Supplementary Information for

TBK1 Recruitment to STING Activates Both IRF3 and NF-κB that Mediate Immune Defense against Tumors and Viral Infections

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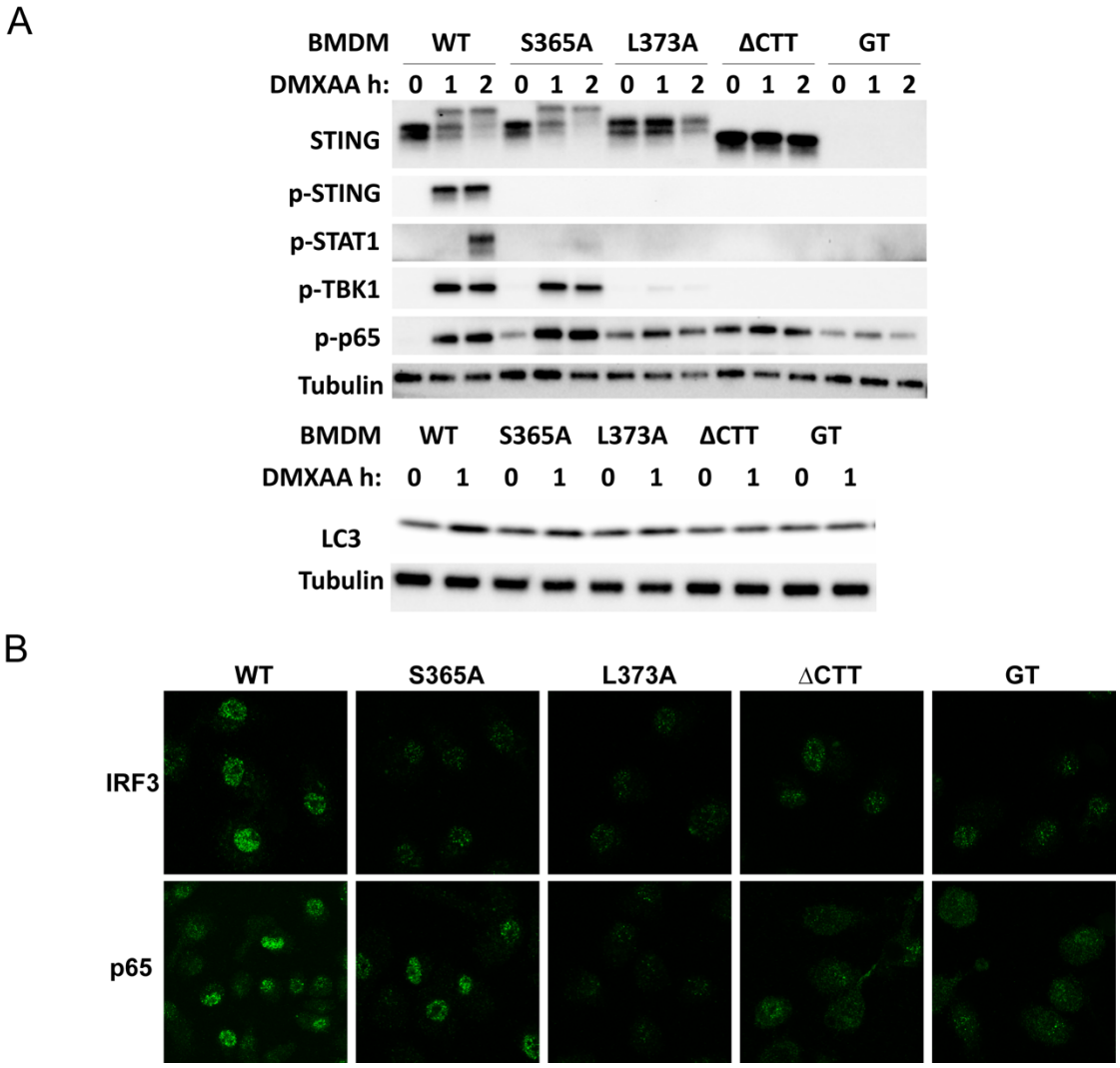


Figure S1. STING mutants with distinct signaling defects. BMDM was isolated from mice with the indicated STING mutations. (*A*) Western blots of BMDM lysates treated with 75 μM DMXAA for the indicated hours. (*B*) Immunofluorescence staining of IRF3 and p65 in BMDMs treated with 75 μM DMXAA for an hour.

