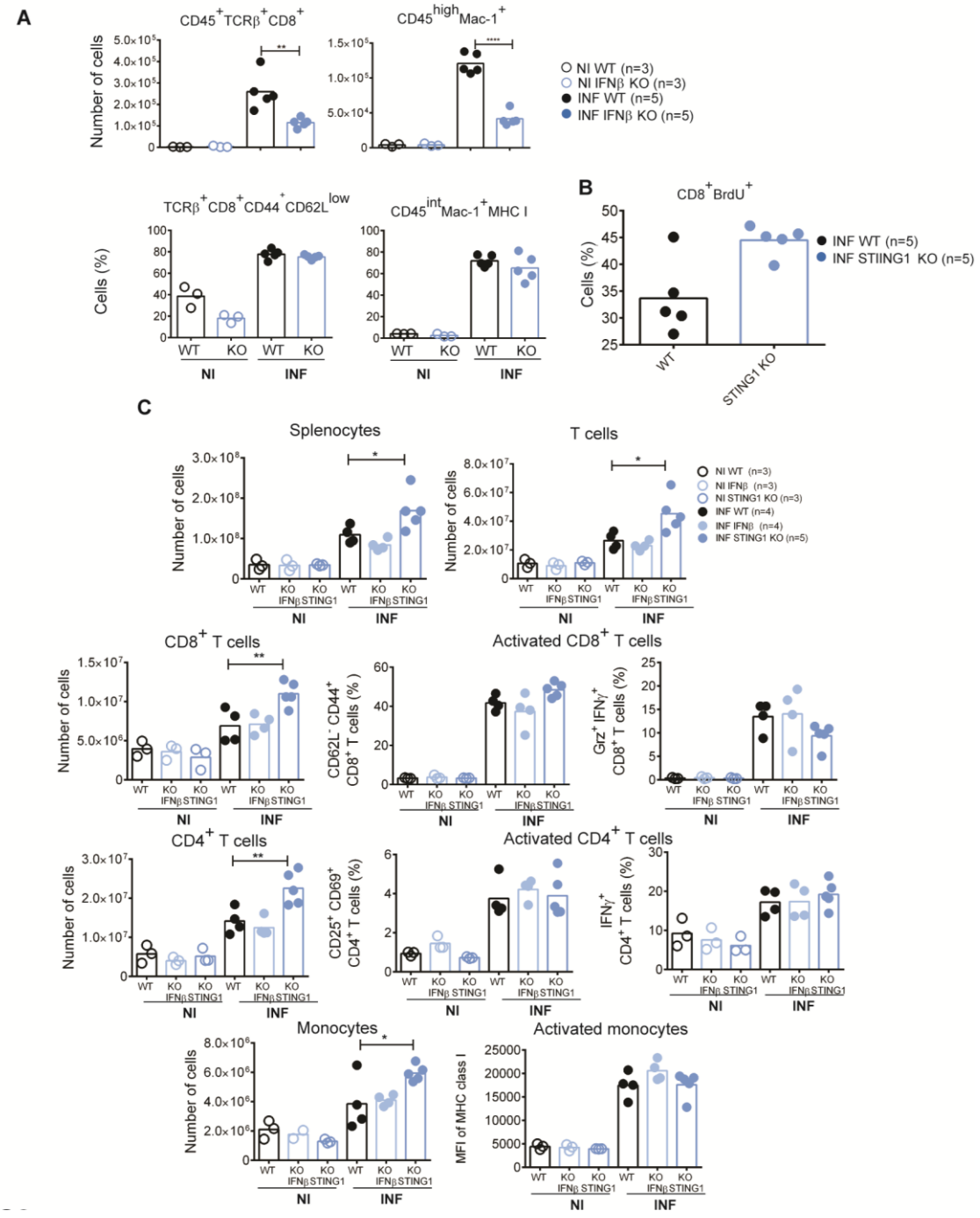

Fig. S2. Decreased leukocyte infiltration in the brain is not associated with deficient cell activation. (A) Flow cytometry analysis of brain leukocyte infiltration in WT and

IFNβ KO mice infected with *Pba*. The number of infiltrating $CD8^+$ T cells and monocytes are lower in IFNβ KO while cell activation profiles are maintained.

