SUPPLEMENTARY INFORMATION FOR

αvβ3-int regulates PD-L1 expression and is involved in cancer immune evasion

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MATERIALS AND METHODS

Cells.

SK-OV-3, MDA-MB-453, SK-BR-3 and 4T1 cells were purchased from American Type Culture Collection (ATCC) and cultured in RPMI 1640-GlutaMAX medium containing 10% heat-inactivated fetal bovine serum (FBS). SK-N-SH, U251, HT29, EO771 and B16 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% or 10% FBS. HaCaT cells were received from Deutsch Krebs Forschung Zentrum (Heidelberg) and grown in high-glucose (4%) DMEM containing 10% FBS. β3-int-silenced HaCaT and SK-N-SH cells (named HaCaT shβ3 and SK-N-SH shβ3, respectively) were grown as previously described (1). Wt GBM23 cells were derived from a female patient as described (2). the GBM shβ3 were derived by lentivirus transduction as described (1). Four different lentiviral stocks, each expressing a 29-mer shRNA for mouse β3-int, were produced by the transfection of 293FT cells with lentiviral packaging plasmids (TR30037, OriGene) plus one of the four lentiviral vectors expressing shRNA for mouse β3-int, GFP protein and the selection cassette (TL50112, OriGene). The same lentiviral vector expressing a noneffective 29-mer of scrambled shRNA (negative control, TR30021) was used to generate the lentiviral control stock. B16 cells were transduced with the lentiviral preparations and selected with 1 μg/ml puromycin for two weeks to obtain B16shβ3 and B16shctrl respectively. Pure clones obtained by limited dilution were monitored for GFP and β3-int expression by flow cytometry or reverse transcription and qRT-PCR. To transiently deplete β3-int, B16 and 4T1 cells were transfected twice with ON-TARGET plus SMART pool siRNA (Dharmacon) for mouse β3-int or nontargeting pool siRNA (control) to generate B16siRNAβ3, 4T1siRNAβ3, B16siRNActrl and 4T1siRNActrl cells, respectively. To transiently deplete β6 and β8 integrin, SK-N-SH and HT29 cells were transfected with ON-TARGET plus SMART pool siRNA for human β6 and human β8 integrins (Dharmacon) or human control siRNA (IBA Life Sciences) to obtain siRNAβ6, siRNAβ8 and siRNActrl cells, respectively (3). To generate SK-OV-3 cells expressing wt or mutant αvβ3-int, cells were transfected with plasmids encoding αv_{wt} plus either $\beta 3_{wt}$ or $\beta 3_{Y747F,Y759F}$, selected with G418 for two weeks, and tested for integrin heterodimer expression before use. Plasmids expressing the αv, $\beta 3_{wt}$ and $\beta 3_{Y747F,Y759F}$ integrins were a generous gift from Dr. S. Blystone (4).

Antibodies, soluble proteins and inhibitors.

Abs against P-STAT1 (Y701), P-JAK1 (Y1022, Y1023), P-MEK1/2 (S217/221) P-SRC(Y416), P-FAK (Y397), STAT1, SOCS1 and tubulin were purchased from Cell Signaling. Anti-mouse and anti-rabbit horseradish peroxidase-conjugated Abs were purchased from Sigma-Aldrich. APC-conjugated anti-human (clone MIH1) and anti-mouse (clone MIH5) PD-L1, PE-Anti-Mouse CD274 (clone MIH5), APC Mouse IgG1 κ (clone MOPC-21) and APC- an PE-Rat IgG2a, λ (clone B39-4) isotype controls, Alexa Fluor 488-conjugated anti-mouse and PE-conjugated anti-mouse Abs were purchased from Becton Dickinson Pharmingen. Anti-CD4-FITC (clone GK1.5), anti-CD8a-PE (clone 53–6.7), anti-CD45-Percp-Cy7 (clone 30-F11), anti-CD61 and anti-CD61-APC (clone HMbeta3-1), anti-mouse-IFNAR1-PE (clone MAR1-5A3), anti- mouse -CD119-PE (clone 2E2),anti- human-CD119-PE (clone GIR-208), anti- human-IFNAR1-PE (clone MA5-23630), PE- Mouse IgG1 kappa isotype control (clone P3.6.2.8.1), PE- and APC- Armenian Hamster IgG isotype controls (cloneBio299Arm) monoclonal antibodies (mAbs) were purchased from eBioscience. The R1.302 mAb against human nectin1 was a gift from M. Lopez (5). The L230 mAb against human integrin αvβ3 was purchased from Santa Cruz Biotechnology. Recombinant human IFNβ (8499-IF/CF), IFNγ (285-IF/CF), universal type I IFN (IFNα, 11200), murine IFNγ (485-MI/CF) and IFNβ (8234-MB/CF) were supplied by R&D Systems. Cyclic [L-arginyl-glycyl-L-α-aspartyl-D-phenylalanyl-N-methyl-L-valyl] peptide, which is named *cln*, was supplied by Chematek SpA. Vitronectin from human plasma (V8379) and P-STAT1 inhibitor fludarabine were purchased from Sigma-Aldrich. P-MEK1/2 inhibitor U0126 was purchased form Selleckchem.

Western blot (WB).

Cells depleted of β3-int or pretreated with *cln* (200 ng/ml) for 18 h were or were not exposed to 50 U/ml of human or murine IFNα, IFNβ, or IFNγ at 37°C for 10 min to detect P-JAK1; for 30 min to detect P-STAT1, T-STAT1 and P-MEK1/2; or for 4 h to detect SOCS1. Wt or silenced HaCaT cells were unexposed or treated to L230 (80 ng/ml), vitronectin (400 ng/ml) for 24 h, or induced with IFNα, β or γ (50IU) for 30 min to detect P-SRC and P-FAK. For P-MEK1/2 inhibition, SK-N-SH cells were unexposed or exposed to U0126 for 24 h, and then treated with IFNγ. For P-STAT1 inhibition, HaCaT cells were unexposed or exposed to fludarabine for 24 h, and then treated with IFNβ. SDS-PAGE and WB were performed as detailed (6).

Reverse transcription and qRT-PCR.

Total RNA was extracted by using a Total RNA Isolation kit (Macherey-Nagel) and reverse transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time

PCR assays were performed using the following TaqMan probes (Thermo Fisher Scientific): human CD274 (Hs01125301_m1), IFNAR1 (Hs01066116_m1), IFNGR1 (Hs00166223_m1), ITGB3 (Hs 1001469_m1), ITGB6 (Hs00982345_m1), ITGB8 (Hs00174456_m1), IRF7 (Hs01014809_g1), SOCS1 (Hs00705164_s1) and GAPDH (Hs99999905_m1) and mouse Cd274 (Mm00452054_m1), Itgb3 (Mm00471209_m1), IFNγ (Mm01168134_m1) and Rpl13a (Mm01612987_g1). Relative changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method, and gene expression levels were normalized to those of GAPDH (human genes) or Rpl13a (mouse genes).

