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Supplemental Information

Combination of Asiatic Acid and Naringenin Modulates NK Cell Anti-cancer Immunity by Rebalancing Smad3/Smad7 Signaling

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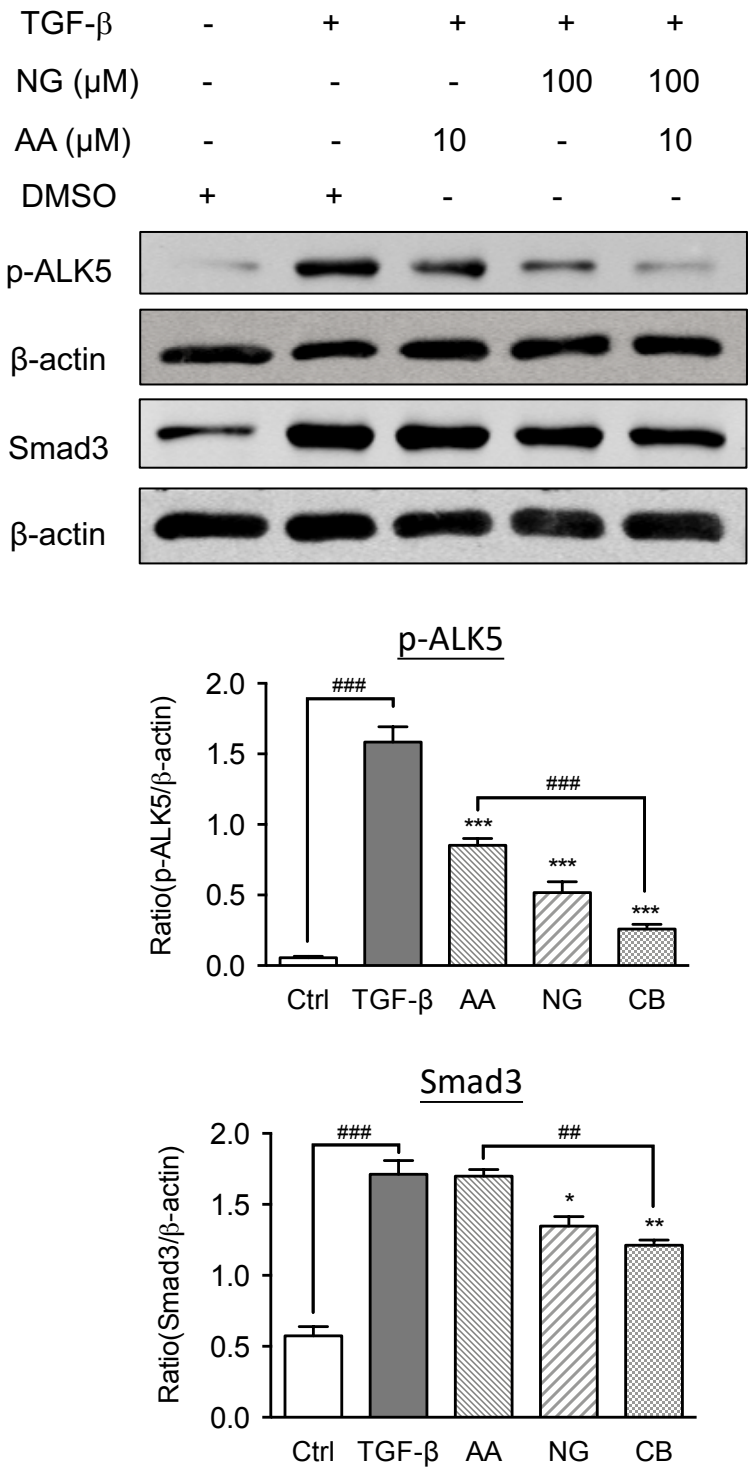

Figure S1. Combination of AA and NG produces a better suppressive effect on TGF-β1-induced p-ALK5 and Smad3 expression in bone marrow-derived NK (BM-NK) cells. NK cells were pre-incubated with AA, NG or their combination for overnight before being stimulated with TGF-β1 (5ng/ml) for p-ALK5 at 15 minutes or expression of Smad3 protein at 24 hours. Each bar represents the mean ± SEM for groups of three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to TGF-β; ## $p<0.05$, ### $p<0.001$ as indicated.

