SI Appendix, Materials and Methods

Selective neutralization of IL-12 p40 monomer induces death in prostate cancer cells via IL-12-IFN-γ

Madhuchhanda Kundu[1], Avik Roy[1], Kalipada Pahan[1, 2]

[1]Department of Neurological Sciences, Rush University Medical Center, Chicago, IL, USA;
[2]Division of Research and Development, Jesse Brown Veterans Affairs Medical Center, 820 South Damen Avenue, Chicago, USA

Animals and Reagents: All murine and human cancer cell lines were purchased from ATCC. Male C57 BL/6 mice (Harlan) were used for this study. Mouse p40 (cat# 554594) and p70 (cat# 554592) were purchased from BD Biosciences. Mouse $p40_2$ (cat# 499-ML) was purchased from R&D. Hamster IgG (cat# IR-HT-GF) was obtained from Innovative Research. Mouse IgG (cat# sc-2025) was purchased from Santa Cruz Biotechnology. Chloropromazine (cat # C8138), filipin (cat# F9765), MTT assay kit (cat# CGD1), and LDH assay kit (cat# TOX7) were purchased from Sigma. IFNγ neutralizing antibody (cat# 16-7311-81) was purchased from eBioscience. TUNEL assay kit (cat# QIA39) was purchased from Calbiochem and Annexin V assay kit (cat# K101-25) was purchased from Biovision.

Sandwich ELISA: Sandwich ELISA was used to quantify mouse $p40_2$ and p40 as described by us (1, 2). Briefly, for $p40_2$, mAb a3-1d (1.3 mg/mL) was diluted 1:3000 and added to each well (100 μL/well) of a 96-well ELISA plate for coating. The biotinylated $p40_2$ mAb d7-12c (2 mg/mL) was diluted 1:3000 and used as detection antibody. Similarly for p40, mAb a3-3a (1.3 mg/mL) and biotinylated p40 mAb a3-7g (2 mg/mL) were also diluted 1:3000 and used as coating and detection antibodies, respectively (1). Concentrations of IFN-γ, IL-12 and IL-10 were measured in serum free supernatants of different treatment groups by ELISA (eBioscience), according to the manufacturer's instructions.

Flow cytometry analysis with live dead exclusion: Briefly, TRAMP-C2 cells were plated in T25 flasks and cells were detached using accutase (Sigma, Cat# A6964) solution. Next, cells were washed and resuspended in PBS. One µL of the reconstituted fluorescent reactive dye (Fisher Scientific, Cat # L34965) was added to the cell suspension and mixed well. Then cells were incubated on ice for 30 minutes at dark. After washing, cells were treated with fixation/permeabilization buffer (Fisher Scientific, cat# 00-5123-43, cat# 00-5223-56) for intracellular staining. Cells were then incubated with mAb against p40 (2.5 µg, a3-3a) and $p40_2$ (2.5 µg, a3-1d) for 90 min followed by incubation with secondary antibody (Anti hamster PE, BD Biosciences, 12-4112-83) for 1h at dark. Flow cytometry was performed in BD FACS Canto II Flow Cytometer (3 laser - 8 "color") and data were analyzed using the FlowJo software. For IFNγ staining, cells were stained with PE tagged IFNγ antibody and detected by flow cytometry analysis. Only PE-treated and unstained cells served as control.

Tumor development and measurement: Animal maintaining and experiments were in accordance with National Institute of Health guidelines and were approved by the Institutional Animal Care and Use committee (IACUC#14-019) of the Rush University of Medical Center, Chicago, IL. Tumors were generated subcutaneously in male C57 BL/6 mice. Mice were injected with 1×10^6 TRAMP-C2 cells in their flank for tumor generation. Mice were maintained in our temperature-controlled animal vivarium with adequate food and water.

Tumor growth was measured with a caliper and tumor cross-sectional area was determined with the formula (mm^2 = longest diameter X shortest diameter). Treatment with p40 mAb started when the tumor sizes reached 0.8-1mm^2. The p40 mAb a3-3a was injected once a week intraperitonially in 0.1ml volume of sterile PBS-1% normal mouse serum. The tumors were then measured to determine regression or progression. Infrared dye (Alexa 800-conjugated 2DG dye; Licor) was injected via tail-vein on the day before imaging analysis. Mice were sacrificed at the

end of the study and tumor tissues were collected appropriately for western blot, mRNA expression and immunohistochemical analysis.

Tissue preparation and Immunohistochemistry: Paraffin embedded tissue sections were prepared and tissue sections were cut 5 micron in size as described (2, 3). To eliminate endogenous peroxidase activity, tissue sections were deparaffinized, rehydrated and incubated with 3% H_2O_2 in methanol for 15 min at room temperature. Antigen retrieval was performed at 95°C for 20 min by placing the slides in 0.01 M sodium citrate buffer (pH 6.0). After blocking, the slides were then incubated with the primary antibodies (SI Appendix, Table S1) at 2h room temperature followed by washing and incubation with Cy2, Cy3 or Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies at RT for 1h. Mouse IgG was used as an isotype control (4).

Semi-quantitative RT-PCR: Total RNA was isolated and semi-quantitative RT-PCR analyses for IFNγ, IL-10, T-bet, GATA3, FoxP3, and GAPDH were performed as described earlier (5-7) using primers (SI Appendix, Table S2).

Real-time quantitative PCR: The mRNA quantification was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems) using SYBR GREEN (Applied Biosystems) as described earlier (5-7). The mRNA expressions of respective genes were normalized to the level of GAPDH mRNA. Data were processed by the ABI Sequence Detection System 1.6 software and analyzed by ANOVA.

FACS: Surface expression of IL-12Rβ1 and IL-12Rβ2 were monitored as described earlier (5, 8, 9). Briefly, after treatment, cells were incubated for 10 min with Accutase (BD Bioscience) for detachment of adherent cells. After washing with FACS buffer, cells were incubated with PE-tagged IL-12Rβ1 and IL-12Rβ2 antibodies at 4°C for 1 h. For intracellular staining, permeabilization was done before incubation with p40 and $p40_2$ mAbs. APC-conjugated anti-

hamster secondary antibodies were used. After washing, the cells were analyzed through FACS (BD Biosciences, San Jose, CA). Cells were gated based on morphological characteristics. Apoptotic and necrotic cells were not accepted for FACS analysis.

Membrane isolation: After treatment, cells were scraped in PBS and cell pellets were dissolved in homogenization buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.2, protease inhibitors and phosphatase inhibitors) and then homogenized with hand homogenizer. Cell debris was removed by centrifugation at 500g for 10 min at 4ºC followed by centrifugation of supernatant at 100,000 g for 1h. Supernatants were discarded and the pellet containing membrane fractions were dissolved in SDS-PAGE sample buffer.

