Absence of cGAS-mediated type I IFN responses in HIV-1-infected T cells

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Supplementary Methods

Supplementary References

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Supplementary Figures 1-8

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Supplementary Methods

Animals

cGAS KO mice on the C57BL/6 background (Mb21d1tm1d(EUCOMM)Hmgu/J, (1)) were kindly obtained from Charles Rice. TREX1 KO mice (2) were kindly obtained from Tomas Lindahl, Francis Crick Institute. C57BL/6 (wildtype), cGAS KO and TREX1 KO mice were bred under specific pathogen-free conditions in the central mouse facility of the Helmholtz Centre for Infection Research, Brunswick, and at TWINCORE, Centre for Experimental and Clinical Infection Research, Hanover, Germany. Mouse experimental work was carried out using 8-week-old to 14-week-old mice in compliance with regulations of the German animal welfare law.

Cell Lines and Primary Cells

A3.01, SUPT1, Jurkat, CEM T-cells were purchased from ATCC. PM1 T-cells were obtained from the NIH AIDS Reagent Program. S1A.TB, R1.1, TIMI.4, were kindly obtained from Oliver Keppler. Parental YAC-1 and L1210 cells were kindly obtained from Roland Jacobs, Hanover Medical School. CRISPR/Cas9-mediated knockouts were generated by transient electroporation of an EF1alpha-Cas9-2A-EGFP/U6-sgRNA expression plasmid, followed by FACS-Sorting and single-cell cloning by limiting dilution. CRISPR target sequences were: hs-cGAS – GGCCGCCCGTCCGCGCAACT(GGG); mm-cGAS - GCTGGGGCTCCCGTACGGC(GGG); hsTREX1 – GAGAGCTTGTCTACCACACG(CGG); mmTrex1 - GTCCACCACACGGGGCGGTC(TGG). Knockouts were validated by Sanger sequencing of PCR-amplified genomic loci and Immunoblot. Primer-Sequences (fw/rev): mm-cGAS genomic - GATCGCGGCGGGCGGAGCGT/ACAACTTTATTCACCGTCTCGGC; hs-cGAS genomic – GCGGCTGAGCTTCAACTTCTC/TGCTGCTGGCTCTTCCTCTTG; mm-Trex1

genomic – ATGGGCTCACAGACCCTGC/CTGCCCAGGTGAGGCCAG; hsTREX1 genomic – CCATGCAGACCCTCATCTTTTTC/GGGGGAAGTCGTAGCGGT. Parental HEK293T cells and HEK293T cells expressing cGAS (clone 17) are described in (3). Parental THP-1, cGAS KO THP-1 and Jurkat T cells stably expressing wildtpye cGAS and cGAS (G212A/S213A) are described in (4).

Withdrawal of blood samples from healthy humans and cell isolation were conducted with approval of the full study of the local ethics committees (Ethical review committee of Hannover Medical School, vote ID 3025-2016; Ethical review committee of Charité – Universitätsmedizin Berlin, vote ID EA4/167/19). Human PBMCs were isolated from buffy coats by Ficoll-Hypaque centrifugation. PBLs were generated by IL-2 (10 ng/ml)/PHA (1 µg/ml) stimulation of PBMCs for 3-4 days, resulting in >90% $CD3^+$ T-cell cultures. Purification of human $CD4^+$ T-cells was performed by negative selection using EasySep Direct Human $CD4^+$ T-Cell Isolation kit (Stemcell Technologies). Isolated cells were either lysed directly after purification or activated by IL-2 (10 ng/ml)/PHA (1 µg/ml) in RPMI 1640 (Sigma-Aldrich) containing 10% heat-inactivated fetal calf serum (Sigma-Aldrich), 1% penicillin-streptomyin (Thermo Fisher Scientific) and 2 mM L-glutamine (Thermo Fisher Scientific). Infection assays were performed in the presence of IL-2 (10 ng/ml).

Single-cell suspensions of mouse splenocytes were prepared by pushing spleen tissue pieces through a 70 µm pore size nylon mesh screen (Fisher Scientific). Washed splenocytes were subjected to $CD4^+$ T-cell isolation using the EasySep Mouse $CD4^+$ T-Cell Isolation kit (Stemcell Technologies). $CD4^+$ T-cells were cultured at 2×10^6/ml in RPMI 1640 containing 10% heat-inactivated fetal calf serum (Sigma-Aldrich), 1% penicillin-streptomyin (Thermo Fisher Scientific) and 2 mM L-glutamine (Thermo Fisher Scientific). Isolated cells were either lysed directly after purification or were stimulated for 3 to 4 days with mouse anti-CD4/CD28 beads (Gibco) using a bead-to-cell ratio of 0.5:1 and IL-2 (50 ng/ml).

Viruses

HIV-1_{Ba-L} was obtained from the NIH AIDS Reagent Program and propagated on PM1 T-cells. Virus-containing supernatant was filtered through a nylon filter of 0.2 µm pore size (VWR International) and stored at -80°C in aliquots.

HSV-1 Δ*UL41*N (HSV-1(KOS) UL41NHB) was kindly provided by David A. Leib (5). It encodes a truncated version of pUL41, which fails to induce the degradation of cellular mRNAs and is unable to counteract cGAS. To prepare concentrated stocks, extracellular virions were pelleted from the medium of cells infected with a multiplicity of infection (MOI) of 0.01 PFU/cell for 3 days (6, 7). Virus stocks were plaque-titrated on Vero cells (6, 8). To determine the genome/pfu ratio of HSV-1 stocks, we measured the number of HSV-1 genomes by quantitative PCR as described previously (7, 9).