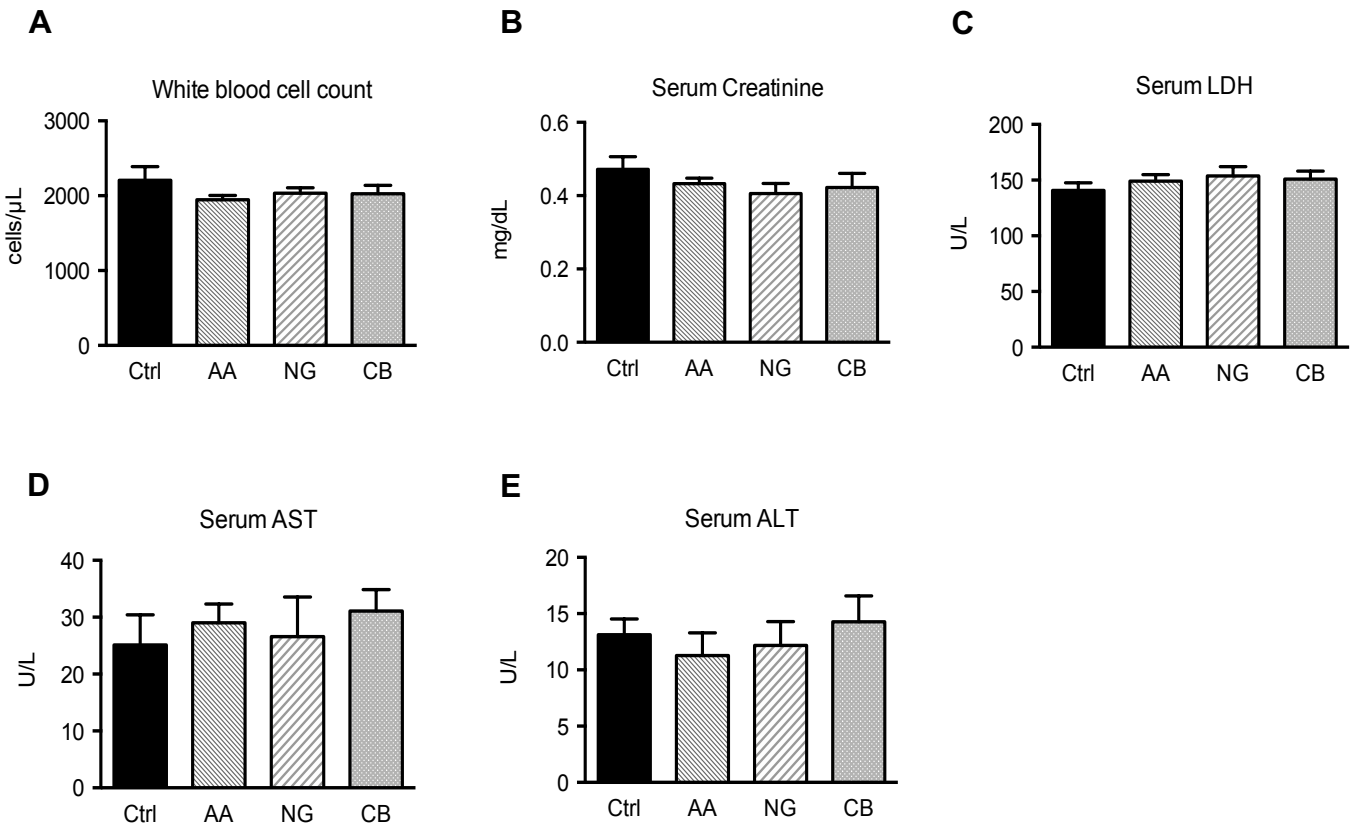

Figure S2. Treatment with AA, NG, or their combination does not induce side effects on LLC- bearing mice. (A) White blood cell counts, serum levels of **(B)** creatinine **(C)** LDH **(D)** AST and **(E)** ALT from LLC bearing mice at 27 days after tumor inoculation. Note that either individual or combination therapy with AA and NG does not cause toxicity to LLC-bearing mice. Each bar represents the mean ± SEM for groups of three to four mice.

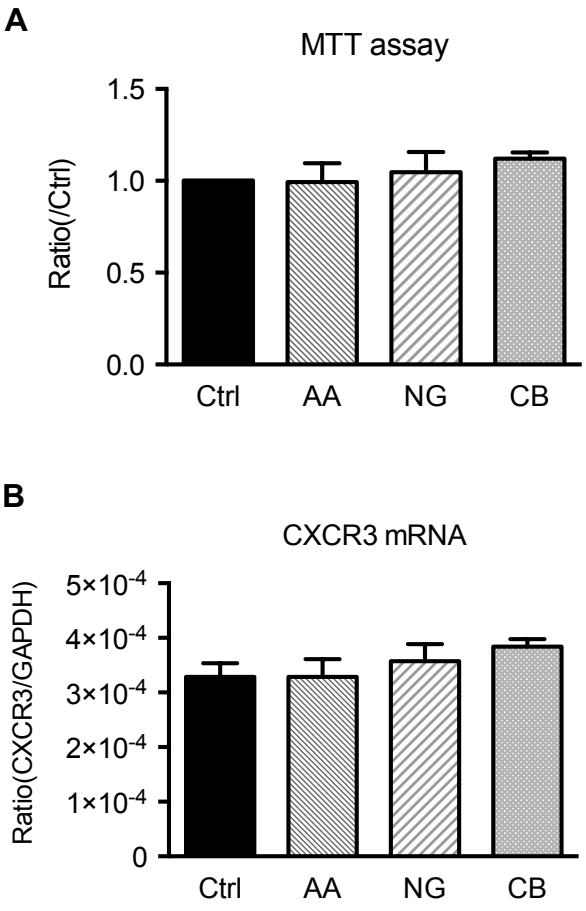

Figure S3. Treatment with AA, NG, or their combination does not influence NK cell proliferation and expression of CXCR3. (A) MTT assay shows that treatment with AA, NG or their combination does not alter BM-NK cell proliferation. **(B)** mRNA levels of CXCR3, a chemokine receptor critical for NK infiltration in tumor microenvironment detected by real-time PCR. Each bar represents the mean ± SEM for groups of three independent experiments.

