

PD-L1 couples with LT β R signaling to accelerate tumor growth and metastasis

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ABSTRACT:

Tregs accumulate in the tumor microenvironment, constrain anti-tumor immunity, and are critical targets for anti-tumor strategies. We previously showed that Tregs use surface lymphotoxin (LT α) and PD-1 to signal LT β R and PD-L1 on lymphatic endothelial cells (LECs), thereby promoting Treg lymphatic transendothelial migration (TEM)^{1,2}. Most tumor cells express LT β R and PD-L1, yet tumor interactions with LT α and PD-1 on Tregs are poorly studied. Here we investigate whether PD-L1 couples with LT β R signaling on tumors or LECs to regulate cell migration and tumor metastasis. Results show PD-L1 bound to LT β R in resting B16F10 melanoma cell and PD-L1 deficiency enhanced LT β R expression and apoptosis. Blocking LT β R-nonclassical NF κ B-NIK signaling increased melanoma PD-L1 expression but decreased tumor TEM. RNASeq analysis of B16F10 revealed that genes regulated by LT β R nonclassical NF κ B signaling were increased by PD-L1 depletion. LT β R activation promoted B16F10 TEM, and PD-L1 deficiency abolished the enhancement, indicating PD-L1 is required for LT β R signaling mediated tumor TEM. Tregs but not effector T cells can directly activate B16F10 LT β R-nonclassical NF κ B signaling, suggesting a critical role of Treg LT-tumor LT β R signaling for tumor metastasis. In vivo, PD-L1 blockade combined with LT β R classical or nonclassical NF κ B blocking peptides inhibited tumor growth and metastases, and enhanced host survival. Overall, PD-L1 couples with LT β R-nonclassical NF κ B signaling to regulate tumor growth and migration. Blocking both arms of tumor LT β R-NF κ B-signaling enhanced immune checkpoint blockade efficacy and tumor bearing mouse survival. These observations provide a rational strategy to modulate Treg activities to prevent tumor spread.

METHODS:

Mice: C57BL/6J (B6), B6.Foxp3GFP mice were used for animal experiments and were performed in accordance with Institutional Animal Care and Use Committee approved protocols. **Cells:** Primary dermal LECs of B6 mice (C57-6064L, Cell Biologics, Inc. Chicago, IL). Human melanoma A375 and murine B16F10 melanoma, CRISPR/Cas9 LT β R KO and PD-L1KO of B16F10 and mouse LECs were made in our laboratory. **Peptides:** Synthesized by GenScript, and labeled with gold nano particles (NP) in our Laboratory.

Flow Cytometry and Immunoblotting: See reference #3. **In vitro transendothelial migration (TEM):** LEC cell layers in Boyden Chambers were loaded with 1×10^5 migrating cells to the upper chamber, while the lower chamber contained 50 ng/mL CCL19, 200 ng/mL CCL21, or 200 μ M S1P. Cells migrated to the lower chamber were counted after 3 hours (T cells) or 16 hours (tumor cells) at 37°C. **RNA sequencing and data analysis (Novogen.com).** **Statistical Analysis:** A p-value of <0.05 was considered significant for one-way ANOVA and Student t-tests using Prism 5 software.