In vivo experiments.

C57BL/6 mice were obtained from The Jackson Laboratories. BALB/c mice were obtained from Charles River Laboratories. NOD-scid mice were provided by PLAISANT, Rome. Mice were bred in a facility at the Department of Veterinary Medical Sciences, University of Bologna. Animal experimentation was carried out at the Department of Veterinary Medical Sciences or at PLAISANT, Rome. B16siRNAβ3, B16siRNActrl, 4T1siRNAβ3, 4T1siRNActrl, B16shβ3 and B16shctrl cells in 100-250 μL of PBS each were implanted subcutaneously in the left flank of six- to eight-week-old mice at 1×10^6 cells/mouse for B16 cells and 1×10^5 cells/mouse for 4T1 cells. Tumor volumes were scored twice weekly as described (7). Mice that did not develop B16siRNAβ3 primary tumors received a contralateral tumor 18 d after primary tumor implantation. The contralateral tumor consisted of B16siRNActrl cells implanted subcutaneously in the right flank at 1×10^6 cells/mouse. The mice were killed 24 d later or when tumor volumes exceeded 1000–2000 mm^3, ulceration occurred, or the animals exhibited distress or pain. For the combination experiments, mice were implanted with wt or shβ3 B16 cells and intraperitoneally (i.p.) administered anti-PD-1 (200 μg/mouse) (Bio X Cell) after 7, 12, or 18 d.

Splenocyte reactivity to B16 and 4T1 cells.

Splenocytes were obtained as described earlier (7), counted, seeded in 24-well plates (1×10^6 cell/well), incubated with 1×10^5 B16, 4T1 in 0.5 ml medium, and cocultured for 48 h. The media were collected, and the amount of secreted IFNγ was quantified by ELISA (IFN-γ Mouse ELISA Kit, Thermo Fisher).

Intratumoral IFNγ.

Tumors in PBS were smashed through a 70 μm cell strainer with a sterile 5 ml syringe plunger to isolate the cells. A portion of the tumor cells was centrifuged and resuspended in 500 μL of lysis buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 0.02%

NaN_3, 0.5% sodium deoxycholate, 1% NP-40, 0.3 mM N^{α}-p-tosyl-L-lysin chloromethyl ketone hydrochloride, 0.3 mM N^{α}-p-tosyl-L-phenylalanine chloromethyl ketone, and 1 mM phenylmethane sulfonyl fluoride (PMSF)). Samples were incubated at 4°C for 1 h and subsequently centrifuged for 30 min at 11000 × g. The protein content of the supernatants was determined with a Bio-Rad protein assay (Bio-Rad). The amount of IFNγ per 1 mg of total protein was quantified by a Mouse IFNγ ELISA kit.

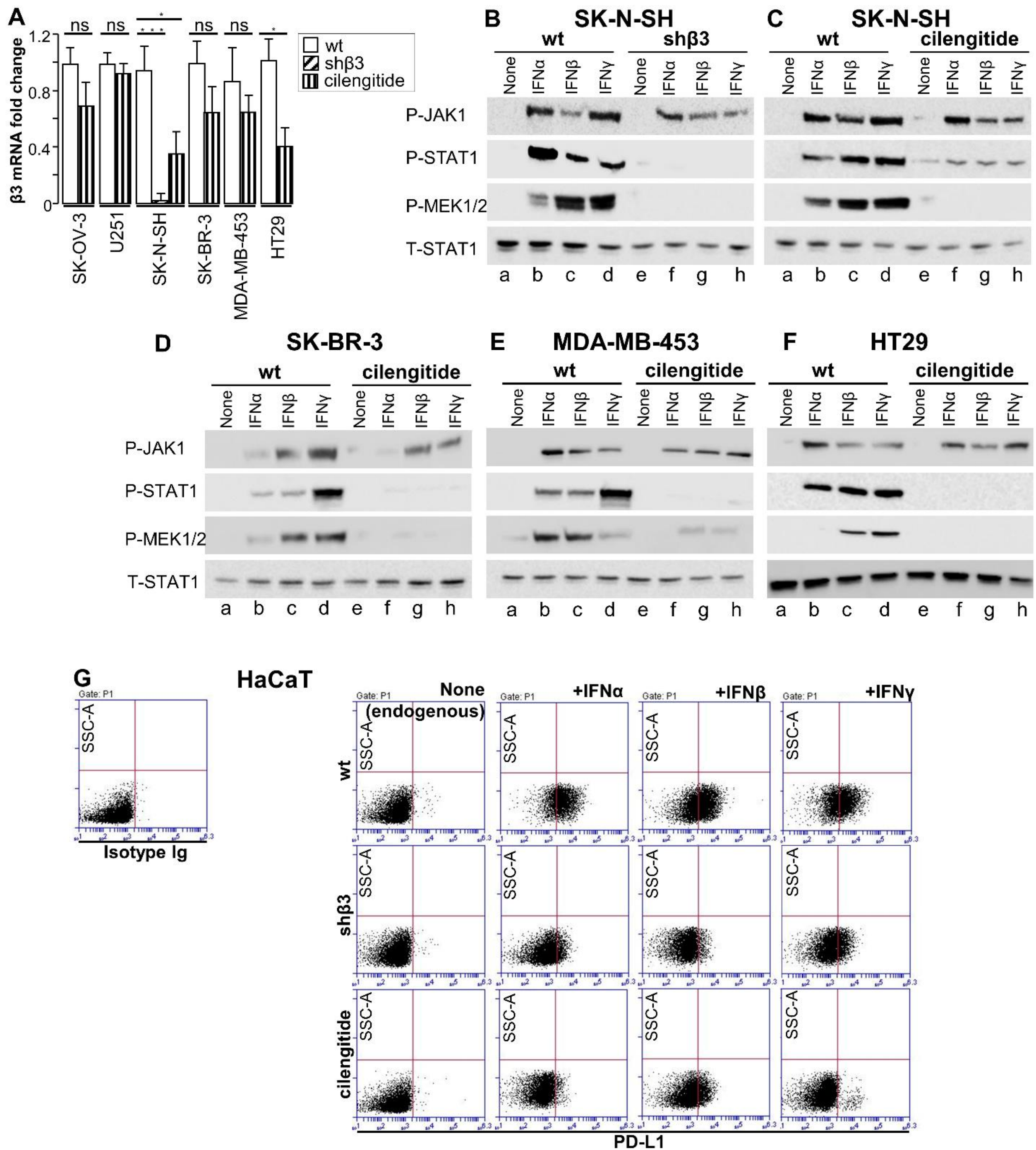
Serum reactivity.

Trypsinized B16, 4T1 and EO771 cells were incubated for 1 h with sera (1:60 dilution) from mice at the end of the tumor challenge experiments (7). The cells were washed with FACS buffer and incubated with anti-mouse PE (1:400). The MFI of B16, 4T1 and EO771 cells that reacted to the mouse sera were quantified by flow cytometry by means of a BD C6 Accuri flow cytometer.

