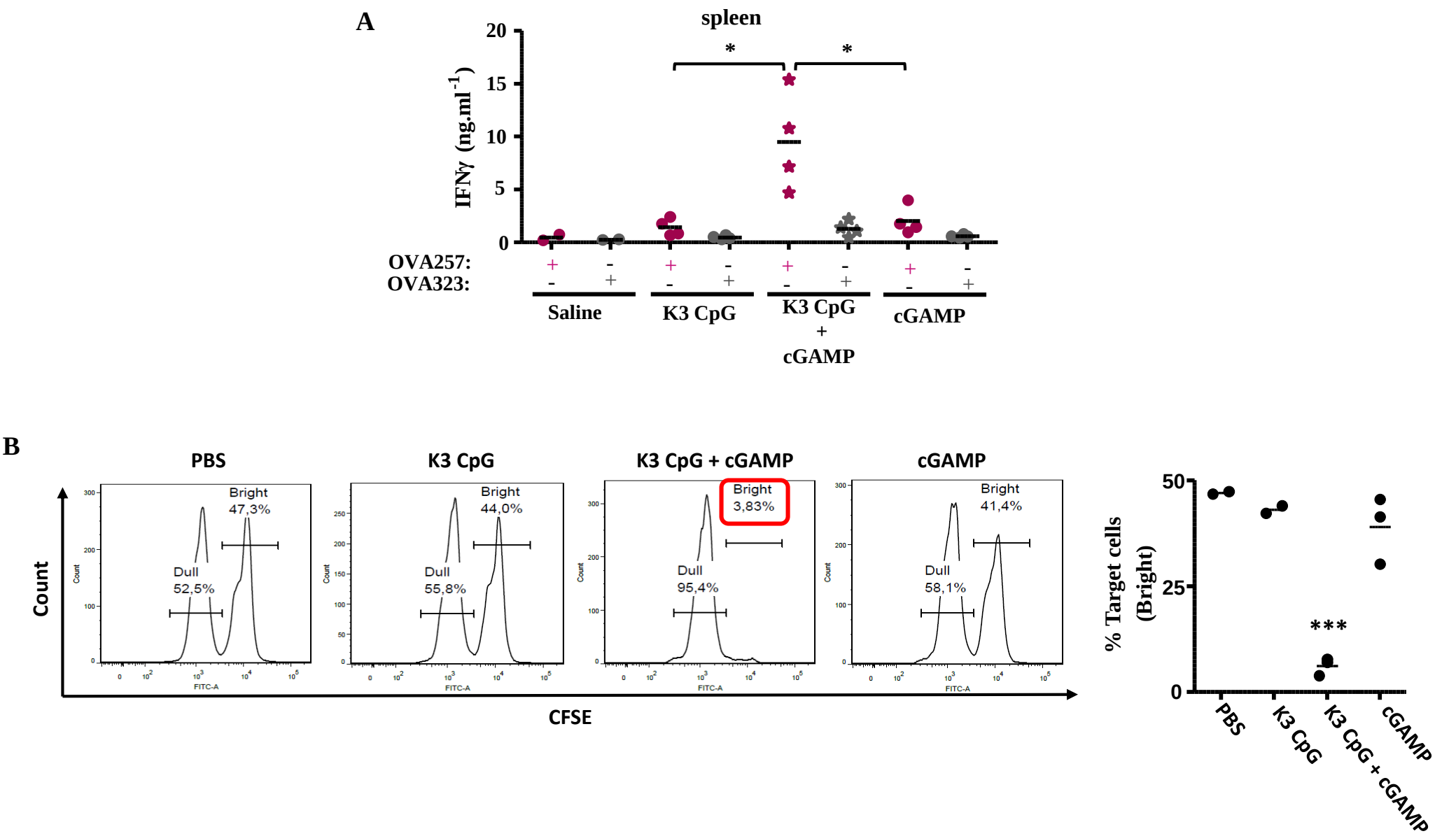
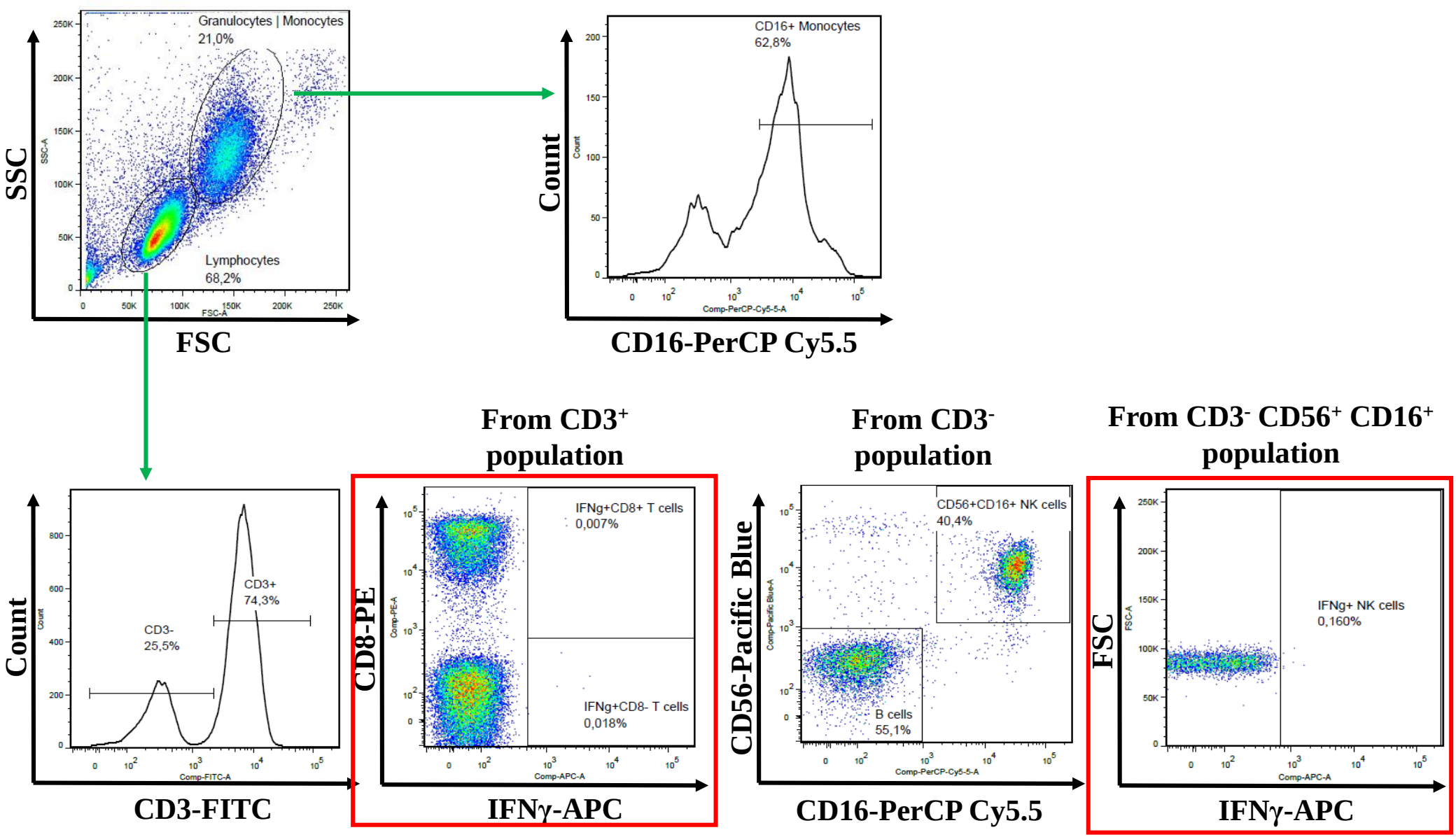