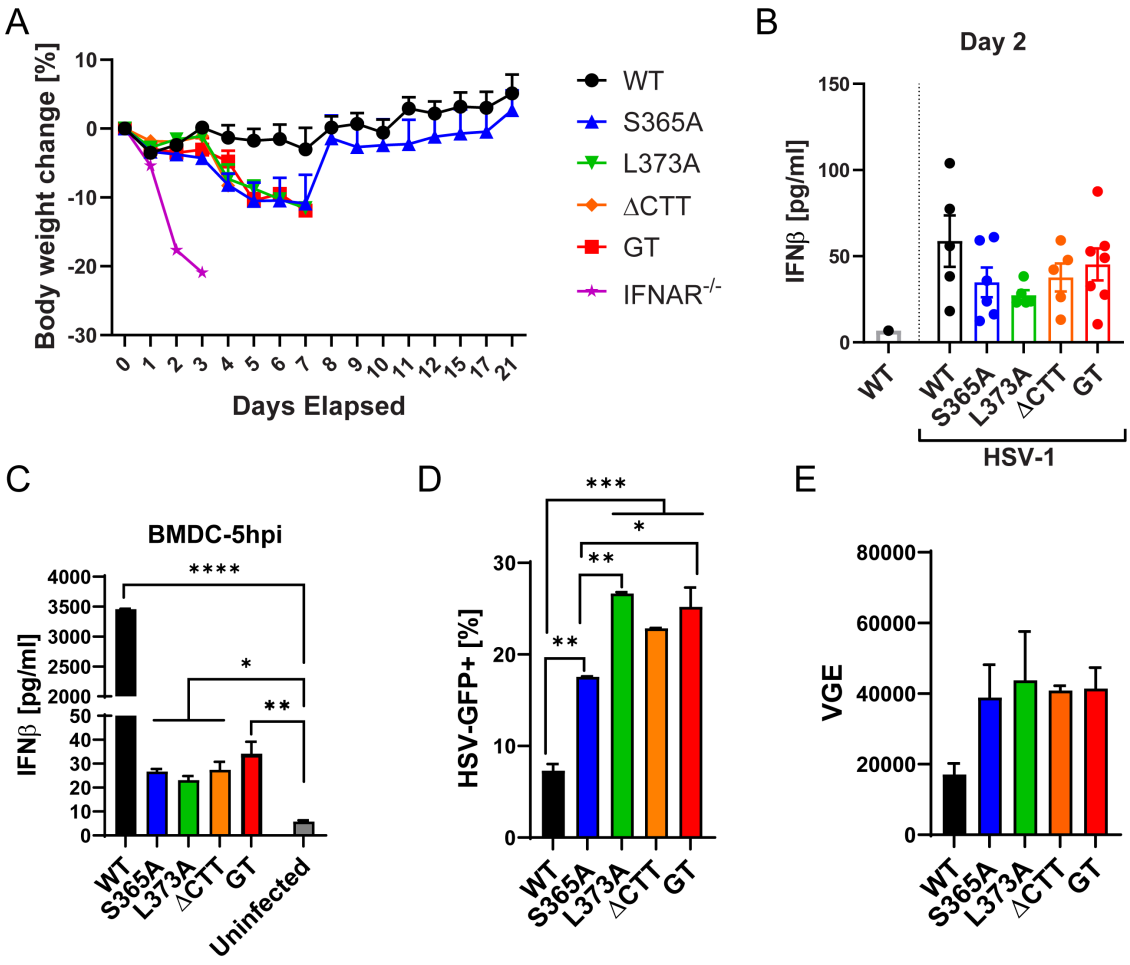


Figure S2. STING-S365A mice, but not L373A or ΔCTT mice, are resistant to HSV-1 infection. (*A-B*) Mice were retro-orbitally infected with HSV-1 (5 x 10^6 pfu per mouse). (*A*) Body weight change. (*B*) Serum IFNβ levels measured by ELISA 2 days post-infection. (*C*) IFNβ levels in the BMDC culture media 5 hours post-infection with HSV-1 (MOI 10). (*D*) Flow cytometric analysis of GFP^+ BMDCs infected with HSV-GFP (MOI 20) for 24 hours. (*E*) VGE counts in BMDCs 12 hours post-infection with HSV-1 (MOI 10) were measured by qPCR. Error bars represent SEM. *P<0.05. **P<0.01. ***P<0.001. ****P<0.0001.