Lentiviral and Gammaretroviral Particles

VSV-G-pseudotyped HIV-1 GFP particles were generated by calcium phosphate-based transfection of HEK293T cells with the packaging plasmid pCMV ΔR8.91 (10) expressing wildtype capsid or CA(P90A) or pCMV ΔR8.2 (CA N74D), the GFP-encoding transfer plasmid pHR.GFP (11) and the pCMV-VSV-G plasmid (12). VSV-G-pseudotyped MLV GFP particles were generated by calcium phosphate-based transfection of HEK293T cells with the packaging plasmid pCMVi gag-pol (13), the GFP-encoding transfer plasmid pSER S11 SF GFP (14), and the pCMV-VSV-G plasmid. Virus-containing supernatants were harvested 40 hours and 64 hours post transfection and subjected to ultracentrifugation through a 20% sucrose/PBS cushion. Virus-containing pellets were resuspended in medium and digested by DNase I (Promega) twice for 90 min at 37 °C. Particles were then pelleted by ultracentrifugation, resuspended in medium, aliquoted and stored at -80°C in aliquots.

Infections and Transductions

30 min prior to lenti- or retroviral transduction, T-cells were left untreated or treated either with EFV (100 nM), azidothymidine (AZT) (10 μM) or PF74 (2 μM). 30 min prior to HSV-1 inoculation, T-cells were left untreated or treated with ACV (20 μM). Inhibitor treatment was maintained during the subsequent virus inoculation. Transduction and HSV-1 inoculation occurred by spinoculation of 0.5 mio cells/24-well for 60 min at 32°C. Following spinoculation, cells were resuspended and individual wells were harvested at indicated time points.

Intracellular HIV-1 p24CA and HSV-1 VP5 Immunostaining

PBS-washed cells were PFA-fixed and immunostained for intracellular HIV-1 p24CA using FITC-conjugated mAb KC57 (Beckman Coulter) in 0.1% Triton in PBS. VP5 immunostaining was performed with rabbit anti-HSV-1 VP5 (#SY4563 (15)) and an appropriate fluorochrome-conjugated secondary antibody in 0.1% Triton in PBS. A FACS Calibur with BD CellQuest Pro 4.0.2 Software (BD Pharmingen) and a FACS Lyric with FlowJo software were used for analysis.

Reagents and Inhibitors

IL-2 and PHA were purchased from Miltenyi Biotec and Oxoid, respectively. Anti-mouse CD3/28 beads were purchased from Gibco. Human IFN-α2a (Roferon) and mouse IFN-α1 were purchased from Roche and PBL Assay Science, respectively. 2'-3'-cGAMP, c-di-UMP and ISD were purchased from Invivogen. EFV was purchased from Brisol-Myers Squibb. ACV, AZT and PF74 were purchased from Sigma-Aldrich.

Quantitative RT-PCR

Total RNA extraction from cells and DNase treatment were performed with Maxwell LEV simplyRNA purification Kit (Promega), followed by cDNA synthesis (NEB, Invitrogen). Quantification of relative mRNA levels was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems) or the ABI7500 Real Time PCR System using Taq-Man PCR technology with the following oligonucleotide primers from MWG: For human *cGAS* (gene ID 115004), *IFN-β* (gene ID 3456), *IFIT1*(gene ID 3434) , *MX2* (gene ID 4600) and mouse *Ifit1* (gene ID 15957), and *Mx2* (gene ID 17858) premade primer-probe kits were used (Applied Biosystems). Relative mRNA levels were determined using the ΔΔCt method with human *RNASEP* mRNA or mouse *Gapdh* mRNA (Applied Biosystems) as internal references. Each sample was analyzed in triplicates. Data analysis was performed using Applied Biosystems 7500 Fast System Software.

Quantitative PCR of viral DNA

DNA extraction from cells were performed with Maxwell 16 Blood DNA purification kit (Promega). Quantification of absolute copy numbers of HIV-1 late RT products was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems) using a published Taq-Man-based PCR (16).

The number of HSV-1 genomes was quantified as described previously (7, 9) using the LightCycler FastStart DNA Master HybProbe kit (Roche Diagnostics, Mannheim, Germany), the forward sense primer 5′-CCACGAGACCGACATGGAGC-3′, the reverse antisense primer 5′-GTGCTYGGTGTGCGACCCCTC-3′, the fluorescein-coupled donor probe 5′-TGTTGGCGACTGGCGACTTTG-3′-fluorescein, and the R640-coupled acceptor probe R640-5′-TACATGTCCCCGTTTTACGGCTACCGG-3′-phosphate, which are all specific to the coding region of HSV-1 gB (gene UL27), and the Roche Light-Cycler 1.5. After one cycle

of denaturation (95°C for 10 min), 55 cycles of amplification were performed (95°C for 10 s, 58°C for 15 s, and 72°C for 15 s). During the 58°C phases, the acceptor fluorescence was measured at 640 nm, and from these data the DNA concentrations of the samples were calculated using standards of known DNA concentrations.

Human and Mouse Type I IFN Bioactivity Assays

Secretion of human type I interferon bioactivity was quantified using the human reporter cell line HL116 that carries the luciferase gene under the control of the IFN-inducible 6-16 promoter ((17), a kind gift from Sandra Pellegrini, Institut Pasteur, France). Human IFN-alpha2a (Roferon) was diluted for generation of a standard curve.

Secretion of murine, bioactive type I IFN was quantified using the mouse reporter cell line MEF that expresses the luciferase gene under the control of the mouse *Mx2* promoter (18), a kind gift of Mario Köster. Mouse IFN-α1 was diluted for generation of a standard curve.

Electroporation of T-cells

T-cell lines and primary $CD4^+$ T-cells were challenged with plasmid DNA, cGAMP and c-di-UMP, and ISD by electroporation. Typically, 10 million cells were pulsed with 24 µg plasmid DNA, 6 µg cGAMP or c-di-UMP, and 4 µg ISD. The electroporation was conducted at 250 V for 1 ms using a Gene Pulser Xcell Electroporation System (BioRad).