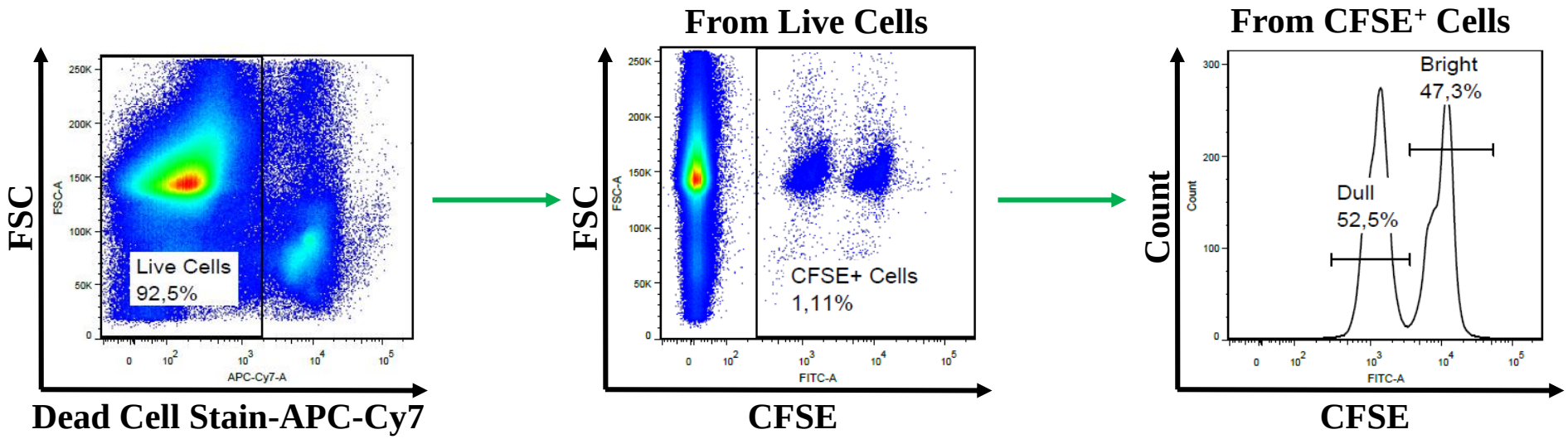

