Supplementary Materials for

Interferon ε protects the female reproductive tract from viral and bacterial infection

Ka Yee Fung, Niamh E Mangan, Helen Cumming, Jay C Horvat, Jemma R Mayall, Sebastian Stifter, Nicole De Weerd, Laila C Roisman, Jamie Rossjohn, Sarah Robertson, John Schjenken, Belinda Parker, Caroline Gargett, Hong PT Nguyen, Daniel J Carr, Philip M Hansbro, Paul J Hertzog

correspondence to: paul.hertzog@monash.edu

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Materials and Methods

Mice

Six to eight week old C57BL/6 (WT) mice were obtained from Monash Animal Services. *Ifnar 1*$^{-/-}$ and *Ifnar 2*$^{-/-}$ mice on a C57BL/6 background were maintained in-house. *Ifnε*$^{-/-}$ mice were generated by targeting the single exon of Ifnε with flanking Lox P sites in C57BL/6 ES cells, which were screened and confirmed by Southern blots as shown in Supplementary Figure 2. The neomycin cassette was removed by electroporation of Flp recombinase. *Ifnε*$^{fl/fl}$ mice were mated with CMV-Cre mice to generate *Ifnε*$^{+/-}$ mice. In breeding studies, 3 female *Ifnε*$^{-/-}$ mice of 6-8 weeks were bred with WT and *Ifnε*$^{-/-}$ C57BL/6 males and vice versa. After delivery of pups, the number and sex of the offspring were determined and the litters were inspected daily. The ability of the females to nurse their offspring was monitored, and the weights of pups were measured weekly from 3-6 weeks of age.

Pseudopregnant mice were generated by mating virgin female C57BL/6 mice of 6-8 weeks with vasectomized C57BL/6 males, and confirmation of vaginal plug designated day 0.5 of pseudo pregnancy. At the appropriate time points, mice were sacrificed and uterine tissues were harvested for RNA analysis.

Ovariectomized mice were generated at the University of Adelaide as previously described *(28)*. Following ovariectomy, all mice were primed with 100ng of 17β-estradiol (Sigma) for 2 consecutive days. After 2 days rest, mice were maintained on daily doses of vehicle (sesame oil), or 25 ng 17β-estradiol (Sigma) for 6 days. On day 11, all mice were sacrificed and uteri were excised for RNA analysis.

The intact and vasectomized mouse mating study was carried out at the University of Adelaide. CBAF1 (F1) females and BALB/c males were used in this study. Estrous cycle of F1 female mice was identified by vaginal lavage cytology. Proestrus F1 females mice were mated with either intact (sperm + seminal plasma) or vasectomized (seminal plasma alone) BALB/c male mice. Proestrus F1 females were used as no mating controls. Tissues were collected either 8h, 16h, d1.5, d2.5 or d4.5 after mating with the exact time of mating determined by video recording. At the appropriate time points, mice were sacrificed and uterine tissues were harvested for RNA analysis.

Animals were housed in individually ventilated and filtered cages under positive pressure in an SPF facility. All experiments were conducted in accordance with approval from institutional animal ethics committees.

Recombinant IFNs

Recombinant murine Ifns were expressed via baculoviral infection of suspension *Trichoplusia ni* (Hi5) insect cells using Bac-to-Bac expression system (Invitrogen). All Ifns were purified from culture supernatants by dialysis into TBS (10 mM Tris-HCl, pH 8.0, 150 mM sodium chloride), affinity chromatography over either a nickel-charged Sepharose 6 Fast Flow column (Ifnα4 and Ifnβ; GE Healthcare) or a Sepharose column coupled with an in-house anti-mIFNε monoclonal antibody (IFNε), followed by size exclusion chromatography over an S200 16/60 size exclusion column (GE Healthcare). Purity was demonstrated by the absence of non-IFN bands/peaks on Coomassie-stained

SDS PAGE gels and chromatography traces, respectively. Biological antiviral activity was determined using L cells and Semliki Forest Virus as the challenge virus and calibrated relative to NIH reference standards for type I IFNs. IFN preparations were tested for endotoxin using the Genscript ToxinSensor Chromogenic LAL assay. IFN preparations of 1μg/ml contained <0.1EU/ml endotoxin (i.e. undetectable).

Anti-mIFNε monoclonal antibody

Anti-mIFNε were generated at Monash Antibody Technologies Facility and purified from hybridomas in-house.

DNA constructs

The murine Ifnε, Ifnβ and Ifnα4 luciferase reporter constructs containing the ATG to 1000 nucleotides of the respective promoter were cloned into the luciferase-reporter plasmid, promoter-less pGL3 Basic vector (Promega, Corporation). p125 luciferase reporter construct was a kind gift from Kate Fitzgerald (University of Massachusetts, Boston, USA), as were the IRF3, IRF7 and IRF5 expression plasmids, constructed in the expression plasmid, pCMVs port (Life Technologies), driven by the cytomegalovirus promoter. The Renilla luciferase reporter plasmid (Promega) is driven by the thymidine kinase promoter and was used as an internal control in transfection experiments.

Promoter analysis

Promoter sequences were extracted from Ensembl release 68, 1,000 bp upstream from the annotated transcription start sites. Computational transcription factor site prediction was performed using STAT, IRF, NF-κB and ISGF3 matrices as annotated in the Transfac transcription factor database. Analysis of putative promoters was performed with the MATCH algorithm applying false positive correction.

Cell isolation and cell lines

Mouse embryonic fibroblasts (MEFs), were generated as previously described *(29)*. In brief, embryos were obtained from pregnant female C57BL/6 mice 13.5 days post-coitum. The head and internal organs of the embryos were removed, and the remaining carcasses were minced and incubated with 0.25% trypsin, EDTA for 10min at 37°C. DMEM (Gibco), supplemented with 10% fetal bovine serum (FCS; Gibco), 5 mm L-glutamine (Gibco) and 1% (v/v) penicillin/streptomycin (Gibco), was added to the cell suspensions, cells were disaggregated, and tissue clumps were allowed to settle to the bottom of the tube. The remaining cell suspensions were then transferred onto T75 flasks. Fibroblasts were grown at 37°C under a 5% CO_2 atmosphere to confluence.

Bone marrow macrophages (BMMs) were isolated by flushing the marrow cavities of mouse femora with complete RPMI (Gibco), consisting of 10% FCS, 5 mm L-glutamine, and 1% (v/v) penicillin/streptomycin using a 23-gauge needle. Cells were centrifuged at 1,000 rpm for 5 mins and resuspended in complete RPMI supplemented with 20% conditioned media from L929 cell lines as a source of macrophage colony

stimulating factor (M-CSF). Cells were plated at ½ femur per 20 ml RPMI complete media in 10cm square low adherence plates. BMMs were cultured at 37°C under a 5% CO_2 atmosphere. On day 3, additional aliquots of 10 mls of complete RPMI medium supplemented with 20% L929-cell conditioned media were added to the plate.

Peritoneal exudate cells were isolated by flushing the peritoneal cavity with ice cold PBS. Cell pellets were obtained by centrifugation at 1,000 rpm for 5 mins.

The murine macrophage cell line, RAW264.7 was grown in complete RPMI. Human kidney fibroblast, HEK293; Monkey kidney epithelial cell line, Vero (ATCC) and human cervical cell line, HeLa (ATCC) were grown in complete DMEM. Human endometrial cell line, Hec1A was grown in McCoy's 5A (Gibco), consisting of 10% FCS, 5 mm L-glutamine, and 1% (v/v) penicillin/streptomycin. Immortalized cervical cell line, Ect1 was grown in keratinocyte-Serum Free medium (Gibco) with 0.1 ng/ml human recombinant EGF, 0.05 mg/ml bovine pituitary extract, and additional calcium chloride 44.1 mg/L (final concentration 0.4 mM). All cell lines were maintained at 37^{o}C in 5% CO_2.