(B) Flow cytometry analysis of brain $CD8^+$ cells that incorporated BrdU *in vivo* in mice with CM. **(C)** Flow cytometry analysis of splenocytes in *Pba*-infected WT, IFNβ KO and STING1 KO mice at day 5 PI. The number of peripheral $CD8^+$ T cells, $CD4^+$ T cells and monocyte are increased in STING KO mice but the activation profile is not affected. Non-infected (NI) and *Pba*-infected mice of the different genotypes were compared using two-way ANOVA (* $p<0.05$, ** $p<0.01$ and **** $p<0.0001$).

Supplemental Figure 3

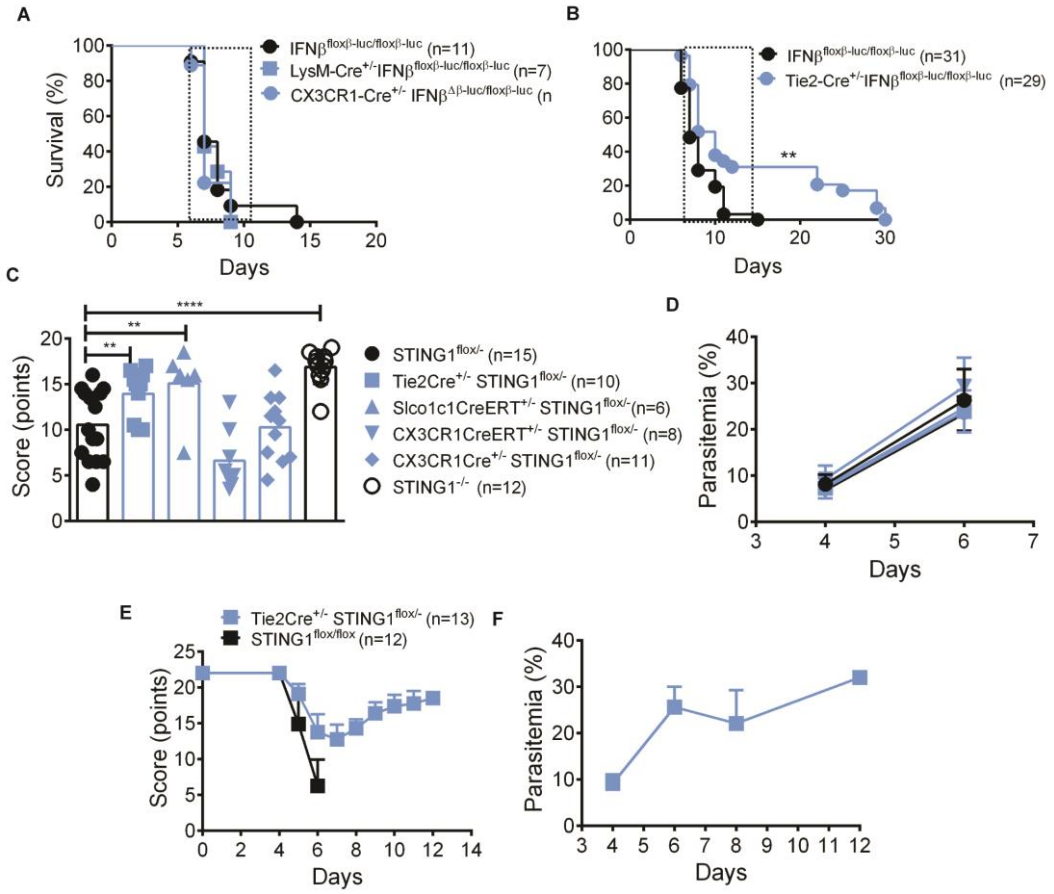

Fig. S3. **Development of CM in mice with *Ifnβ1* or *Sting1* conditional deletion in myeloid and endothelial compartments**.

(A) Survival of *Pba*-infected mice with conditional IFNβ deletion targeting macrophages (LysM-Cre), microglia (CX3CR1-Cre) and **(B)** endothelial cells (Tie2-Cre). Gehan-Breslow-Wilcoxon test, **p<0.01 **(C)** Health/neurological score of mice with STING-specific deletion in the different cellular compartments. Non-infected animals have a score of 22 that decreases in mice with CM at day 6 PI. Data were compared by unpaired, two-tailed *t*-test. **p<0.01, ***p<0.001 by Mann Whitney test. **(D)** Parasitemia at day 4 and day 6 in conditional STING1 KO mice. **(E)** Kinetics of the health/neurological score, and **f**, parasitemia in Tie2^{+} cell-specific conditional STING1 KO mice.