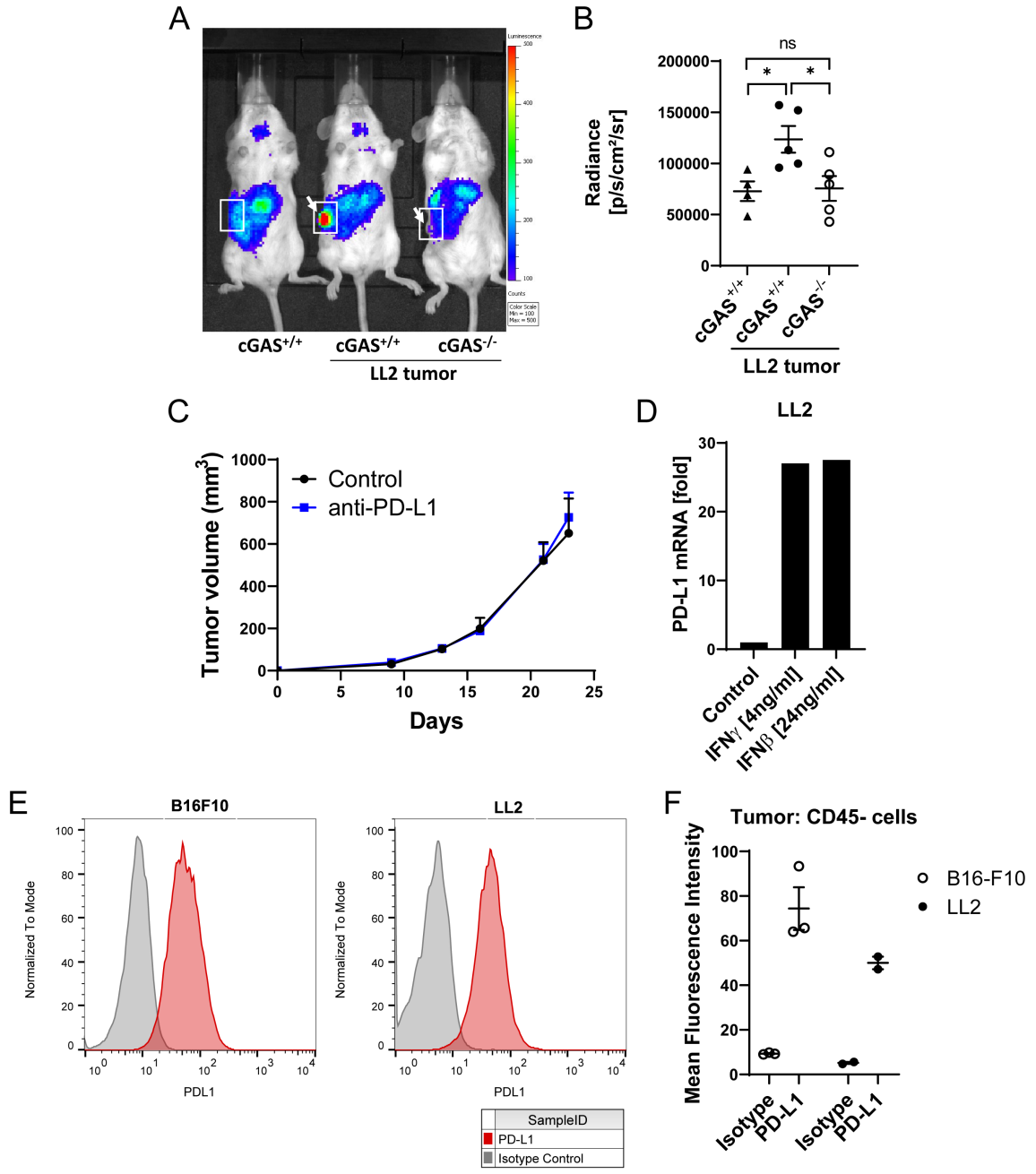


Figure S3. The LL2 tumor is detected by cGAS but does not respond to the anti-PD-L1 treatment. (*A-B*) LL2 tumor cells were injected in *Ifnb1*$^{\Delta\beta\text{-luc}/\Delta\beta\text{-luc}}$ mice containing (cGAS$^{+/+}$) or lacking (cGAS$^{-/-}$) the cGAS gene, and luciferase activity was imaged 9 days after tumor implantation. (*A*) Representative luciferase activity in the reporter mice. Regions of Interest (ROI) are marked with white boxes. Tumors are marked with white arrows. Basal luciferase signal in the *Ifnb1*$^{\Delta\beta\text{-luc}/\Delta\beta\text{-luc}}$ mouse represents tonic IFNβ expression. (*B*) Quantification of luciferase activity at the ROI. (*C*) Tumor growth with anti-PD-L1 treatments. LL2-implanted WT mice (n=5) were intraperitoneally treated with 200 µg of anti-PD-L1 on days 6, 9, 13, and 16. (*D*) PD-L1 mRNA levels in LL2 cells 24 hours after the indicated treatments. (*E-F*) Isotype control and PD-L1 surface staining of the CD45^{-} population in LL2 tumors 14 days post-implantation. (*E*) Histogram plots. (*F*) Mean Fluorescence Intensity of PD-L1. Error bars represent SEM. **P<0.01.