PD-L1 binds LT β R in resting B16F10, and dissociates from activated LT β R

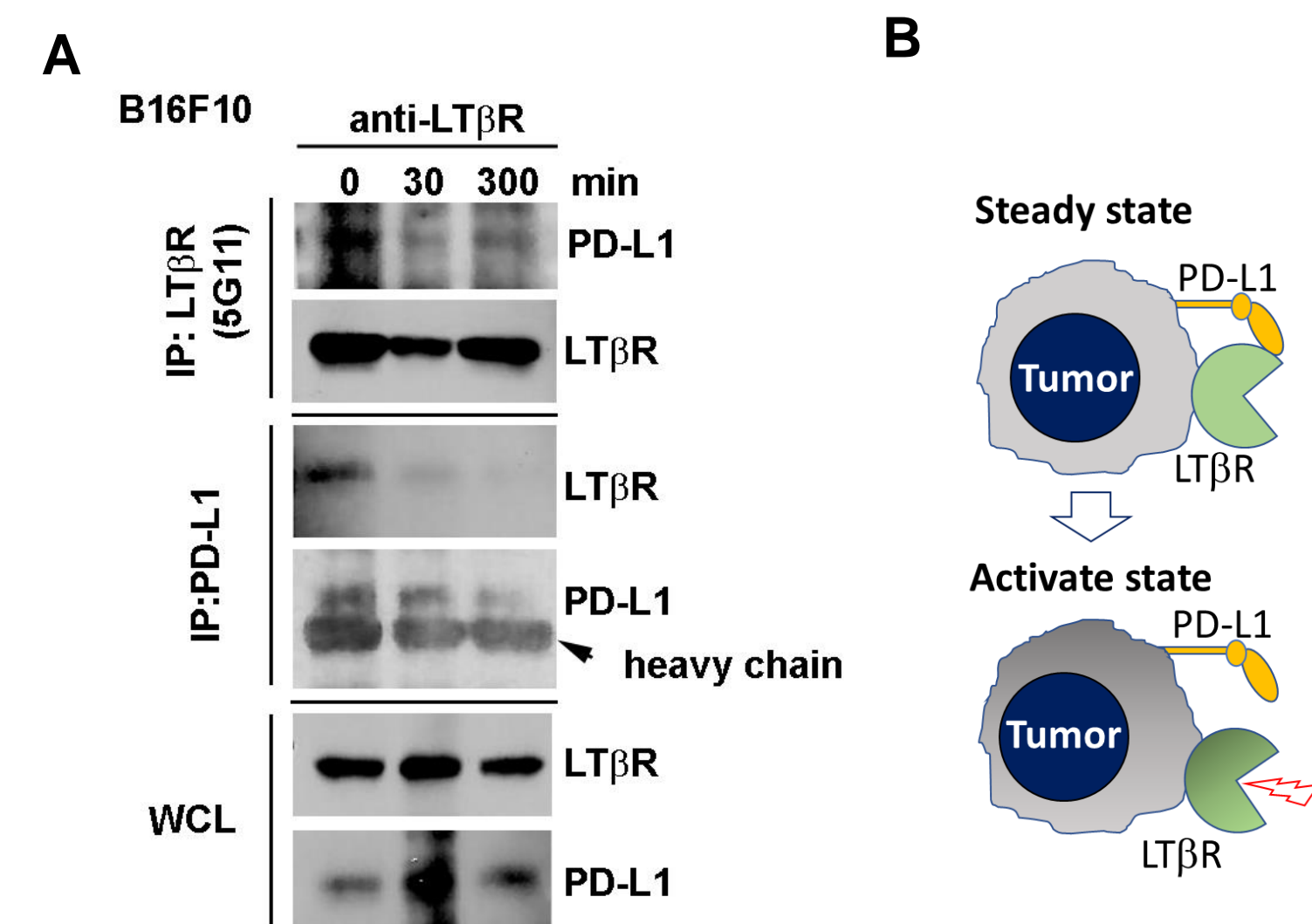


Figure 1. A.. Co-Immunoprecipitation with anti- LT β R Ab (5G11) or rat anti-PD-L1 Ab of B16F10 stimulated with 2 μ g/ml anti-LT β R Ab(3C8) as indicated. B. Diagram of dissociation of PD-L1 and LT β R on tumor cell upon LT β R signaling. One representative shown (A)..

Blocking LT β R-nonclassical NF κ B but not classical NF κ B signaling increases B16F10 PD-L1 expression and inhibits tumor growth

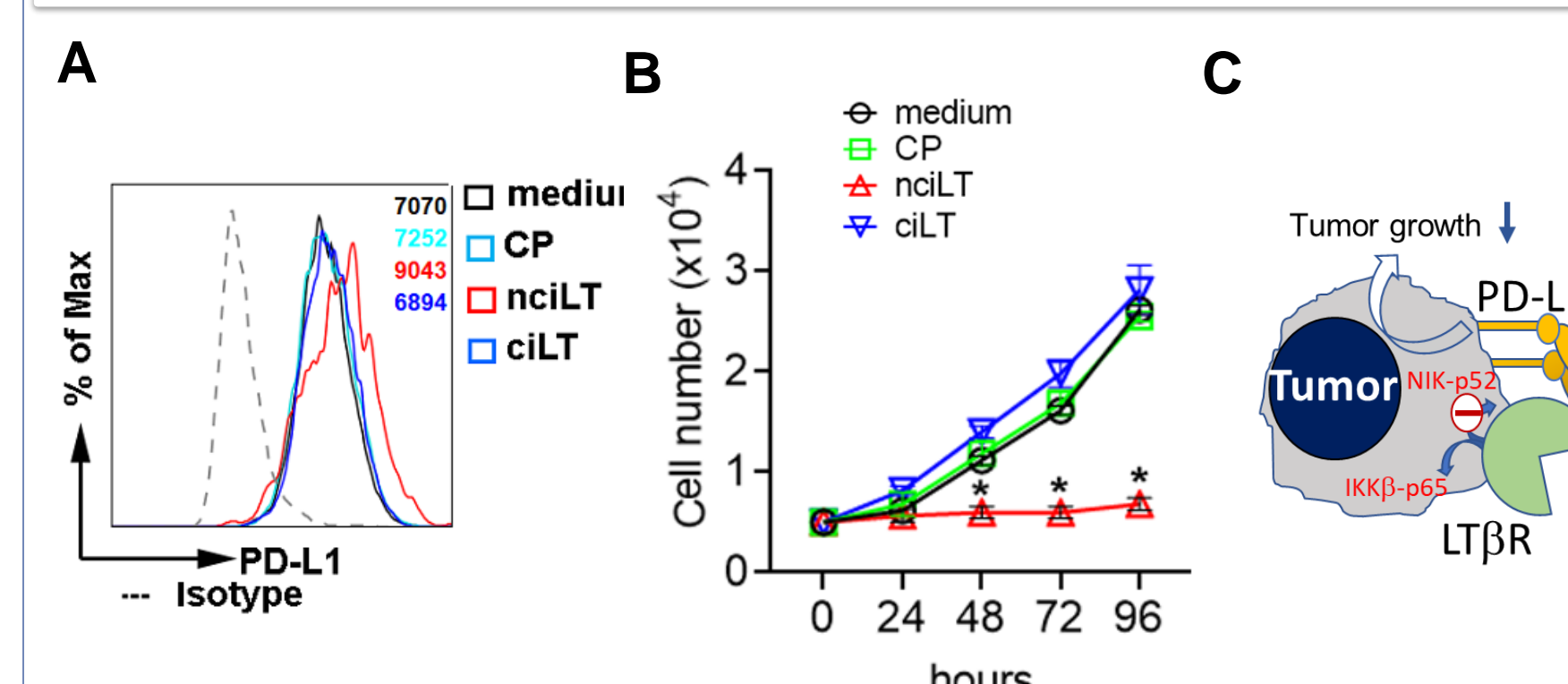


Figure 5. A and B. WT B16F10 treated with 20 μ M CP, cILT, or nciLT for 1 hour, washed, 16 hours later PD-L1 expression analyzed with flow cytometry (A), Δ MF1 shown. Peptide-treated cells monitored for cell growth in culture plate at 37°C as indicated (B). C. Diagram of increased PD-L1 expression and inhibited cell growth of LT β R-nonclassical NF κ B blockade tumor cells. B: Mean \pm SEM. *p < 0.05, by one-way ANOVA.