Immunoblotting

Cells were lysed with M-PER Mammalian Protein Extraction Reagent (Pierce). Proteins were run on a 7.5% SDS-PAGE and transferred onto nitrocellulose using a semi-dry transfer system (Biometra). Blocked membranes were incubated with the following primary antibodies (rabbit anti-MAPK (#SC-153, Santa Cruz), rabbit-anti-β-actin (#ab8227, Abcam), rabbit-anti-human

cGAS (#66546) and rabbit anti-mouse cGAS (#31659) (both Cell Signaling Technology), rabbit anti-pIRF3 (Ser396) (#4947S, Cell Signaling), rabbit anti-STING (#13647, Cell Signaling Technology), rabbit anti-human TREX1 (#ab185228, Abcam) and mouse anti-mouse TREX1 (#611987, BD Bioscience). Secondary antibodies conjugated to Alexa680/800 fluorescent dyes were used for detection and quantification by Odyssey Infrared Imaging System (LI-COR Biosciences).

RNA Sequencing

Total RNA extraction from primary cells and DNase treatment were performed with Maxwell LEV simplyRNA purification Kit (Promega). Quality and integrity of total RNA was controlled on Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). The RNA sequencing library was generated from 100 ng total RNA using NEBNext® Single Cell/Low Input RNA Library following the manufacturer´s protocol. The libraries were sequenced on Illumina NovaSeq 6000 using NovaSeq 6000 S1 Reagent Kit (100 cycles, paired end run 2x 50 bp) with an average of 3 x 10^7 reads per RNA sample. Data generated from individual samples (>30 million read per sample, paired-end read 50-mers) were mapped separately against the hg38 human reference genome or the HIV-1_{BaL} genome (GenBank: AY713409). Gene expression was calculated for individual transcripts as reads per kilobase per million bases mapped (RPKM). All transcriptomic analyses were performed using CLC Genomics Workbench 12.0.3 (Qiagen, Aarhaus). Differentially expressed genes (DEGs) were identified by calculating fold changes in expression, p-values were corrected by taking false discovery rate (FDR) for multiple comparison into account.

Data Presentation and Statistical Analysis

If not otherwise stated, bars and symbols show the arithmetic mean of indicated amount of repetitions. Error bars indicate S.D. from one representative experiment out of at least three or S.E.M. from the indicated amount of individual experiments. Statistical significance was calculated by performing Student´s t-test using GraphPad Prism. *P* values <0.05 were considered significant (*) and <0.01 very significant (**); n.s. = not significant (≥0.05).

Supplementary References

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Supplementary Legends

Figure S1. cGAS Expression in Primary T-cells

(**A**) Immunoblotting of lysates of primary IL-2-activated, human $CD4^+$ T-cells and indicated THP-1 cells

(**B**) Immunoblotting of lysates of mouse $CD4^+$ T-cells of indicated genotypes

(**C**) cGAS mRNA expression in indicated cells

(**D**) cGAS mRNA expression in resting and IL-2/PHA-stimulated, human $CD4^+$ T-cells

Error bars indicate S.E.M. from values obtained from cells from three-four individual experiments.

Figure S2. Mock Electroporation of Primary CD4+ T-Cells and PM1 T-Cells Triggers Neglectable Levels of *IFIT1* mRNA Expression

Primary $CD4^+$ T-cells (**A**) and parental PM1 T-cells (**B**) were left untouched, mock-electroporated or electroporated with plasmid DNA. Cultures were monitored for relative *IFIT1* mRNA expression 48 hours post electroporation by Q-RT-PCR. Error bars indicate S.E.M. from values obtained from cells from three individual experiments.

Figure S3. ISD triggers induction of *IFIT1* and *IFN-β* mRNA expression in THP-1 in a cGAS-dependent fashion

Undifferentiated THP1 cells were mock-transfected or transfected with ISD and cGAMP. Cultures were monitored at 5 hours post electroporation for relative *IFIT1* and *IFN-β* mRNA expression by Q-RT-PCR. Error bars indicate S.E.M. from values obtained from cells from four individual experiments.

Figure S4. Absence of PF74-induced innate immune responses in primary CD4$^+$ T-cells infected with HIV-1

(**A-B**) Primary human CD4$^+$ T-cells were infected with HIV-$1_{Ba\text{-}L}$ in the absence and presence of PF74 (2 μM) and monitored for:

(**A**) HIV-1 p24 capsid expression by intracellular immunostaining by FACS analysis and assessment by viability (FSC/SSC) three days post-infection

(**B**) Relative expression of *IFIT1*, *MX2* and *IFN-β* at indicated time points by Q-RT-PCR.

Error bars show S.E.M. from values obtained from CD4$^+$ T-cells from three individual donors.

Figure S5. Analysis of the cGAS/STING pathway in human T-cell lines

Indicated human T-cell lines were either mock-electroporated or electroporated with plasmid DNA (**A**), and electroporated either with c-di-UMP or cGAMP (**B**). Cultures were monitored at indicated time points post challenge for relative *IFIT1* mRNA expression by Q-RT-PCR (top panels). Error bars indicate S.E.M. from values obtained from cells from three individual experiments.

Figure S6. Absence of innate immune responses in parental PM1 T-cells upon transduction with lentiviral vectors containing capsid-destabilizing mutations in capsid

(**A**) Representative immunoblot of lentivirus vector inocula

(**B**) Reporter GFP expression in parental PM1 T-cells 72 hours post transduction by FACS analysis

(**C**) Relative expression of *IFIT1* and *MX2* by Q-RT-PCR

Error bars indicate S.E.M. from values obtained from cells from three individual experiments.

Figure S7. Analysis of the cGAS/STING pathway in mouse T-cell lines

Indicated mouse T-cell lines were either mock-electroporated or electroporated with plasmid DNA (**A-B**), and electroporated either with c-di-UMP or cGAMP (**C-D**). Cultures were monitored at indicated time points post challenge for relative *Ifit1* mRNA expression by Q-RT-PCR (**A, C**) and for release of bioactive IFN into the culture supernatant (**B, D**). Error bars indicate S.E.M. from values obtained from cells from three individual experiments.