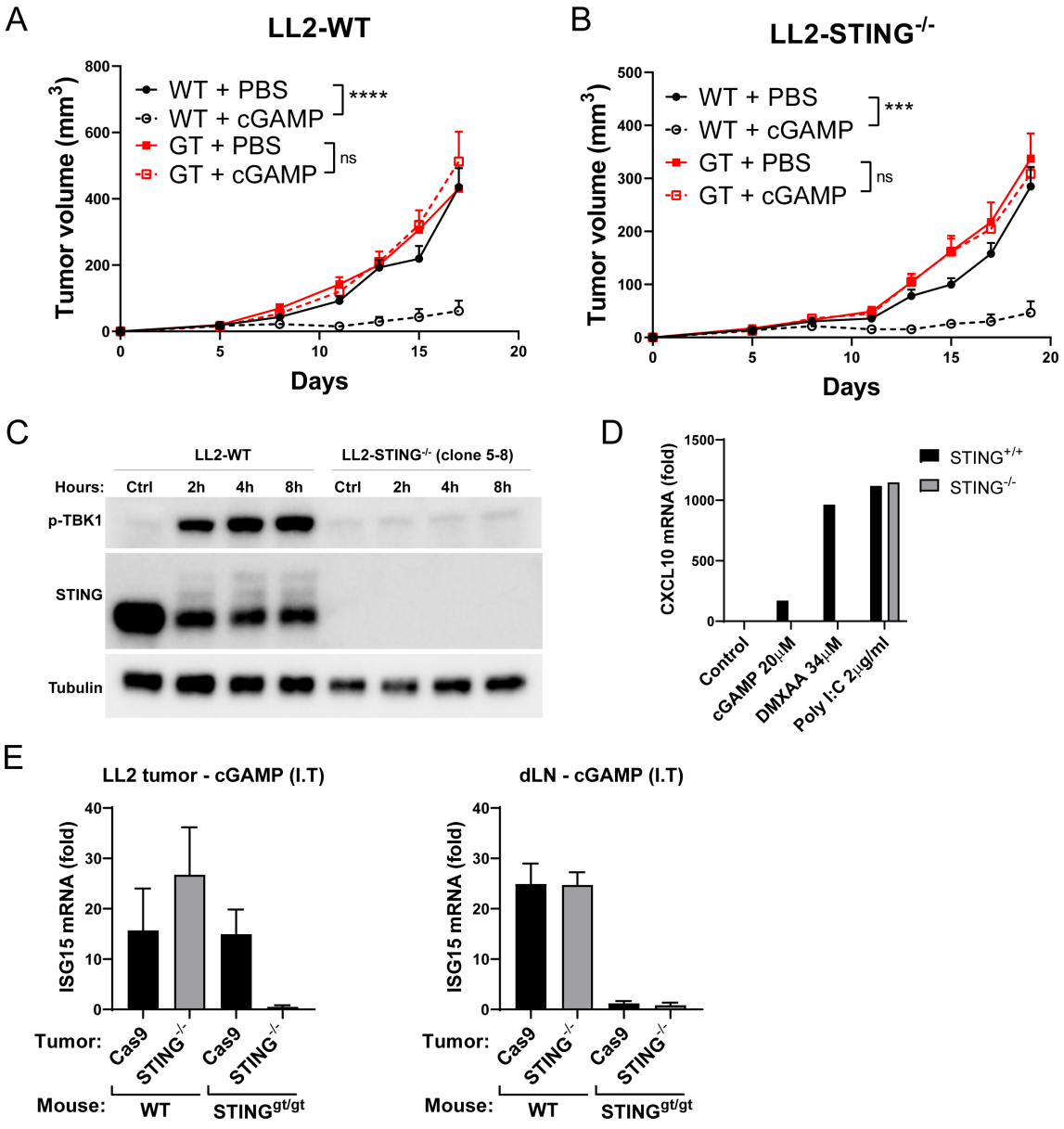


Figure S4. Host STING activation is necessary and sufficient for the anti-tumor effect of cGAMP on LL2 tumors. (*A-B*) WT or STING^{gt} mice (n=4~5) were implanted with LL2-WT (*A*) or LL2-*STING*$^{-/-}$ (*B*) cells, followed by intratumoral injections of 10 μg of cGAMP on days 5, 8, and 11. (*C*) Western blot of cell lysates from LL2-WT and -*STING*$^{-/-}$ cells that were treated with 34 μM DMXAA for the indicated times. (*D*) qRT-PCR analysis of CXCL10 expression 6 hours after the indicated treatments. (*E*) qRT-PCR analysis of LL2 tumors (left) and tumor draining lymph nodes (dLN, right) 6 hours post-cGAMP treatment. 12 days after LL2 cell implantation, mice were intratumorally injected with 10 μg of cGAMP. Expression levels were normalized by tissues from a PBS-injected mouse. Error bars represent SEM. ***P<0.001. ****P<0.0001.