Intratumoral and spleen-infiltrating lymphocytes.

Single-cell suspensions were prepared from freshly isolated B16 and 4T1 tumors and spleens at sacrifice. Tumors and spleens were processed as described above. For each sample, 2×10^6 cells were reacted with the following antibodies: CD4-FITC, CD8a-PE, CD45-Percp-Cy7 and PD-L1-APC. Data were acquired on a BD C6 Accuri flow cytometer. Only samples that provided at least 100000 events were included in the subsequent analysis.

SUPPLEMENTARY FIGURE



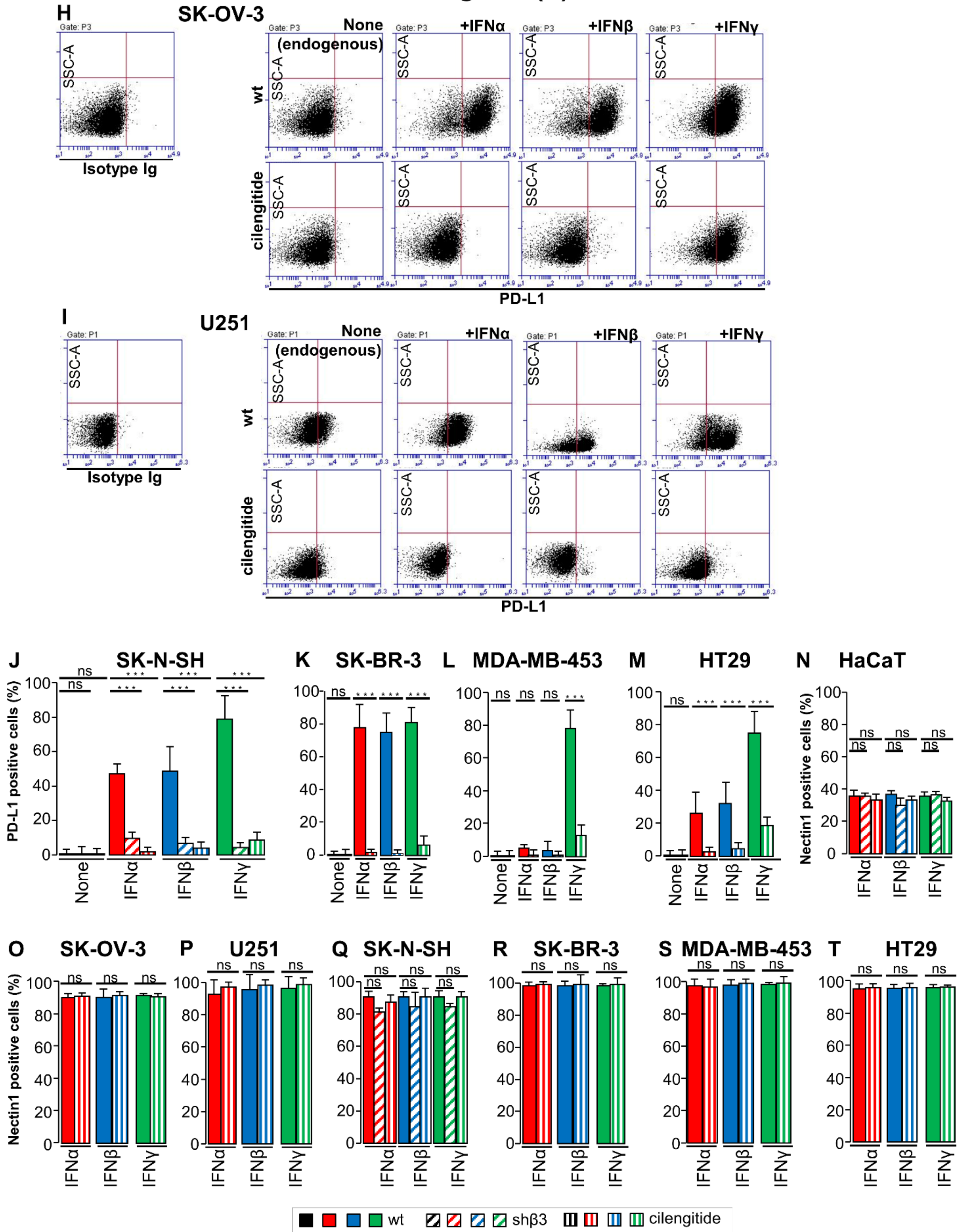
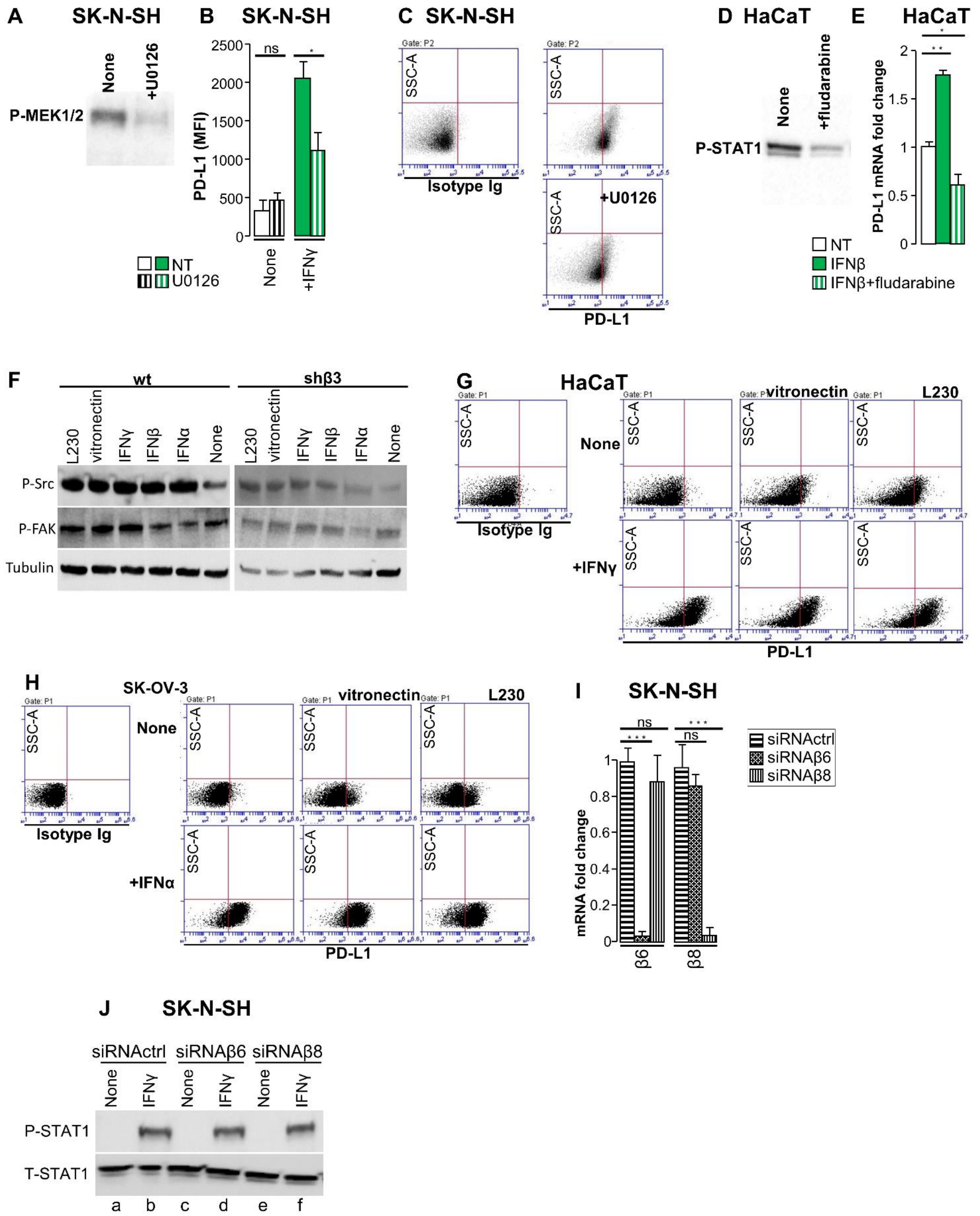