A
spleen
IFNγ (ng.ml-1)
20
15
10
5
0
*
*
OVA257:
OVA323:
Saline
K3 CpG
K3 CpG + cGAMP
cGAMP
B
PBS
K3 CpG
K3 CpG + cGAMP
cGAMP
Count
Bright 47,3%
Dull 52,5%
Bright 44,0%
Dull 55,8%
Bright 3,83%
Dull 95,4%
Bright 41,4%
Dull 58,1%
FITC-A
CFSE
% Target cells (Bright)
50
25
0
***
PBS
K3 CpG
K3 CpG + cGAMP
cGAMP

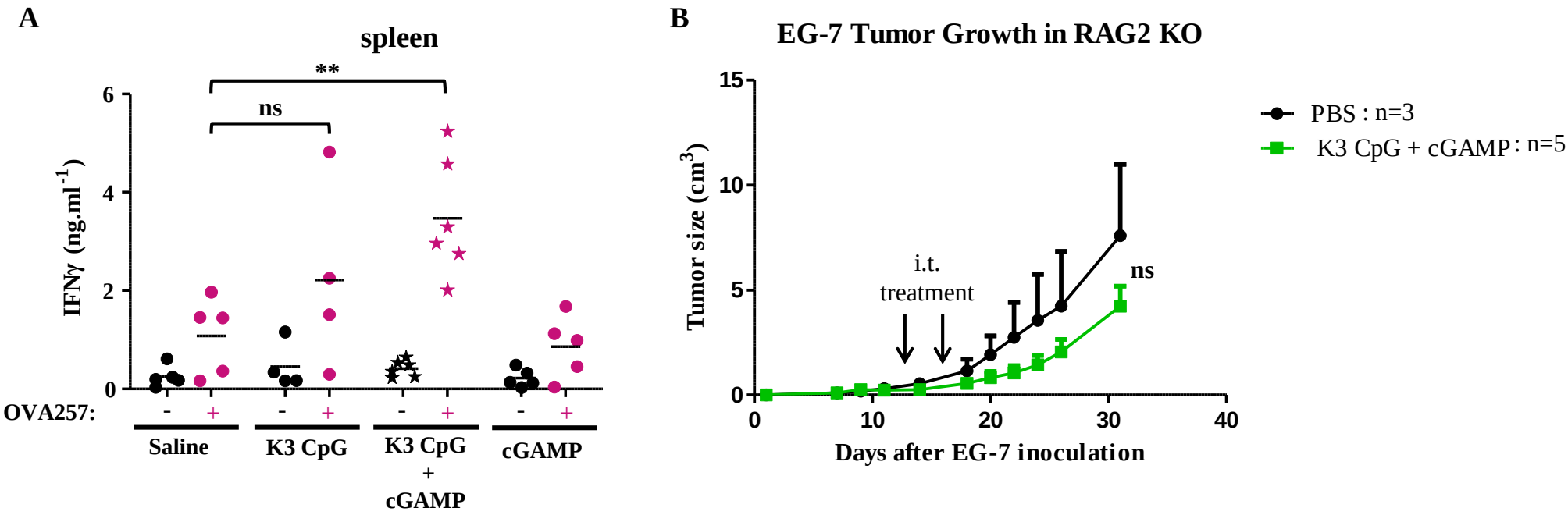
Supporting Information Figure 1. Combination of K3 CpG and cGAMP induces potent $CD8^+$ T cell activity in vivo. (**A**) Mice were immunized with OVA only (n=2), or OVA and either K3 CpG (n=4), cGAMP (n=4) or K3 + cGAMP (n=4) at days 0 and 10, via the intramuscular route. On day 17, spleen cells were isolated and stimulated with OVA, or OVA peptides that are specific for MHC class I (OVA 257) or MHC class II (OVA 323) for 48 h. Production of IFN-γ was measured by ELISA. Data are representative of at least two independent experiments. *$p < 0.05$; **$p < 0.01$ (Student's t test). (**B**) Seven days after immunization with OVA only (n=2), or OVA and either K3 CpG (n=2), cGAMP (n=3) or K3 + cGAMP (n=3) via the intramuscular route, in vivo CTL assay was performed. Data are representative of two independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (One-way ANOVA with Bonferroni's multiple comparison test).



