Science Advances
AAAS

# Supplementary Materials for

## When killers become thieves: Trogocytosed PD-1 inhibits NK cells in cancer

Mohamed S. Hasim, Marie Marotel, Jonathan J. Hodgins, Elisabetta Vulpis, Olivia J. Makinson, Sara Asif, Han-Yun Shih, Amit K. Scheer, Olivia MacMillan, Felipe G. Alonso, Kelly P. Burke, David P. Cook, Rui Li, Maria Teresa Petrucci, Angela Santoni, Padraic G. Fallon, Arlene H. Sharpe, Giuseppe Sciumè, André Veillette, Alessandra Zingoni, Douglas A. Gray, Arleigh McCurdy, Michele Ardolino*

*Corresponding author. Email: m.ardolino@uottawa.ca

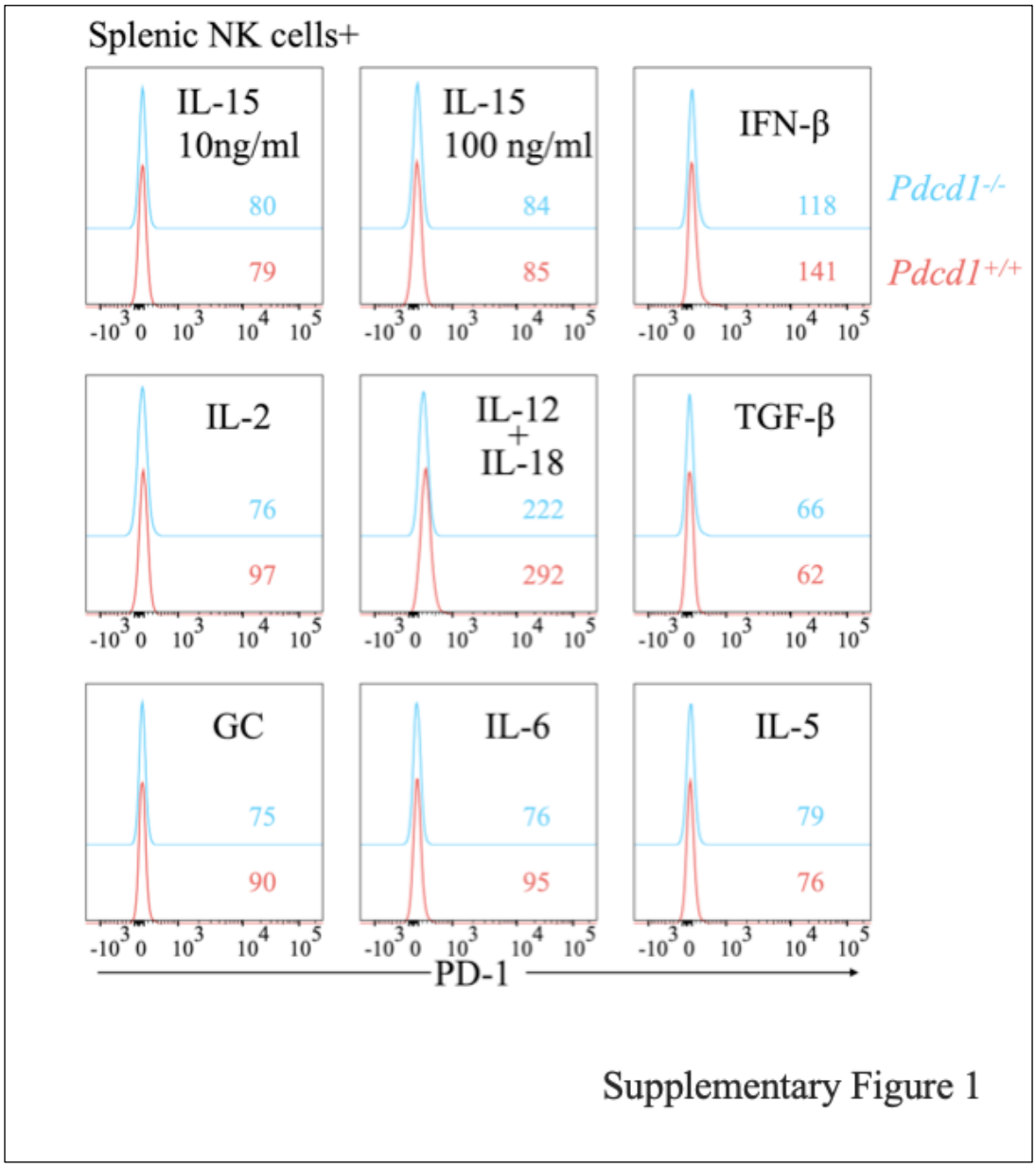
Published 13 April 2022, *Sci. Adv.* **8**, eabj3286 (2022)
DOI: 10.1126/sciadv.abj3286