Fig. S1. β3-int depletion or inhibition hinders the signaling cascade of IFNα/β and γ receptors and decreases PD-L1 expression. (A) β3-int expression in SK-OV-3, U251, SK-N-SH, SK-BR-3, MDA-MB-453 and HT29, untreated, treated with *cln* or silenced by means of shβ3 lentiviral transfection. mRNA levels were measured by qRT-PCR. (B-F) Effects of β3-int depletion (shβ3) or inhibition with *cln* on the signaling pathway of IFNα/β and γ receptors in SK-N-SH, SK-BR-3, MDA-

MB-453, and HT29 cells. Details as in the legend to Fig. 1 B-E. (G-I) Representative raw plots of flow cytometry data presented in Fig. 1, F-H. HaCaT, SK-OV-3 and U251 cells were unexposed or exposed to IFNα, IFNβ or IFNγ (see Fig. 1 legends for the details) for 24 h and stained with isotype Ig-APC or anti-PD-L1-APC. (J-M) Effect of β3-int depletion (shβ3) or inhibition with *cln* on PD-L1 expression in SK-N-SH, SK-BR-3, MDA-MB-453 and HT29 cells, as detected by flow cytometry. Details as in the legend to Fig. 1 F-H. (N-T) Effect of β3-int depletion (shβ3) or inhibition with *cln* on expression of nectin1, as detected by flow cytometry. The amounts of IFNs for PD-L1 expression assay were as follows: 100 IU in SK-N-SH and SK-BR-3 cells; 500 IU in HT29 cells; 1000 IU in MDA-MB-453 cells. In panels A, J-T, histograms represent the average of triplicates ± SD. Statistical significance was calculated by means of t-test (panels A, K-M, O, P, R-T) or one-way ANOVA (panel A, J, N, Q).

Fig. S2 (a)



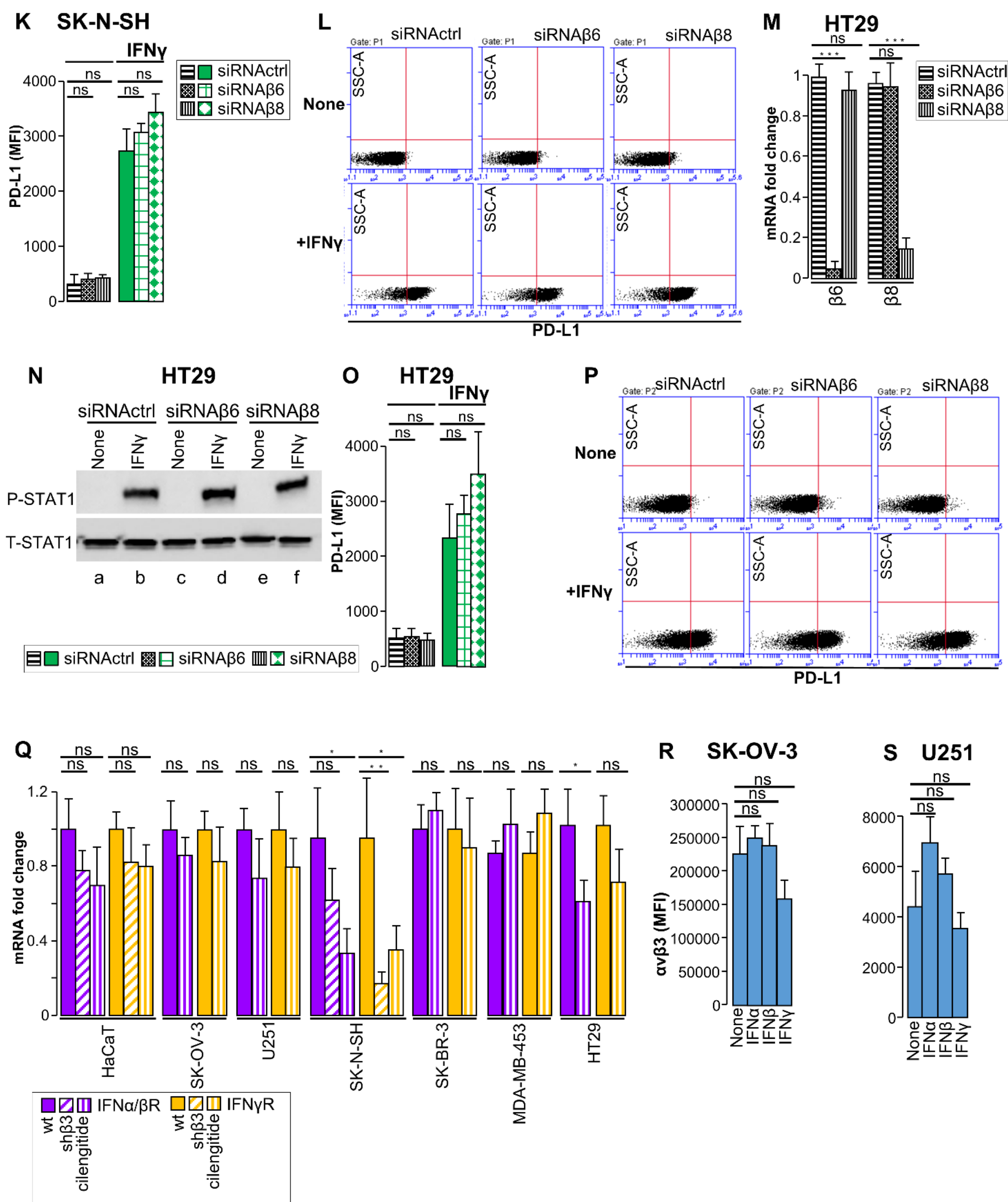


Fig. S2. β3-int depletion or inhibition hinders the signaling cascade of IFNα/β and γ receptors and decreases PD-L1 expression. (A-C) Effects of P-MEK1/2 inhibition on PD-L1 expression. (A) SK-N-SH cells were unexposed (None) or exposed to U0126 for 24 h. Then, samples were exposed or not to IFNγ for 24 h. Cell lysates were extracted as detailed in the legend to Fig. 1. Lysates were subjected to SDS-PAGE and P-MEK1/2 was detected by WB. (B) Cells treated with U0126 and/or with IFNγ were stained with Ig-APC or anti-PD-L1-APC, and MFI values were measured by flow cytometry. APC-MFI values are plotted. (C) Representative raw plots of flow cytometry data presented in panel B. (D-E) Effects of P-STAT1 inhibition on PD-L1 expression. (D) HaCaT cells