Supplemental Figure 4

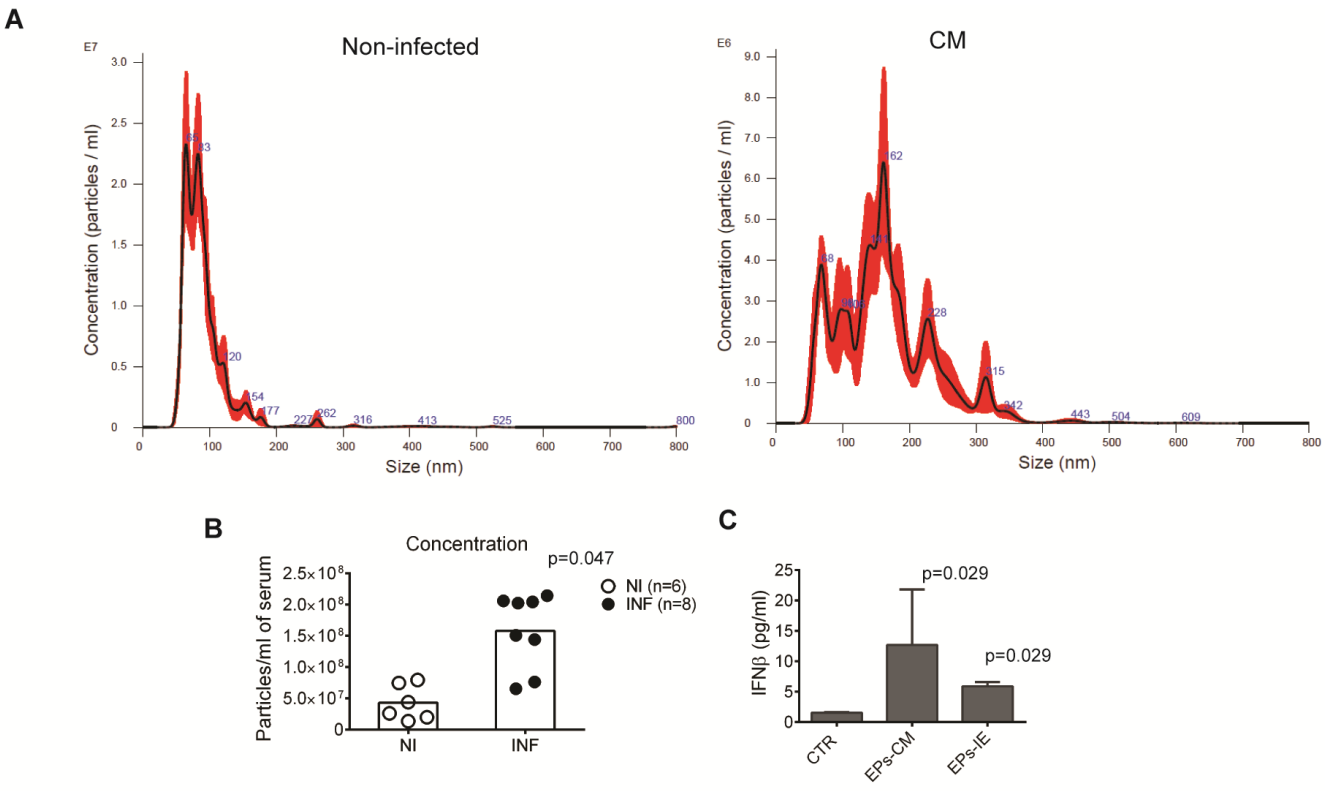

Fig. S4. Characterization of extracellular particles obtained from serum of CM affected mice and from *Pba*-IE cultures.

(A) Representative histogram of the size and concentration of EPs obtained from non-infected and CM wild-type mice. **(B)** NanoSight analysis of concentration of particles within the range of 100.5-265.5 nm in EPs collected from symptomatic *Pba*-infected mice (day 6 PI) and non-infected control mice. **(C)** In vitro IFNβ secretion in brain endothelial cells stimulated with particles obtained from the serum of *Pba*-infected animals (EPs-CM) or *Pba*-IE cultures (EPs-IE), measured by ELISA in cell culture supernatants. Data were compared by unpaired, two-tailed *t*-test.