SI Appendix, Supplementary Tables

Table S1: Antibodies used in this study

Antibody	Manufacturer	Catalog	Host	Application	Dilution
IFNγ	eBioscience	16-7311-81	Rat	Blocking, IF	0.25-1µg/mL (BL), 1:100 (IF)
Tbet	Santacruz	sc-21003	Rabbit	IF	1:100
IL-12	eBioscience	16-7123-81	Rat	Blocking	0.25-1µg/mL (BL)
IL-12Rβ1	BD Bioscience (PE-tagged)	551974	Mouse	FACS	0.5-1 µg/ 10^6 cells
	Santacruz	sc-658	Rabbit	WB	1:200
IL-12Rβ2	BD Bioscience	552819	Hamster	FACS	0.5-1 µg/ 10^6 cells
Caveolin-1	Cell Signaling Technology	3238	Rabbit	WB, IC	1:1000 (WB) 1:400 (IC)
Clathrin	Cell Signaling Technology	2410	Rabbit	WB	1:1000
P-cadherin	Cell Signaling Technology	4068	Rabbit	WB	1:1000
β-actin	Abcam	ab6276	Mouse	WB	1:2000

IF, immunofluorescence; WB, Western blot; FACS, fluorescence-activated cell sorting; IC, immunocytochemistry; BL, blocking

Table S2: List of primers used in this study

Gene	Directions	Sequence (5'…3')
IFNγ	Sense	CGGCACAGTCATTGAAAGCC
	Antisense	TGCATCCTTTTTCGCCTTGC
IL10	Sense	TAAGGCTGGCCACACTTGAG
	Antisense	GTTTTCAGGGATGAAGCGGC
Tbet	Sense	ATTGGTTGGAGAGGAAGCGG
	Antisense	TGTGCACCCTTCAAACCCTT
GATA3	Sense	TGGCGCCGTCTTGATAGTTT
	Antisense	CCTCTTCCGTCAGCGGATAC
Foxp3	Sense	TGTGCCTGGTATATGCTCCC
	Antisense	GTTCTTGTCAGAGGCAGGCT
GAPDH	Sense	GCATCTTCTTGTGCAGTGCC
	Antisense	TACGGCCAAATCCGTTCACA
PDL-1	Sense	TCACTTGCTACGGGCGTTTA
	Antisense	TGCCAATCGACGATCAGAGG
PDL-2	Sense	GGTGTGTGATTGGTAGGCCA
	Antisense	CATCCAGCAGGTAACCAGGG
PD-1	Sense	ATCTACCTCTGTGGGGCCAT
	Antisense	GAGTGTCGTCCTTGCTTCCA
CTLA-4	Sense	TACTCTGCTCCCTGAGGACC
	Antisense	CCGTGTCAACAGCTCTCAGT
Cytochrome C	Sense	CCCCCAGCCTCCCTTATCTT
	Antisense	GGTCTGCCCTTTCTCCCTTC
Caspase 3	Sense	GAGCTTGGAACGGTACGCTA
	Antisense	CCGTACCAGAGCGAGATGAC
Caspase 8	Sense	AACATTCGGAGGCATTTCTGT
	Antisense	AGAAGAGCTGTAACCTGTGGC
Caspase 7	Sense	TTTTCCCAAAGCTGCCCTCG
	Antisense	GCGTCAATGTCGTTGATGGG
Caspase 9	Sense	CTCTGAAGACCTGCAGTCCC
	Antisense	CTGCTCCACATTGCCCTACA
P53	Sense	ACCAGGGCAACTATGGCTTC
	Antisense	AGTGGATCCTGGGGATTGTG
BAD	Sense	CAGCGTACGCACACCTATCC
	Antisense	CGGGATCGGACTTCCTCAAG
BID	Sense	TCTGAGGTCAGCAACGGTTC
	Antisense	TTTGTCTTCCTCCGACAGGC
BAX	Sense	CTGGATCCAAGACCAGGGTG
	Antisense	CCTTTCCCCTTCCCCCATTC
BCL2	Sense	AGCATGCGACCTCTGTTTGA
	Antisense	GCCACACGTTTCTTGGCAAT
BCL-XL	Sense	TTGTACCTGCTTGCTGGTCG
	Antisense	CCCGGTTGCTCTGAGACATT
BAK	Sense	CCTGGGCCAACACGC
	Antisense	CTGTGGGCTGAAGCTGTTCTA