Figure S8. MLV-based retroviral transduction fails to trigger cGAS-dependent innate immune reactions in mouse YAC-1 T-cells

Parental and cGAS KO T-cell lines were transduced with VSV-G MLV GFP vectors in the absence and presence of AZT and monitored, at indicated time points, for:

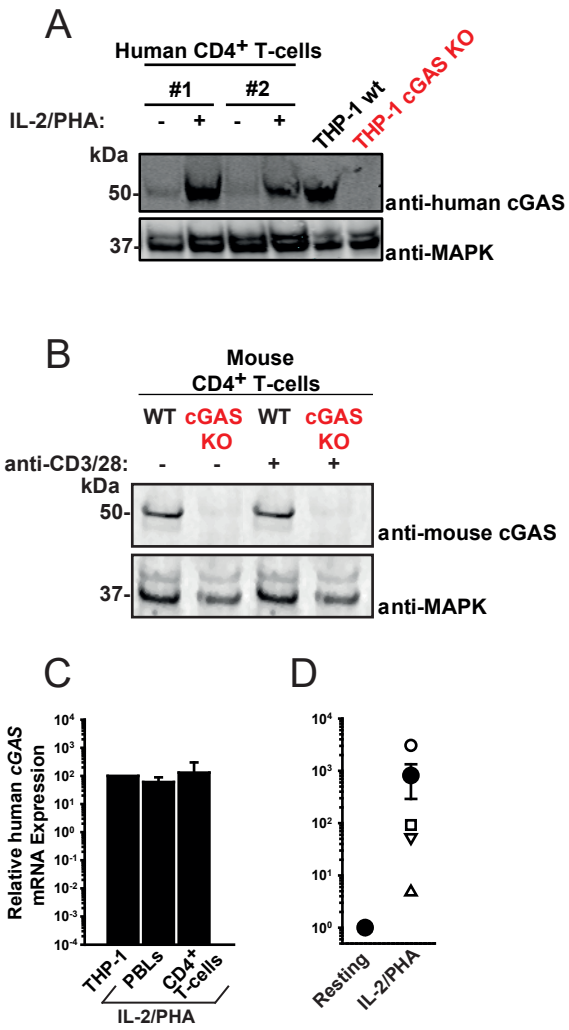
(**A**) Reporter GFP expression by FACS analysis

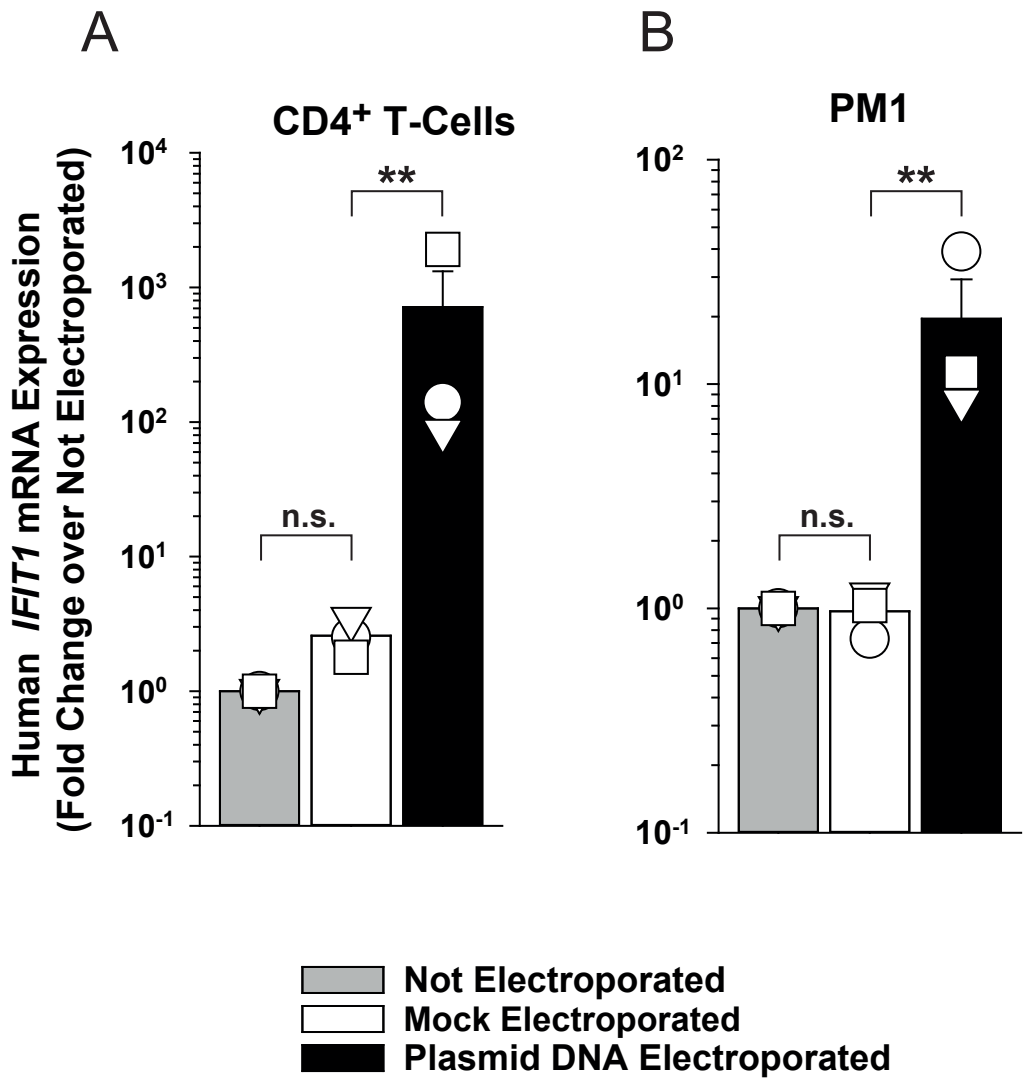
(**B**) Relative expression of *Ifit1* mRNA by Q-RT-PCR

