Supporting Information

The GRA15 protein from Toxoplasma gondii enhances host defense responses by activating the interferon stimulator STING

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This article contains Supplementary Figure 1-7 and Tables S1–S2:

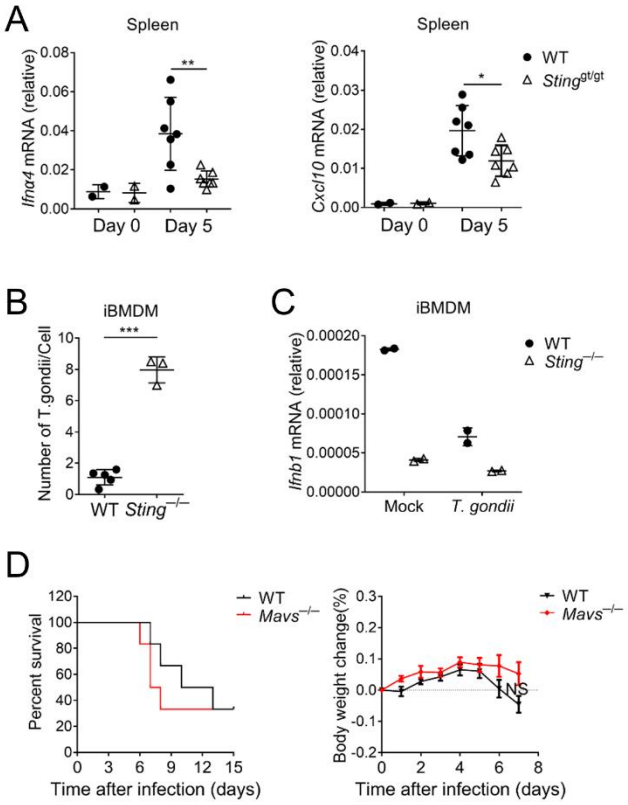


Figure S1. cGAS/STING axis is critical for anti-*T. gondii* immunity in vivo and in vitro. **A**, RNA was isolated after spleens were harvested as in figure 1B. *Ifnα4* and *Cxcl10* mRNA were quantified by qRT-PCR. **B**, Isogenic wild type and Sting$^{-/-}$ iBMDM cells were infected by *T. gondii* and at 24h stained for nucleus (DAPI, blue) and visualized by confocal microscopy (Data not shown). For each sample, number of parasites in 2-4 cells was counted and the mean ± SD values are shown. **C**, qRT-PCR analysis of *Ifnb1* mRNA in wild type or *Sting*$^{-/-}$ iBMDM cells after 24 h of infected by *T. gondii*. **D**, 129 wild type (WT, black lines, n=6) and Mavs$^{-/-}$ (red lines, n=6) mice were infected with 10^5 *T. gondii* tachyzoites per mouse, survival and body weight were monitored. Statistical analysis was performed by log rank test for (D) and *t* test for (A - C). Data were from three independent experiments. NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001. Error bars, S.D.