Genes regulated by LT β R - nonclassical NF κ B signaling are increased by PD-L1 depletion

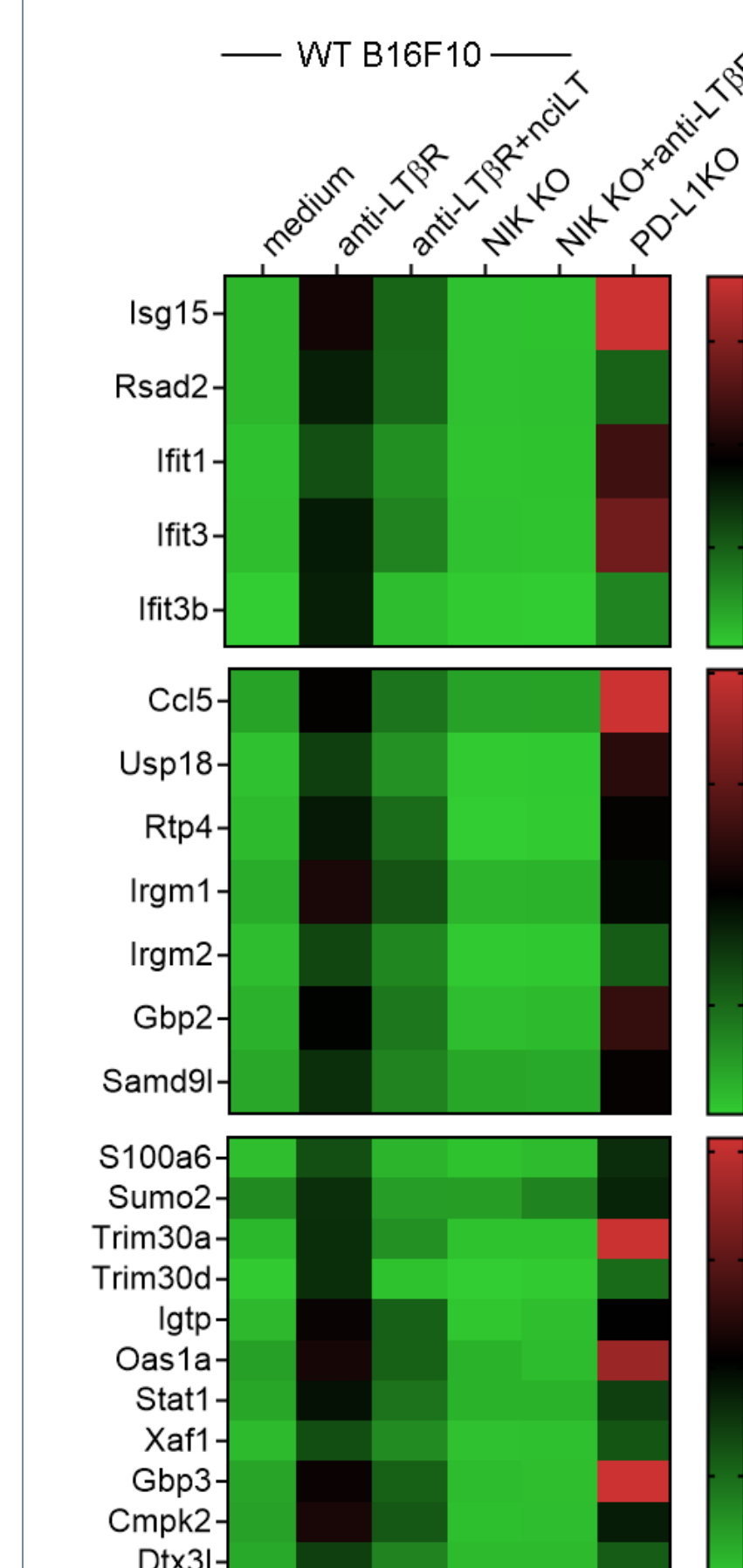


Figure 8. Heatmap of the 23 genes most highly upregulated by LT β R-nonclassical NF κ B signaling. Bulk RNA-Seq of B16F10 treated with 6-hour agonistic anti-LT β R Ab (3C8) or pretreated with LT β R-nonclassical NF κ B blocking peptide (nciLT, 1h), compared with CRISPR/Cas-9 NIK or PD-L1 KO B16F10.