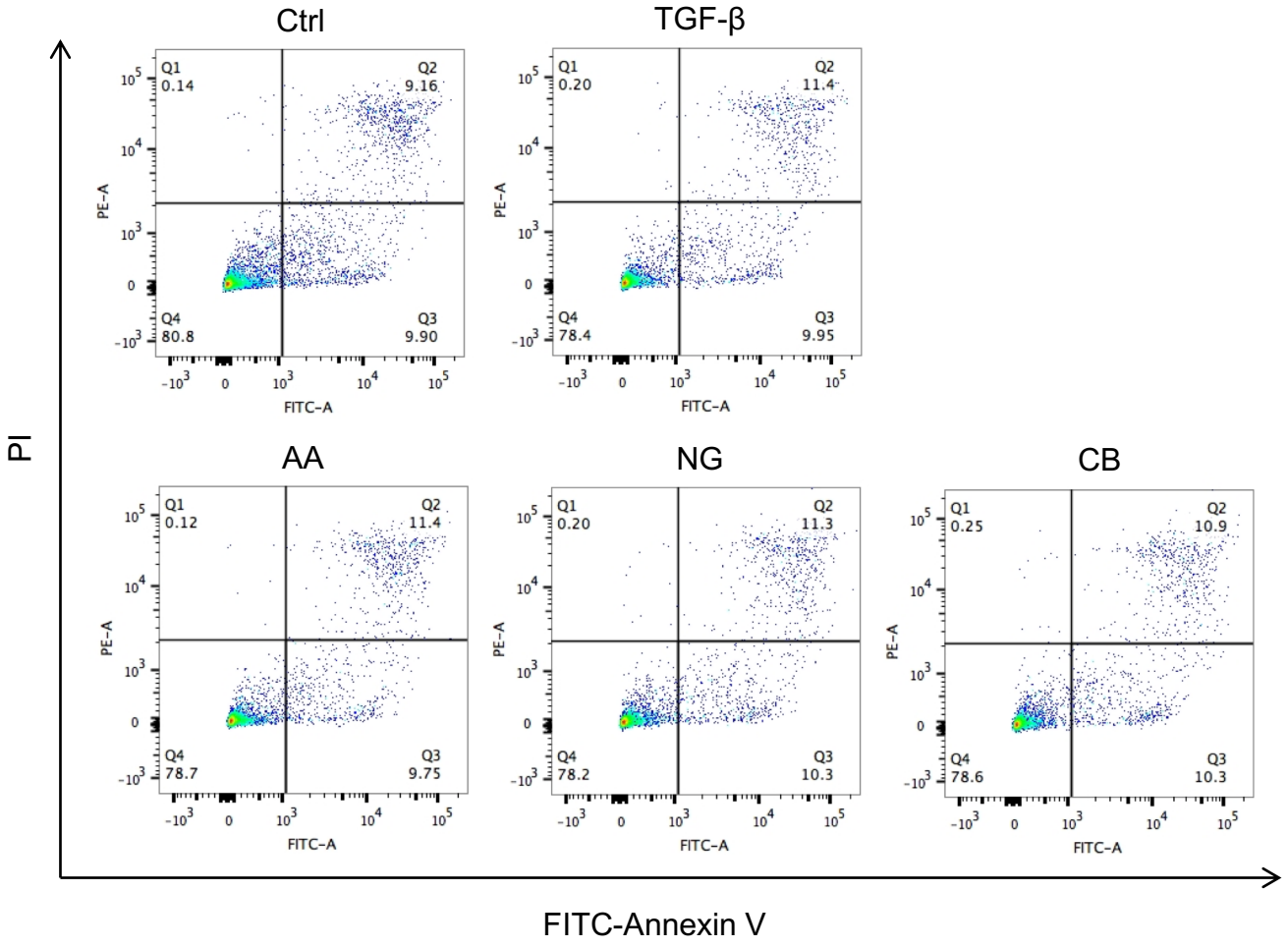

Figure S4. Neither individual nor combination therapy with AA and NG influences NK cell apoptosis. Individual or combination of AA and NG pre-treated BM-NK cells were stimulated with 5ng/ml TGF-β1 for 24 hours and then stained with FITC-Annexin V and PI to identify apoptotic NK cells. Results show that treatment with AA, NG or combination of AA and NG does not alter TGF-β1-induced NK cell apoptosis. Results represent three independent experiments.