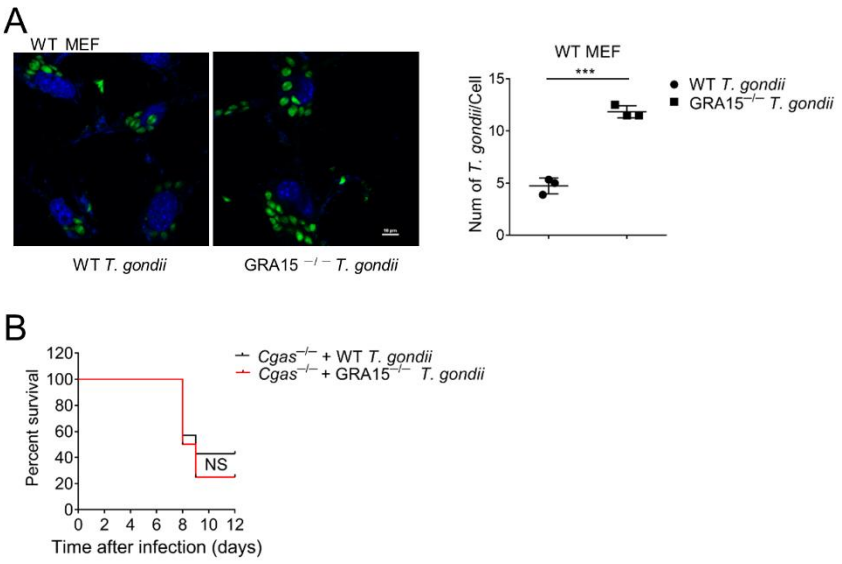


Figure S2. GRA15 is required for mounting innate immune response during *T. gondii* infection. A, Left: Immortalized MEF cells was infected by wild type or GRA15$^{-/-}$ *T. gondii*, and at 24 hours post-infection stained for nucleus (DAPI, blue), *T. gondii* (green) and visualized by confocal microscopy. The scale bar length is 10 μm. Right: For samples in immunofluorescence, number of parasites in 10-12 cells was counted and the mean ± SD values are shown. **B**, C57BL/6 *Cgas*$^{-/-}$ mice were infected with 10^5 wild type (black lines, n=7) or GRA15$^{-/-}$ (red lines, n=8) *T. gondii* tachyzoites, survival and body weight were monitored. Statistical analysis was performed by *t* test for (A) and log rank test for (B). Data were from three independent experiments. NS, not significant; ***P < 0.001. Error bars, S.D.