SI Appendix, Supplementary Figures

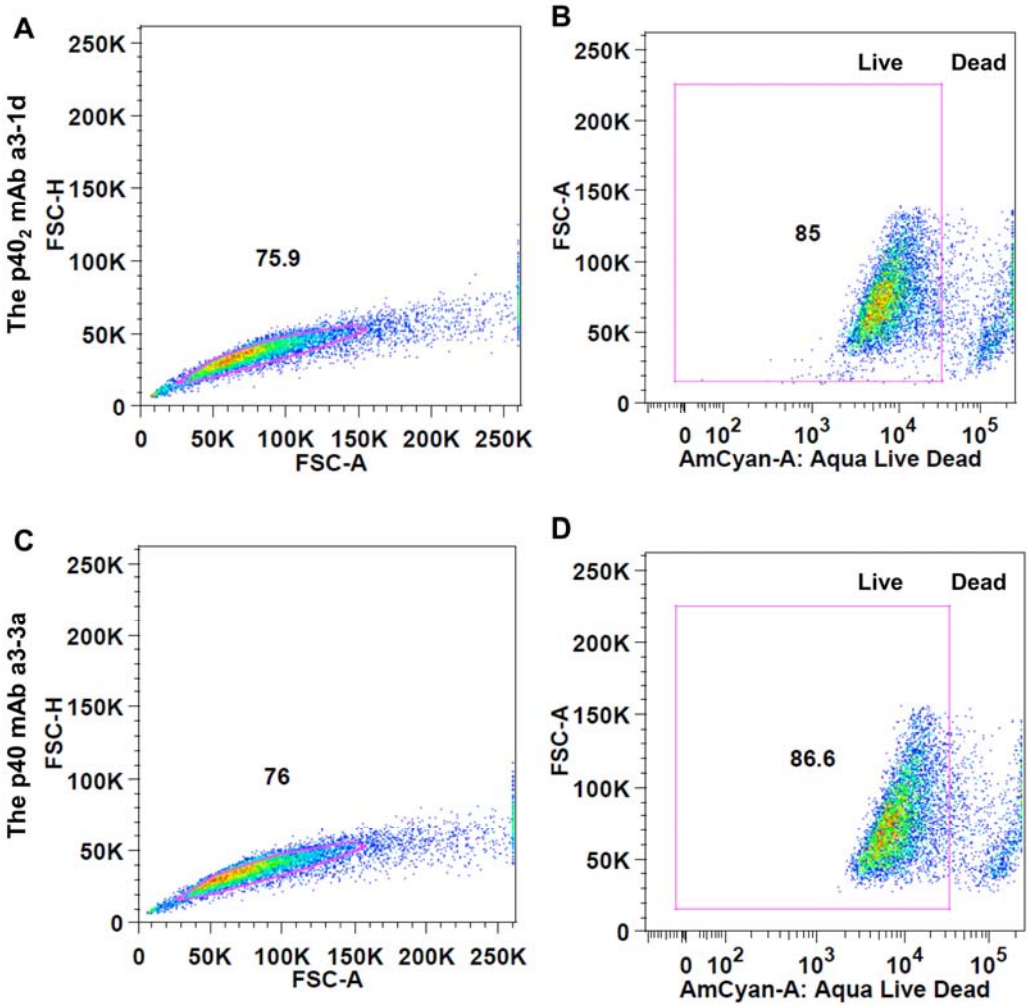

Figure S1. FACS analysis of p40 and p40$_2$ in TRAMP-C2 cells. TRAMP cells were suspended in PBS and stained with aqua fluorescent reactive dye (Fisher Scientific, Cat# L34965) for live-dead exclusion. Cells were then solubilized in FACS buffer for intracellular staining. For analysis, cells were gated to exclude dead cells (A-B, p40$_2$; C-D, p40). Live cells were then analyzed for p40$_2$- and p40-specific population by FlowJo software (Fig. 1F-G).

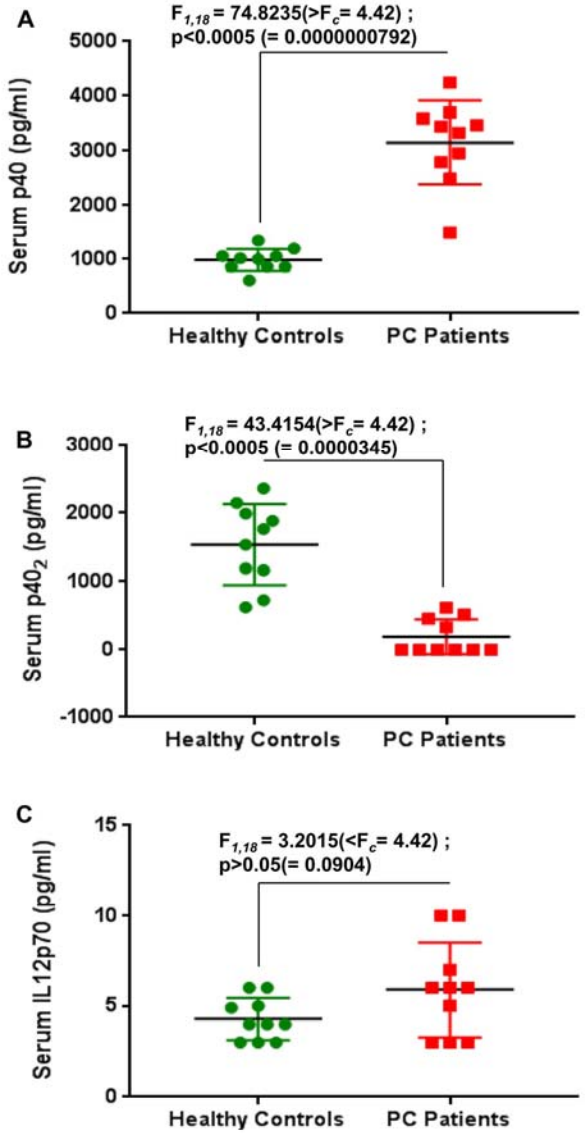

Figure S2. Levels of p40, $p40_2$ and IL-12 in serum of prostate cancer patients and healthy controls. Serum of prostate cancer patients (n=11) and age-matched healthy controls (n=10) was analyzed for p40 (A), $p40_2$ (B) and IL-12 (C) by sandwich ELISA. The p40 (A) was significantly elevated in serum of prostate cancer cases compared with healthy controls *[$F_{1,18}$ = 74.8235 (>F_c= 4.42); p<0.0001 (= 0.0000000792)]* whereas the $p40_2$ (B) was significantly higher in healthy controls relative to prostate cancer cases *[$F_{1,18}$ = 43.4154 (>F_c= 4.42); p<0.0005 (= 0.0000345)]*.