Supplemental Figure 5

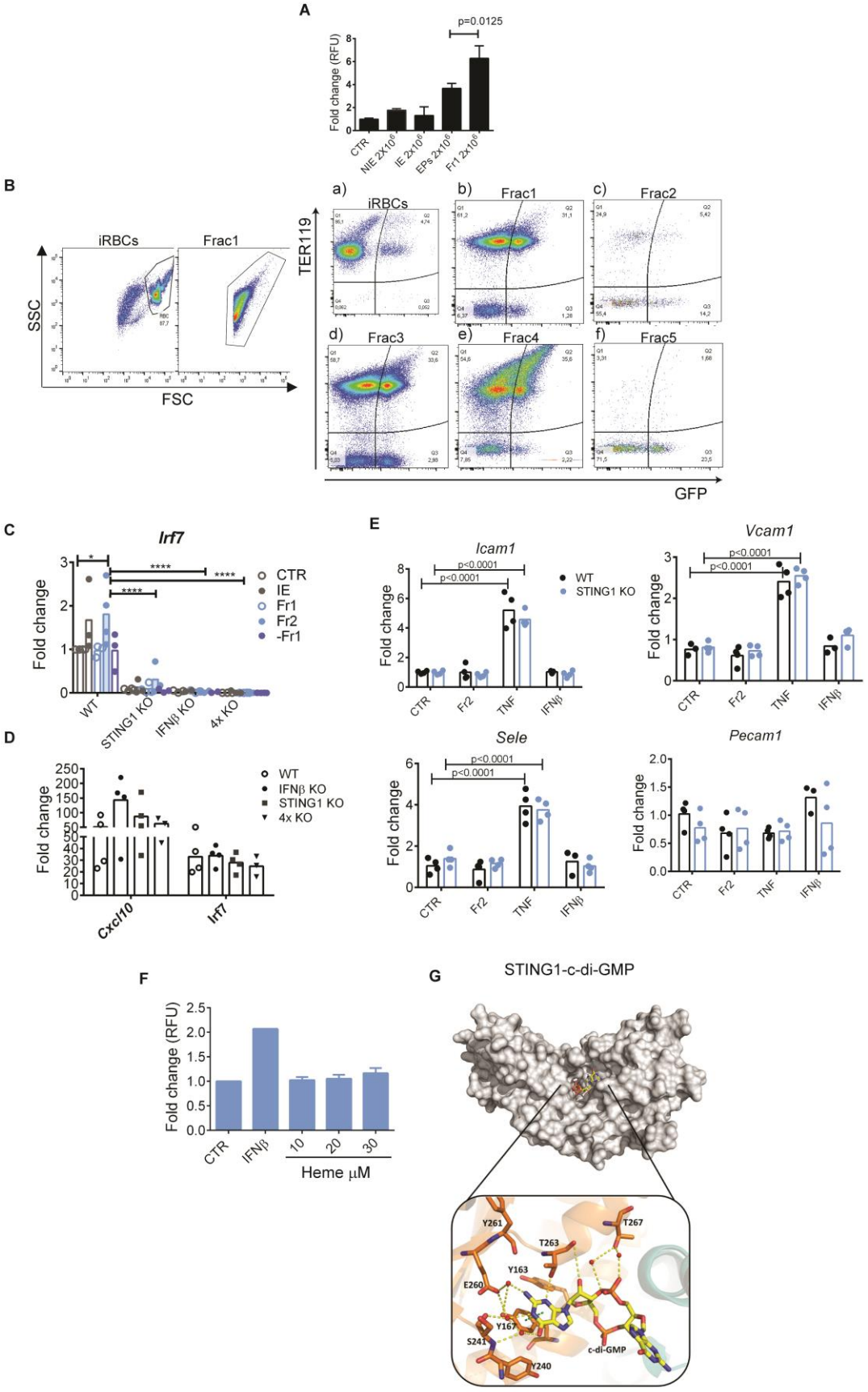

Fig. S5. Identification of IFNβ-inducing factors in fractions derived from EPs collected from *Pba*-IE cultures.