Human endometrium specimens were obtained with approval from the Institutional Human Research Ethics Committee from women undergoing hysterectomy. Endometrial cells were isolated by mincing, digestion with collagenase and DNase I and filtration, as previously described (*30)*. Anti-human EpCAM (BerEP4 clone) antibody-coated magnetic Dynabeads (Invitrogen) were used to positively select epithelial cells from glandular epithelial cell suspensions using Dynabead Epithelial Enrich kits (Invitrogen) according to the manufacturer's instructions.

Transient transfection and reporter gene assays

HEK293 cells ($2x10^4$/well) were plated in a 96-well plate 24h prior to co-transfection with 80 ng of *Ifnε*, *Ifnβ* and *Ifnα4* promoter reporter and different Irf3, Irf7 and Irf5 expression vectors using FuGENE6 (Roche Diagnostics). TK Renilla was used to normalize for transfection efficiency and an appropriate pEF-BOS empty vector plasmid was used to maintain a constant amount of DNA. Transfected cells were lysed using Reporter Lysis Buffer (Promega) and assayed for luciferase and Renilla activity using luciferase assay reagent (Promega) and Renilla substrate. Luminescence readings were detected using FLUOstar Optima (BMG Technologies) corrected for Renilla and expressed as fold induction over empty vector control values.

In vitro PAMP and Semliki Forest Virus stimulation

BMM, MEF and RAW264.7 cells ($1x10^6$/well) were seeded in a 6-well plate at 37^{o}C overnight before stimulation for 3h with TLR ligands: LPS, 1 μg/ml (Sigma); Pam3Cys, 1 μg/ml (EMC Microcollections GmbH,); poly (I:C), 25 μg/ml (Amersham/GE Healthcare); CpG oligonucleotide, 1 μM (Gene works); or Loxoribine, 100 μM (Invitrogen).

RAW264.7 cells ($1x10^6$/well) were seeded in a 6-well plate at 37^{o}C overnight before stimulation. These cells were then stimulated for 3h with 300 TCID50 of Semliki Forest Vrius (tissue culture infective dose causing 50% lethality).

dsRNA and dsDNA transfection

BMM, MEF and RAW264.7 cells ($1x10^6$/well) were seeded in a 6-well plate at 37^oC overnight before stimulation. The synthetic dsRNA, poly (I:C) and synthetic dsDNA, poly (dA:dT) were transfected at a concentration of 10 μg/ml and 1 μg/ml respectively, into BMM, MEF and RAW264.7 cells for 3h at 37^0C using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Genotyping PCR

10 ng of genomic DNA extracted from mouse tail clips was used in 25μl PCR reactions using primers (F' GATCCCTGTGTGCCCTCCACC, R' GCAAGAGCCAACAGGGGATTTG) with GoTaq® DNA Polymerase (Promega).

In vivo LPS adminstration

Female (6-8 weeks old) C57BL/6 WT and *Ifnε*$^{-/-}$ mice were cheek bled (0h time point) then injected intraperitoneally with re-purified LPS (10mg/kg) from *Escherichia coli* (K-235; Sigma-Aldrich, St Louis, MO, USA) as previously described *(31).* After 24h mice were culled, peritoneal exudate cells were isolated by peritoneal lavage (described above) and blood was collected via cardiac puncture.

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assay kits for murine IL-6 and TNF-α (Pharmingen, San Diego, CA, USA) were used according to the manufacturer's instructions.

HSV-2 infection

A clinical isolate of HSV-2 strain 186 was a kind gift from Prof. Anthony Cunningham (University of Sydney, NSW, Australia). HSV-2 was propagated three times in Vero cells. Virus stocks, $3.87x10^6$ Plaque Forming Unit/ml (PFU/ml), were stored at -80^oC and diluted in PBS immediately before infection. Female (6-8 weeks old) WT and *Ifnε*$^{-/-}$ C57BL/6 mice were pretreated with 2 mg Depo-ralovera (Kenral) subcutaneously on day -5 then intra-vaginally infected with HSV-2 (24 or 2400 PFU/mouse in 10 μl) on day 0 and monitored daily to assess clinical signs of disease: 0, no apparent signs of disease; 1, genital erythema; 2, moderate genital inflammation and swelling; 3, mucus, swelling and redness with presence of genital lesions; 4, severe genital lesions and/or hind limb paralysis.

Viral plaque assay

Tissues (vagina, spinal cord and brain stem) were obtained from HSV-2 infected mice and homogenized using a tissue homogenizer. Supernatants were clarified and subsequently assessed for virus titres by plaque assay on Vero cells as previously described *(12*).

Chlamydia muridarum infection

Female (6-8 weeks old) WT and *Ifnε*$^{-/-}$ C57BL/6 mice were pretreated with 2.5 mg medroxyprogesterone acetate (Troy Laboratories) subcutaneously on day -7. On day 0, mice were anesthetized with ketamine and xylazine (1.8 mg/mouse and 0.04 mg/mouse, respectively; Troy Laboratories) and then infected via intra-vaginal inoculation with $5x10^4$ inclusion forming units (IFU) *C. muridarum* (ATCC VR-123) per mouse in 10 µl sucrose phosphate glutamate (SPG) buffer, as described previously*(15)*. Infection was allowed to progress for 30 days. Mice were monitored on a daily basis to assess body weight, signs of disease, and survival. Clinical scores were assigned based on swelling, erythema and mucus production in the vagina and poor coat condition.

In vivo intra-vaginal rIfnα, rIfnβ and rIfnε treatment

Female (6-8 weeks old) WT and *Ifnε*$^{-/-}$ C57BL/6 mice were pretreated with 2 mg Depo-ralovera (Kenral) subcutaneously on day -5 then intra-vaginally treated with 840 IU/mouse rIfnα, rIfnβ and rIfnε on day 0. Mice were culled 6 hrs post-treatment for analysis.

Histology and immunohistology

Uteri were fixed overnight in 10% formalin and then washed in 70% ethanol. All tissues were paraffin embedded and sectioned (4-µm thickness) at the Monash Institute of Medical Research histology facility. Tissues were stained with haematoxylin and eosin and standard immunohistology techniques were performed as described previously *(32)*. In brief, heat antigen retrieval was carried out in citrate buffer (10 mM trisodium citrate, pH 6.0) at 125°C for 5 min under pressure. Mouse Ifnε antibody and its IgG control were generated in-house. All antibodies were used at a concentration of 10 µg/ml.

Determination of estrus cycle

The estrous stage of cycle in mice was determined by analysis of Diff-Quick (Lab Aids) stained vaginal smears, which were stained according to the manufacturer's instructions. Stained cells were carefully examined and scored for the presence of leukocytes, nucleated or non-nucleated epithelial cells. The estrous stage for each mouse was identified as diestrus, proestrus, early estrus, estrus and postestrus according to previously established protocols *(33)*.