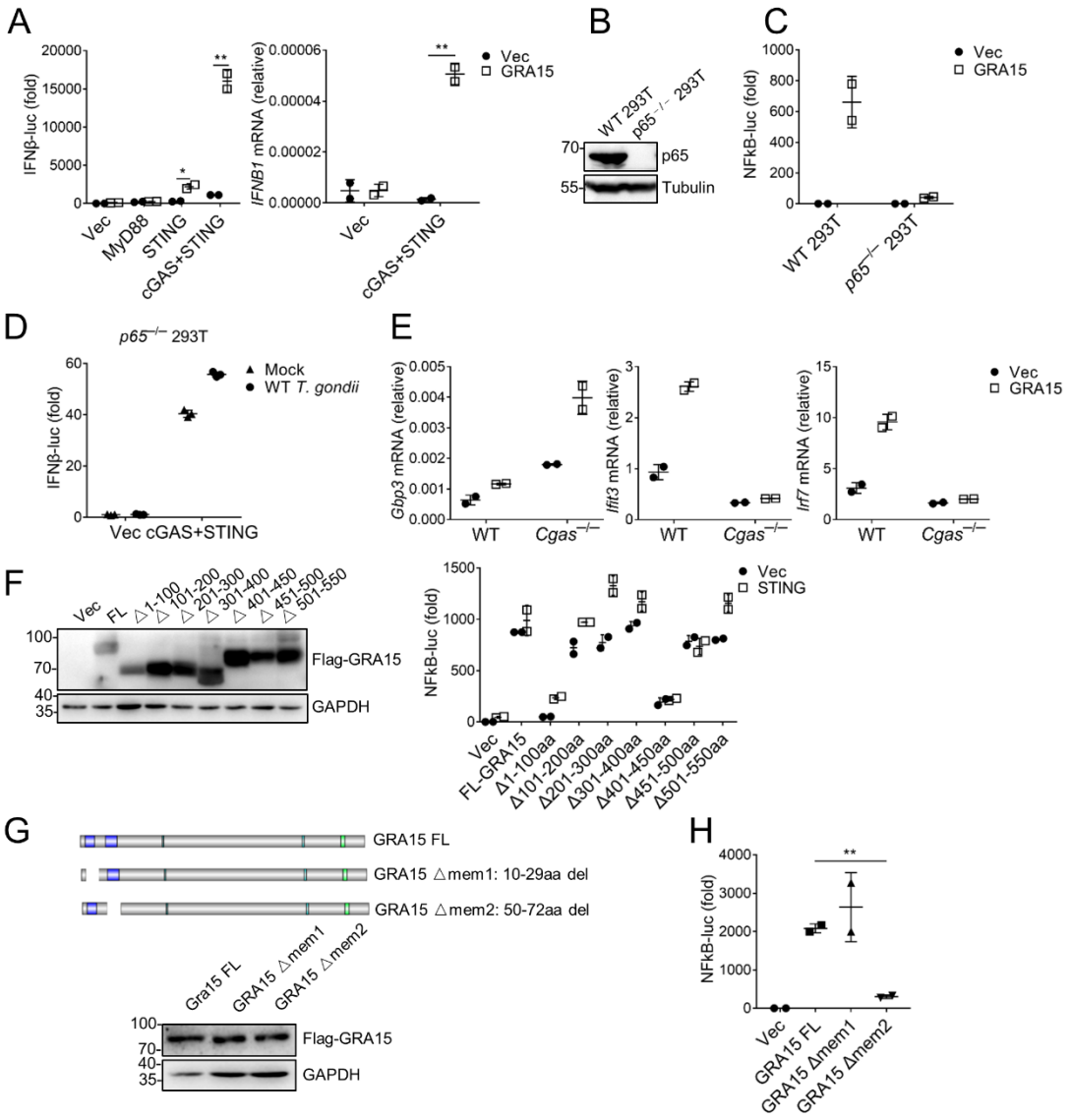


Figure S3. GRA15 enhances STING mediated anti-*Toxoplasma gondii* immunity. A, Luciferase activity (left) and qRT-PCR (right) in HEK293 cells 24h after transfection with indicated reporter plasmids and empty vector (Vec) or GRA15. **B**, Total cell proteins of wild type or *p65*$^{-/-}$ HEK293 cells were analyzed by immunoblotting to detect p65. **C**, Luciferase activity of NFκB-luc was measured in wild type or *p65*$^{-/-}$ HEK293 cells overexpressing GRA15. **D**, Luciferase activity of IFNβ-luc was measured 24h after wild type *T. gondii* infection in *p65*$^{-/-}$ HEK293 cells overexpressing cGAS and STING for 24h. **E**, qRT-PCR experiment in WT and *Cgas*$^{-/-}$ iBMDM cells overexpressing GRA15. **F**, Left: Immunoblot analysis of HEK293 cells overexpressing FL (GRA15 full length) and different deletions of GRA15. Right: luciferase activity of NFκB-luc was measured in HEK293 cells overexpressing FL (GRA15 full length) and different deletions of GRA15 with STING. **G**, Diagram detailing GRA15 domains (transmembrane domain 1, 2) and constructs used in this study. Immunoblot analysis of HEK293 cells transfected with plasmids encoding GRA15 full length (GRA15 FL), GRA15 without transmembrane domain 1 (GRA15 △mem1) or GRA15 without transmembrane domain 2 (GRA15 △mem2). **H**, Luciferase activity of NFκB-luc was measured in HEK293 cells transfected with plasmids encoding Vec, GRA15 FL, GRA15

△mem1 or GRA15 △mem2. Statistical analysis was performed by *t* test for (A, C, D - F, H). Data were from three independent experiments. **P < 0.01. Error bars, S.D.