(A) IFNβ gene expression measured by luciferase activity in wild IFNβ-reporter BECs stimulated with $2x10^6$ of total EPs or of Fr1. Results are representative of two independent experiments. One-way ANOVA, Tukey's multiple comparisons test. **(B)** Flow cytometry profiles of EPs fractions obtained from *PbaGFP*-IE cultures and stained for the erythrocytic marker TER119. **(C)** Induction of *Irf7* expression in BEC of the indicated genotypes upon stimulation with IE and EPs fractions. 4XKO refers to quadruple KO (*Myd88*$^{-/-}$, *Trif*$^{-/-}$, *Mavs*$^{-/-}$ and *Sting1*$^{-/-}$). *Irf7* expression is induced by Fr2 in WT cells but is ablated in absence of endogenous IFNβ expression. Data comprise 3-4 experiments. Comparison between genotypes were performed by two-way ANOVA using Tukey's multiple comparisons test, *$p<0.05$, **** $p<0.0001$. Each spot represents a cell culture well. **(D)** Induction of *Cxcl10* and *Irf7* expression in BEC of indicated genotypes stimulated with IFNβ (1000 U/ml) showing response to IFNAR signaling in the different genotypes. **(E)** Gene expression of adhesion molecules in WT and STING1 KO BECs stimulated with IFNβ (1000 U/ml), TNF (10 ng/ml) and Fr2 (~$2x10^5$) of EPs.

(F) Luciferase activity of STING1 KO IFNβ-reporter BECs exposed to different concentrations of hemin for 20 hours. Data comprise two different experiments. **(G)** Surface view of c-di-GMP pocket. Close-up view of specific recognition of c-di-GMP by STING1 and detailed interactions between ribose-phosphate of GMP and water, as well as c-di-GMP and T263 from STING1 monomer. Hydrogen bonds between c-di-GMP and water are shown as yellow dashed lines and red spheres, respectively. π-π interaction of c-di-GMP and Y167 is shown as a green dashed line. Residues from STING1 monomer

A that interact with c-di-GMP are shown as orange sticks. c-di-GMP is shown as yellow sticks.