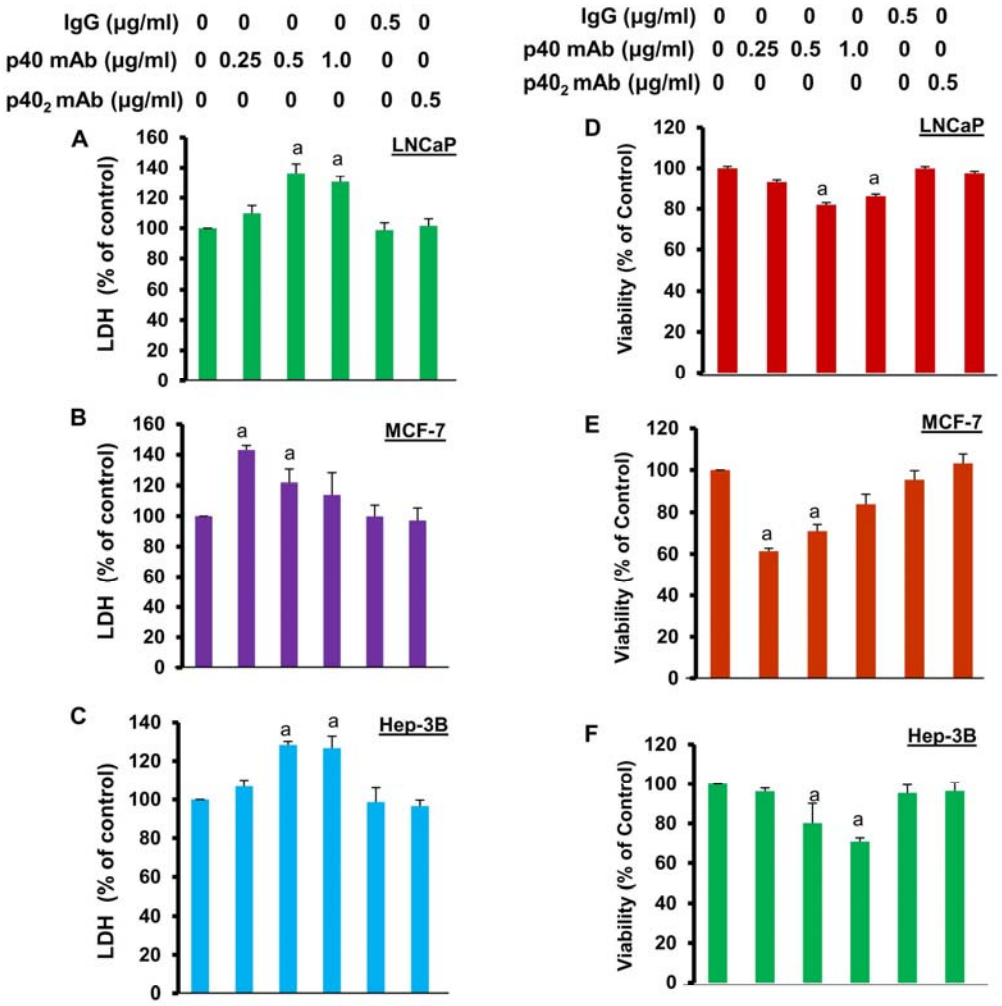

Figure S3. Neutralization of p40, but not p40$_2$, induces death of different human cancer cells. LnCaP (A & D), MCF-7 (B & E) and Hep3B (C & F) cells were treated with neutralizing mAbs against p40 and p40$_2$ for 48 h under serum-free condition followed by monitoring cell death by LDH release (A-C) and MTT (D-F). Results are mean ± S.D. of three separate experiments. **p<0.05 vs. control.*

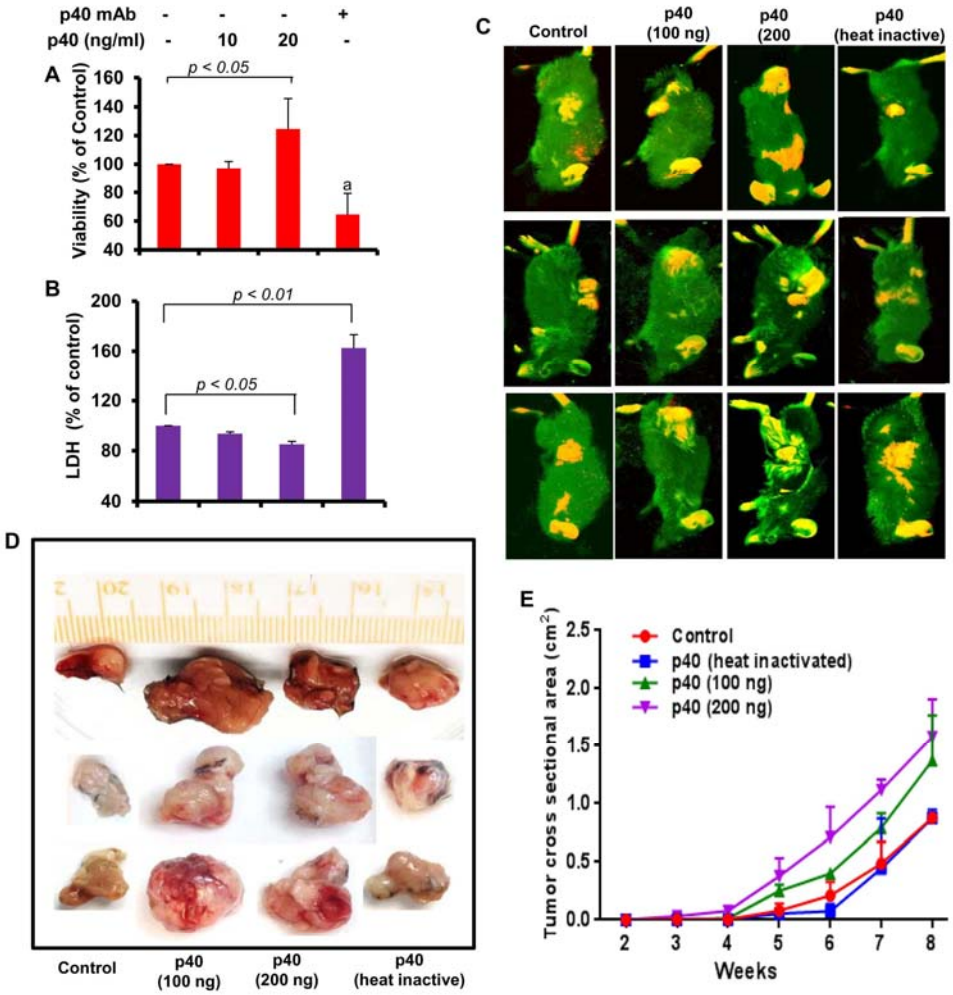

Figure S4. Effect of recombinant p40 monomer on the survival of TRAMP cells and the size of TRAMP tumor. TRAMP cells were treated with different concentrations of p40 for 48 h under serum free condition followed by monitoring cell survival by MTT (A) and LDH (B). Results are mean ± SD of three independent experiments. [a] *p<0.01 vs control.* C) Eight to ten weeks old male C57BL/6 (n=3 per group) were injected with 1 million of TRAMP cell suspension subcutaneously. After one week, p40 monomer (100 or 200 ng/mouse) was injected i.p. every 5 d interval for eight weeks. Heat-inactivated (boiled for 5 min) p40 monomer was included as control. Tumors were labeled with Alexa 800-conjugated 2DG dye (injected via tail-vein) and then imaged in Licor Odyssey infrared scanner after 24 h of dye injection. Results were compared with no treatment control group (left panel). D) Tumors were excised from the flank of all animals. E) Tumor size was monitored every week for all three groups and plotted vs number of weeks. Results are mean ± SEM of three different animals per group.