Immunophenotyping

Surface marker expression was assessed by flow cytometry with data collected on BD FACS Canto II analyzer (BD Bioscience). Single cell suspensions were obtained from bone marrow, spleen, thymus and uterus of WT and *Ifnε*$^{-/-}$ mice. Non-specific binding was blocked with anti-mouse CD16/CD32 Fcγ III/II. Monocyte/macrophage and granulocyte populations in the bone marrow were defined by surface marker expression

of CD11b (Mac-1; clone M1/70; APC-Cy-7 conjugated) and Ly6G (Gr-1; clone IA8; PE conjugated). Distinct cell populations in the bone marrow were further characterized using biotin-conjugated CD31 (390; detected with streptavidin PE anti-mouse IgG), or FITC-conjugated Ly6C (AL-21) expression: blast cells ($CD31^{hi}Ly6C^{-}$), lymphocyte ($CD31^{med}Ly6C^{-}$), erythroid cells ($CD31^{-}Ly6C^{-}$), myeloid progenitor cells ($CD31^{+}Ly6C^{+}$), granulocytes ($CD31^{-}Ly6C^{med}$) and monocytes ($CD31^{-}Ly6C^{hi}$). B cell subsets in the bone marrow and spleen were assessed using FITC-conjugated B220 (RA3-6B2), PE-Cy7-conjugated IgM (R6-60.2) and PE-conjugated IgD (11-26C). Immature B cells were defined as $B220^{+}IgD^{-}IgM^{+}$ and mature B cells as $B220^{+}IgD^{+}IgM^{+}$. Spleen and thymus cells were assessed for T cell subsets - $CD3^{+}CD4^{+}CD8^{-}$ T helper cells, $CD3^{+}CD8^{+}CD4^{-}$ cytotoxic T cells, or immature $CD3^{+}CD4^{+}CD8^{+}$ T cells using PE-conjugated CD3 (145-2C11), Pacific Blue-conjugated CD4 (RM4-5), or APC-Cy7-conjugated CD8a (53-6.7). NK cells in uterine horn tissue were identified by expression of CD3 and NK1.1 ($CD3^{-}NK1.1^{+}$) using APC-conjugated CD3 (145-2C11 from BioLegend) and biotin-conjugated NK1.1 (PK136) detected with streptavidin PE-Cy7 (from BioLegend). All antibodies were from BD PharMingen unless otherwise stated. Results are presented as % population (or total numbers for NK cells) with quadrant gates set using isotype controls and/or fluorescence minus one control staining. Analyses were performed using FlowJo or FACSDiva software.

Statistical analyses

All statistical analyses were performed using GraphPad PRISM software package. All studies were assumed as normally distributed; therefore Student's two-tailed un paired *t*-test was used for differences between treated and non-treated groups.

Fig. S1.

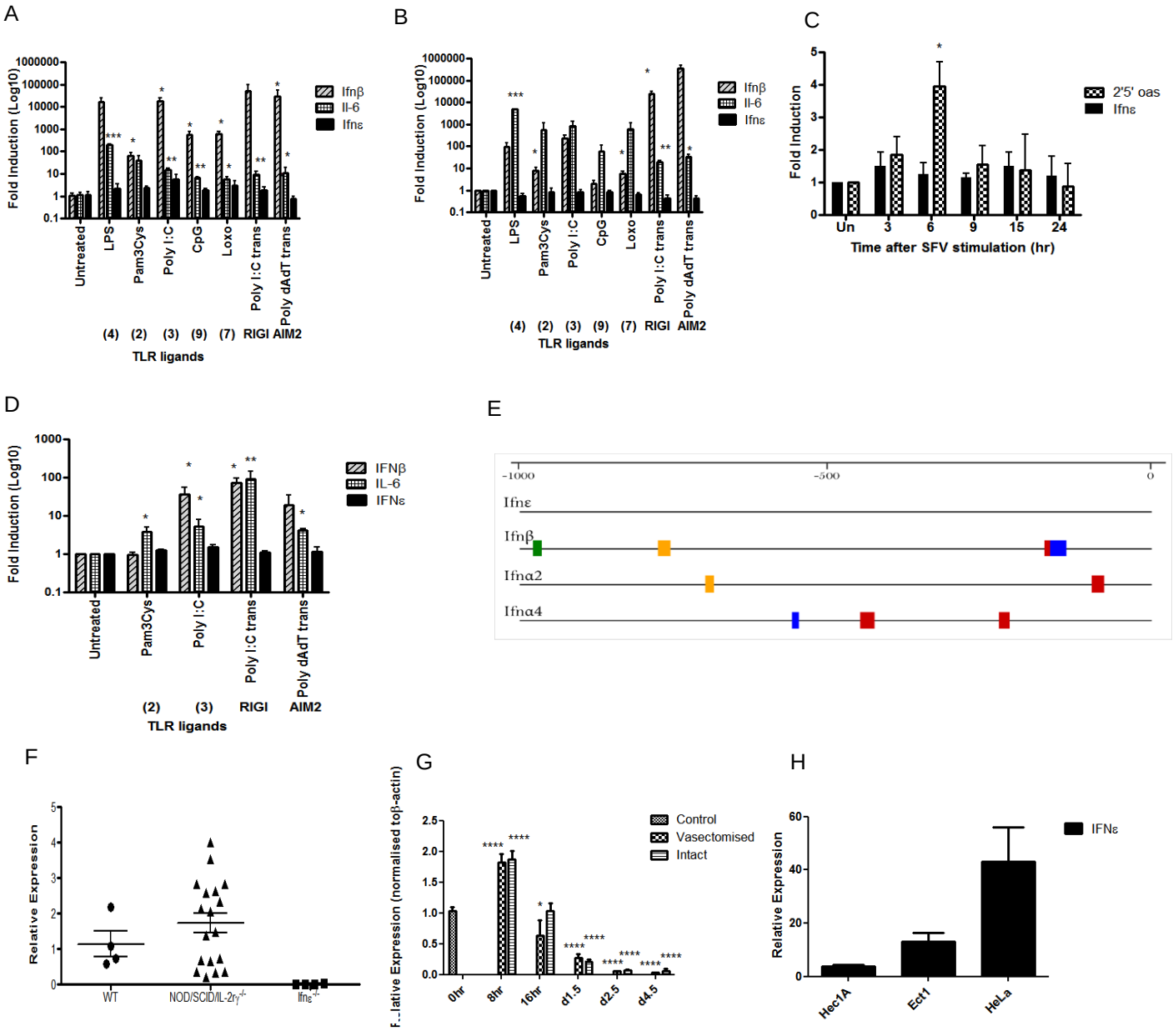
A
Fold Induction (Log10)
Ifnβ
Il-6
Ifnε
Untreated
LPS
Pam3Cys
Poly I:C
CpG
Loxo
Poly I:C trans
Poly dAdT trans
(4) (2) (3) (9) (7) RIGI AIM2
TLR ligands
B
Fold Induction (Log10)
Ifnβ
Il-6
Ifnε
Untreated
LPS
Pam3Cys
Poly I:C
CpG
Loxo
Poly I:C trans
Poly dAdT trans
(4) (2) (3) (9) (7) RIGI AIM2
TLR ligands
C
Fold Induction
2'5' oas
Ifnε
Un 3 6 9 15 24
Time after SFV stimulation (hr)
D
Fold Induction (Log10)
IFNβ
IL-6
IFNε
Untreated
Pam3Cys
Poly I:C
Poly I:C trans
Poly dAdT trans
(2) (3) RIGI AIM2
TLR ligands
E
-1000
-500
0
Ifnε
Ifnβ
Ifnα2
Ifnα4
F
Relative Expression
WT
NOD/SCID/IL-2rγ-/-
Ifnε-/-
G
Control
Vasectomised
Intact
0hr 8hr 16hr d1.5 d2.5 d4.5
H
Relative Expression
IFNε
Hec1A
Ect1
HeLa