PD-L1 deficiency increases LT β R expression on B16F10

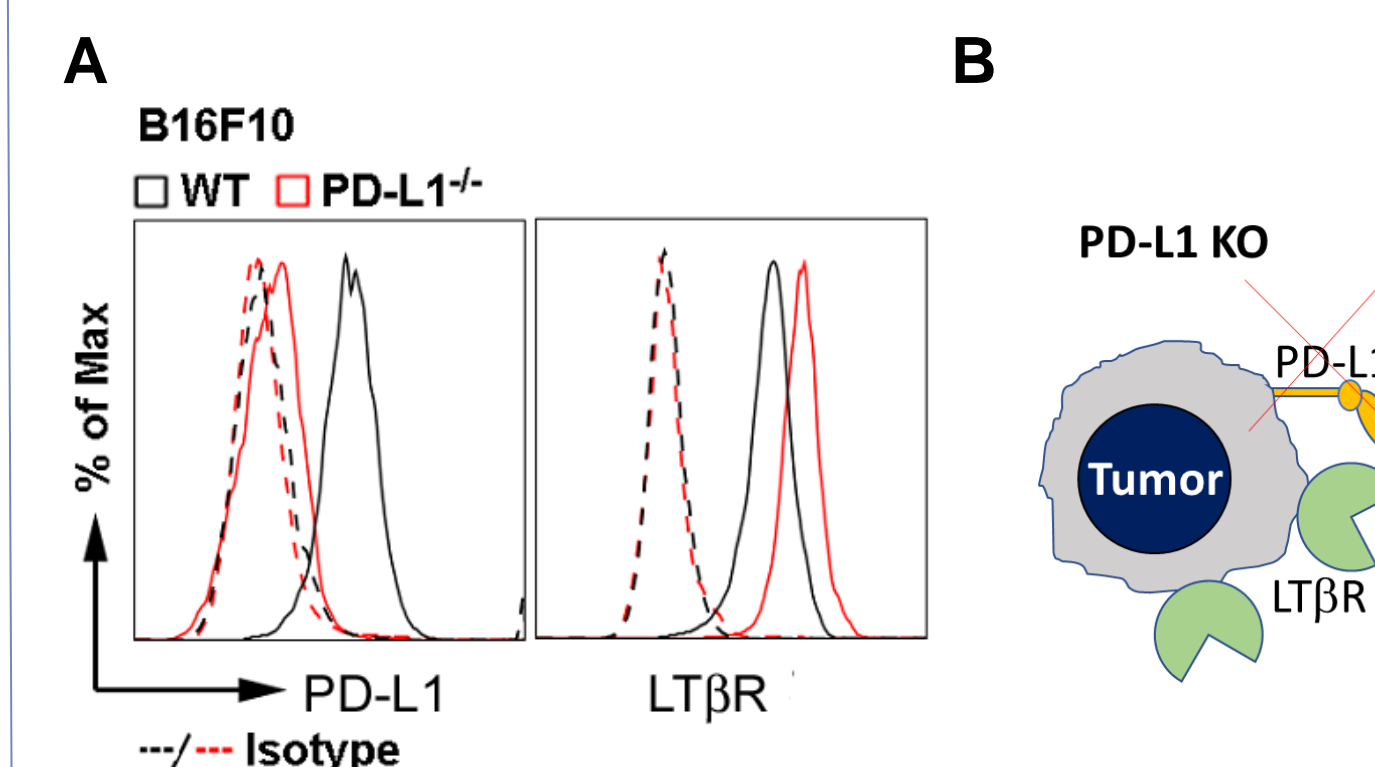


Figure 2. A. Flow cytometry analysis of LT β R expression in wild type (WT) and CRISPR/Cas9 PD-L1 knock out (PD-L1^{-/-}) B16F10. B. Diagram of LT β R expression on PD-L1-deficient tumor cell. One representative FACS shown (A).

Tumors preferentially use LT β R nonclassical NF κ B signaling for TEM across LEC