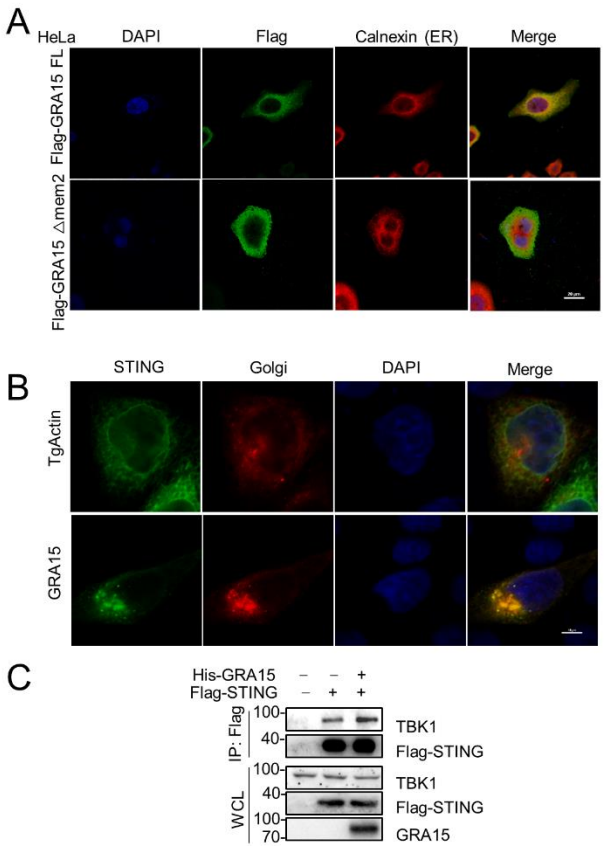


Figure S4. GRA15 anchors to endoplasmic reticulum (ER) of host cells. A, HeLa cells were transfected with plasmids encoding Flag-GRA15 FL or Flag-GRA15 △mem2, and at 24h stained for nucleus (DAPI, blue), Flag (green) and ER (Calnexin, red). The scale bar length is 20 μm. **B**, HeLa cells were transfected with plasmids encoding Flag-TgActin or Flag-GRA15, and at 24h stained for nucleus (DAPI, blue), STING (green) and Golgi apparatus (red). The scale bar length is 10 μm. **C**, Immunoblot analysis of HEK293 cells transfected with indicated plasmids, lysed and immunoprecipitated with anti-Flag antibody. Whole cell lysates (WCL) were immunoblotted with antibodies to indicated proteins.

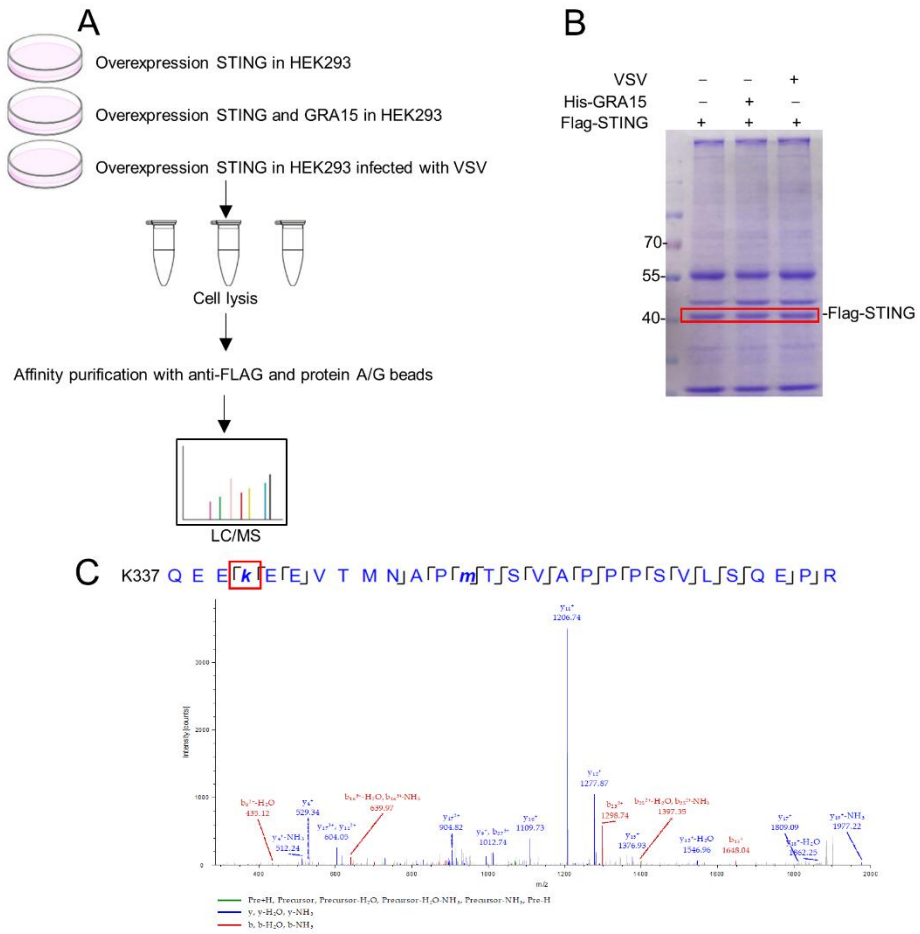
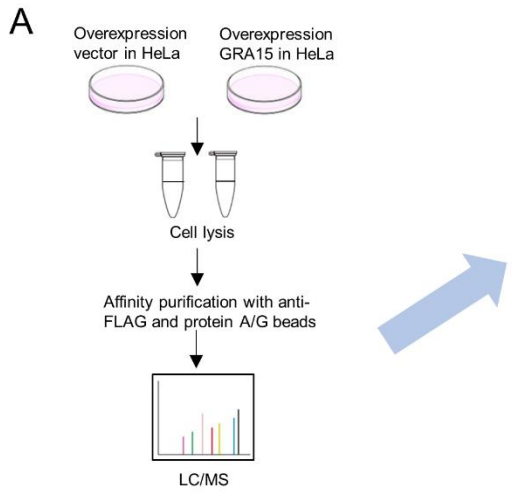
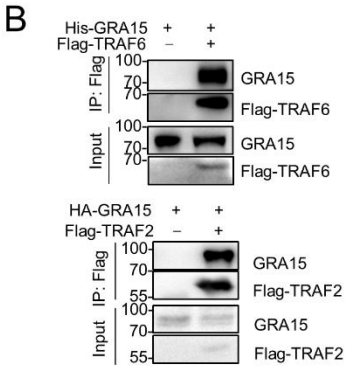
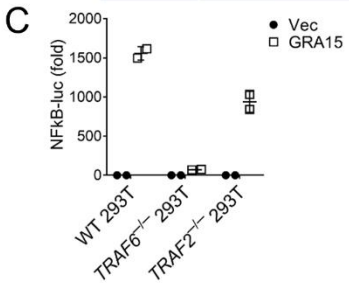