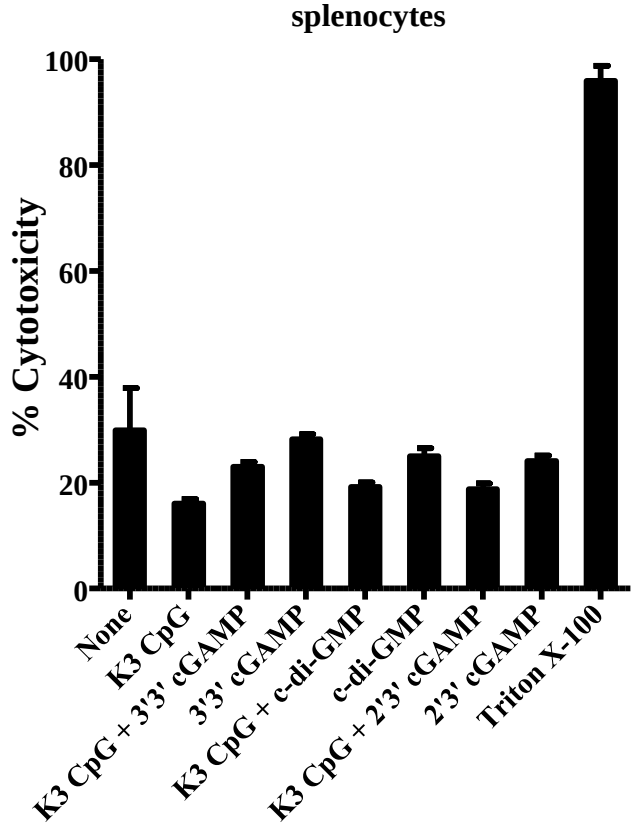
Supporting Information Figure 2. Gating strategy for intracellular IFN-γ staining experiment in hPBMCs. First, $CD3^{+/-}$ cells were gated from the lymphocyte gate. Then, from the $CD3^{+}$ population, IFN-γ^{+} $CD8^{+/-}$ cells were gated and are shown in the results. NK cells were gated from the $CD3^{-}$ $CD56^{+}$ $CD16^{+}$ population and IFN-γ^{+} NK cells are shown in the results.