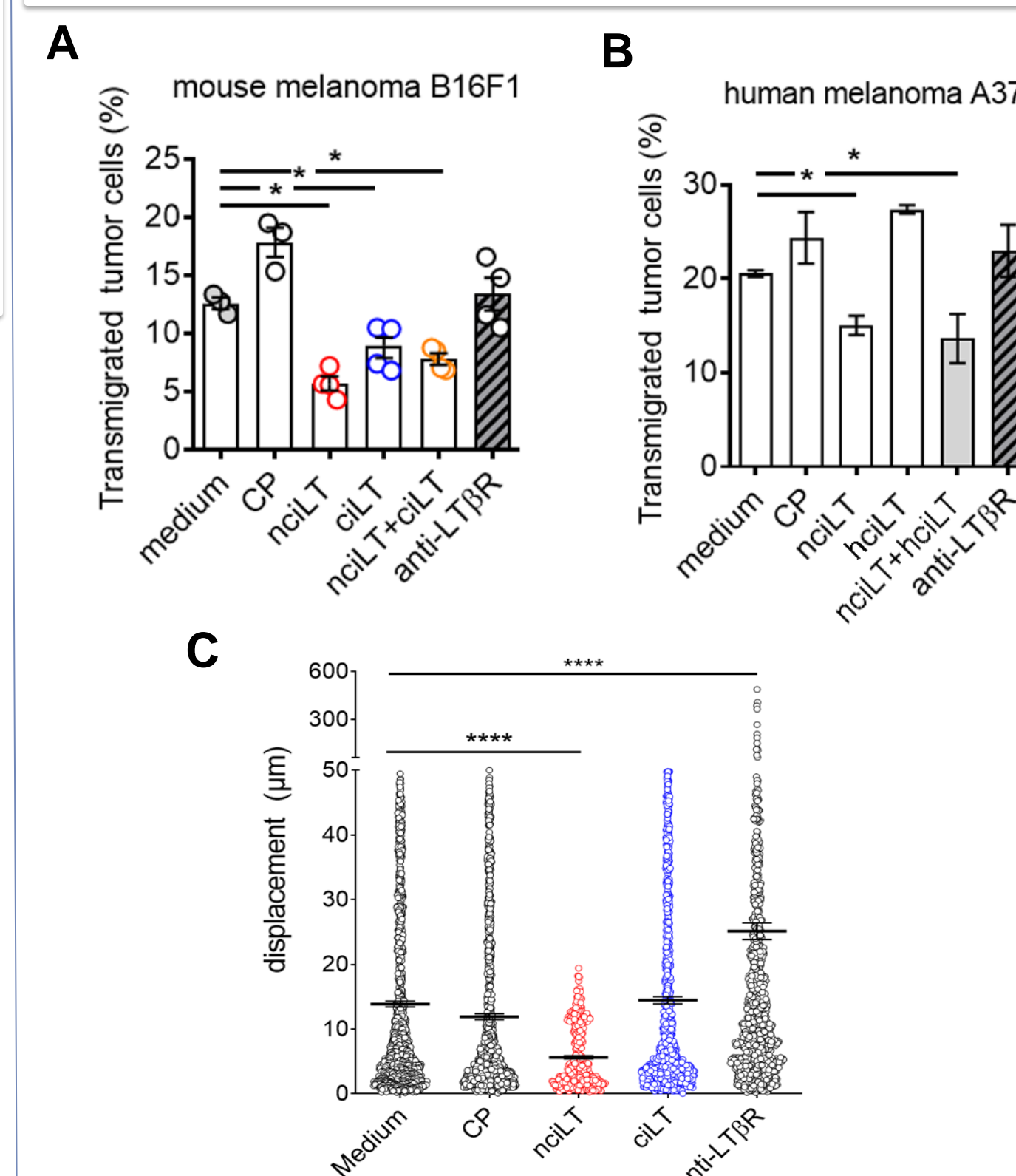


Figure 6. A-B. Trans well TEM assay of mouse (A) or human (B) melanoma cells. Tumor cells pretreated with 20 μ M nciLT, cILT (or huciLT), or CP for 1 hour, washed and loaded for TEM across mouse or human LECs toward 200 μ M S1P for 16 hours. C. Time-lapse microscopy of B16F10-eGFP pre-treated with nonclassical blocking peptide nciLT inhibited migrating B16F10 mobility. Mean \pm SEM. *p < 0.05, by one-way ANOVA.

Checkpoint inhibitor combined with LT β R-NF κ B blocking peptides inhibits tumor growth and enhances host survival.

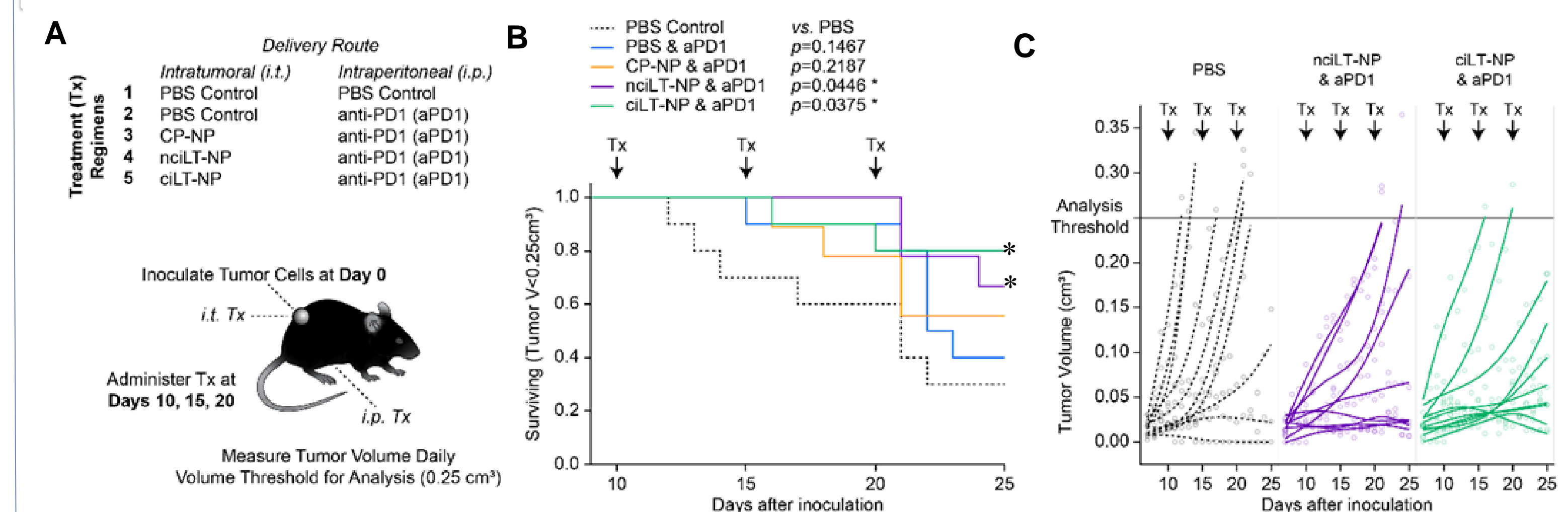


Figure 10. A. Schematic of intratumor nanoparticle peptides together with checkpoint inhibitor anti-PD-1 at days 10, 15, and 20. B. Kaplan-Meier survival curve (tumor volume threshold of 0.25 cm³). P values: Wilcoxon test. C. Individual mice traced by a cubic spline algorithm using a λ value of 1 to smooth the growth trajectory. PBS, n=10; PBS & +aPD-1, n=10; CP-NP & +aPD-1, n=9; nciLT-NP & +aPD-1, n=9; and cILT-NP & +aPD-1, n=10. All tumor volumes calculated based on caliper measurements and the equation $V=(W2 \times L)/2$.