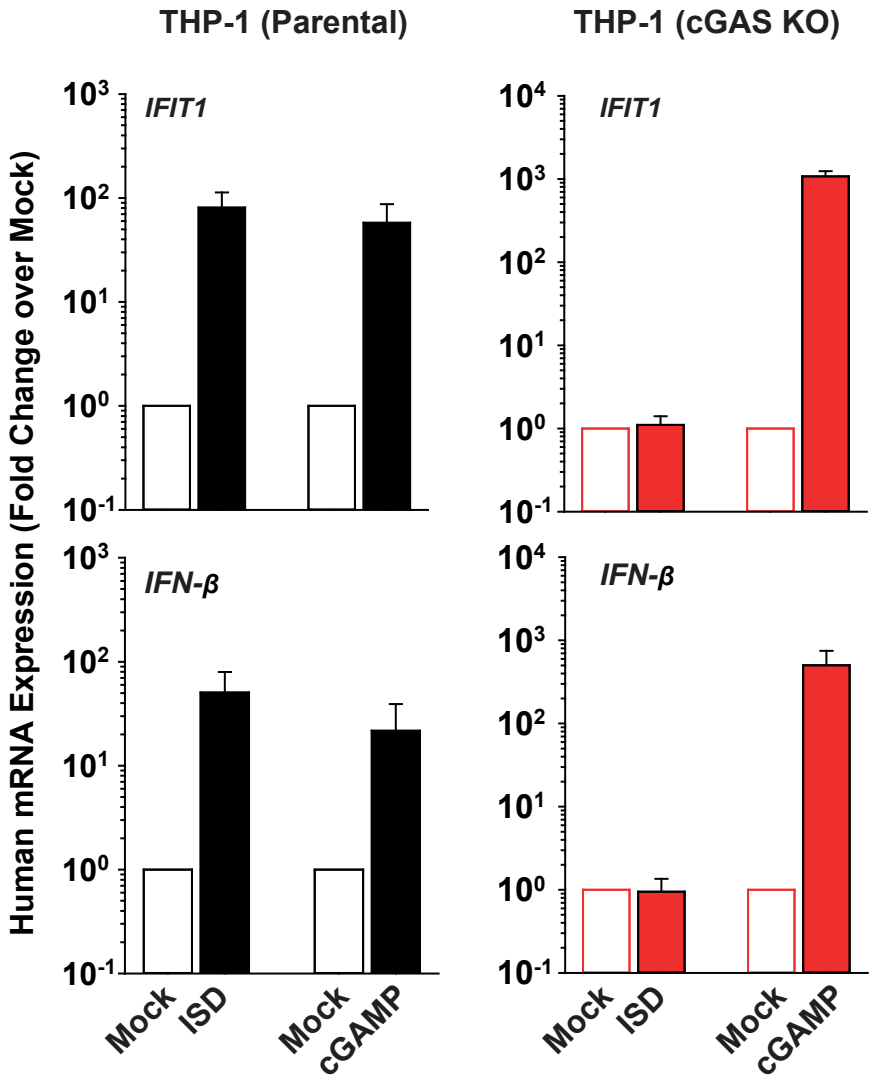
(**C**) Relative expression of *Mx2 mRNA* by Q-RT-PCR

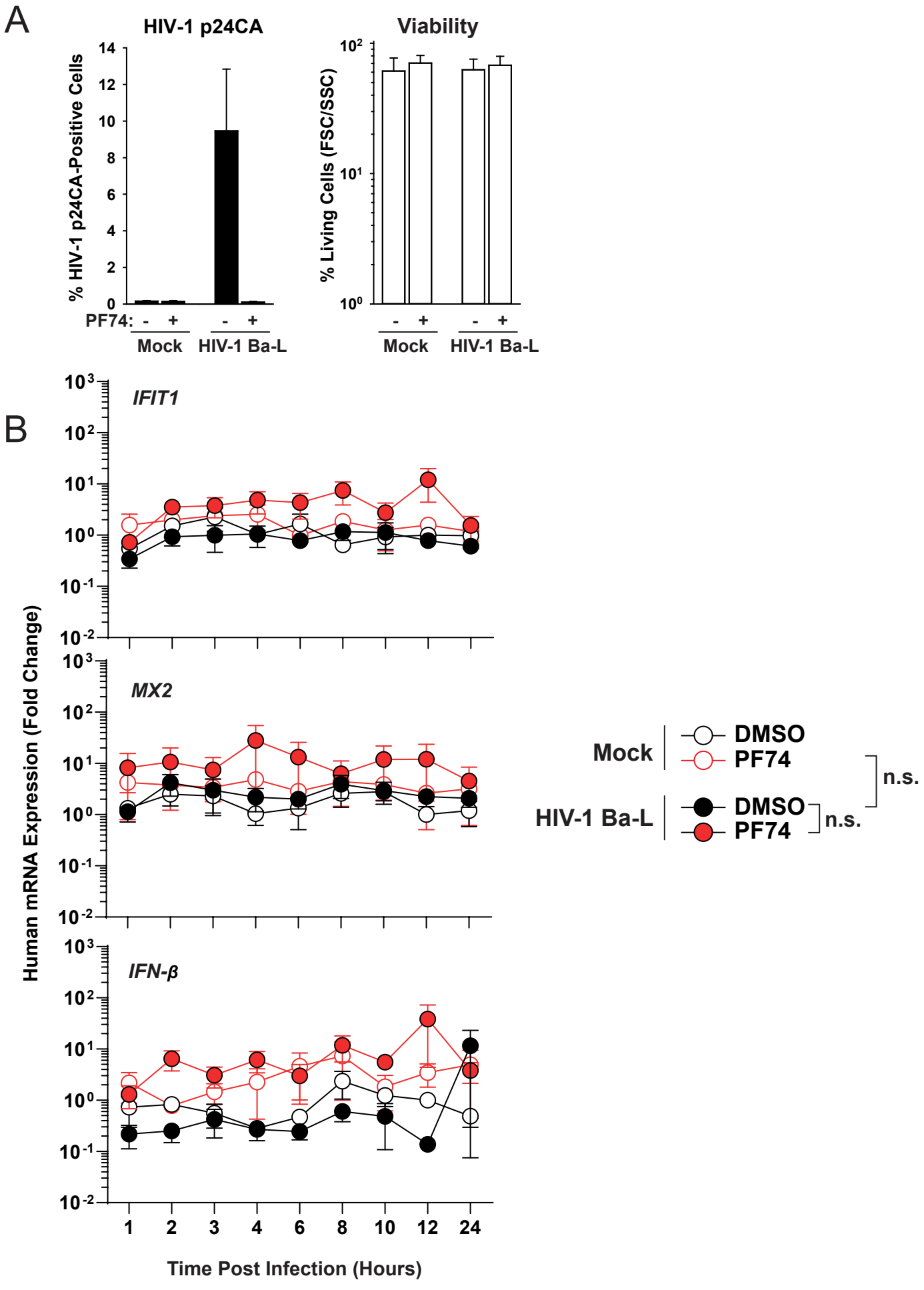
Error bars indicate S.E.M. from values obtained from cells from three individual experiments.