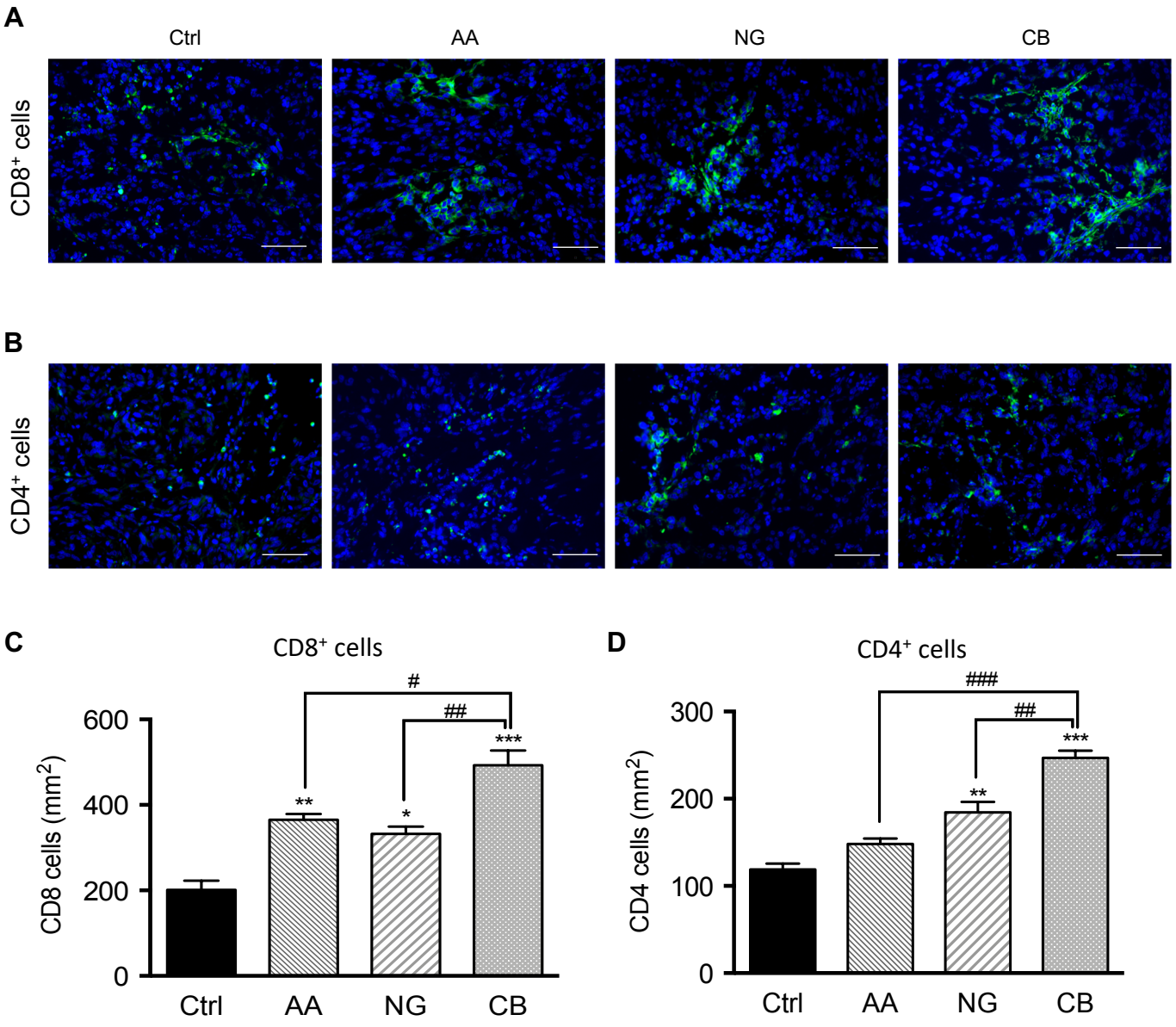

Figure S5. Combination therapy enhances the accumulation of CD8⁺ and CD4⁺ cells in tumor microenvironment. (A) Infiltrated CD8⁺ cells detected with FITC-CD8 and **(B**) infiltrated CD4⁺ cells detected with FITC-CD4 in LLC tumor microenvironment. Each bar represents the mean ± SEM for groups of three mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to Ctrl; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ as indicated. Scale bar, 100µm.