Fig. S1

Ifnε is not induced by PRRs and Semliki Forest virus stimulation. (A) MEFs and (B) Raw264.5 cells were treated with different TLR ligands or transfected with Poly (I:C) and Poly (dA:dT) for 3h, respectively, and *Ifnβ*, *Il-6* and *Ifnε* expression was measured by qRT-PCR. All values are means + SEM of at least three independent experiments.. (C) RAW264.5 cells were treated with Semilki Forest virus (SFV). Total RNA was extracted and *Ifnε* and *2'5' oas* expression was measured by qRT-PCR. . Data are expressed as mean + SEM of at least three independent experiments. (D) HeLa cells were treated with different TLR ligands or transfected in Poly (I:C) and Poly (dA:dT) for 3h, respectively, and *Ifnβ*, *Il-6* and *Ifnε* expression was measured by qRT-PCR. . Data are expressed as mean + SEM of at least three independent experiments. (E) Promoter analysis of mouse type 1 IFNs comparing predicted IRF (Red), ISRE (Green), NF-κB (Blue) and STAT (Orange) sites in the upstream promoter region of Ifnε to the equivalent promoter regions upstream of Ifnβ, Ifnα2 and Ifnα4. (F) *Ifnε* expression was measured in WT, NOD/SCID/IL-2rγ$^{-/-}$ and *Ifnε* $^{-/-}$ C57BL/6 mice. . Data are expressed as mean $\pm$ SEM of four to fourteen individual mice. (G) *Ifnε* expression was measured in the endometrial tissue following mating with either intact or vasectomized males. . Data are expressed as mean + SEM of at least four individual mice. (H) *IFNε* expression was measured in the indicated endometrial cell lines. . Data are expressed as mean + SEM of at least three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 (unpaired Student's t-test).

Fig. S2

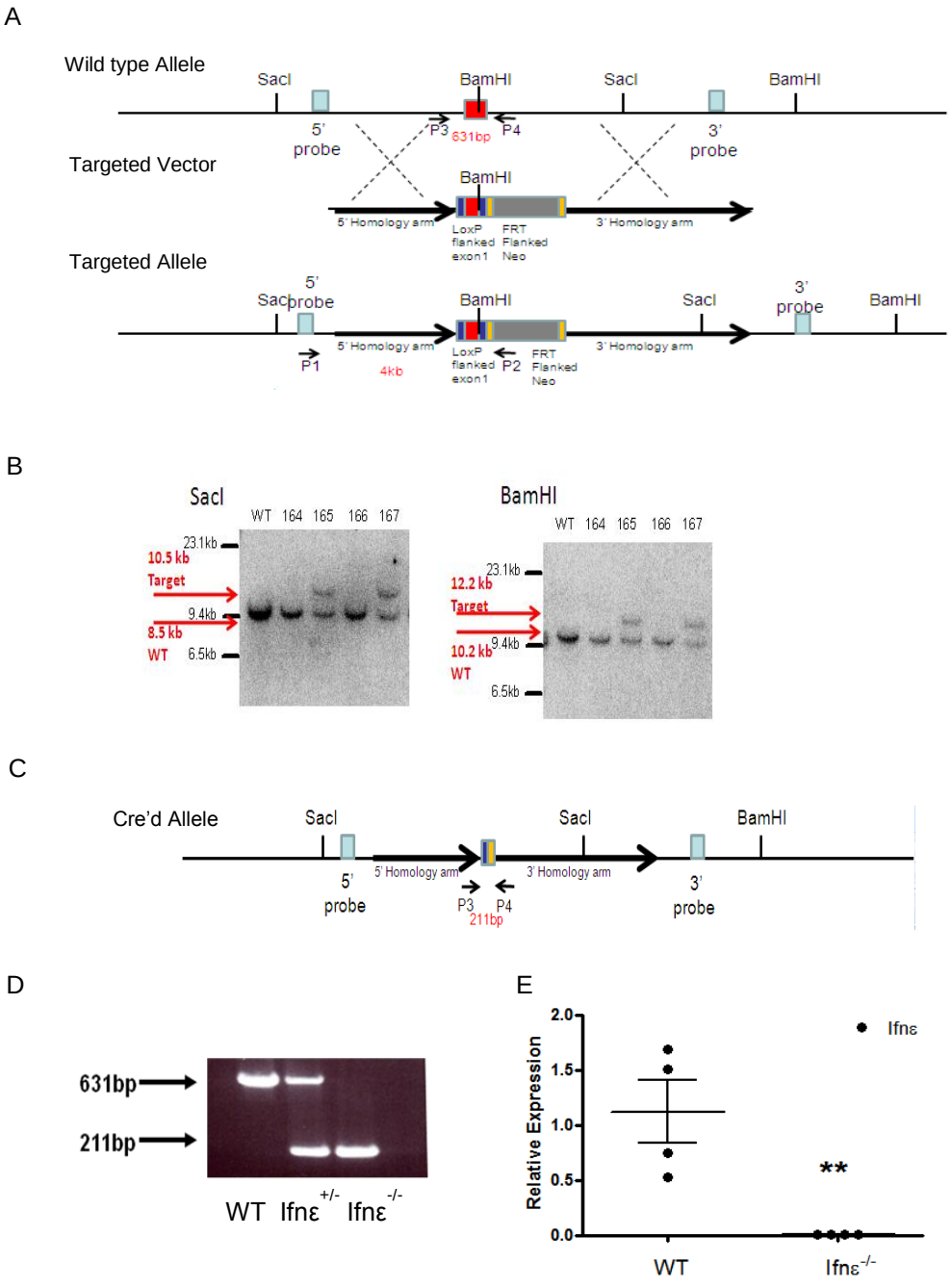
A
Wild type Allele
SacI
BamHI
SacI
BamHI
5' probe
P3
631bp
P4
3' probe
Targeted Vector
BamHI
5' Homology arm
3' Homology arm
LoxP flanked exon1
FRT Flanked Neo
Targeted Allele
5' probe
SacI
BamHI
SacI
3' probe
BamHI
5' Homology arm
3' Homology arm
P1
4kb
LoxP flanked exon1
P2
FRT Flanked Neo
B
SacI
WT 164 165 166 167
23.1kb
10.5 kb
Target
9.4kb
8.5 kb
WT
6.5kb
BamHI
WT 164 165 166 167
23.1kb
12.2 kb
Target
9.4kb
10.2 kb
WT
6.5kb
C
Cre'd Allele
SacI
SacI
BamHI
5' probe
5' Homology arm
3' Homology arm
P3
P4
211bp
3' probe
D
631bp
211bp
WT
Ifnε+/-
Ifnε-/-
E
Ifnε
Relative Expression
2.0
1.5
1.0
0.5
0.0
**
WT
Ifnε-/-

Fig. S2

Generation of *Ifnε*$^{-/-}$ mice. (A) The wild type allele, targeting vector and the targeted allele are depicted. The *Ifnε* exon is represented by a red rectangle. The blue and grey rectangles indicate the loxP sites and neo cassette, respectively, flanked by FRT sites (yellow rectangle). *Bam*HI and *Sac*I restriction sites used for assessing 5' and 3' homologous recombination are shown. (B) Southern Blot analysis of targeted ES cells. (C) The knockout allele with a LoxP site (blue rectangle) and FRT site (yellow rectangle). (D) PCR of genomic DNA to confirm genotypes generated using the P3 and P4 primers as indicated. (E) *Ifnε* expression in uterus tissue of WT and *Ifnε*$^{-/-}$ C57BL/6 mice was determined by qRT-PCR. Data are expressed as mean $\pm$ SEM of four individual mice. $^{**}P<0.01$ (unpaired Student's t-test).