Figure S5. GRA15 promotes ubiquitination of STING. A / **B**, SDS-PAGE of HEK293 cells expressing Flag-STING (in red frame) treated with VSV or co-expressing His-GRA15 and harvested 36h after transfection followed by immunoprecipitation using anti-Flag antibody. Gels containing Flag-STING peptides from all three samples were cut off and analyzed the posttranscription modification by mass spectrometry. **C**, STING modification identified by mass spectrometry. Flag-STING was found to be ubiquitinated at lys-337 (in red frame) in both GRA15 overexpressing and VSV infected cells according to the results from mass spectrometry.

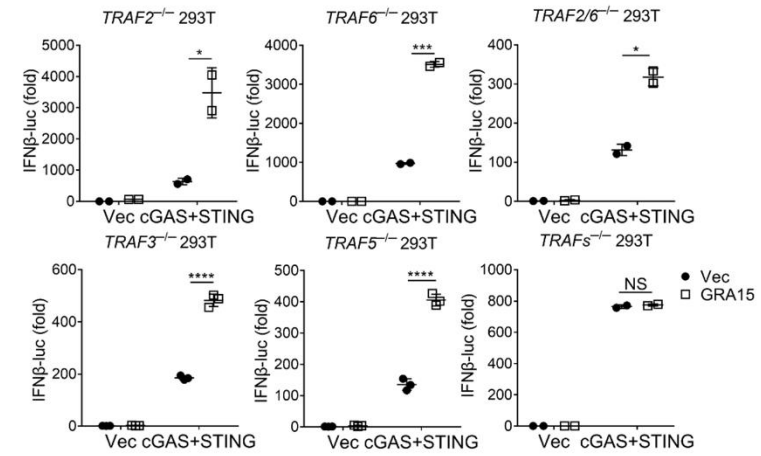


protein	peptides
TRAF2	NEEDAAELVALAQAVNAR
	KPLLESGTLGTK
	AAVATFLQSVQVPEFTPK
	YDGQVAVFGSDLQEK
	YFLVGAGAIGcELLK
	VGEFcHNR
	ATLPSPDKLPGFK
	LQTSSVLVSGLR
	QFLFRPWDVTK
	FEVQGLQPNGEEMTLK
	LDQPMTEIVSR
	AVTLHDQGTAQWADLSSQFYLR
	IYDDDFFQNLDGVANALDNVDAR
	ALPAVQQNNLDEDLIR
	QPAENVNQYLTDPK
	NIILGGVK
	DEFEGLFK
	GNVQVVIPFLTESYSSSQDPPEK
	LVVADTR
	QFLDYFK
TRAF6	NLDLDSIIAEVK
	YEDEINKR
	ISISTSGGSFR
	YEELQQTAGR
	LLEGEEcR
	QNLEPLFEQYINNLR
	FASFIDK