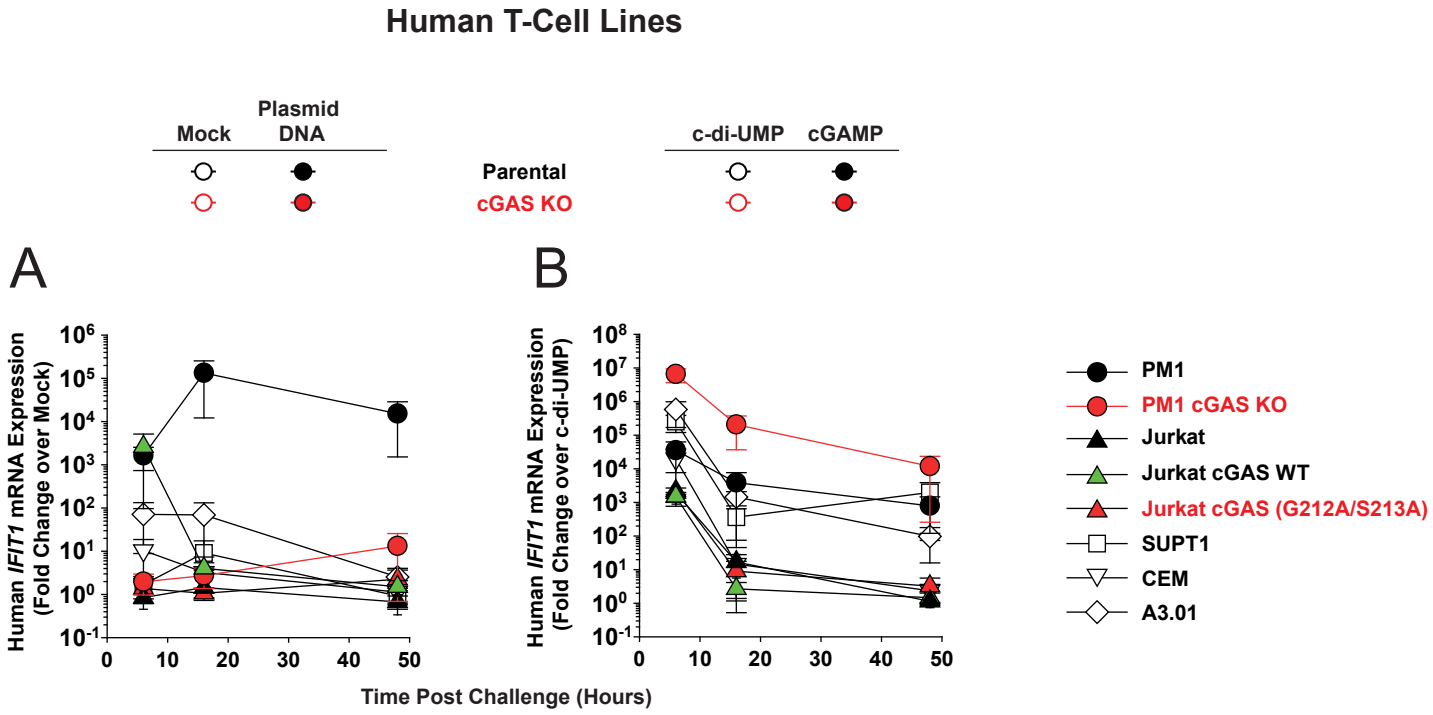
Elsner and Ponnurangam *et al.*, Suppl. Fig. 1




A
B
CD4+ T-Cells
PM1
Human IFIT1 mRNA Expression
(Fold Change over Not Electroporated)
10^4
10^3
10^2
10^1
10^0
10^-1
10^2
10^1
10^0
10^-1
**
**
n.s.
n.s.
Not Electroporated
Mock Electroporated
Plasmid DNA Electroporated


THP-1 (Parental)
THP-1 (cGAS KO)
Human mRNA Expression (Fold Change over Mock)
IFIT1
IFN-β
Mock
ISD
Mock
cGAMP


A
HIV-1 p24CA
% HIV-1 p24CA-Positive Cells
PF74: - + - +
Mock
HIV-1 Ba-L
Viability
% Living Cells (FSC/SSC)
B
IFIT1
MX2
IFN-β
Human mRNA Expression (Fold Change)
Time Post Infection (Hours)
Mock
DMSO
PF74
n.s.
HIV-1 Ba-L
DMSO
PF74
n.s.