were unexposed (None) or exposed to fludarabine for 24 h. Then, samples were exposed or not to IFNβ for 24 h. Cell lysates were extracted as detailed in the legend to Fig. 1. Lysates were subjected to SDS-PAGE and P-STAT1 was detected by WB. (E) Cells treated with fludarabine and/or with IFNβ were stained with Ig-APC or anti-PD-L1-APC, and MFI values were measured by flow cytometry. APC-MFI values are plotted. (F) Wt or silenced HaCaT cells were unexposed (None) or treated to L230 (80 ng/ml), vitronectin (400 ng/ml) for 24 h, or induced with IFNα, β or γ (50IU) for 30 min. Cell lysates were extracted as detailed in the legend to Fig. 1. Lysates were subjected to SDS-PAGE and P-SRC, P-FAK, and tubulin (control) were detected by WB. (G, H) Representative raw plots of flow cytometry data presented in Fig. 1, panels I and J, respectively. (I-P) SH-N-SH or HT29 cells were transiently depleted of β6-integrin (siRNAβ6), β8-integrin (siRNAβ8), or mock-depleted (siRNActrl) for 48 h by means of ON-TARGET plus (Dharmacon). (I, M) Integrins silencing was quantified by qRT-PCR. (J, N) Effect of β6 or β8 integrin silencing on STAT1 phosphorylation. Wt or silenced SH-N-SH and HT29 cells were exposed to 50 IU IFNγ for 30 min. Cell lysates were extracted as detailed in the legend to Fig. 1. Lysates were subjected to SDS-PAGE and P-STAT1 and T-STAT1 were detected by WB. (K, O) Effect of β6 or β8 integrin silencing on PD-L1 levels. Cells were untreated or exposed to IFNγ for 48 h. Amounts of IFNγ were 100 IU and 500 IU for SK-N-SH or HT29 cells, respectively. Cells were stained with Ig-APC or anti-PD-L1-APC, and MFI values were measured by flow cytometry. APC-MFI values are plotted. (L, P) Representative raw plots of flow cytometry data presented in panels K and O. (Q) Expression of IFNAR1 or IFNGR1 mRNA in HaCaT, SK-OV-3, U251, SK-N-SH, SK-BR-3, MDA-MB-453 or HT29 cells, depleted of β3-int, or treated with *cln*. (R, S) Expression of αvβ3 in SK-OV-3 and U251, untreated (None) or treated with IFNα, IFNβ or IFNγ. Cells were stained with isotype Ig-APC or anti-αvβ3-APC, and APC-MFI values are plotted. In panels B, E, I, K, M, O, Q, R, S, histograms represent the average of triplicates ± S.D. Statistical significance was calculated by means of the t-test (B, Q) or one-way ANOVA (panel E, I, K, M, O, Q, R, S).

Fig. S3

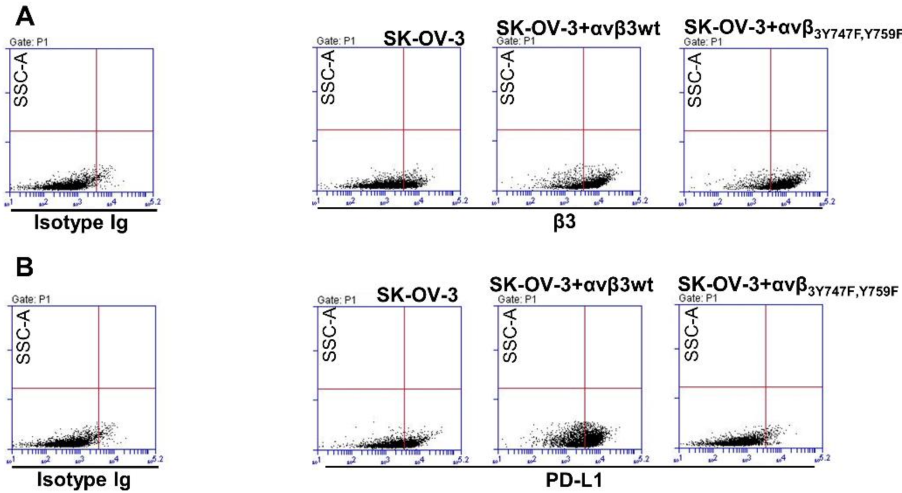


Fig. S3. Mutations in β3 integrin C-tail hinder the signaling cascade of IFNα receptor. (A-B) Representative raw plots of flow cytometry data presented in Fig. 3. (A) SK-OV-3 wt cells, or cells overexpressing αv plus wt-β3 integrin subunit ($\alpha v\beta 3_{wt}$), or mutant, $\beta 3_{Y747F,Y759F}$ integrin subunit ($\alpha v\beta 3_{Y747F,Y759F}$) were stained with isotype Ig-APC or anti-β3-APC. The APC-MFI values are plotted in Fig. 3, A. (B) SK-OV-3 wt, $\alpha v\beta 3_{wt}$, or $\alpha v\beta 3_{Y747F,Y759F}$ were exposed to IFNα (100 IU) for 48 h and stained with isotype Ig-APC or anti-PD-L1-APC. The APC-MFI values are plotted in Fig. 3, C.