	TRAF6	TRAF2	STING
GRA15	+	+	–



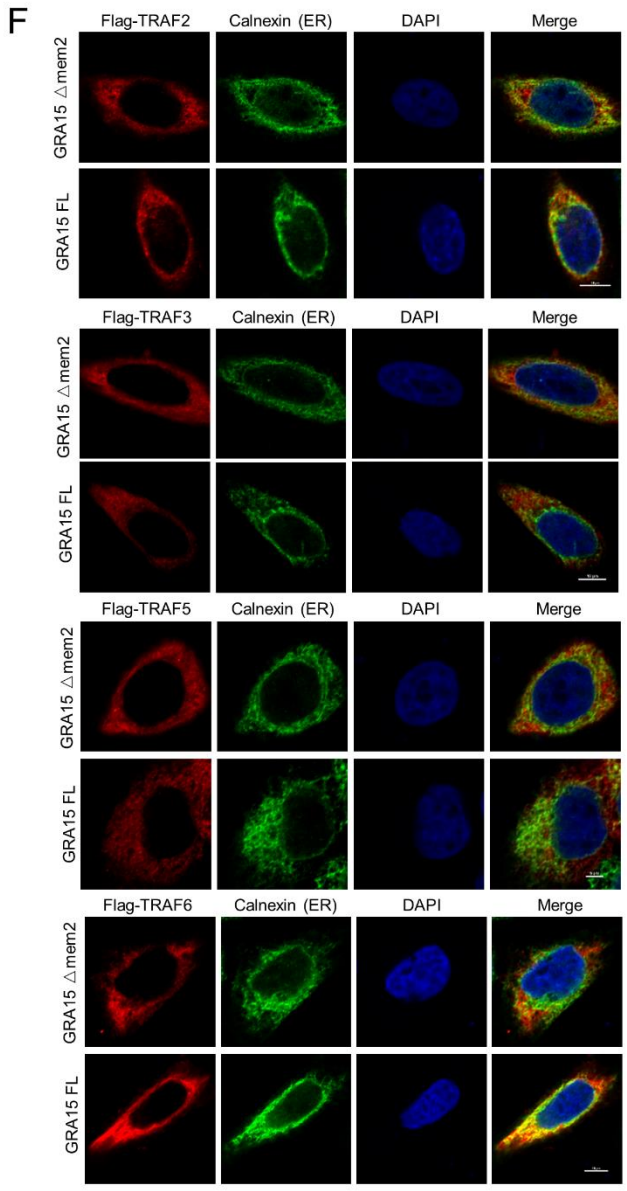


Figure S6. GRA15 depends on TRAFs to activate STING. A, HeLa cells were transfected with empty vector or Flag-GRA15 and harvested 24h after transfection, followed by immunoprecipitation with anti-Flag antibody. TRAF2 and TRAF6 were identified by mass spectrometry. **B**, Immunoblot analysis of HEK293 cells transfected with indicated plasmids, lysed and immunoprecipitated with anti-Flag antibody. Whole cell lysates (WCL) were immunoblotted with antibodies to indicated proteins. **C**, Luciferase assay of NFκB-luc in wild type, *TRAF6*$^{-/-}$ or *TRAF2*$^{-/-}$ HEK293 cells overexpressing GRA15. **D**, Luciferase assay of IFNβ-luc in *TRAF2*$^{-/-}$, *TRAF6*$^{-/-}$, *TRAF2/6*$^{-/-}$, *TRAF3*$^{-/-}$, *TRAF5*$^{-/-}$ or *TRAFs*$^{-/-}$ HEK293 cells overexpressing GRA15, cGAS and STING. **E**, Detection of the interaction between GRA15 and TRAF6, TRAF2, STING by yeast two hybridization. **F**, HeLa cells were transfected with plasmids encoding Flag-TRAFs, His-GRA15 FL or His-GRA15 △mem2, and at 24h stained for nucleus (DAPI, blue), Flag (red) and ER (Calnexin, green). The scale bar length of each figure is 10 μm. Statistical analysis was performed by *t* test for (C, D). Data were from three independent experiments. NS, not significant; *P < 0.05; ***P < 0.001; ****P < 0.0001. Error bars, S.D.