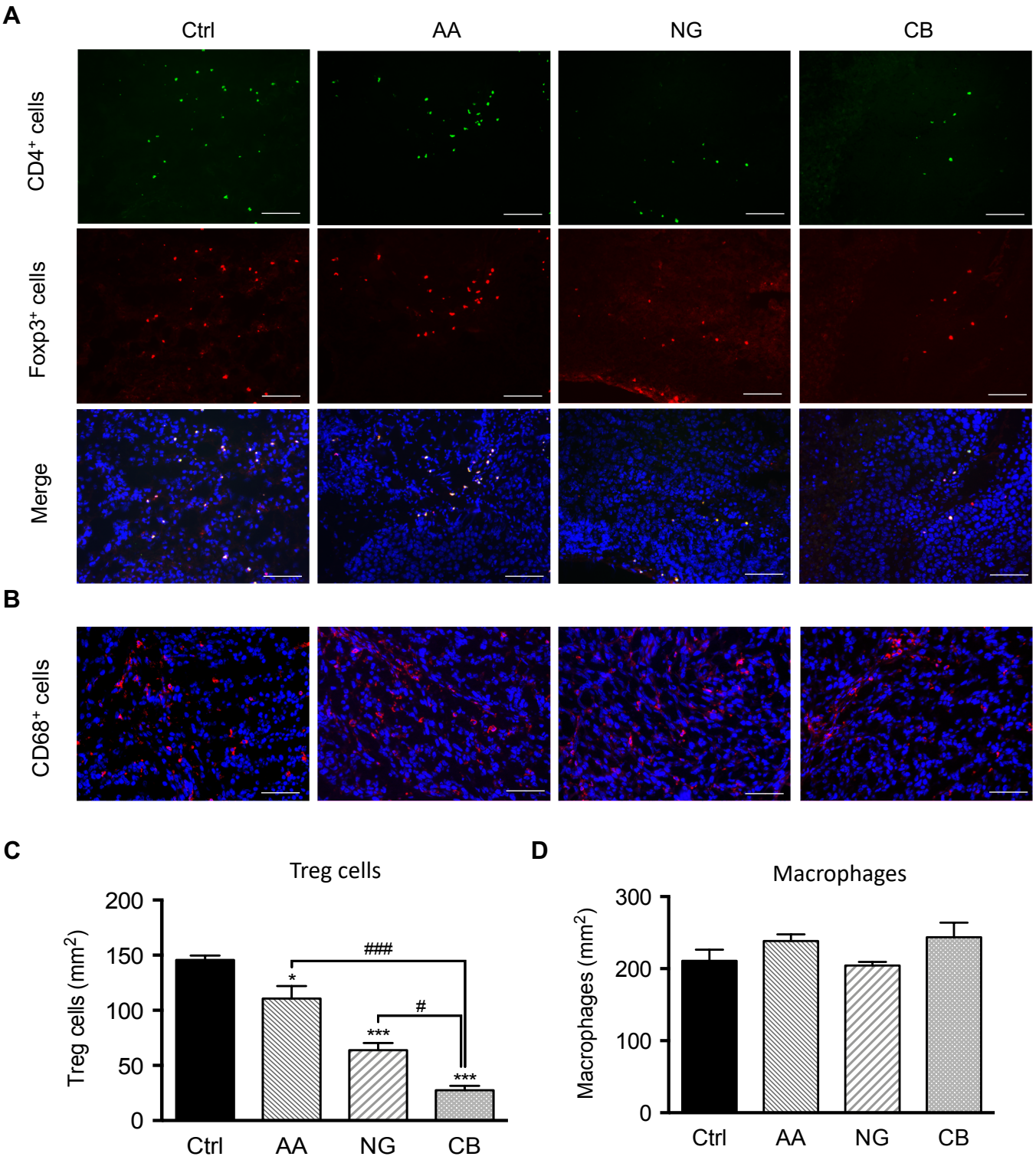

Figure S6. Combination therapy inhibits the accumulation of regulatory T cells (Tregs) while does not influence the accumulation of macrophages in tumor microenvironment. (A) Infiltrated Tregs were detected with FITC-CD4 and PE-Foxp3 and **(B)** infiltrated Tregs were detected with PE-CD68 in LLC tumor microenvironment. Each bar represents the mean ± SEM for groups of three mice. * $p<0.05$, *** $p<0.001$ compared to Ctrl; # $p<0.05$, ### $p<0.001$ as indicated. Scale bar, 100μm.

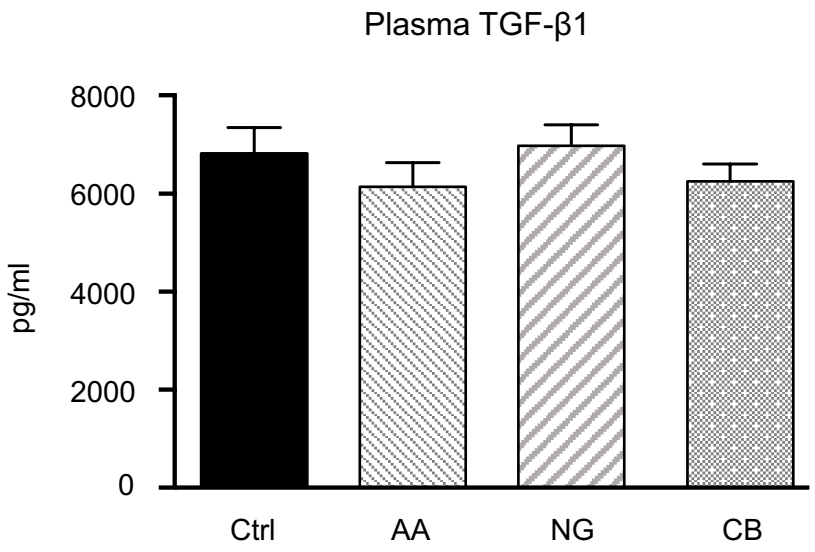

Figure S7. Combination therapy does not influence plasma levels of TGF-β1 in LLC-bearing mice. Each bar represents the mean ± SEM for groups of four to five mice.