Human T-Cell Lines
Plasmid
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c-di-UMP
cGAMP
A
B
Human IFIT1 mRNA Expression
(Fold Change over Mock)
Human IFIT1 mRNA Expression
(Fold Change over c-di-UMP)
Time Post Challenge (Hours)
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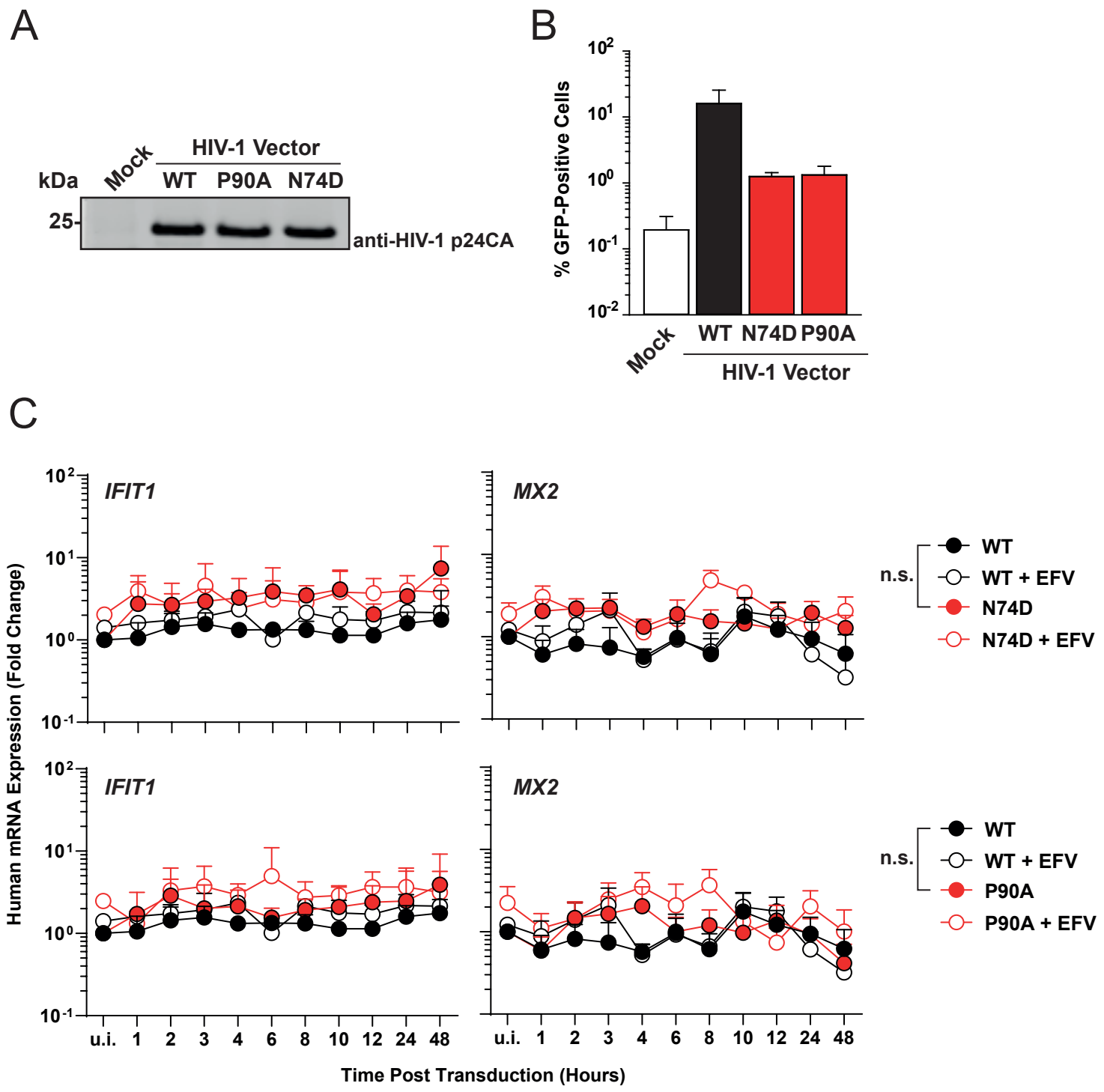
PM1 cGAS KO
Jurkat
Jurkat cGAS WT
Jurkat cGAS (G212A/S213A)
SUPT1
CEM
A3.01