PD-L1 deficiency induces B16F10 apoptosis which is promoted by LT β R activation

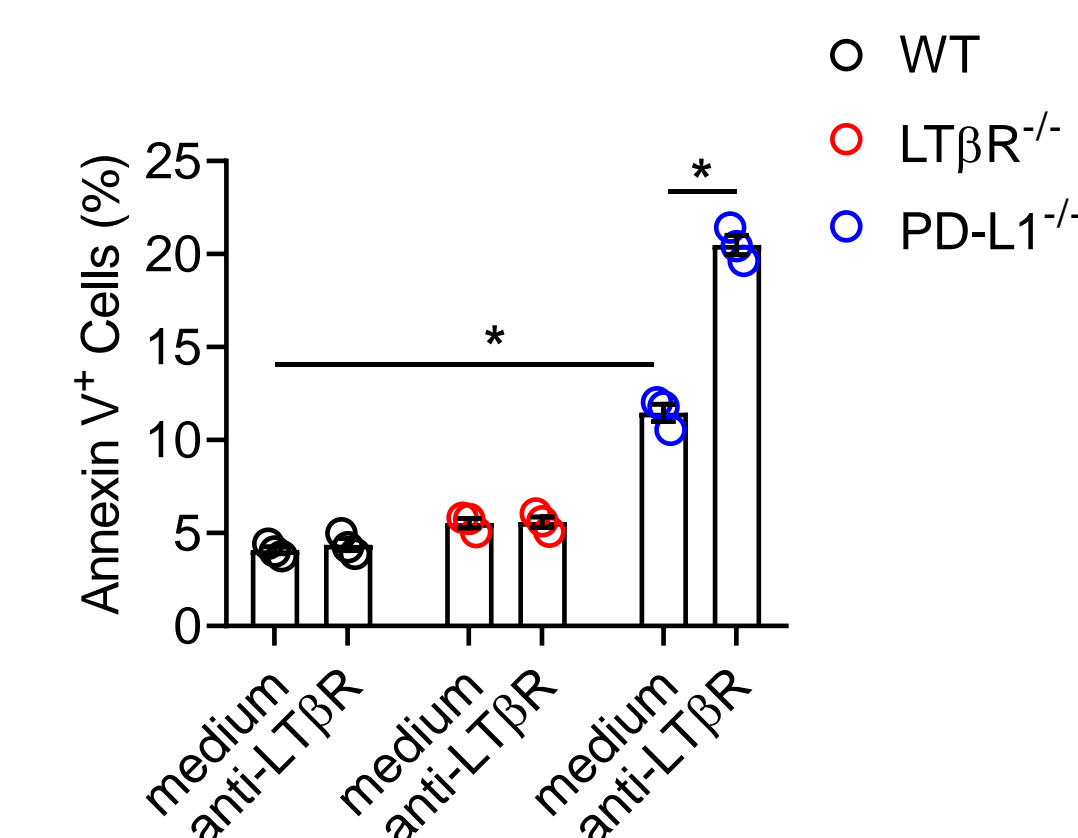
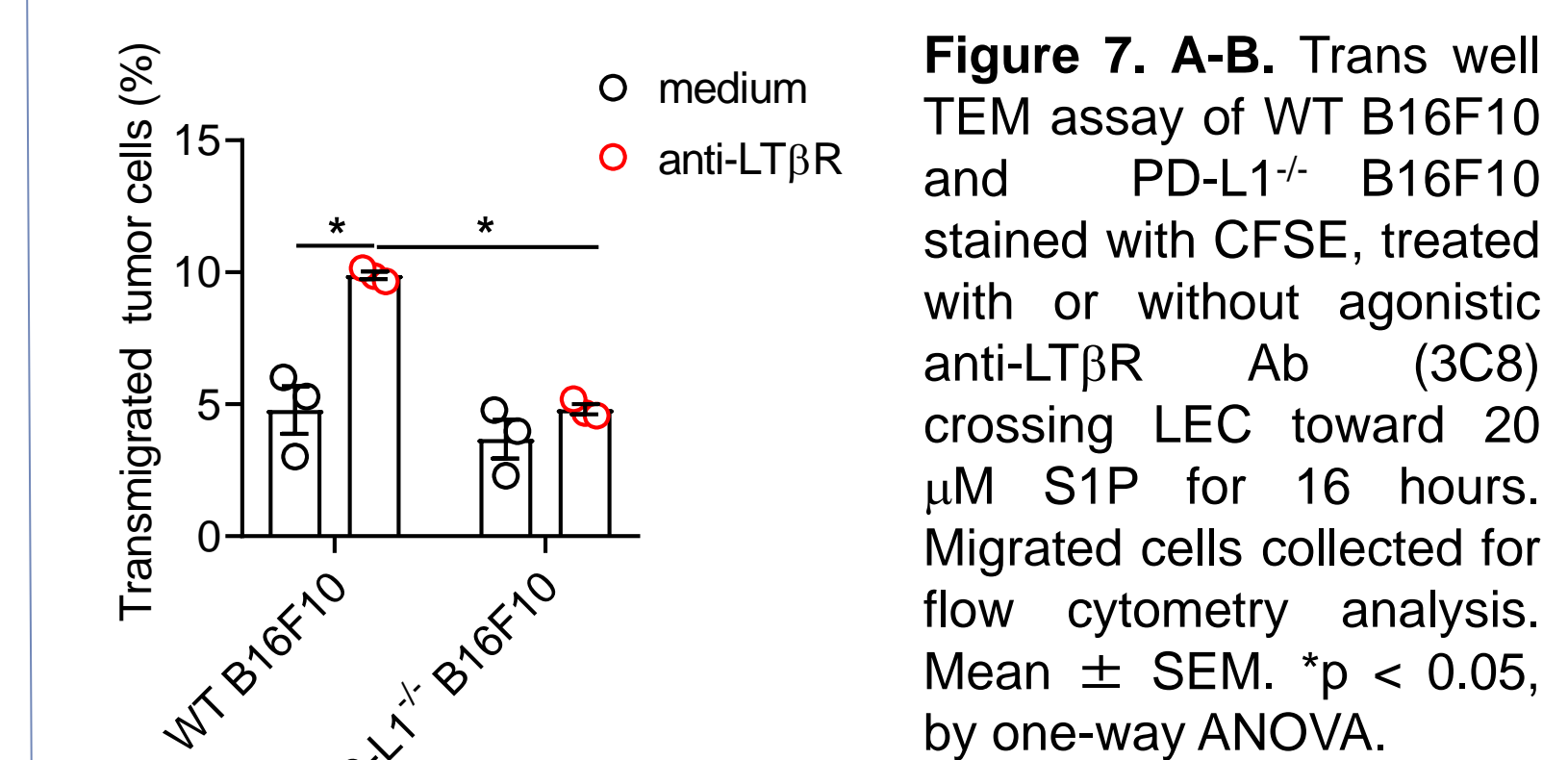