Fig. S3

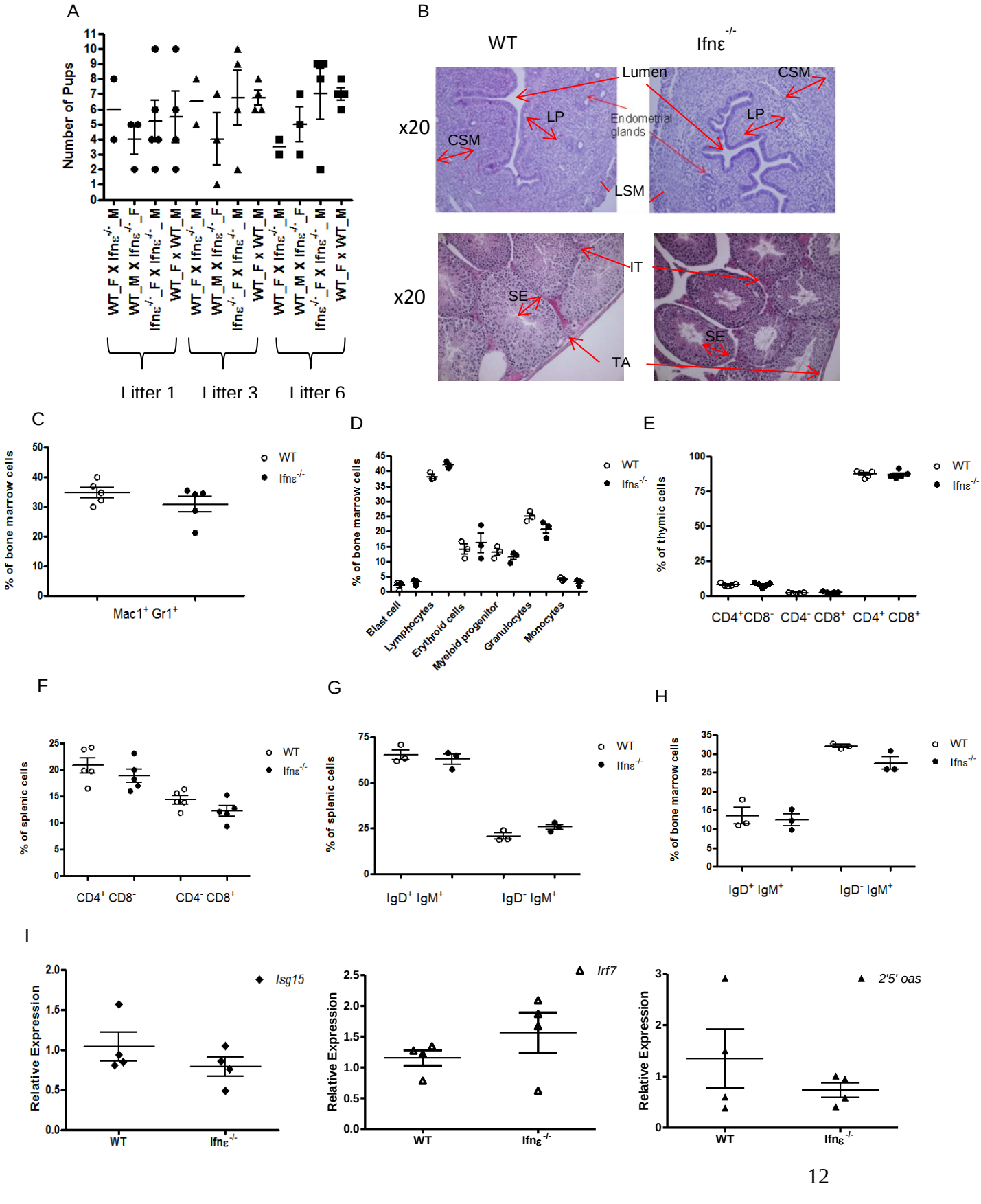
A
Number of Pups
WT_F X Ifnε-/-_M
WT_M X Ifnε-/-_F
Ifnε-/-_F X Ifnε-/-_M
WT_F x WT_M
WT_F X Ifnε-/-_M
WT_M X Ifnε-/-_F
Ifnε-/-_F X Ifnε-/-_M
WT_F x WT_M
WT_F X Ifnε-/-_M
WT_M X Ifnε-/-_F
Ifnε-/-_F X Ifnε-/-_M
WT_F x WT_M
Litter 1
Litter 3
Litter 6
B
WT
Ifnε-/-
x20
Lumen
CSM
LP
Endometrial glands
LSM
x20
IT
SE
TA
C
% of bone marrow cells
WT
Ifnε-/-
Mac1+ Gr1+
D
% of bone marrow cells
Blast cell
Lymphocytes
Erythroid cells
Myeloid progenitor
Granulocytes
Monocytes
E
% of thymic cells
CD4+CD8-
CD4-CD8+
CD4+CD8+
F
% of splenic cells
CD4+ CD8-
CD4- CD8+
G
% of splenic cells
IgD+ IgM+
IgD- IgM+
H
% of bone marrow cells
IgD+ IgM+
IgD- IgM+
I
Relative Expression
Isg15
Irf7
2'5' oas
WT
Ifnε-/-

Fig. S3

Ifnε is not required for fertility, uterine development or immune cell development. (A) Scatter plot showing the numbers of pups produced from different mated pairs from different litters. Data are expressed as mean of two individual matings or mean ± SEM of five individual matings. (B) Representative images from H&E staining of uteri (upper panel) and testis (lower panel) from WT and *Ifnε*$^{-/-}$ C57BL/6 mice. LP, lamina propria; CSM, circular smooth muscle; LSM, longitudinal smooth muscle; IT, interstitial tissue; SE, seminiferous epithelium; TA, tunica albuginea. This is representative of at least five individual mice. (C-H) Immunophenotyping of *Ifnε*$^{-/-}$ mice by FACS analysis. (C) Myeloid lineages (Mac1^{+}Gr^{+}) in bone marrow cells harvested from WT and *Ifnε*$^{-/-}$ C57BL/6 mice. (D) CD31 and Ly6C analysis of immune cell types in bone marrow cells harvested from WT and *Ifnε*$^{-/-}$ C57BL/6 mice. . Data are expressed as mean ± SEM of at least three individual mice. Cells extracted from (E) thymus and (F) spleen were analyzed for T cell populations, CD4^{+} CD8^{-}, CD4^{-} CD8^{+}and CD4^{+} CD8^{+}. . Data are expressed as mean ± SEM of at least three individual mice. (G) Bone marrow and (H) spleen cells of WT and *Ifnε*$^{-/-}$ C57BL/6 mice were analyzed for B cell populations: IgD^{+} IgM^{+} (mature B cells) and IgD^{-} IgM^{+} (immature B cells). . Data are expressed as mean ± SEM of at least three individual mice. (I) *Isg15, Irf7* and *2'5' oas* expression were measured in kidney of WT and *Ifnε*$^{-/-}$ C57BL/6 mice by qRT-PCR. Data are expressed as mean ± SEM of four individual mice.