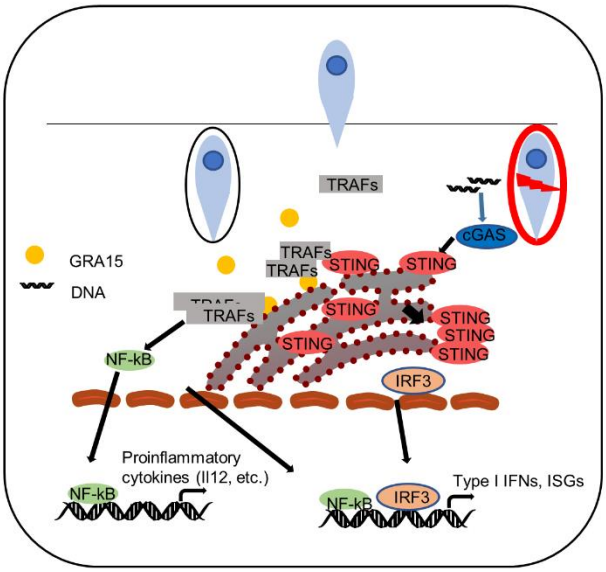


Figure S7. Graphic summary

Table S1. List of primers for RT-PCR and qRT-PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Murine *Ifnβ1*	TCCGAGCAGAGATCTTCAGGAA	TGCAACCACCACTCATTCTGAG
Murine *Ifnα4*	CCTGTGTGATGCAGGAACC	TCACCTCCCAGGCACAGA
Murine *Cxcl10*	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA
Murine *Isg15*	AGCAATGGCCTGGGACCTA	AGCCAGAACTGGTCTTCGTG
Murine *Il12a*	CTTAGCCAGTCCCGAAACC	GCTCCCTCTTGTTGTGGAAG
Murine *Ccl22*	GATGCAGGTCCCTATGGTGC	CTTGCGGCAGGATTTTGAGG
Murine *Ifng*	GGAGGAACTGGCAAAAGGAT	TTCAAGACTTCAAAGAGTCTGAGG
Murine *Gbp2*	GATGAACAAGCTAGCTGGGAAGAG	CCTTGGTGTGAGACTGCACAGT
Murine *Gbp3*	TGGAGGCACCCATTTGTCTGGTG	GACAAAGGTGCTGCTCAGAAGCACAG
Murine *Sqstm1*	GATGTGGAACATGGAGGGAAGAG	AGTCATCGTCTCCTCCTGAGCA
Murine *Irf7*	CAGCGAGTGCTGTTTGGAGAC	AAGTTCGTACACCTTATGCGG
Murine *Ifit3*	GCCGTTACAGGGAAATACTGG	CCTCAACATCGGGGCTCT
Murine *Irgm1*	TGGCAATGGCATGTCATCTT	AGTACTCAGTCCGCGTCTTCGT
Murine *Lcn2*	TGCCACTCCATCTTTCCTGTT	GGGAGTGCTGGCCAAATAAG
Murine *Ccl5*	AGATCTCTGCAGCTGCCCTCA	GGAGCACTTGCTGCTGGTGTAG
Murine *Ddit3*	GCGACAGAGCCAGAATAACAGC	TTCTGCTTTCAGGTGTGGTGGT
Murine *Usp18*	CGTGCTTGAGAGGGTCATTTG	GGTCGGGAGTCCACAACTTC
Murine *Tubulin*	GGAACATAGCCGTAAACTGC	TCACTGTGCCTGAACTTACC
Human IFNB1	AGGACAGGATGAACTTTGAC	TGATAGACATTAGCCAGGAG
Human GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
TgB1	AACGGGCGAGTAGCACCTGAGGAGA	TGGGTCTACGTCGATGGCATGACAAC

Table S2. List of guiding RNA

Gene	guiding RNA sequence (5'—3')
Murine *Sting*	CAGTAGTCCAAGTTCGTGCG
Murine *Cgas*	TGACTCAGCGGATTTCCTCG
Human STING	CAGCTGGGACTGCTGTTAAA
Human RELA	GTAGGAAAGGACTGCCGGGA
Human TRAF2	AGCCGGGCTGTAGCAACTCC
Human TRAF3	AGCCCGAAGCAGACCGAGTG
Human TRAF5	TATACTGGGCTCAAAGTCCA
Human TRAF6	TGGGTGGAACTGCCAGCACG
GRA15	AAGCGACTTCTAAACACGTG