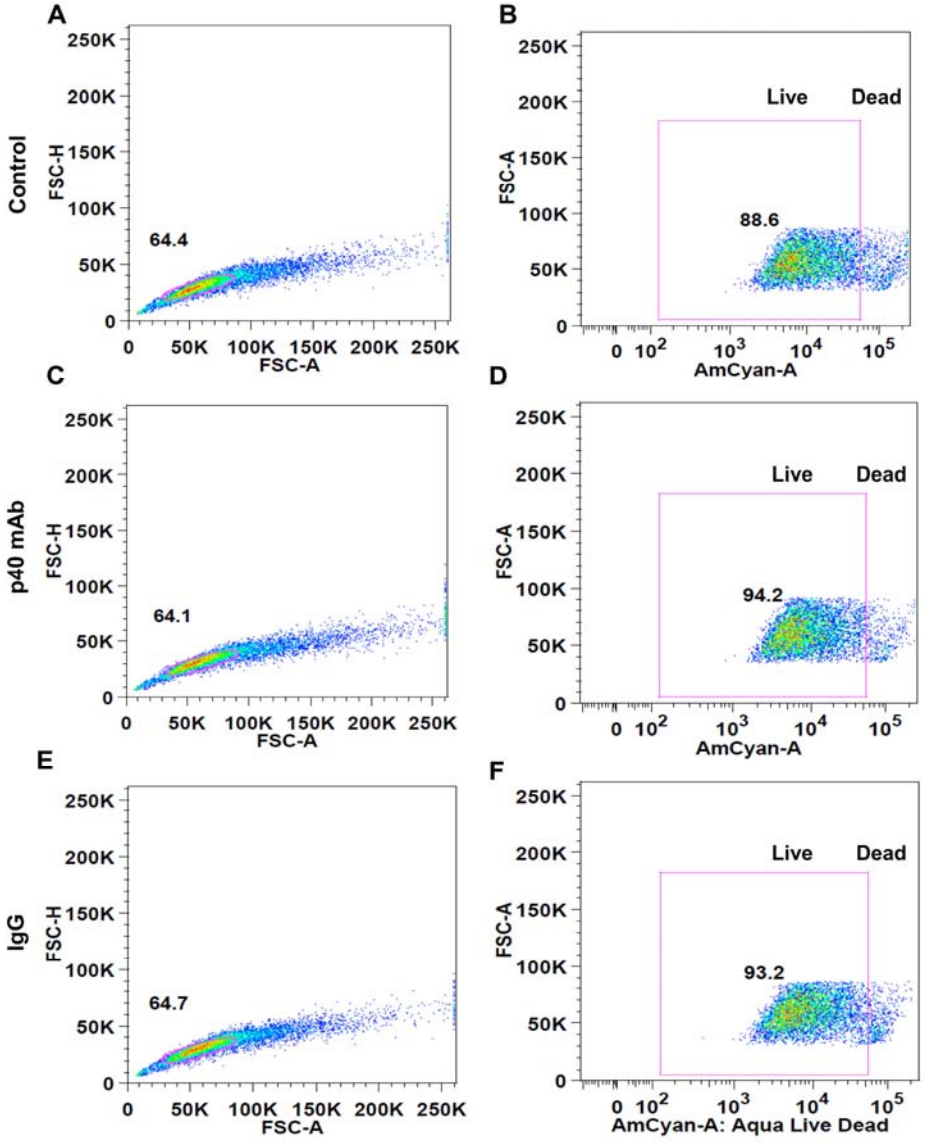

Figure S5. Analyzing IFNγ in TRAMP-C2 cells by flow cytometry. TRAMP cells were cultured and treated with p40 mAb (0.5μg/ml) and IgG (0.5μg/ml) for 18h under serum-free condition. Cells were gated to discriminate live and dead cells using Aqua fluorescent reactive dye (Fisher Scientific, Cat# L34965) (A-B, control; C-D, p40mAb; E-F, IgG). Live cells were then analyzed for IFNγ population (Fig. 4C-D) by FlowJo software.

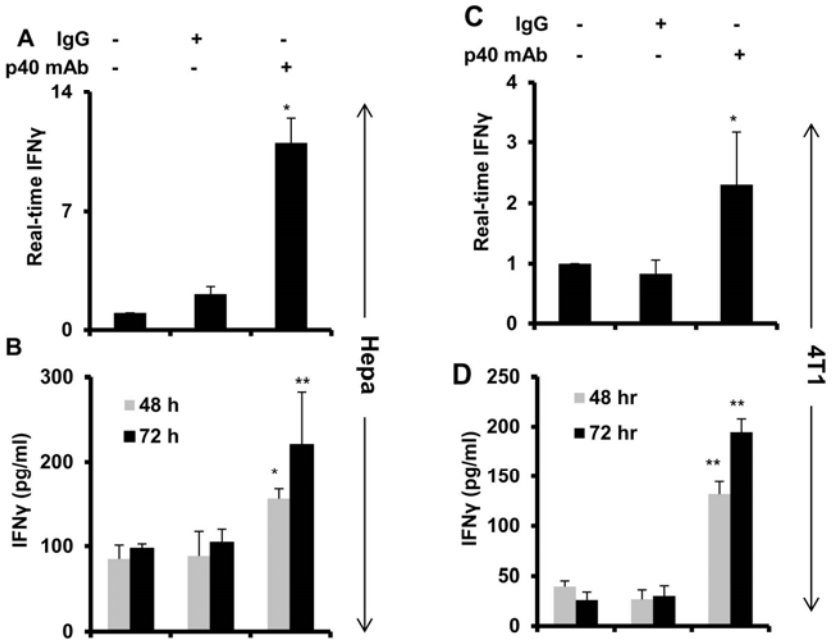

Figure S6. Effect of p40 mAb on the expression of IFNγ protein in Hepa and 4T1 cancer cells. Real-time mRNA (A & C) and ELISA (B & D) analyses of IFNγ in control, IgG-, and p40 mAb-treated Hepa (A & B) and 4T1 (C & D) cells. Results are mean ± SD of three different experiments. *$^{*}p<0.05$ vs. control, and $^{**}p<0.001$ vs. control.*

p40 mAb	-	0.5	-	0.5	0.5	0.5	0.5	-
Ham IgG	-	-	0.5	-	-	-	-	-
IFNγ Ab	-	-	-	0.25	0.5	1	-	0.5
Mouse IgG	-	-	-	-	-	-	0.5	-