Figure 3. WT, CRISPR/Cas9 LT β R KO (LT β R^{-/-}), or PD-L1^{-/-} B16F10s treated with or without agonistic anti-LT β R Ab (2 mg/mL) for 16 hours and stained with Annexin V-PE and 7AAD for flow cytometry analysis of annexin V⁺ apoptosis. Mean \pm SEM. *p < 0.05, by one-way ANOVA.

B16F10 LT β R activation enhances TEM across LEC; PD-L1 deficiency in B16F10 abolishes the enhancement



Tregs directly activate LT β R-nonclassical NF κ B signaling

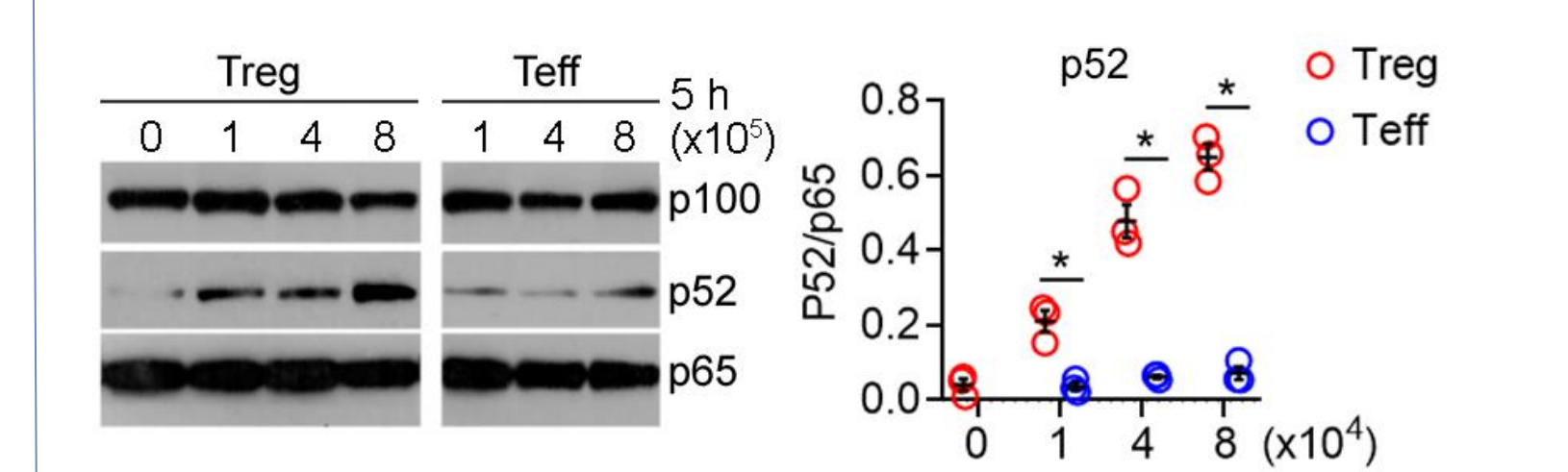


Figure 9. Immunoblotting for p100 to p52 processing in B16F10 cells cocultured with Tregs or Teffs as indicated. One representative blot shown.