Fig. S4

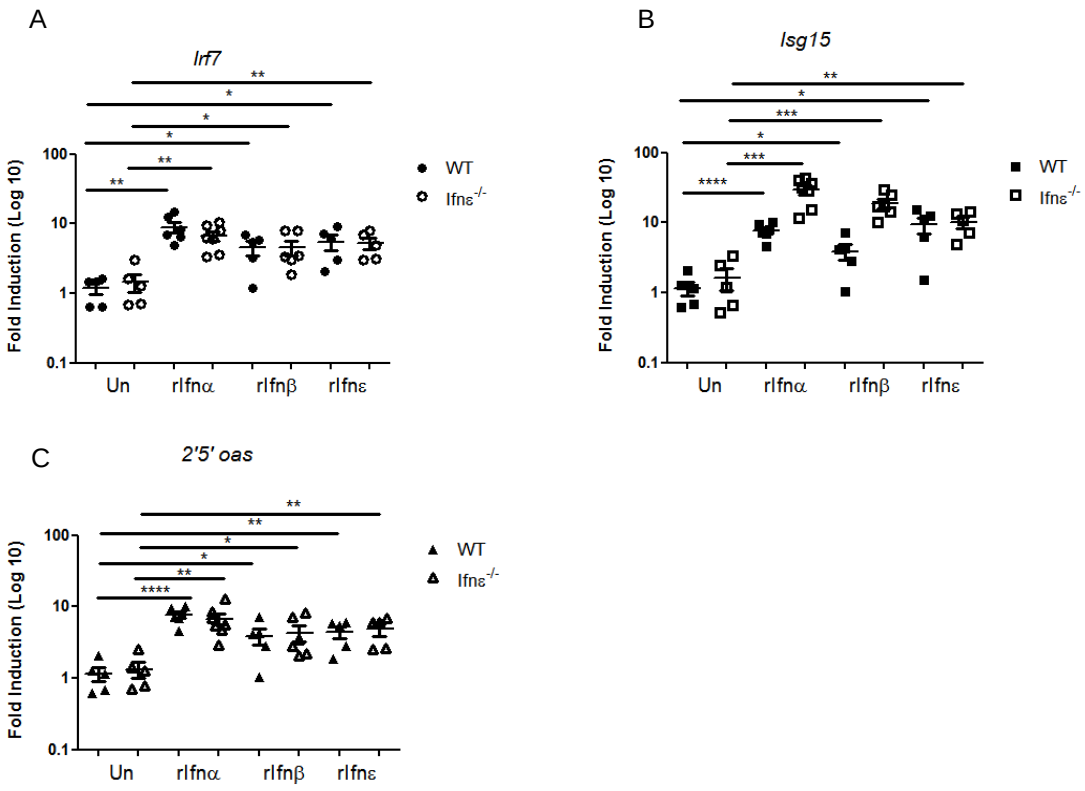

Fig. S4

IRG expression in WT and *Ifnε*$^{-/-}$ mice following rIfnα, rIfnβ and rIfnε treatment. Mice pretreated with Depo-ralovera at day -5 were treated with rIfnα, rIfnβ and rIfnε for 6h intra-vaginally on day 0. *Irf7, Isg15 and 2'5' oas* expression were measured by qRT-PCR. . Data are expressed as mean ± SEM of five individual mice. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (unpaired Student's t-test).

Fig. S5

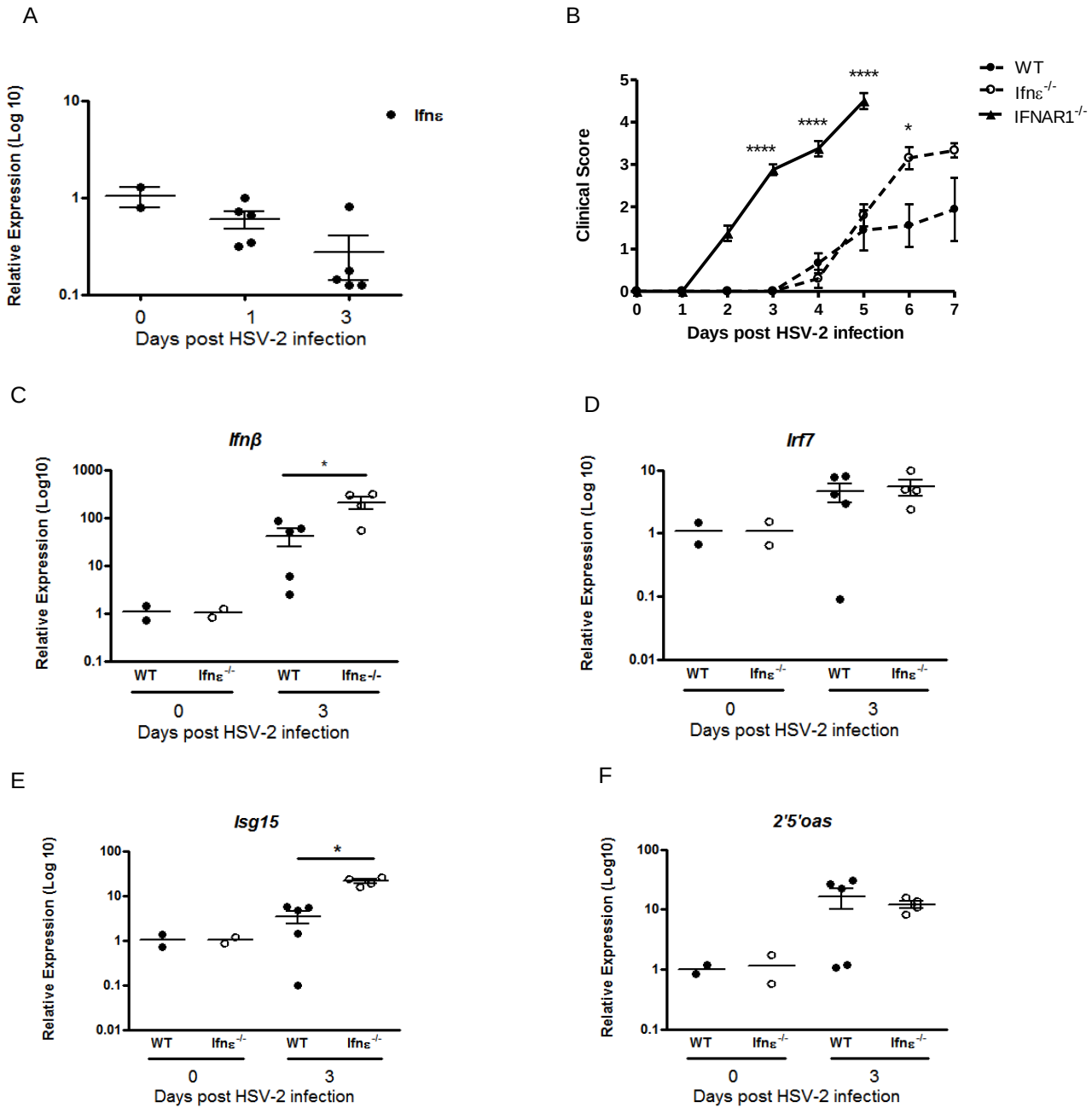
A
Relative Expression (Log 10)
Ifnε
Days post HSV-2 infection
B
Clinical Score
WT
Ifnε-/-
IFNAR1-/-
Days post HSV-2 infection
C
Ifnβ
Relative Expression (Log10)
WT
Ifnε-/-
Days post HSV-2 infection
D
Irf7
Relative Expression (Log 10)
E
Isg15
F
2'5'oas

Fig. S5

Ifn and IRG expression following HSV-2 vaginal infection and comparison of *Ifnε*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice susceptibility to HSV-2 vaginal infection. Mice pretreated with Depo-ralovera at day -5 were infected with 2400 PFU/mouse of HSV-2 on day 0. (A) Uteri from HSV-2 infected WT C57BL/6 mice were harvested and *Ifnε* expression was measured by qRT-PCR. Data are expressed as mean ± SEM of two to five individual mice. (B) Clinical scores of WT, *Ifnε*$^{-/-}$ and *Ifnar1*$^{-/-}$ C57BL/6 mice during 7 day course of infection. Data are expressed as mean ± SEM of five individual mice and are representative of at least three separate experiments.. (C-F) Vaginal tissue from HSV-2 infected WT and *Ifnε*$^{-/-}$ C57BL/6 mice were harvested and *Ifnβ, Irf7, Isg15 and 2'5' oas* expression were measured by qRT-PCR. Data are expressed as mean of two individual mice or mean ± SEM of five individual mice. *P<0.05, ****P<0.0001 (unpaired Student's t-test).