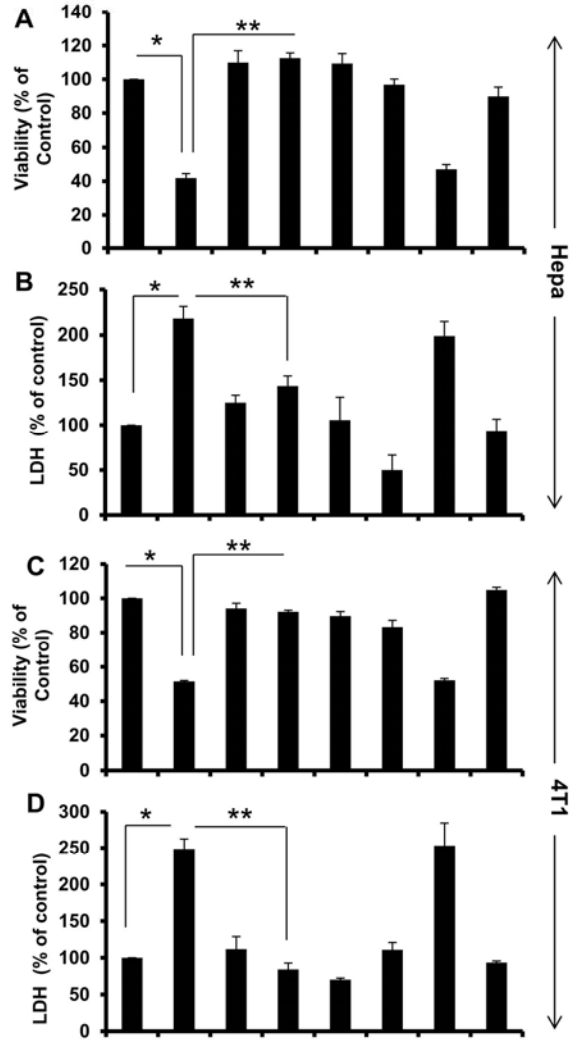

Figure S7. Role of IFNγ in p40 mAb-mediated death in Hepa and 4T1 cancer cells. MTT (A & C) and LDH (B & D) assays were performed in p40 mAb- (0.5 µg/mL) or p40 mAb together with increasing doses of IFNγ Ab (0.25, 0.5, and 1 µg/mL)-treated Hepa (A & B) and 4T1 (C & D) cells. Results are mean ± SD of three different experiments. *$^{*}p<0.001$ vs. control, and $^{**}p<0.001$ vs. p40 mAb only.*

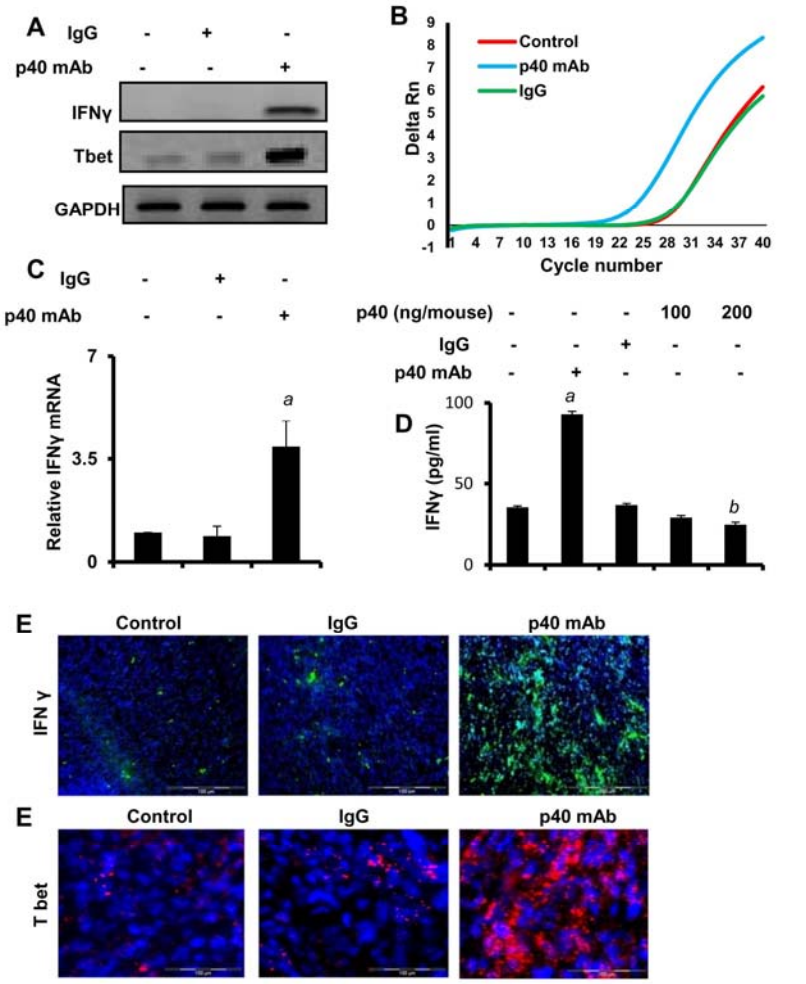

Figure S8. Effect of p40 mAb on the expression of IFNγ in TRAMP tumor tissue. A) Semi-quantitative RT-PCR of IFNγ and Tbet is shown. B) Graphical representation of real-time PCR of IFNγ where Rn (the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye) is plotted against PCR cycle number. C) Relative IFNγ mRNA is shown from real-time PCR. D) IFNγ ELISA was performed in control, p40 mAb-, IgG-, and p40-treated TRAMP tumor tissues. *[a]p<0.001 vs. control; [b]p<0.05 vs. control.* Immunohistochemical analyses of IFNγ (E) and T-bet (F) in different groups of tumor tissues. Results represent analysis of two sections of each of five mice per group.