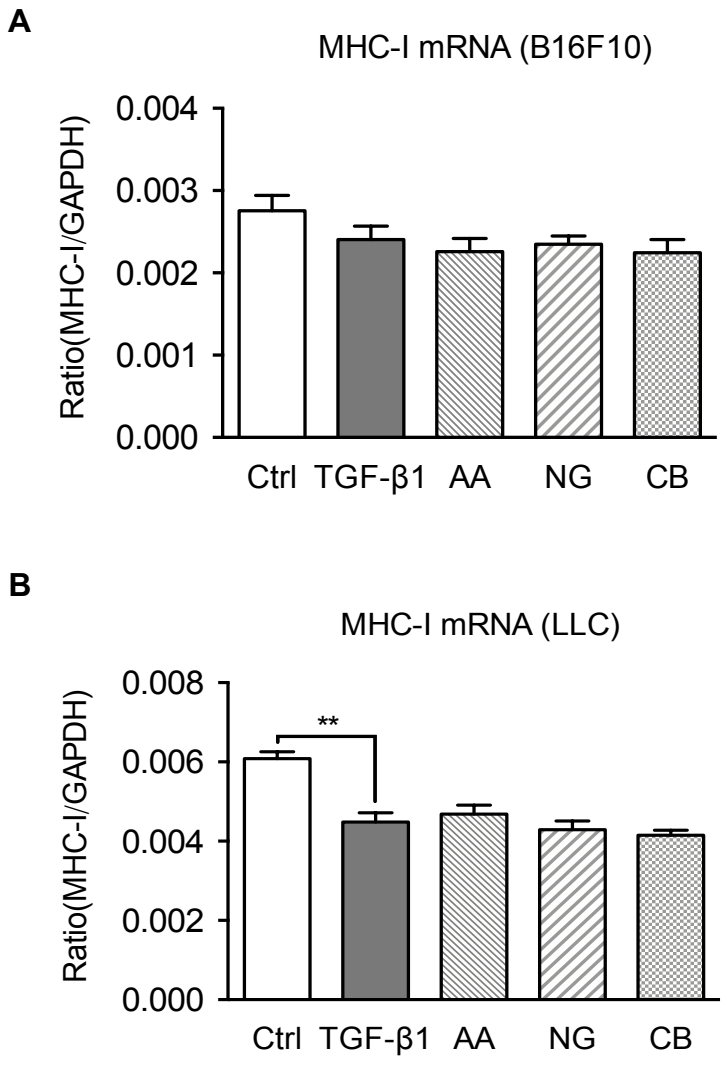

Figure S8. Treatment with AA, NG or their combination does not influence TGF-β1-induced inhibition on MHC-I expression on B16F10 and LLC cells. mRNA levels of MHC-I in **(A)** B16F10 and **(B)** LLC cells. Note that pre-treatment with AA, NG or their combination (CB) does not influence MHC-I expression in response to TGF-β1 (5ng/ml) stimulation in both B16F10 melanoma and LLC lung carcinoma cells. Each bar represents the mean ± SEM for groups of three independent experiments. ** $p<0.01$ compared to TGF-β1.