Supplemental Figure 6

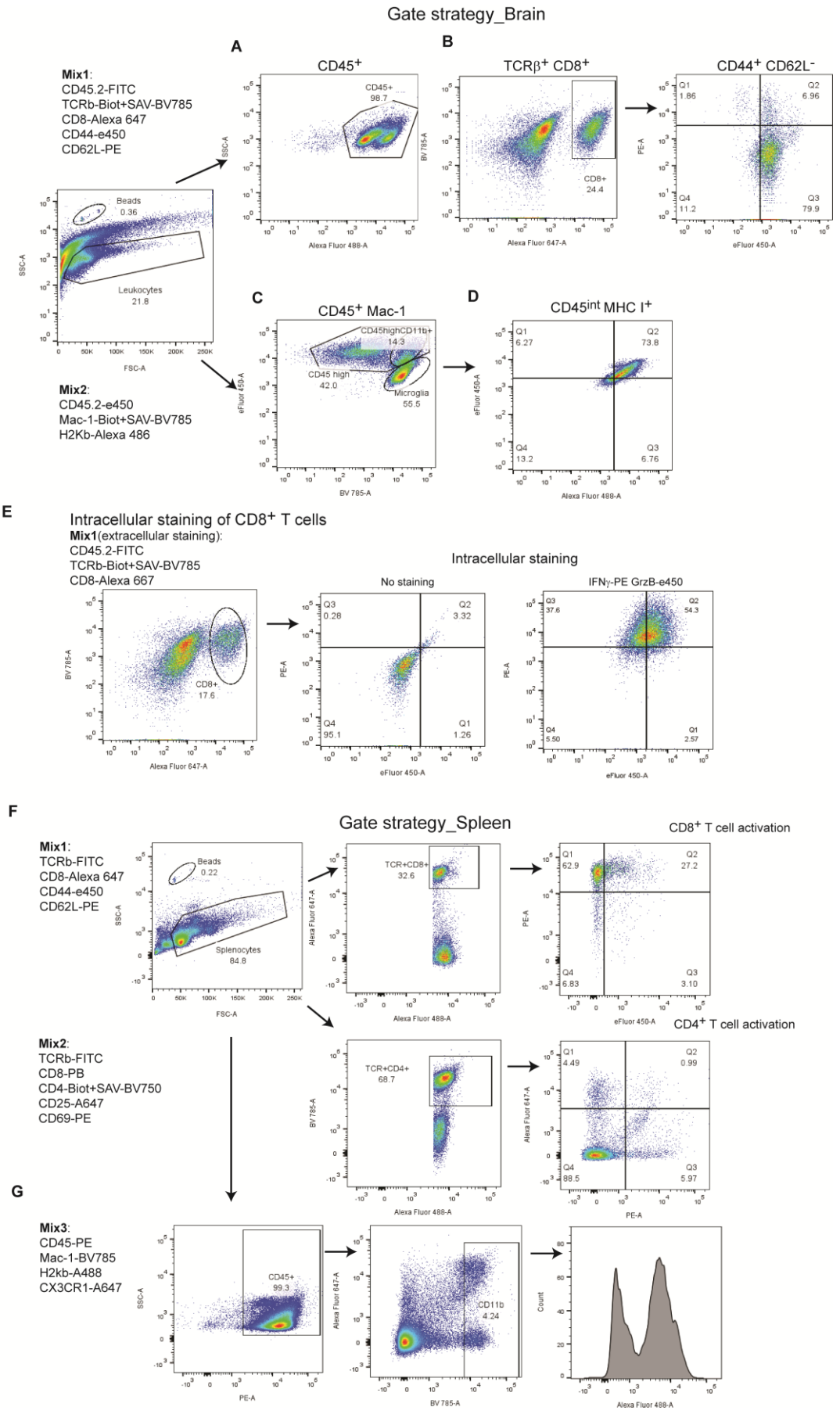

Fig. S6. Flow cytometry gating strategy for the identification of immune cells.

Brain cells were initially gated on forward scatter (FSC)-A vs side scatter (SSC)-A and then **(A)** gated as $CD45^+$ cells and identified as $TCR\beta^+CD8^+$. **(B)** $TCR\beta^+CD8^+$ gated cells were examined using cell surface markers for activation (CD44 and CD62L). **(C)** Monocytes and microglia, distinguished based on CD45 expression and Mac-1, were characterized as $CD45^{high}$ Mac-1^+ and $CD45^{int}$ Mac-1^+, respectively. **(D)** Within microglial cell population, cellular activation was examined based on MHC class I expression. **(E)** Activation of $CD8^+$ T cells was further studied using positivity for IFNγ and Granzyme B. **(F)** Analysis of cell activation profile in splenic $CD8^+$ and $CD4^+$ T cells and **(G)** monocytes (Mac-1^+) of experimental mice.