**The PDF file includes:**

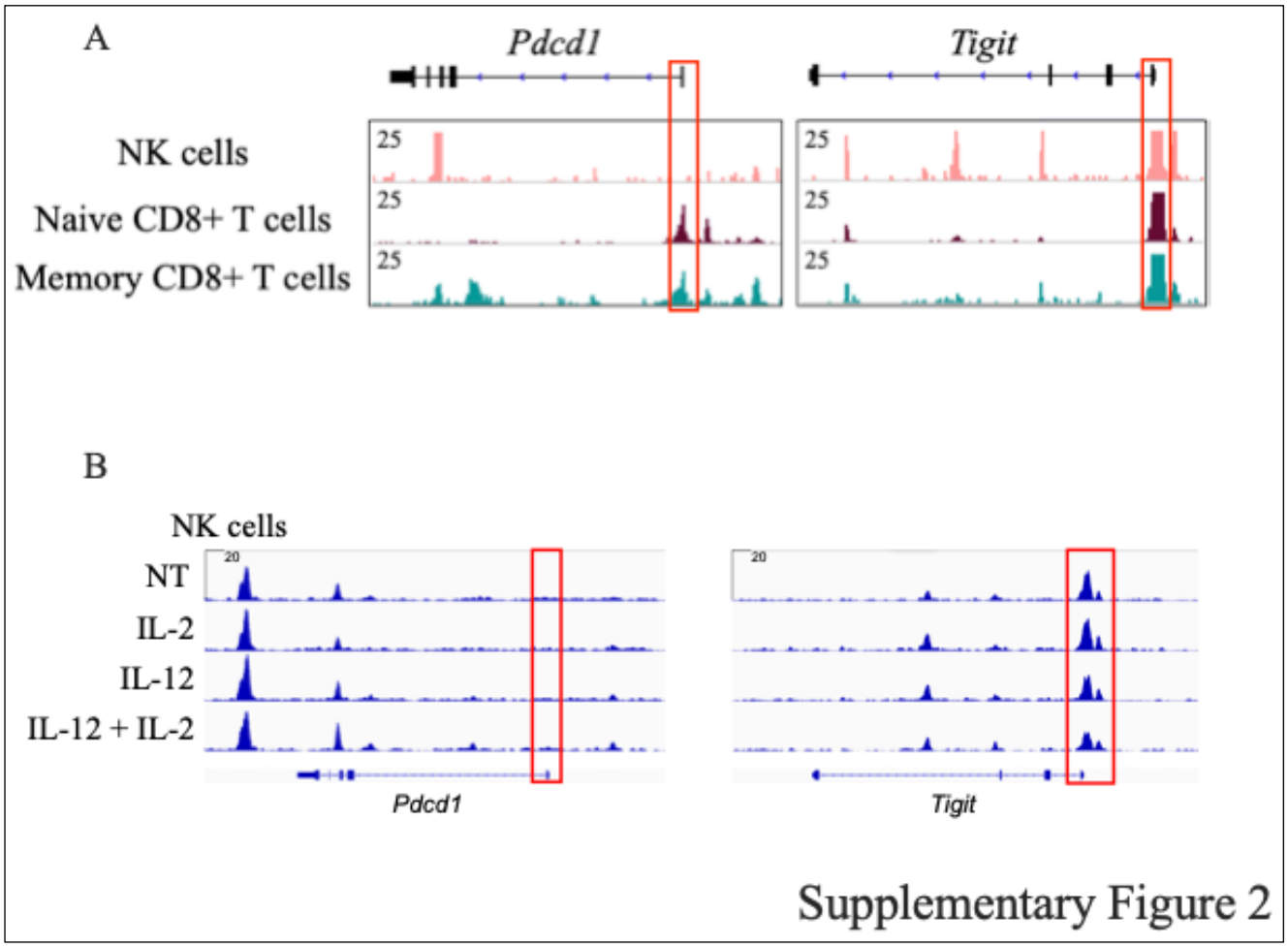


**Other Supplementary Material for this manuscript includes the following:**

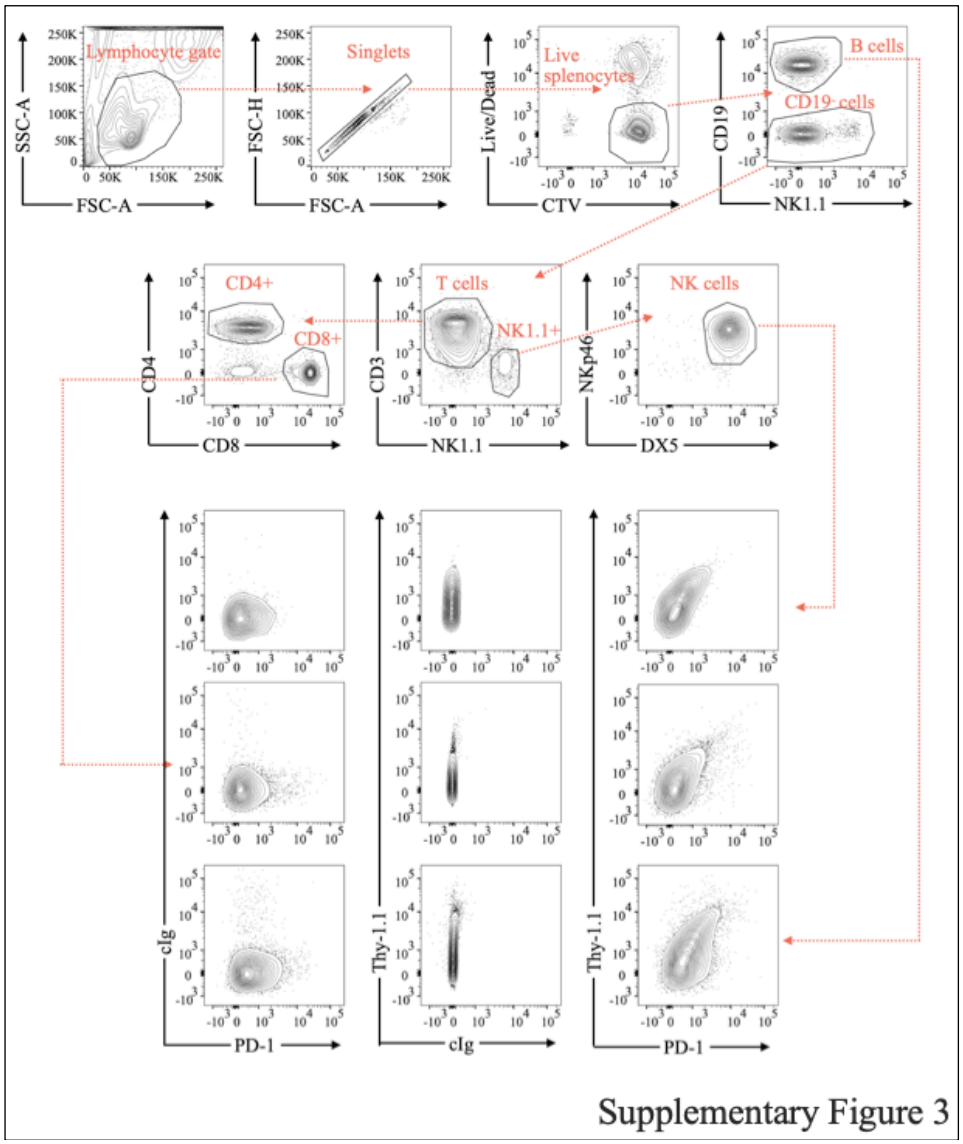
**Supplementary Figure 1: Inflammatory signals fail to induce PD-1 upregulation in purified NK cells in vitro.** Negatively isolated splenic NK cells from *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ mice were cultured for 72hrs with the inflammatory mediators indicated in the figure. GC=glucocorticoid (Corticosterone). The numbers on the plots refer to PD-1 geometric MFI. NK cells from 3 mice/genotype where pooled. The experiment depicted is representative of 3 performed.



**Supplementary Figure 2: The *Pdcd1* locus is closed in resting NK cells.** Genomic snapshots of normalized ATAC-seq signals in NK cells, naïve and memory CD8+ T cells across *Pdcd1* and *Tigit* loci.