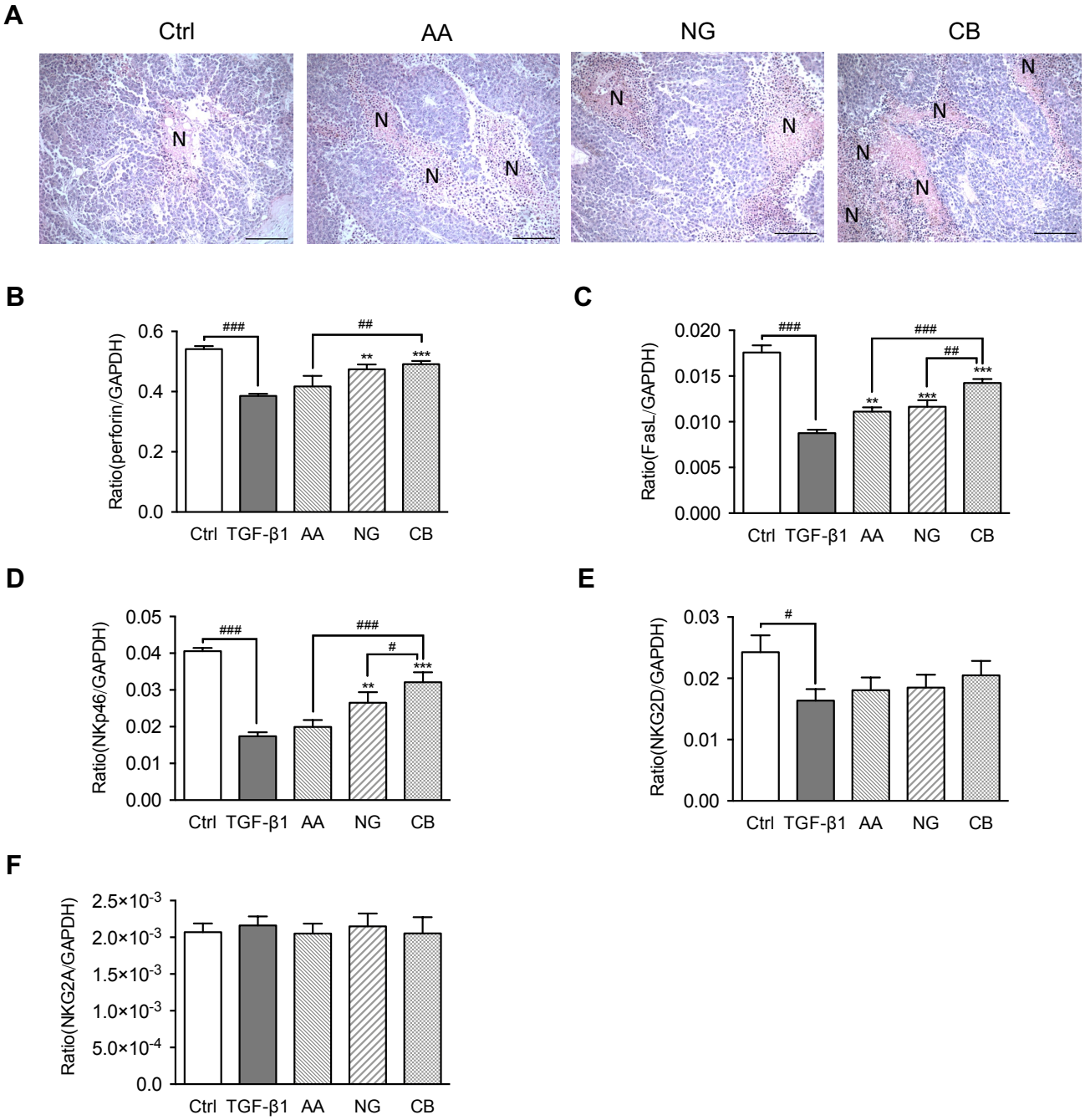

Figure S9. Combination therapy promotes NK-mediated anti-cancer activity via enhancing the expression of cytotoxic mediators and activation receptors in NK cells. (A) Periodic acid–Schiff (PAS) staining shows an increment of intratumoral necrosis (N indicated) in melanoma induced by combination therapy compared with control. mRNA levels of **(B)** perforin, **(C)** Fas ligand (FasL), **(D)** NKp46, **(E)** NKG2D and **(F)** NKG2A in splenic NK cells with TGF-β1 stimulation were detected by real-time PCR. Note that the combination treatment with AA and NG increases tumor necrosis in melanoma and produces a better anti-tumor effect compared to monotherapy by promoting NK cell activation (NKp46) and cytotoxicity (perforin, Fas ligand) in response to TGF-β1, although no alteration is found on expression of NKG2A and NKG2D expression. Each bar represents the mean ± SEM for groups of three independent experiments. ** $p<0.01$, *** $p<0.001$ compared to TGF-β1; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ as indicated. Scale bar, 200μm.

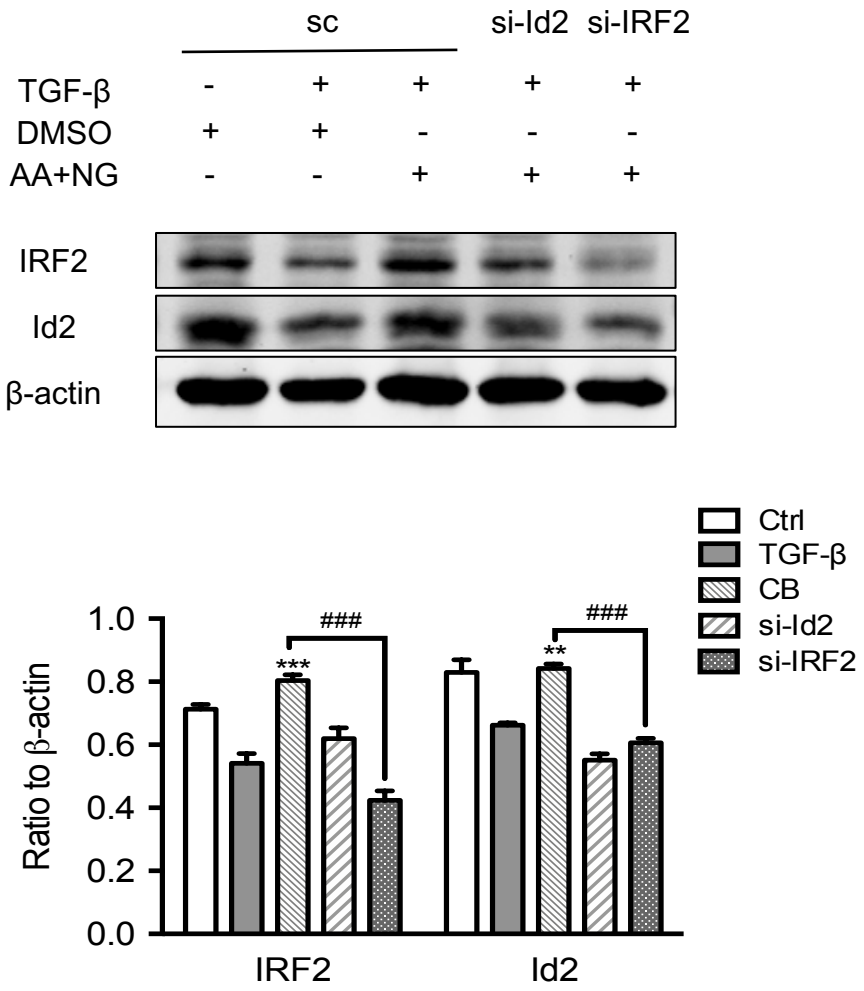

Figure S10. Knocking down Id2 and IRF2 prevents the counter-regulatory effect of AA and NG on TGF-β1-induced suppression of IRF2 and Id2 expression in BM-NK cells. BM-NK cells were transfected with scramble sequence (sc), si-Id2 or si-IRF2 and then cultured with AA (10μM) and NG (100μM) under TGF-β1 (5ng/ml) conditions for 9 days. Each bar represents the mean ± SEM for groups of three independent experiments. ** p<0.01, *** p<0.001 compared to TGF-β1; ### p<0.001 as indicated.