Supporting Information Figure 3. Gating strategy for in vivo CTL assay. $CFSE^+$ cells were gated from the live cells, which are negative for the dead cell stain. Histograms, which were drawn with the $CFSE^+$ cells, show the target cells (bright) that are pulsed with the OVA 257 (MHC Class I-specific peptide), and non-target cells (dull).

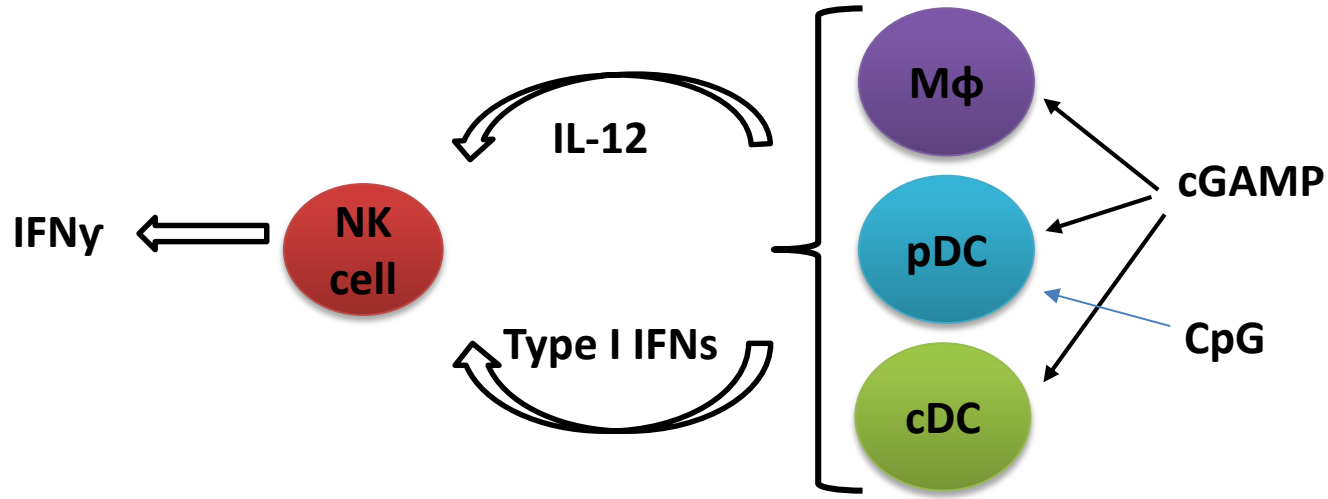


Supporting Information Figure 4. $CD8^+$ T cell activity, but not NK cell activity, is required for the anti-tumor effect of the combination of K3 CpG and cGAMP in the EG-7 tumor model. (**A**) Mice were injected with 1×10^6 EG-7 lymphoma cells (in 100 µl of PBS) subcutaneously on day 0. On days 7 and 10, mice were given intra-tumor injections of PBS (n=5), K3 CpG (n=4), cGAMP (n=5), or K3 CpG + cGAMP (n=6). On day 23, spleen cells were isolated and stimulated with the OVA peptide that is specific for MHC class I (OVA 257) for 48 h. Production of IFN-γ was measured by ELISA. Data are representative of two independent experiments. (**B**) RAG2 KO mice were injected with 1×10^6 EG-7 lymphoma cells (in 100 µl of PBS) subcutaneously on day 0. On days 8 and 11, mice were given intra-tumor injections of PBS (n=3), or K3 CpG + cGAMP (n=5), and mice were monitored for tumor growth for 31 days. Data are shown as the mean + SEMs. *$p < 0.05$; **$p < 0.01$ (Mann–Whitney U test).