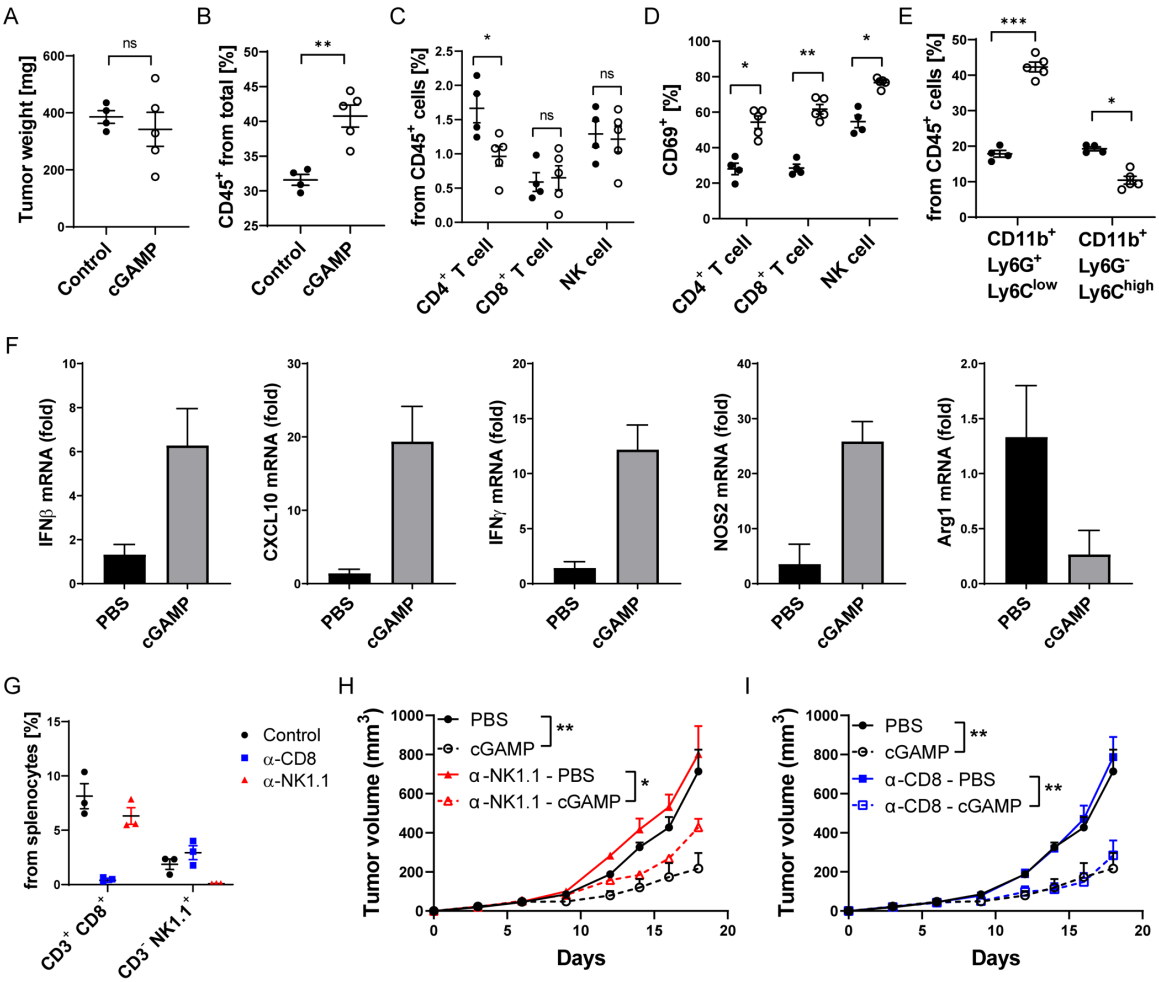


Figure S5. cGAMP treatment recruits immune cells to the tumor to elicit antitumor effects that are partially mediated by NK cells, but not CD8$^+$ T cells. (*A-E*) WT mice were treated with 10 μg of cGAMP (open circle) or vehicle control (closed circle) on days 9 and 13 after LL2 implantation. On day 14, tumors were isolated and analyzed by flow cytometry. (*A*) Tumor weights. (*B*) CD45$^+$ cells in tumors. (*C*) The percentage of CD4$^+$ T cells (CD3$^+$ CD4$^+$), CD8$^+$ T cells (CD3$^+$ CD8$^+$), and NK cells (CD3$^-$ NK1.1$^+$) from tumor-infiltrating CD45$^+$ cells. (*D*) The percentage of CD69$^+$ cells from the parental populations. (*E*) The percentage of neutrophil-like cells (CD11b$^+$ Ly6G$^+$ Ly6C^{low}) and monocyte-like cells (CD11b$^+$ Ly6G$^-$ Ly6C^{high}) from tumor-infiltrating CD45$^+$ cells. (*F*) WT mice were treated with 10 μg of cGAMP on day 14 after LL2 implantation. Tumors were isolated 6 hours after the treatment and analyzed by qRT-PCR. (*G-I*) WT mice (n=5) were treated with 200 μg of anti-CD8 or anti-NK1.1 on days 1, 4, 7, and 10 and with 10 μg of cGAMP on days 4, 7, and 10 after LL2 implantation. (*G*) CD8$^+$ T cells and NK cells in the spleen on day 21 after LL2 implantation were analyzed by flow cytometry. (*H* and *I*) Tumor growth curve. Error bars represent SEM. *P<0.05. **P<0.01. ***P<0.001.

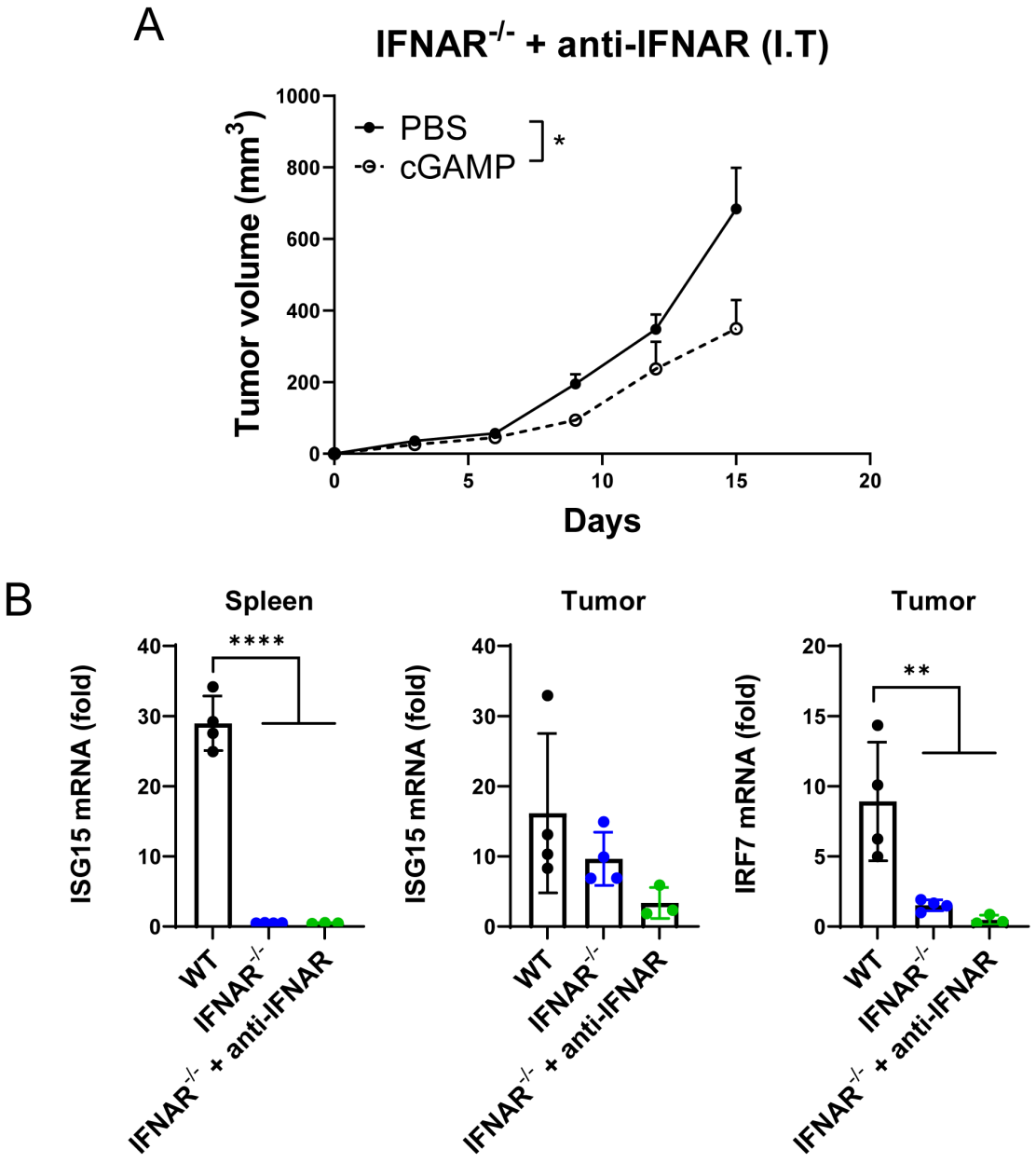
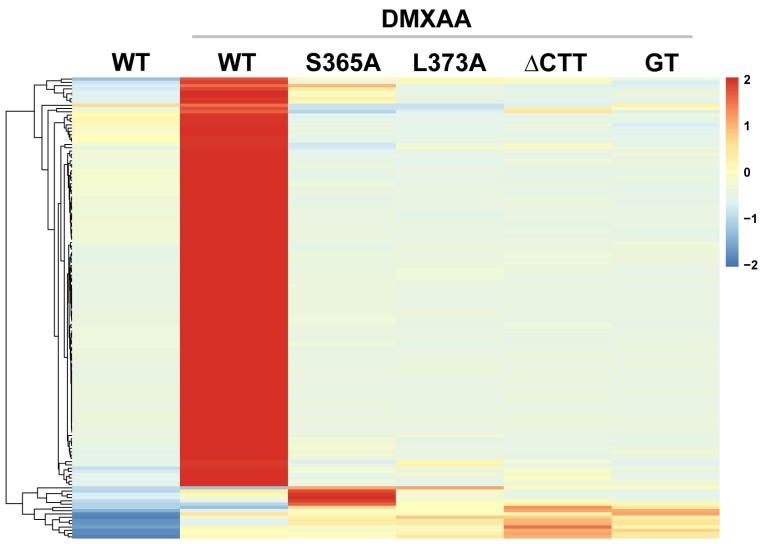