Fig. S6

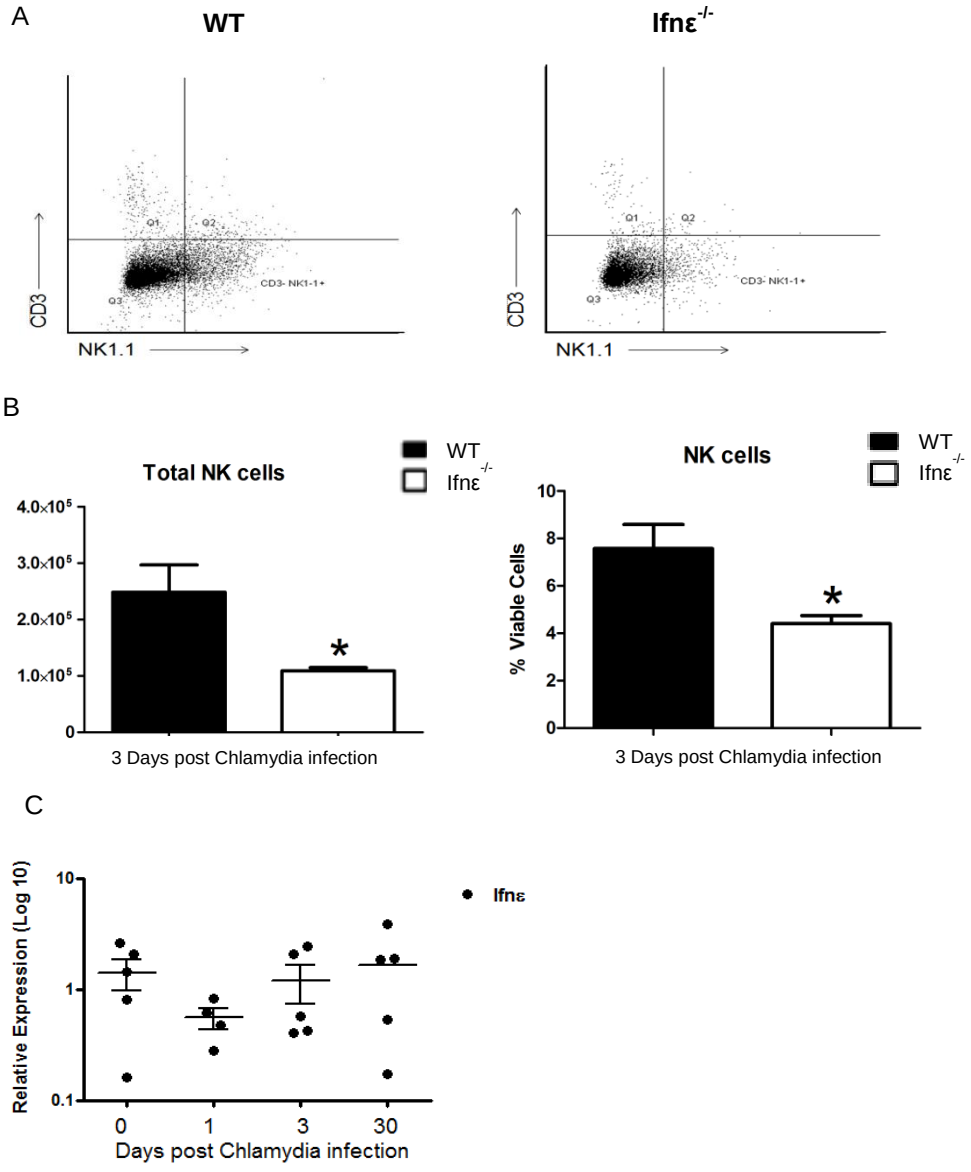
A
WT
Ifnε-/-
CD3
NK1.1
Q1
Q2
Q3
CD3- NK1-1+
B
Total NK cells
WT
Ifnε-/-
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
*
3 Days post Chlamydia infection
NK cells
% Viable Cells
10
8
6
4
2
0
*
3 Days post Chlamydia infection
C
Relative Expression (Log 10)
10
1
0.1
Ifnε
0
1
3
30
Days post Chlamydia infection

Fig. S6

Ifnε$^{-/-}$ mice have reduced numbers of NK cells and *Ifnε* is not induced by *C. muridarum* or HSV-2 infection. (A-B) Uterine tissues were harvested from WT and *Ifnε*$^{-/-}$ C57BL/6 mice day 3 p.i. with *C. muridarum*. The population of NK cells was analyzed by FACS. (A) Dot plot of NK cells ($CD3^{-}$ $NK1.1^{+}$) populations; left and right panels represent WT and *Ifnε*$^{-/-}$ C57BL/6 mice, respectively. This is representative of five individual mice. (B) Histogram showing total numbers and percentages of NK cells in uterine tissues. . Data are expressed as mean + SEM of from ten individual mice. (C) Uteri from *C. muridarum* infected WT C57BL/6 mice were harvested and *Ifnε* expression was measured by qRT-PCR. . Data are expressed as mean $\pm$ SEM of at least five individual mice. *P<0.05, **P<0.01 (unpaired Student's t-test).