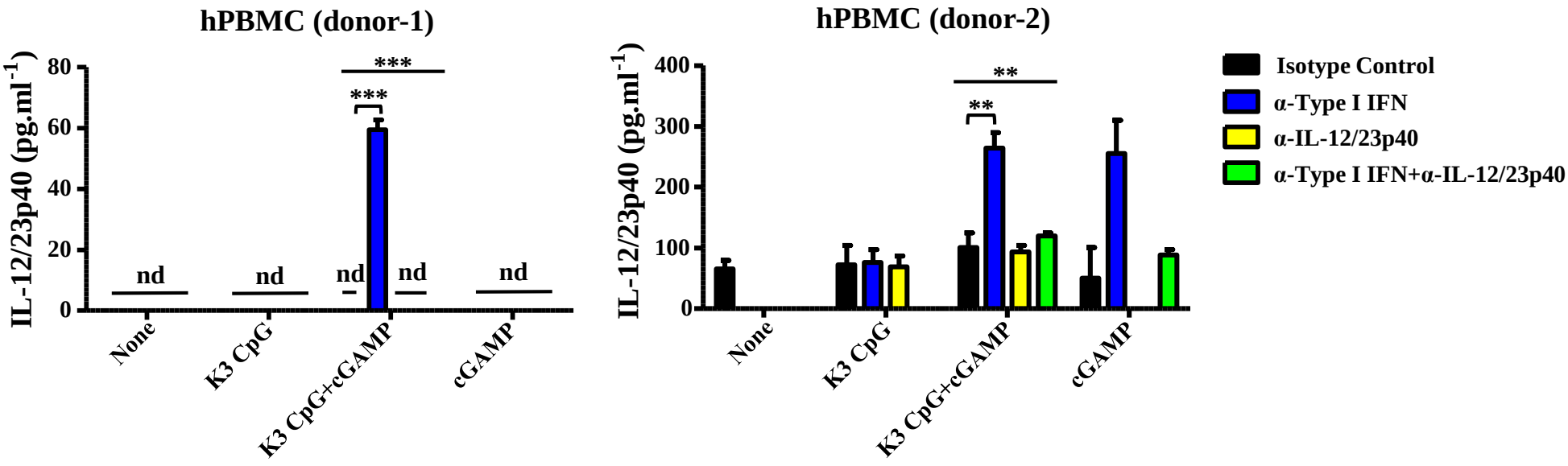


Supporting Information Figure 5. Cytotoxicity of the STING ligands in the splenocytes. Splenocytes were stimulated with K3 CpG (10 μg/ml), STING-agonists (10 μM), or K3 CpG + STING-agonists for 24 h in 96-well round-bottom plates and % cytotoxicity was determined by measuring LDH release with the Non-Radioactive Cytotoxicity Assay Kit (Promega). Triton X-100 treatment was used as the positive control. Data are shown as the mean + SD of duplicates.