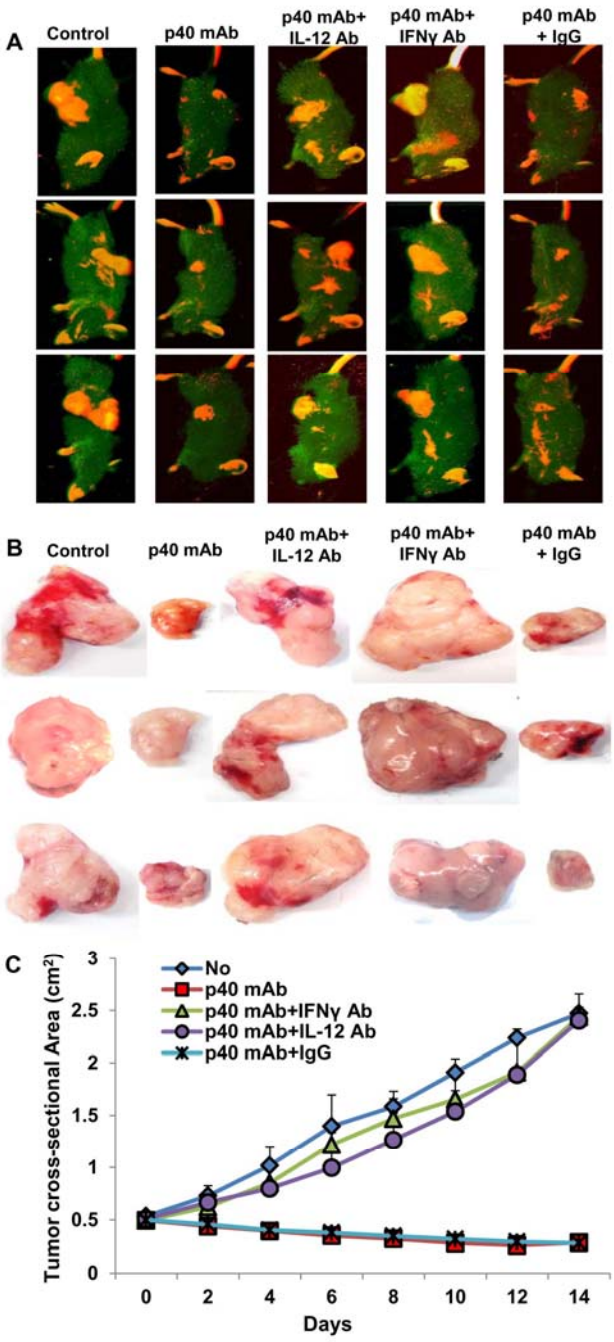

Figure S9. Abrogation of p40 mAb-mediated regression of TRAMP tumor by functional blocking antibodies against IL-12 and IFNγ *in vivo* in mice. A) Eight to ten weeks old male C57BL/6 (n=3 per group) were injected with 1 million of TRAMP cell suspension subcutaneously. After about 6 weeks, when tumors were within 0.8 to 1 mm in size, mice were treated with p40 mAb at a dose of 2 mg/kg body wt twice a week. Next day, mice also received neutralizing antibodies against either IL-12 or IFNγ (25 µg/mouse) once via i.p. injection. After 2 wks, tumors were labeled with Alexa800 conjugated 2DG dye via tail vein injection and then imaged in Licor Odyssey infrared imaging system. Results were compared with no treatment control group (left panel). B) Tumors were excised from the flank of all groups of mice. C) Tumor size was monitored every alternate day for all groups of mice and plotted in a comparative line plot. Results are mean ± SEM of three different mice.

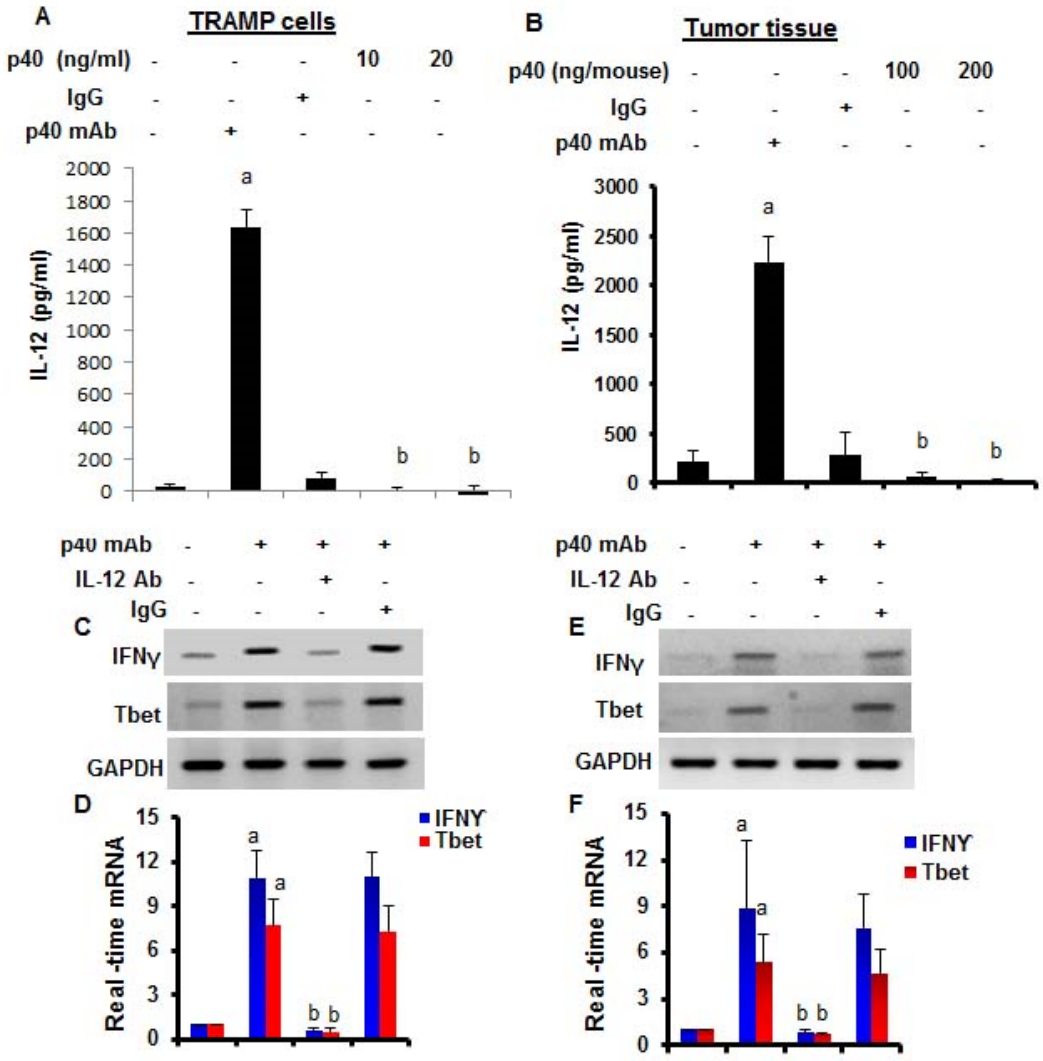

Figure S10. Effect of p40 mAb on the expression of IL-12 in cultured TRAMP cells and TRAMP tumor tissue. (A) ELISA analyses to monitor the levels of IL-12 in cultured TRAMP cells treated with p40 mAb (0.5 µg/mL), mouse IgG (0.5 µg/mL) and recombinant mouse p40 (20 ng/mL) for 24 h under serum-free condition. Results are mean ± SD of three different experiments. *[a]p<0.001 vs. control; [b]p<0.01 vs. control.* (B) Similarly, IL-12 level was monitored in TRAMP tumor tissue (n=3) treated with saline, p40 mAb, IgG, and recombinant p40. Results are mean ± SEM of three mice per group. *[a]p<0.001 vs. control; [b]p<0.01 vs. control.* TRAMP cells were treated with p40 mAb for 1 h followed by addition of IL-12 neutralizing antibodies (0.5 µg/mL) or control IgG (0.5 µg/mL). After 24 h, the mRNA expression of T-bet and IFNγ was examined by semi-quantitative RT-PCR (C) and real-time PCR (D). Results are mean ± SD of three different experiments. *[a]p<0.001 vs. control; [b]p<0.001 vs. p40 mAb.* Male C57BL/6 mice were injected with 1 million of TRAMP cell suspension subcutaneously. After about 6 weeks, when tumors were within 0.8 to 1 mm in size, mice were treated with p40 mAb at a dose of 2 mg/kg body wt twice a week. Next day, mice also received neutralizing antibodies against either IL-12 (25 µg/mouse) once via i.p. injection. After 2 weeks, tumors were excised from the flank of all groups of mice followed by monitoring the mRNA expression of T-bet and IFNγ by semi-quantitative RT-PCR (E) and real-time PCR (F). Results are mean ± SEM of three mice per group. *[a]p<0.001 vs. control; [b]p<0.001 vs. p40 mAb.*