Tumor LT β R signals by classical NF κ B and nonclassical NF κ B pathways

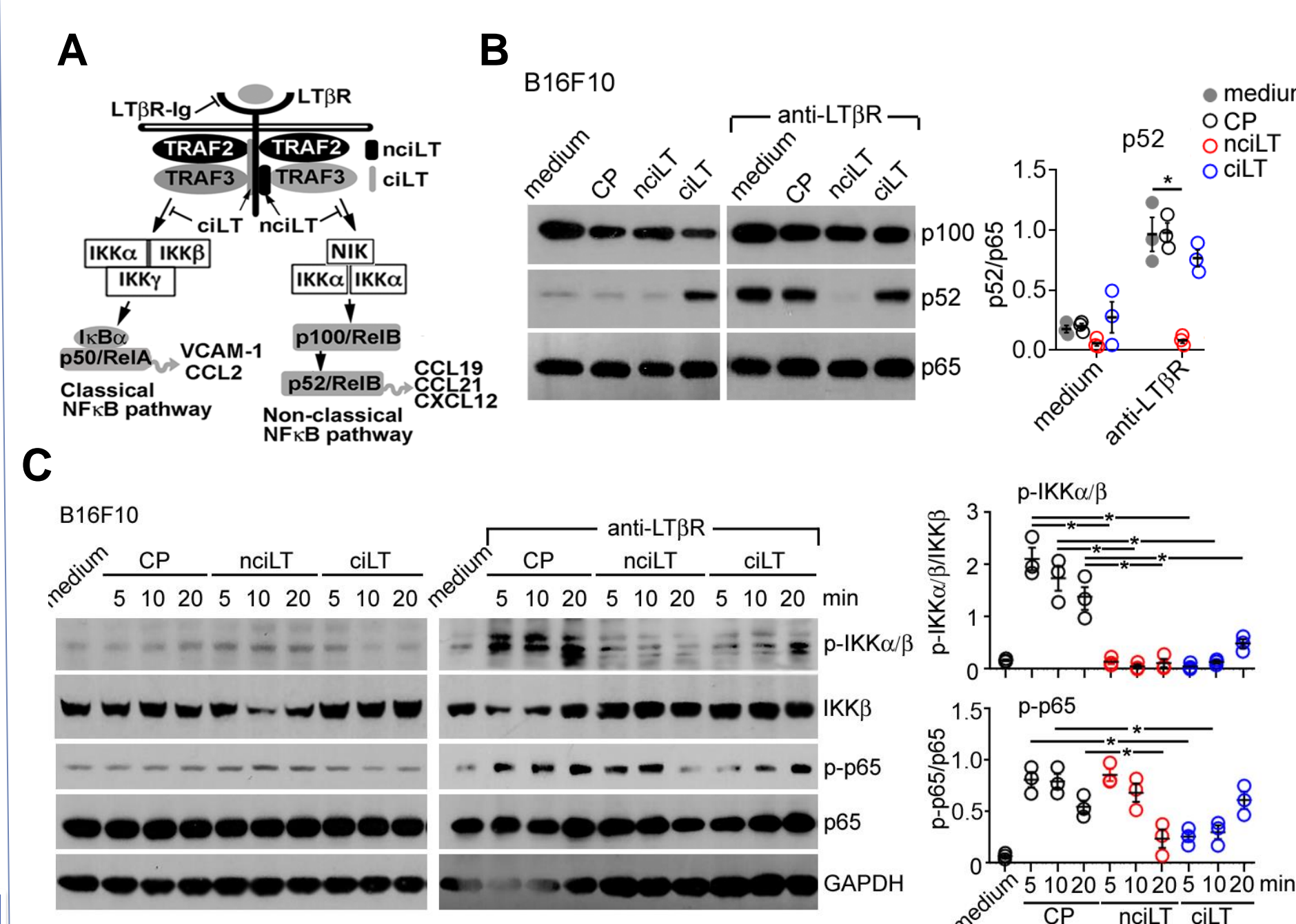


Figure 4. A. Diagram of peptide selective blockade of separate arms of LT β R signaling. cILT, classical NF κ B blocking peptide; nciLT, nonclassical NF κ B blocking peptide. B and C. Immunoblots for nonclassical NF κ B-NIK-p52 (B) and classical NF κ B-p65 and IKK α / β phosphorylation (C) in B16F10 pretreated with 20 μ M cILT, nciLT, or control scrambled peptide (CP) for 1 hour at 37°C; and then stimulated with or without agonistic anti-LT β R mAb for indicated times (C) or 6 hours (B). Mean \pm SEM. *p < 0.05, by one-way ANOVA. Representative blots shown (B and C).

CONCLUSIONS:

1. PD-L1 binds LT β R in resting B16F10 and dissociates from activated LT β R.
2. PD-L1 deficiency increases B16F10 LT β R expression and signaling and induces B16F10 apoptosis which is promoted by LT β R activation.
3. Tumor LT β R signals by classical NF κ B and nonclassical NF κ B pathways, and preferentially uses the nonclassical arm for TEM.
4. Blockade of LT β R-nonclassical NF κ B increases B16F10 PD-L1 expression and inhibits tumor growth.
5. Genes regulated by LT β R -nonclassical NF κ B signaling are increased by PD-L1 depletion.
6. Tregs directly activate LT β R-nonclassical NF κ B signaling
7. Checkpoint inhibitor combined with LT β R-NF κ B blocking peptides inhibits tumor growth and enhances host survival.

Together, our observations provide a rational strategy to modulate Treg activities to prevent tumor spread.

References:

1. Piao W, et al. (2020) Regulatory T Cells Condition Lymphatic Endothelia for Enhanced Transendothelial Migration. *Cell Rep.*, 30(1), 1052-1062.
2. Piao W, et al. (2022) PD-L1 signaling selectively regulates T cell lymphatic transendothelial migration. *Nature communications* 13, 2176.
3. Piao W, et al. (2018) Regulation of T cell afferent lymphatic migration by targeting LT β R-mediated non-classical NF κ B signaling. *Nature communications* 9(1):3020.