Fig. S4

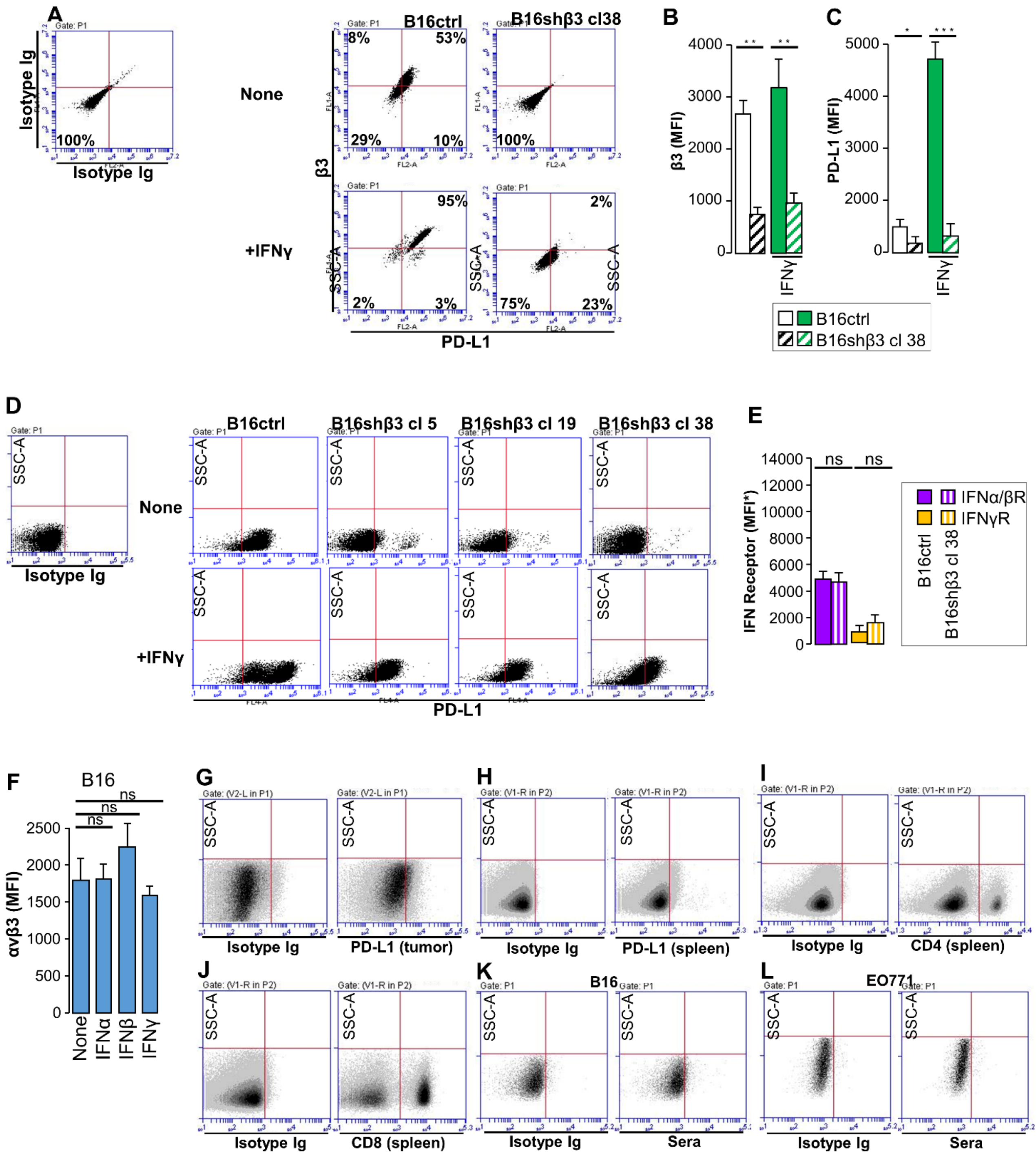


fig. S4. B16 murine cancer cells stably depleted of β3-int exhibit reduced PD-L1 expression *in vitro* and *in vivo*, reduced tumor growth *in vivo* and elicit a durable immune response. (A-L) IFNR MFI, αvβ3 MFI and representative raw plots of flow cytometry data presented in Fig. 4. (A) B16ctrl and B16shβ3 (cl 38) cells were stained with isotype Ig-PE or anti-PD-L1-PE, and at the same time with anti-β3, followed by the staining with Ig-FITC or anti-mouse-FITC (B) β3 (FITC) MFI values of panel A. (C) PD-L1(PE) MFI of panel A. (D) B16ctrl and B16shβ3 cells (cl 5, 19 and 38) cells were unexposed or exposed to IFNγ (100 IU) for 24 h and stained with isotype Ig-APC or anti-PD-L1-APC. The APC-MFI values are plotted in Fig. 4, B. (E) Expression of IFNα/β and γ receptors

in B16 cells, wt or depleted of β3-int. B16 cells were stained with isotype Ig-PE or anti- IFNα/β and γ receptors -PE. The IFNR-PE MFI values subtracted of isotype MFI values are plotted (MFI*). (F) Expression of αvβ3 in B16 cells, untreated (None) or treated with IFNα, IFNβ or IFNγ. Cells were stained with isotype Ig-APC or anti-β3-APC, and APC-MFI values are plotted. (G) Tumor cells were gated on CD45- population (CD45-peCy7, cut-off 3*10^3) and stained with isotype Ig-APC or anti-PD-L1-APC. The APC-MFI values are plotted in Fig. 4, I. (H) Splenocytes were gated on CD45+ population (CD45-peCy7, cut-off 3*10^3) and stained with isotype Ig-APC or anti-PD-L1-APC. The APC-MFI values are plotted in Fig. 4, J. (I) Splenocytes were gated on CD45+ population (CD45-peCy7, cut-off 3*10^3) and stained with isotype Ig-FITC or anti-CD4-FITC. Cut-off line (red vertical line) for FITC-channel is set at 2*10^3 and the percentages of positive cells are plotted in Fig. 4, K. (J) Splenocytes were gated on CD45+ population (CD45-peCy7, cut-off 3*10^3) and stained with isotype Ig-PE or anti-CD8-PE. Cut-off line (red vertical line) for PE-channel is set at 1.2 *10^3 and the percentages of positive cells are plotted in Fig. 4, L. (K-L) B16 and EO771 cells were reacted with mice sera (1:60) and stained with isotype Ig-PE or anti-mouse-PE. The PE-MFI values are plotted in Fig. 4, N. In panels B, C, E, and F, histograms represent the average of triplicates ± SD. Statistical significance was calculated by means of t-test (panels B, C, E) or one-way ANOVA (panel F).