A
kDa
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HIV-1 Vector
WT
P90A
N74D
25-
anti-HIV-1 p24CA
B
% GFP-Positive Cells
Mock
WT
N74D
P90A
HIV-1 Vector
C
IFIT1
MX2
Human mRNA Expression (Fold Change)
n.s.
WT
WT + EFV
N74D
N74D + EFV
P90A
P90A + EFV
u.i.
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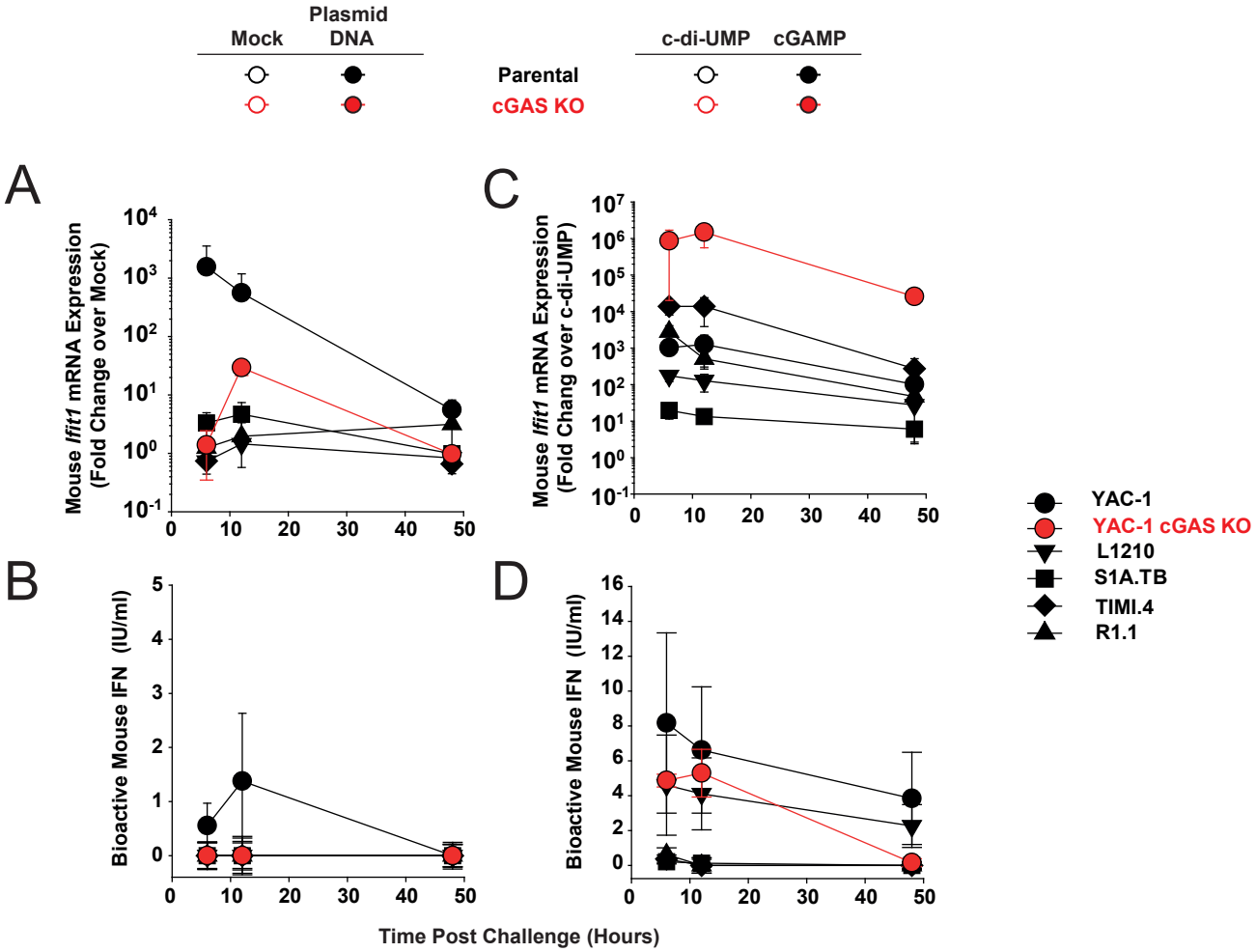
2
3
4
6
8
10
12
24
48
Time Post Transduction (Hours)


Plasmid
Mock
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Parental
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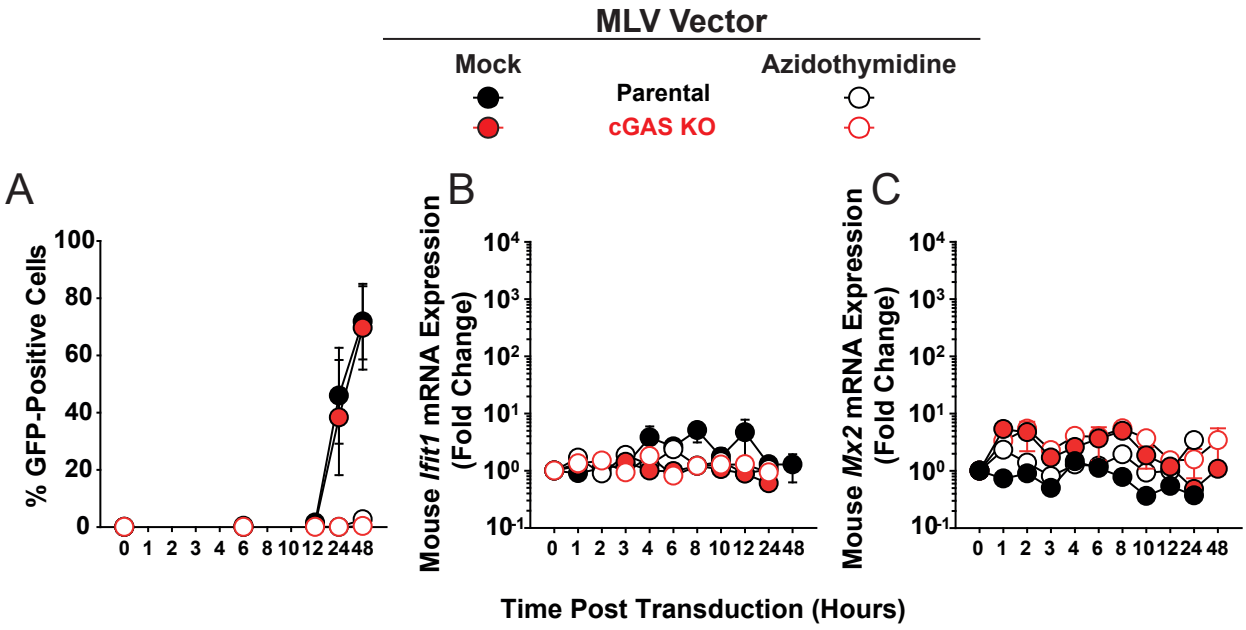
A
C
B
D
Mouse Ifit1 mRNA Expression (Fold Change over Mock)
Mouse Ifit1 mRNA Expression (Fold Chang over c-di-UMP)
Bioactive Mouse IFN (IU/ml)
Bioactive Mouse IFN (IU/ml)
Time Post Challenge (Hours)
YAC-1
YAC-1 cGAS KO
L1210
S1A.TB
TIMI.4
R1.1


MLV Vector
Mock
Azidothymidine
Parental
cGAS KO
A
% GFP-Positive Cells
B
Mouse Ifit1 mRNA Expression (Fold Change)
C
Mouse Mx2 mRNA Expression (Fold Change)
Time Post Transduction (Hours)