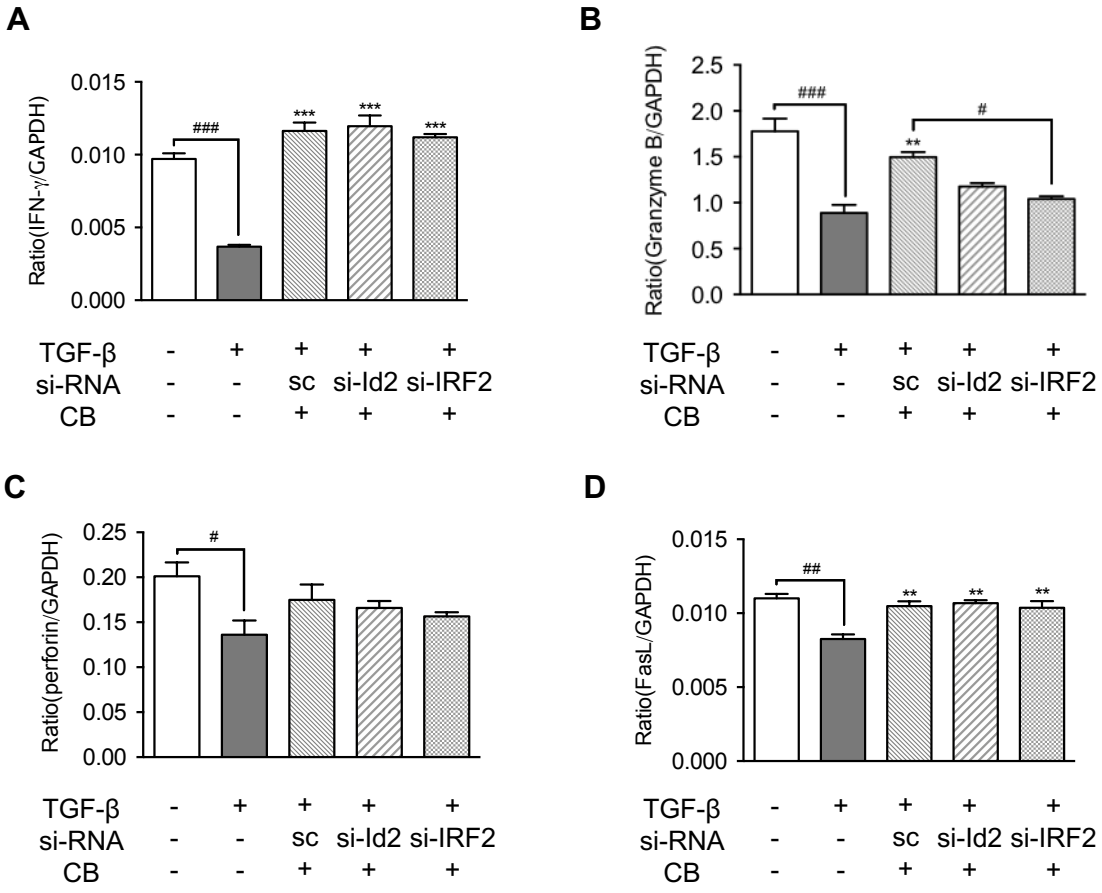

Figure S11. Expression of cytotoxic mediators in BM-NK cells treated with specific siRNA sequence for Id2 and IRF2. mRNA levels of **(A)** IFN-γ, **(B)** granzyme B, **(C)** perforin, **(D)** Fas ligand (FasL) in mature BM-NK cells transfected with scramble sequence (sc), si-Id2 or si-IRF2 with TGF-β1 (5ng/ml) stimulation. Each bar represents the mean ± SEM for groups of three independent experiments. ** $p<0.01$, *** $p<0.001$ compared to TGF-β1; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ as indicated.

Table S1. Sequence of primers for real-time PCR

Primers for Real-time PCR		
Target Gene	Forward Primer	Reverse Primer
Id2	ACCAGAGACCTGGACAGAAC	AAGCTCAGAAGGGAATTCAG
IRF2	CTTATCCGAACGACCTTCCA	CTTGCTGTCCAGATGGGACT
CXCR3	TGCTAGATGCCTCGGACTTT	CGCTGACTCAGTAGCACAATT
MHC-I	GAGGGTGGCTCTCACACATTC	TTGGCCTTCGTAAGCAAACTG
IFN-γ	TTTCGCCTTGCTGTTGCTGA	TGGATATCTGGAGGAACTGGCA
granzyme B	TGCTGCTAAAGCTGAAGAGTAAG	CGTGTTTGAGTATTTGCCCATTG
perforin	GCTCCCACTCCAAGGTAGC	GCTCCCACTCCAAGGTAGC
FasL	GCCCATGAATTACCCATGTCC	ACAGATTTGTGTTGTGGTCCTT
NKp46	ATGCTGCCAACACTCACTG	GATGTTCACCGAGTTTCCATTTG
NKG2D	ACTCAGAGATGAGCAAATGCC	CAGGTTGACTGGTAGTTAGTGC
NKG2A	GCCCCTGCAAAGATACCGAA	TCTGTGGGTTCTAGTCATTGAGG
GAPDH	GCATGGCCTTCCGTGTTC	GATGTCATCATACTTGGCAGGTTT

Table S2. Sequence of primers for ChIP assay

Primers for ChIP Assay		
Target Gene	Forward Primer	Reverse Primer
Id2 SBS1	GGGGTGAGAGAACAGAAGGA	TTTCAGACAACCAGTGCTTTG
Id2 SBS2	CAGCATTCAGTAGGCTCGTG	GCCTTTTCACAAAGGTGGAG
IRF2 5'UTR SBS1	GGTGTCGTGTGTTGTGGGTA	GGTGGCGACAGTGTCTGTAA
IRF2 3'UTR SBS2	GTGTCTCAGCTCCACCCATT	CTCCTATGCTCAGCCTGTCC