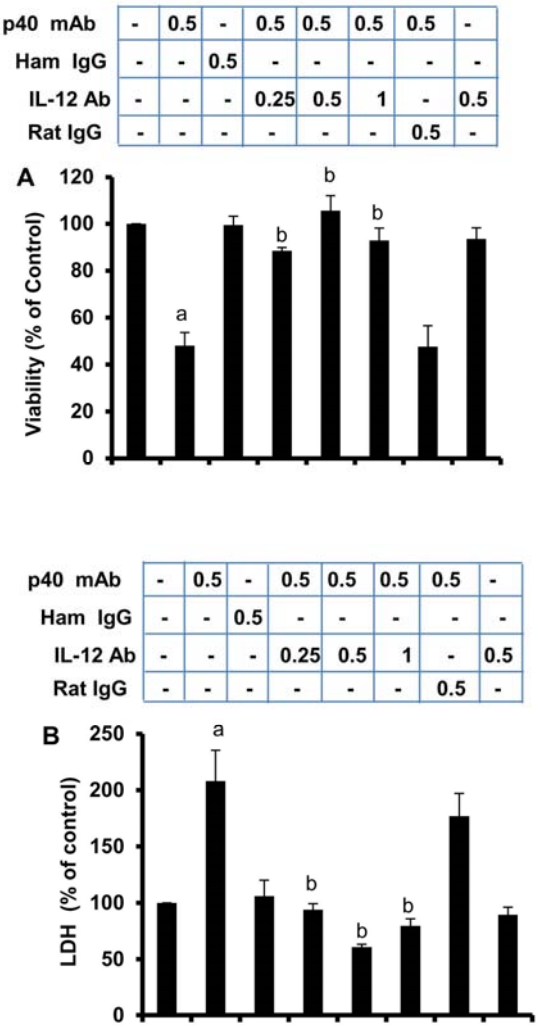

Figure S11. Role of IL-12 in p40 mAb-mediated death in TRAMP cells. MTT (A) and LDH (B) assays were performed in p40 mAb- (0.5 µg/mL) or p40 mAb together with increasing doses of IL-12 Ab-treated TRAMP cells. Results are mean ± SD of three different experiments. *[a]p<0.001 vs. control, and [b]p<0.001 vs. p40 mAb only.*

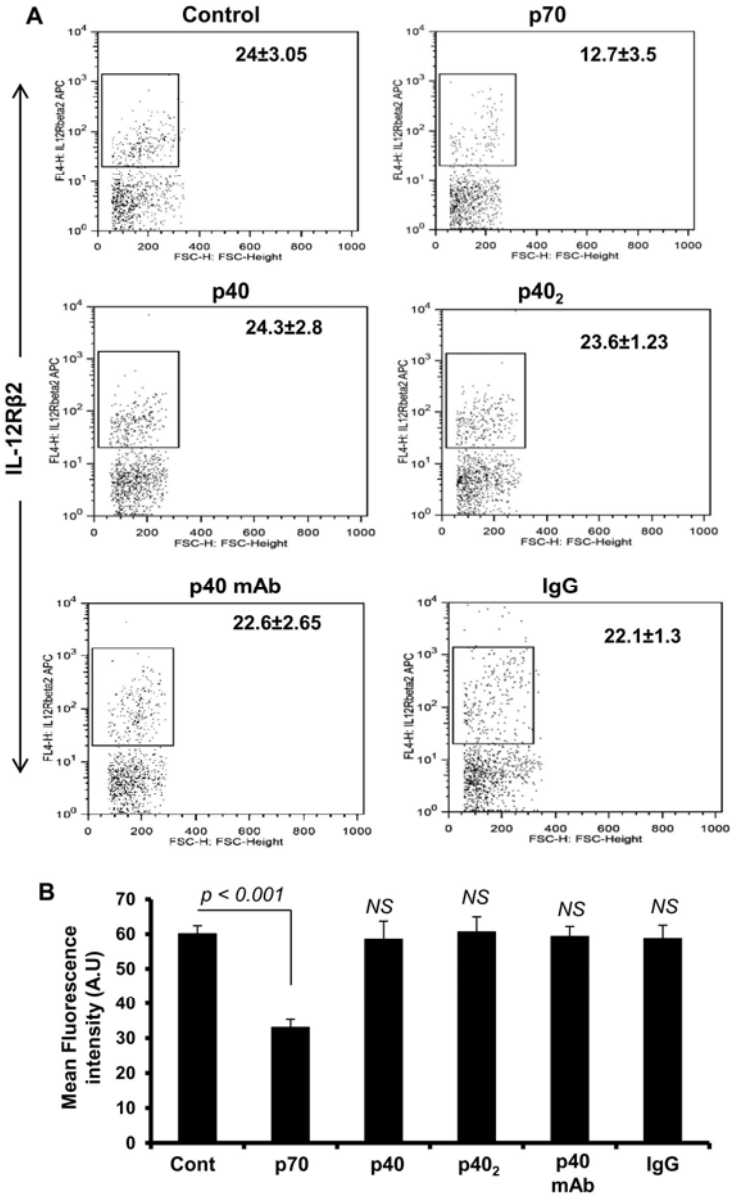

Figure S12. Effect of p40, $p40_2$, p70, p40 mAb, and IgG on the surface expression IL-12Rβ2 in cultured TRAMP cells. (A) FACS analyses to monitor the level of IL-12Rβ2 in cultured TRAMP cells treated with p40 (20 ng/mL), $p40_2$ (20 ng/mL), p70 (20 ng/mL), p40 mAb (0.5 µg/mL), and mouse IgG (0.5 µg/mL) for 2 h under serum-free condition. B) The MFI of Foxp3 in CD4+ population was calculated by using CellQuest software. Data are mean ± SD of three different experiments. NS, not significant.

SI Appendix, References

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