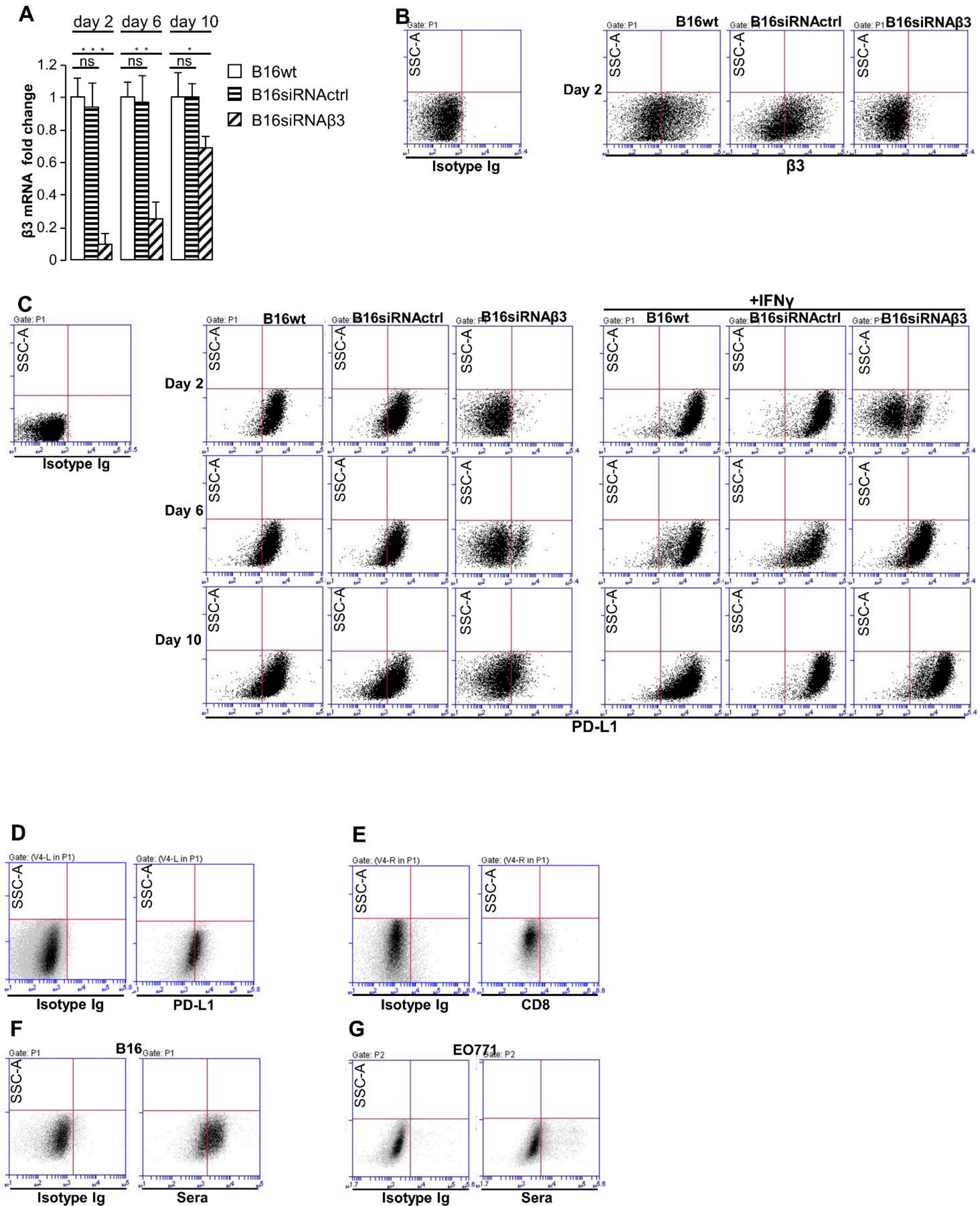


Fig. S5. B16 murine cancer cells transiently depleted of β3-int exhibit a reduction in PD-L1 expression *in vitro*, in the growth of primary and challenge tumor, and the challenge tumors show signs of immunotherapeutic effects. Long term reactivity of splenocyte and serum. (A-G) qRT-PCR and representative raw plots of flow cytometry data presented in Fig. 5. (A) Extent of β3-

int silencing in B16siRNActrl or B16siRNAβ3 cells, measured by qRT-PCR 2, 6, 10 days after siRNA transfection. (B) B16wt, B16siRNActrl and B16siRNAβ3 cells were stained with isotype Ig-APC or anti-β3-APC, 2 days after transfection. The APC-MFI values are plotted in Fig. 5, A (day 2). (C) B16wt, B16siRNActrl and B16siRNAβ3 cells were unexposed or exposed to IFNγ (100 IU) for 24 h and stained with isotype Ig-APC or anti-PD-L1-APC. The APC-MFI values are plotted in Fig. 5, B. (D) Tumor cells were gated on CD45- population (CD45-peCy7, cut-off 3*10^3) and stained with isotype Ig-APC or anti-PD-L1-APC. The APC-MFI values are plotted in Fig. 5, I. (E) Tumor infiltrating leucocytes (TILS) were gated on CD45+ population (CD45-peCy7, cut-off 3*10^3) and stained with isotype Ig-PE or anti-CD8-PE. Cut-off line (red vertical line) for PE-channel is set at 6 *10^3 and the percentages of positive cells are plotted in Fig. 5, J. (F, G) B16 and EO771 cells were reacted with mice sera (1:60) and then stained with isotype Ig-PE or anti-mouse-PE. The PE-MFI values are plotted in Fig. 5, L. In panel A, histograms represent the average of triplicates ± SD and statistical significance was calculated by means of one-way ANOVA.