Figure S6. cGAMP exerts a partial antitumor effect on LL2 tumors in the absence of type-I interferon signaling. (*A*) LL2 tumor growth in *Ifnar1*$^{-/-}$ mice (n=4~5) with intratumoral anti-IFNAR-1 (100 μg) and cGAMP (10 μg) treatments on days 4, 7, and 10 after LL2 implantation. (*B*) qRT-PCR analysis of spleen and tumor tissues 6 hours post-treatment. WT and *Ifnar1*$^{-/-}$ mice were intratumorally injected with cGAMP (10 μg) and anti-IFNAR-1 (100 μg) on day 7 after LL2 implantation. Expression levels were normalized by tissues from PBS-injected WT mice. Error bars represent SEM. *P<0.05. **P<0.01. ****P<0.0001.

A



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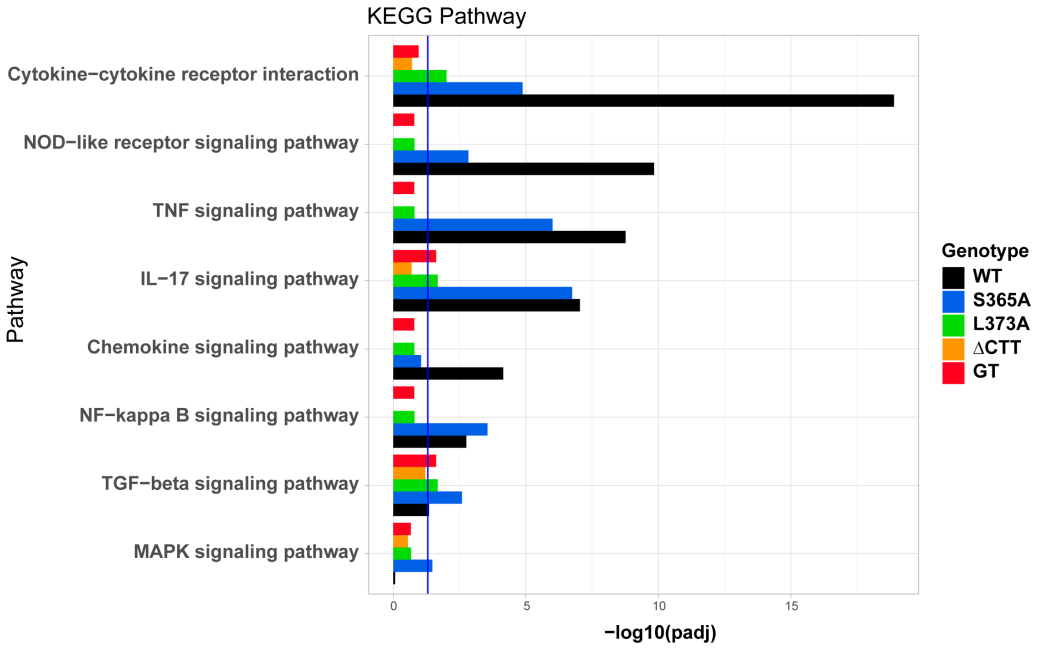

Figure S7. Transcriptome analysis of BMDMs from STING mutant mice. BMDMs were treated with 75 μM DMXAA for 2 hours. (*A*) Heatmap showing differentially expressed genes. (*B*) KEGG pathway analysis with the blue line indicating adjusted p-value<0.05.