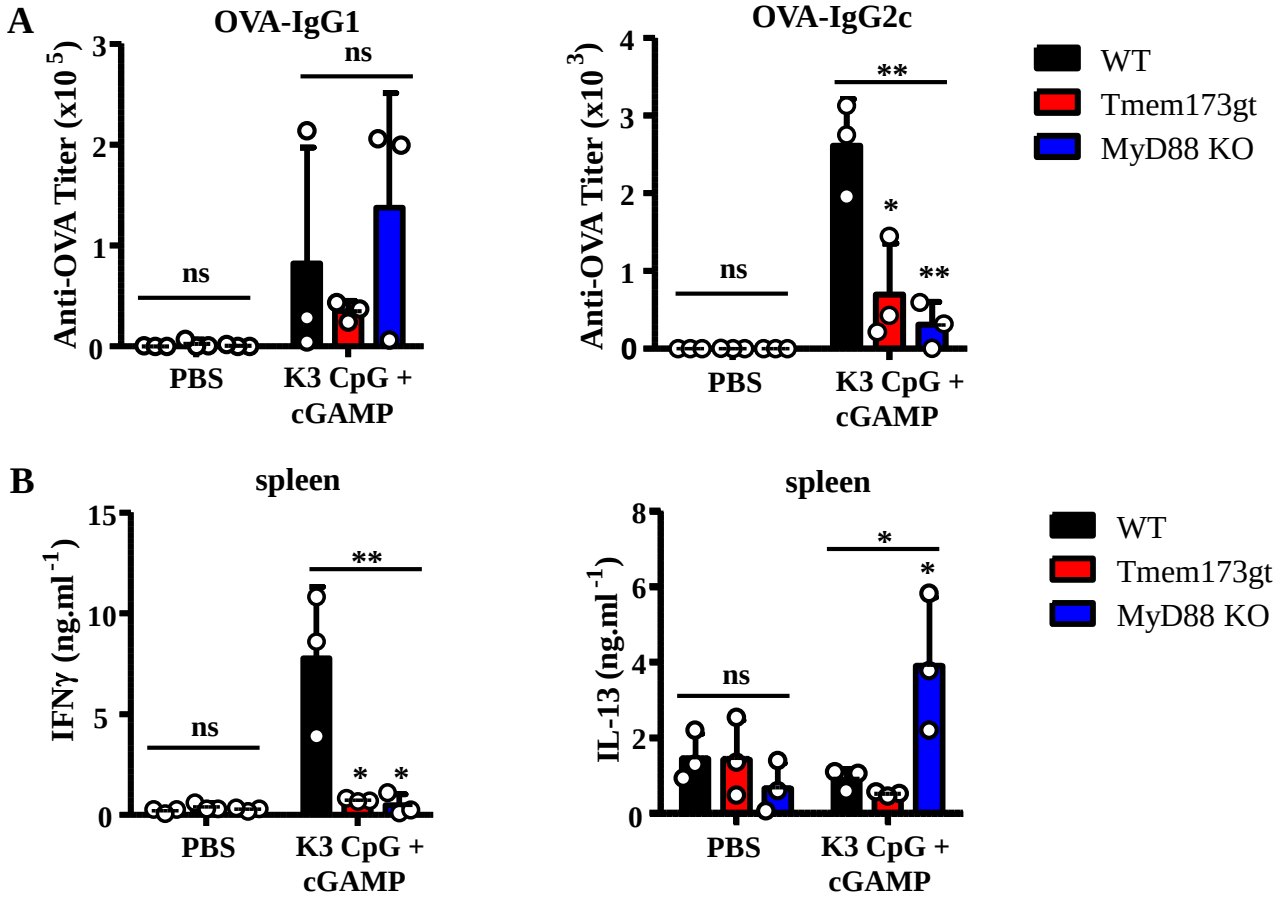
A


IL-12
IFNγ
NK cell
Type I IFNs
Mϕ
pDC
cDC
cGAMP
CpG

B


hPBMC (donor-1)
IL-12/23p40 (pg.ml-1)
80
60
40
20
0
***
***
nd
nd
nd
nd
nd
None
K3 CpG
K3 CpG+cGAMP
cGAMP
hPBMC (donor-2)
IL-12/23p40 (pg.ml-1)
400
300
200
100
0
**
**
None
K3 CpG
K3 CpG+cGAMP
cGAMP
Isotype Control
α-Type I IFN
α-IL-12/23p40
α-Type I IFN+α-IL-12/23p40

Supporting Information Figure 6. (**A**) Proposed mechanisms of innate IFN-γ synergy. pDCs are the main responders to the K3 CpG while cGAMP stimulates cells including cDCs, macrophages and, maybe pDCs, to produce high amounts of IL-12 and type I IFNs. Then, by signaling through IL-12 and type I IFN receptors, these cytokines synergize in the induction of IFN-γ in NK cells. (**B)** IL-12 production is increased in the human PBMCs that are treated with type I IFN neutralizing antibodies. Human PBMCs from two healthy donors were treated with 5 μg/ml of isotype control, Type I IFN neutralizing, IL-12/23p40 neutralizing or Type I IFN + IL-12/23p40 neutralizing antibodies 30 minutes prior to 24 h of stimulation with K3 CpG, cGAMP, or K3 CpG + cGAMP. IL-12 production was measured by ELISA (nd: non-detected). Data are shown as the mean + SD of duplicates. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (One-way ANOVA with Bonferroni's multiple comparison test).



Supporting Information Figure 7. No significant differences were observed between the PBS immunization groups of WT and STING mutant, or MyD88 KO mice. WT, Tmem173gt, and MyD88 KO C57BL/6J mice were immunized with OVA (n=3) only, or OVA and K3 + cGAMP (n=3) at days 0 and 10, via the intramuscular route. (**A**) On day 17, OVA-specific serum IgG2c and IgG1 were measured by ELISA. (**B**) Spleen cells were stimulated with OVA for 48 h. Production of IL-13 and IFN-γ were measured by ELISA. Data are shown as the mean + SD. *$p < 0.05$; **$p < 0.01$ (One-way ANOVA with Bonferroni's multiple comparison test).