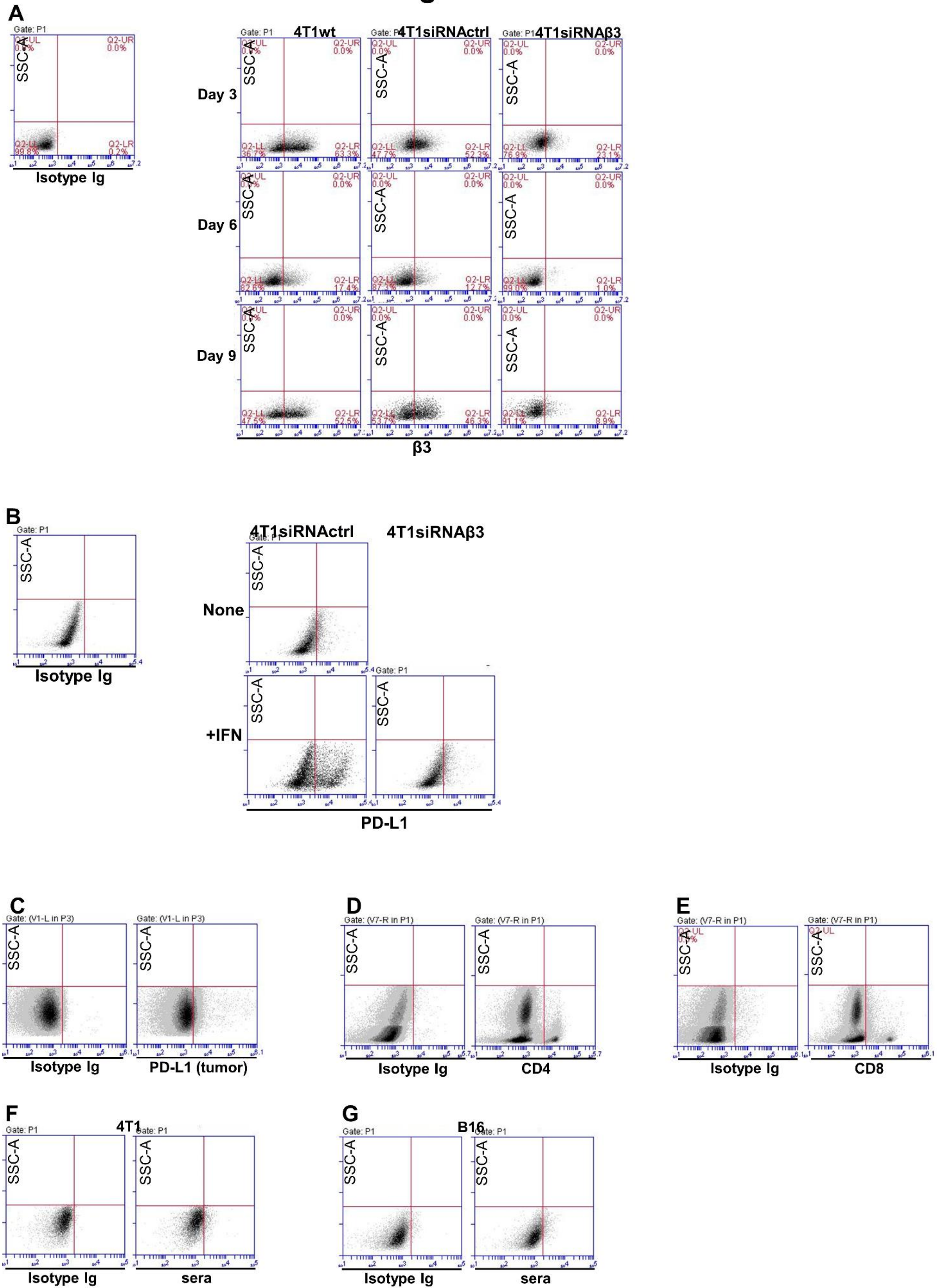


Fig. S6. 4T1 murine cancer cells transiently depleted of β3-int **exhibit a reduction in PD-L1**

expression *in vitro* and in the growth of primary tumor. Long term reactivity of splenocyte and serum. (A-G) Representative raw plots of flow cytometry data presented in Fig. 6. (A-B) Effect of transient β3-int depletion on PD-L1 expression. 4T1 cells were transiently depleted of β3-int by siRNAβ3 (4T1siRNAβ3), or mock depleted by scrambled siRNA control (4T1siRNActrl), by siRNA transfection. (A) Extent of silencing measured by flow cytometry at 3, 6, 9 days after siRNA transfection. 4T1wt, 4T1ctrl and 4T1shβ3 cells were stained with isotype Ig-APC or anti-β3-APC, at the indicated days after transduction. The APC-MFI values are plotted in Fig 6 A. (B) Reduction in PD-L1 expression in β3-int depleted cells. 4T1wt, 4T1siRNActrl or 4T1siRNAβ3 cells were unexposed (no IFN) or exposed to IFNγ (100 IU) for 24 h. Cells were stained with isotype Ig-APC or anti-PD-L1-APC. The APC-MFI values are plotted in Fig 6 B. (C) Tumor cells were gated on CD45- population (CD45-peCy7, cut-off 3*10^3) and stained with isotype Ig-APC or anti-PD-L1-APC. The APC-MFI values are plotted in Fig. 6, G. (D) Tumor cells were gated on CD45+ population (CD45-peCy7, cut-off 3*10^3) and stained with isotype Ig-PE or anti-CD4-PE. Cut-off line (red vertical line) for PE-channel is set at 6 *10^3 and the percentages of positive cells are plotted in Fig. 6, I. (E). Tumor cells were gated on CD45+ population (CD45-peCy7, cut-off 3*10^3) and stained with isotype Ig-PE or anti-CD8-PE. Cut-off line (red vertical line) for PE-channel is set at 6 *10^3 and the percentages of positive cells are plotted in Fig. 6, J. (F, G) 4T1 and B16 cells were reacted with mice sera (1:60) and then stained with isotype Ig-PE or anti-mouse-PE. The PE-MFI values are plotted in Fig. 6, L.

Fig. S7

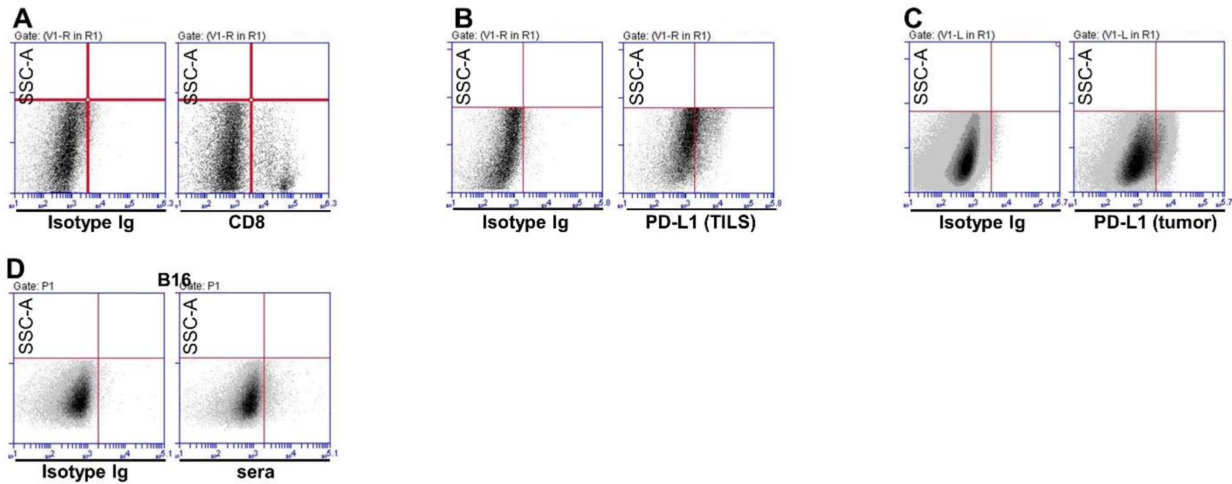


Fig. S7. The combination of β3-int depletion and anti-PD-1 therapy elicits an immunotherapeutic abscopal effect. (A-D) Representative raw plots of flow cytometry data presented in Fig. 7. (A) TILS were gated on CD45+ population (CD45-peCy7, cut-off 3*10^3) and stained with isotype Ig-PE or anti-CD8-PE. Cut-off line (red vertical line) for PE-channel is set at 3.5*10^3 and the percentages of positive cells are plotted in Fig. 7, I. (B) TILS were gated on CD45+ population (CD45-peCy7, cut-off 3*10^3) and stained with isotype Ig-APC or anti-PD-L1-APC. The APC-MFI values are plotted in Fig. 7, K. (C) Tumor cells were gated on CD45- population (CD45-peCy7, cut-off 3*10^3) and stained with isotype Ig-APC and anti-PD-L1-APC. The APC-MFI values are plotted in Fig. 7, L. (D) B16 cells were reacted with mice sera (1:60) and then stained with isotype Ig-PE or anti-mouse-PE. The PE-MFI values are plotted in Fig. 7, N.

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