Strain	Source	JAX mice database stock #	Reference
B6;Ifnβ1$^{-/-}$	Dr. Kalinke TWINCORE		(*49*)
B6;Ifnβ1$^{+/\Delta\beta\text{-luc}}$	Dr. Kalinke TWINCORE		(*50*)
B6;Ifnβ1$^{\text{flox}\beta\text{-luc/flox}\beta\text{-luc}}$	Dr. Kalinke TWINCORE		(*29*)
B6;Trif$^{-/-}$	Dr. Kalinke TWINCORE	005037	
B6(Cg)-*Tmem173*$^{tm1.2Camb}$/J (*Tmem173*$^{-/-}$, STING KO)	Dr. Kalinke TWINCORE	025805	
B6;Myd88$^{-/-}$*Trif*$^{-/-}$*Mavs*$^{-/-}$*Tmem173*$^{-/-}$ (MyTrMaSt KO)	Dr. Kalinke TWINCORE		(*51*)
B6;Myd88$^{-/-}$	Dr. Akira Osaka University		(*52*)
B6;Mda5$^{-/-}$	Dr. Mota IMM	015812	
B6;129-*Mavs*^{tm1Zjc}/J	JAX	008634	
B6;SJL-*Sting1*$^{tm1.1Camb}$/J (STING1$^{\text{flox/flox}}$)	JAX	031670	
Tg(Slco1c1-icre/ERT2)1Mrks (Slco1c1CreERT2)	Dr. Kalinke TWINCORE		(*31*)
GFAPCre in C57BL/6 background	Dr. Veiga Fernandes Champalimaud Foundation	004600	
B6.Cg-Tg(Tek-cre)1Ywa/J (Tie2Cre)	JAX	008863	
B6J.B6N(Cg)-*Cx3cr1*$^{tm1.1(cre)Jung}$/J (CX3CR1Cre)	JAX	025524	
B6.129P2(Cg)-*Cx3cr1*$^{tm2.1(cre/ERT2)Litt}$/*WganJ* (CX3CR1CreERT2)	JAX	021160	
B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J mice (LysMCre)	JAX	004781	
GFAPCre$^{+/-}$IFNβ$^{+/\ \text{flox}\beta\text{-luc}}$	This study		
LysMCre$^{+/-}$IFNβ$^{+/\ \text{flox}\beta\text{-luc}}$	This study		
CX3CR1CreERT$^{+/-}$IFNβ$^{+/\ \text{flox}\beta\text{-luc}}$	This study		
Tie2Cre$^{+/-}$IFNβ$^{+/\ \text{flox}\beta\text{-luc}}$	This study		
Slco1c1CreERT2$^{+/-}$IFNβ$^{+/\ \text{flox}\beta\text{-luc}}$	This study		
LysMCre$^{+/-}$IFNβ $^{\text{flox}\beta\text{-luc / flox}\beta\text{-luc}}$	This study		
CX3CR1Cre$^{+/-}$IFNβ $^{\text{flox}\beta\text{-luc / }\Delta\text{-luc}}$	This study		
Tie2Cre$^{+/-}$IFNβ $^{\text{flox}\beta\text{-luc / flox}\beta\text{-luc}}$	This study		
CX3CR1CreERT$^{+/-}$STING1$^{\text{flox/-}}$	This study		
CX3CR1Cre$^{+/-}$STING1$^{\text{flox/}\Delta}$	This study		
TieCre$^{+/-}$STING1$^{\text{flox/}\Delta}$	This study		
Slco1c1CreERT$^{+/-}$STING1$^{\text{flox/-}}$	This study		

Table S1. Strains used in this study.

Oligonulceotides	Genotyping
5’-CACAGGCCATGAAGGAAGAT-3’	*Ifnb1* wt, floxed and deleted alleles-
5’-TTGGAGCTGGAGCTGCTTAT-3’	
5’-GCCTTATGCAGTTGCTCTCC-3’	
5’-GCGCACACACACTAAAAACTG-3’	*Sting1* wt, floxed and deleted alelles
5’-TTGCTAGTAGCTACTGAAAGGC-3	
5’-TTTCATCTGCCTTCCAGGTC-3’	

Table S2. Oligonucleotides used to genotyping *Ifnβ1* and *Sting1* modified alleles.

Antibody	Supplier	Clone
Anti-mouse-Fc-block/CD16/32	IGC (antibody facility)	2.4G2
Anti-mouse FITC CD45.2	IGC (antibody facility)	104.2
Anti-mouse PE CD45.2	IGC (antibody facility)	104.2
Anti-mouse PE CD41	eBiosciences	MWReg30
Anti-mouse PE CD45	Life Technologies	30-F11
Anti-mouse PE CD105	Life Technologies	MJ7/18
Anti-mouse PE TER119	Life Technologies	TER119
Anti-mouse efluor 450 CD45.2	eBiosciences	104
Anti-mouse biotinylated TCR beta	IGC (antibody facility)	H57-597
Anti-mouse Alexa 648 CD8	IGC (antibody facility)	YTS169.4
Anti-mouse Pacific Blue CD8	IGC (antibody facility)	YTS169.4
Anti-mouse biotinylated CD4	IGC (antibody facility)	GK1.5
Anti-mouse eFluor 450 CD44	IGC (antibody facility)	IM7
Anti-mouse PE CD69	IGC (antibody facility)	H1.2F3
Anti-mouse PE CD62L	IGC (antibody facility)	MEL-14
Anti-mouse Alexa 647 CD25	IGC (antibody facility)	PC61
Anti-mouse biotinylated CD11b/Mac-1	IGC (antibody facility)	M1/70
Anti-mouse Alexa 488 MHC-I/H-2Kb	IGC (antibody facility)	AF6-88.5
Anti-mouse efluor 450 Granzyme B	BioLegend	N6ZB
Anti-mouse PE IFNγ	BioLegend	XMG1.2

Table S3. Antibodies used for flow cytometry.