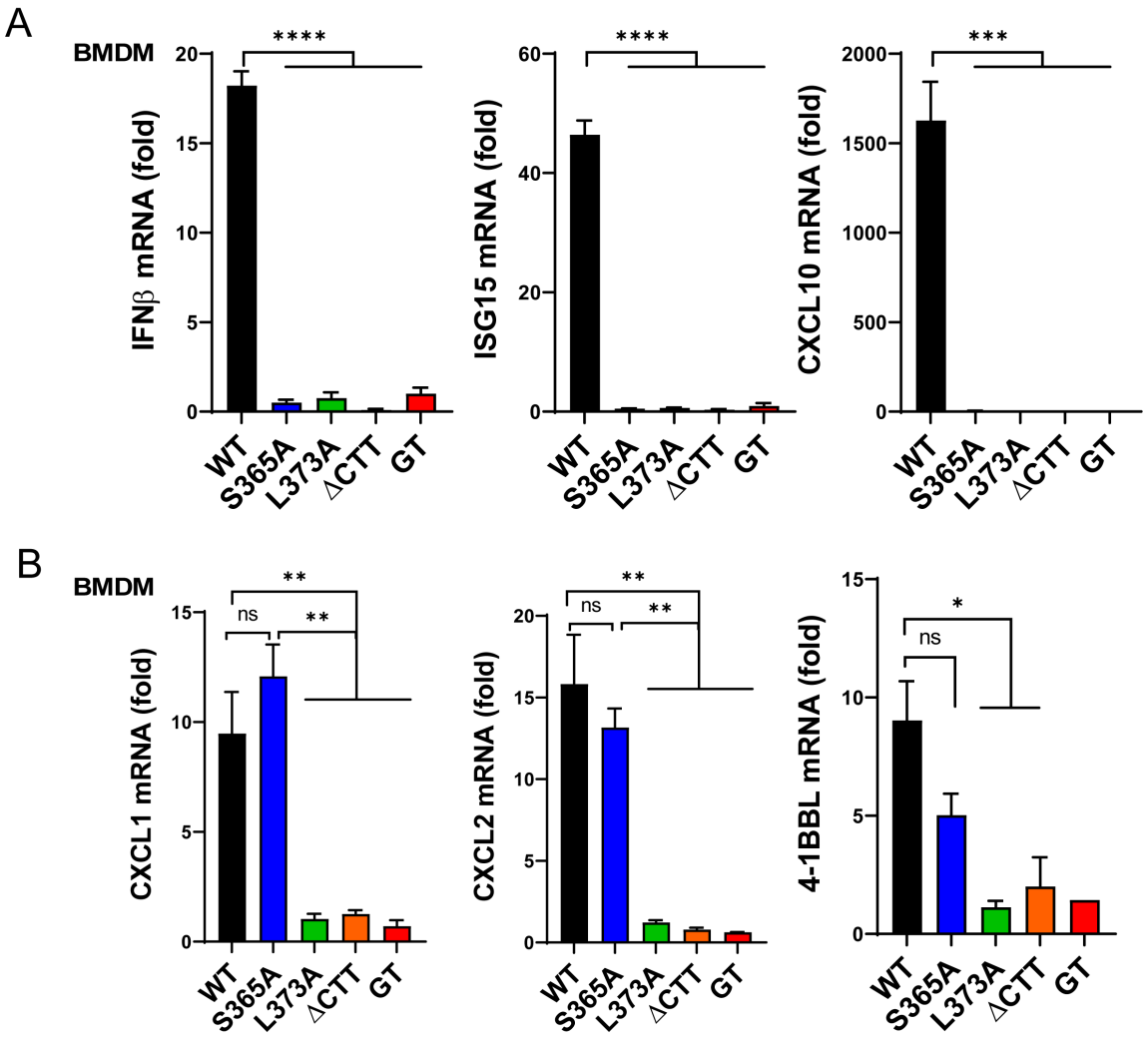

Figure S8. qRT-PCR analysis of BMDMs from STING mutant mice. BMDMs were treated with 75 μM DMXAA for 2 hours. (*A*) IFN and ISGs. (*B*) Genes expressed highly in STING-S365A BMDMs but not in other STING mutant cells. Expression levels were normalized by untreated WT cells. Error bars represent SEM. *P<0.05. **P<0.01. ***P<0.001. ****P<0.0001.