Fig. S7

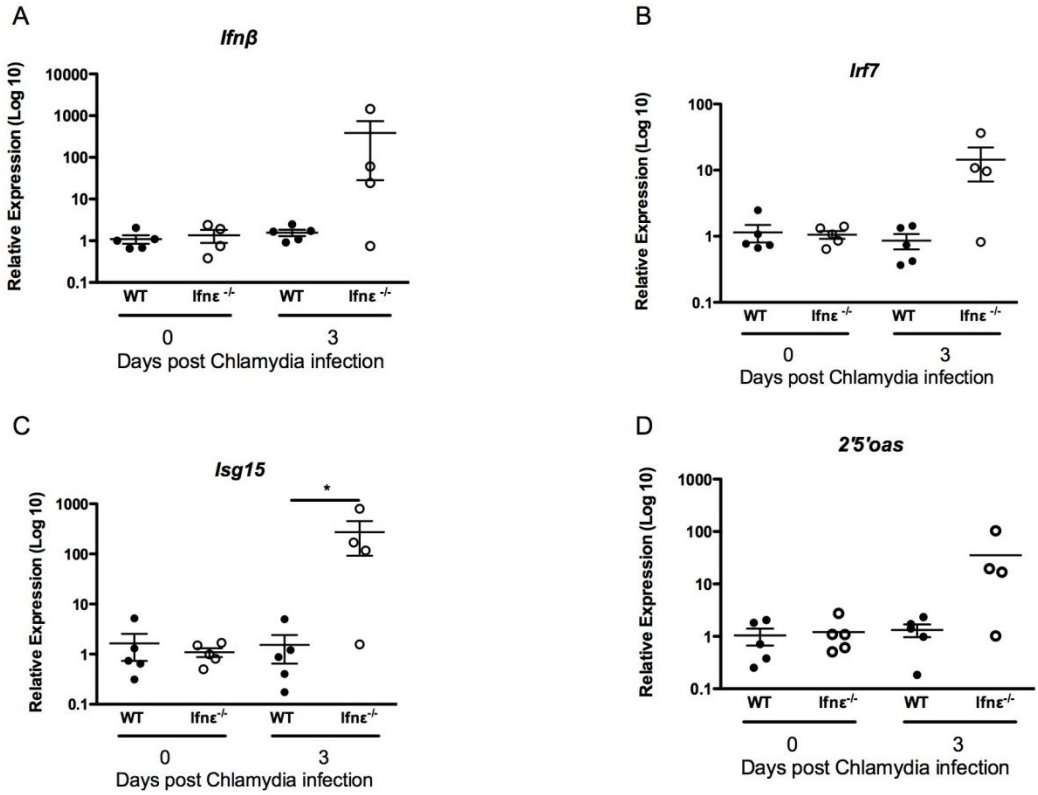

Fig. S7

Ifnβ and IRGs expression in WT and *Ifnε*$^{-/-}$ mice following *Chlamydia* infection. (A-D) Mice were pretreated with progesterone at day -7 and infected intra-vaginally with 5x10^4 IFU *C. muridarum*. Uteri from WT and *Ifnε*$^{-/-}$ C57BL/6 mice were harvested and *Ifnb, Irf7, Isg15 and 2'5' oas* expression was measured by qRT-PCR. . Data are expressed as mean $\pm$ SEM of five individual mice. **P*<0.05, (unpaired Student's t-test).

Fig. S8

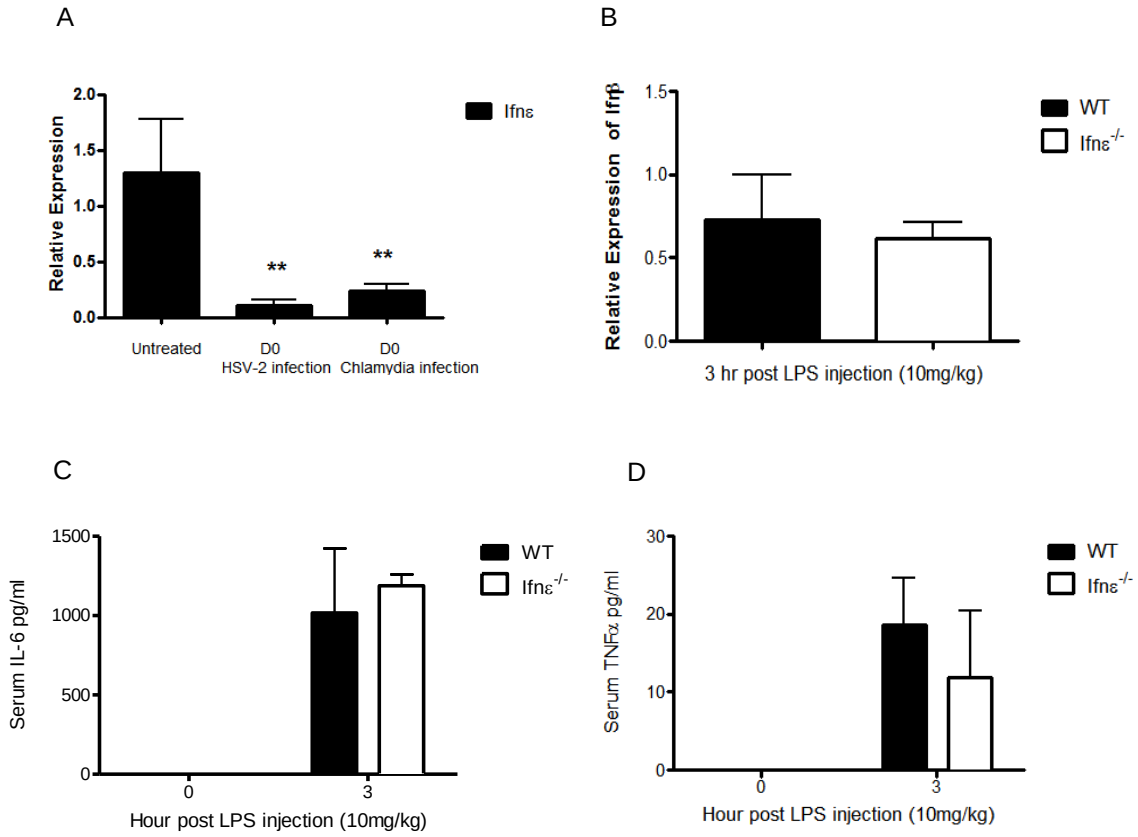

Fig. S8

Ifnε expression following Depo-ralovera treatment and the effect of *Ifnε*on systemic LPS treatment. (A) Uteri were obtained from WT C57BL/6 mice treated with Depo-ralovera for 5 and 7 days respectively and *Ifnε* expression was measured by qRT-PCR and the results were made relative to *Ifnε* expression at estrus stage. . Data are expressed as mean + SEM of at least four individual mice. (B-D) WT and *Ifnε*$^{-/-}$ C57BL/6 mice were treated with LPS through intraperitoneal injection and assessed 3h later. (B) Peritoneal macrophages were harvested and *Ifnβ* expression was measured by qRT-PCR. . Data are expressed as mean + SEM of at least five individual mice. Serum (C) IL-6 and (D) TNFα were measured by ELISA. . Data are expressed as mean + SEM of at least five individual mice. **P<0.01 (unpaired Student's t-test).

Fig. S9

Fig. S9

Ifnε amino acid alignment across different eutherian mammals. Amino acid sequences of Ifnε from different species (sIfnε represents *Sus scrofa*, fIfnε represents *Felis catus*, hIfnε represents *Homo sapiens*, pIfnε represents *Pan troglodytes*, mIfnε represents *Mus musculus* and rIfnε represents *Rattus norvegicus*) were aligned using Clustal W. Red and black boxes represent the predicted binding residues to Ifnar1 and Ifnar2, respectively.

Table S1.

Ratio of WT, *Ifnε*$^{+/-}$ and *Ifnε*$^{-/-}$ mice obtained from breeding *Ifnε*$^{+/-}$ mice.

Genotype	Wild Type (WT)	Heterozygous (Ifnε$^{+/-}$)	Knockout (Ifnε$^{-/-}$)	Total
Number of mice	35	88	41	164
Actual ratio	1.0	2.2	1.1	
Expected ratio	1	2	1	

Table S2.

Comparison of mechanism of action of IFNε and other type I IFNs. IFNε has a characteristic constitutive expression that is not induced by pathogens, persists throughout the course of an infection and is hormonally regulated. It protects from HSV-2 and *Chlamydia* infection, the latter by activating recruitment of NK cells. Conventional type I IFNs are not constitutively expressed, nor hormonally regulated, but transiently expressed after induction by virus or bacteria via PRR signaling pathways. While type I IFNs are protective against HSV-2 infection, they exacerbate *Chlamydi*a infection by activation of cytotoxic T cells.

	IFNε	IFNα/β
Expression - Constitutive	+	-
- PRR induced	-	+++
-Persistent	+	-
-Transient	-	+
-Hormone regulated	+	-
Chlamydia protection	+	-
HSV-2 protection	+	+
Cell Targets	NK	T