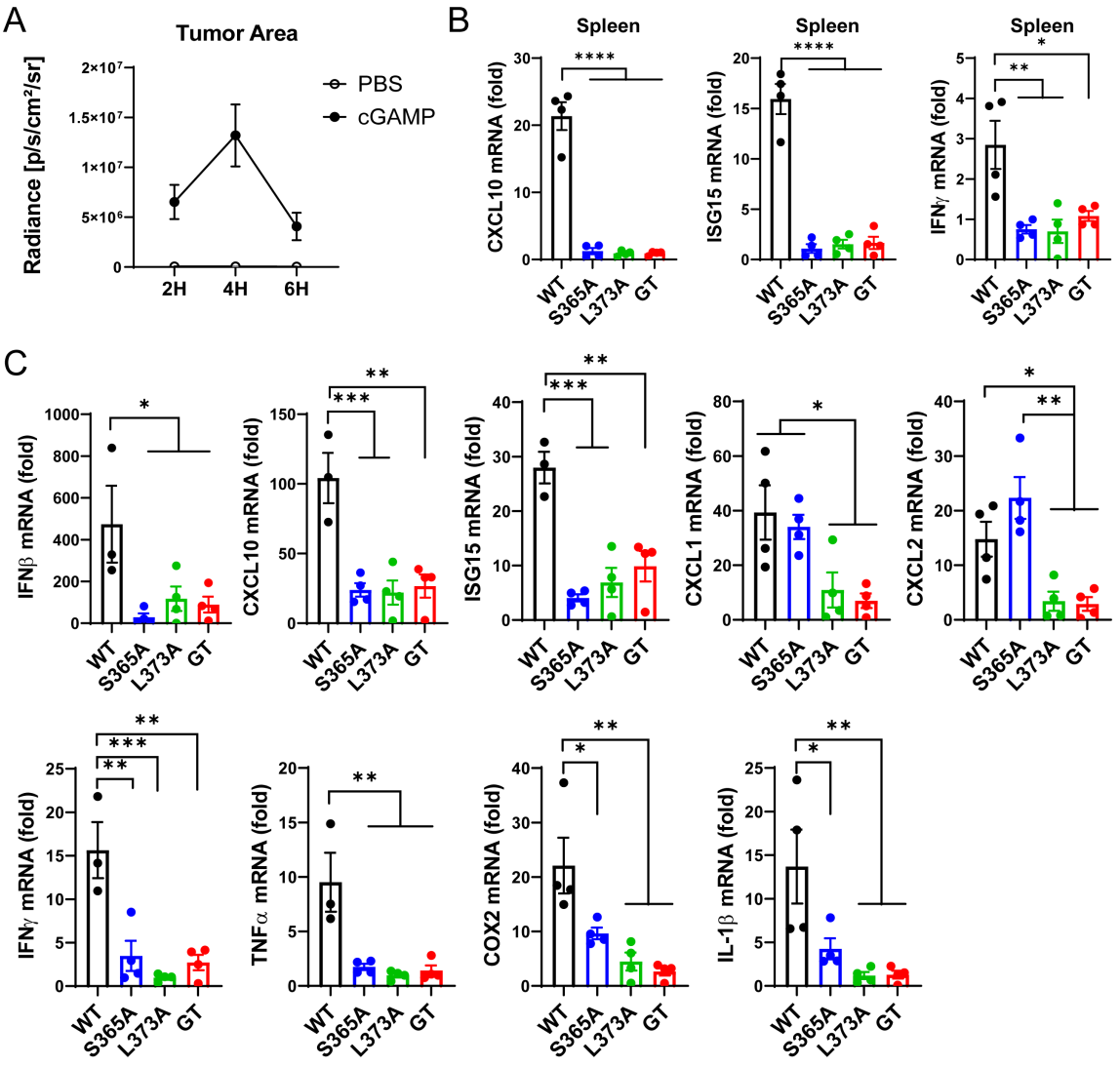

Figure S9. Gene expression in LL2 tumors after cGAMP treatment. (*A*) PBS or cGAMP was injected into *Ifnb1*$^{\Delta\beta\text{-luc}/\Delta\beta\text{-luc}}$ mice 11 days after LL2 implantation, and luciferase activity was imaged in the tumor area at the indicated time points. (*B-C*) Mice were intratumorally injected with 10 μg of cGAMP 7 days after LL2 tumor implantation. Spleen (*B*) and tumor (*C*) tissues were isolated 4 hours after cGAMP treatment for qRT-PCR analysis of the indicated genes. Expression levels were normalized using tissues from PBS-injected WT mice. Error bars represent SEM. *P<0.05. **P<0.01. ***P<0.001. ****P<0.0001.

Table S1. gRNA target site and template DNA

Mut	gRNA target site	Template DNA (5' to 3')
S365A	GAGCCAAGAC TCCTCATCAG	TCTCAGGAGGTGCTCCGGCACATTCGTCAGGAAGAAAAGGAGG AGGTTACCATGAATGCCCCCATGACCTCAGTGGCACCTCCTCCC TCCGTACTGTCCCAAGAGCCTAGACTCCTCATCGCTGGTATGGA TCAGCCTCTCCCACTCCGCACTGACCT
L373A	AGATGAGGTC AGTGCGGAGT	CGTCAGGAAGAAAAGGAGGAGGTTACCATGAATGCCCCCATGA CCTCAGTGGCACCTCCTCCCTCCGTACTGTCCCAAGAGCCAAGA CTCCTCATCAGTGGTATGGATCAGCCTCTTCCAGCCCGCACTGA CCTCATCTGAGGCATGGGACAGCCTTG
ΔCTT	CATTCGTCAG GAAGAAAAGG	CCAAGGGTGCCTGCATCTTGCTGACCACCCCCCATCTCTATTCC AGAACCCACAGACGGAAACAGTTTCTCACTGTCTCAGGAGGTGC TCCGGCACATCCGACAGGAAGAAAAGGAGGAGTGAGTTACCAT GAATGCCCCCATGACCTCAGTGGCACCT

Table S2. Mouse qPCR primers.

Protein	Forward (5' to 3')	Reverse (5' to 3')
RPL19	AAATCGCCAATGCCAACTC	TCTTCCCTATGCCCATATGC
IFNβ	TCCGAGCAGAGATCTTCAGGAA	TGCAACCACCACTCATTCTGAG
CXCL10	GCCGTCATTTTCTGCCTCA	CGTCCTTGCGAGAGGGATC
ISG15	GGAACGAAAGGGGCCACAGCA	CCTCCATGGGCCTTCCCTCGA
TNFα	CACAGAAAGCATGATCCGCGACGT	CGGCAGAGAGGAGGTTGACTTTCT
CXCL1	TCCAGAGCTTGAAGGTGTTG	GTCTGTCTTCTTTCTCCGTTACTT
CXCL2	ATGCCTGAAGACCCTGCCAAG	GGTCAGTTAGCCTTGCCTTTG
4-1BBL	TCCTGTGTTCGCCAAGCTAC	ATCTTGGCTGTGCCAGTTCA
IFNγ	TTTGCAGCTCTTCCTCATGGCTGTTTCTG	TGACGCTTATGTTGTTGCTGATGGCCTG
NOS2	CCAAGCCCTCACCTACTTCC	CTCTGAGGGCTGACACAAGG
Arg1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
IRF7	ATGCACAGATCTTCAAGGCCTGGGC	GTGCTGTGGAGTGCACAGCGGAAGT
IFNα	GGACTTTGGATTCCCGCAGGAGAAG	GCTGCATCAGACAGCCTTGCAGGTC
COX-2	CAGACAACATAAACTGCGCCTT	GATACACCTCTCCACCAATGACC
IL-1β	CCTTCCAGGATGAGGACATGA	TGAGTCACAGAGGATGGGCTC
PD-L1	ATTGCTCCTTGACTGCTGGCTG	TTCTGGGTTCCTCCTCCTTTCC
Adipsin	AGTGTGCGGGGATGCAGT	ACGCGAGAGCCCCACGTA
HSV Pol	CATCACCGACCCGGAGAGGGAC	GGGCCAGGCGCTTGTTGGTGTA