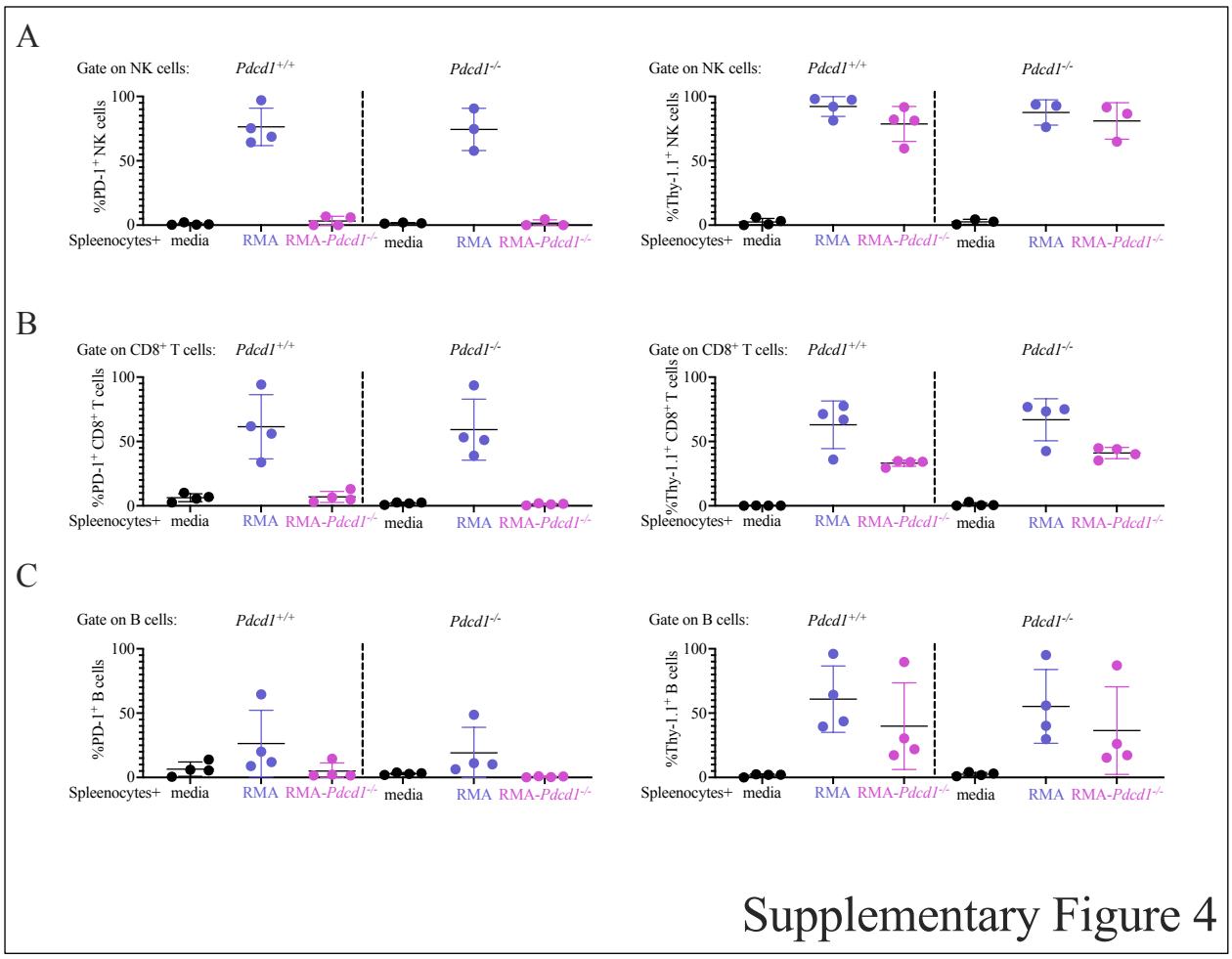


**Supplementary Figure 3: gating strategy for ex vivo experiments.** The gating strategy for ex vivo experiments is depicted. Lymphocytes were gated using an FSC/SSC gate, then singlets were excluded and splenocytes were gated as live cells staining for CTV. B cells were identified by CD19 expression. In the CD19- gate, T cells were gated as CD3+, and CD8/CD4 gating was used to identify CD8+ T cells. In the CD3-NK1.1+ gate, cells expressing NKp46 and DX5 were identified as NK cells. FMO for PD-1 and Thy-1.1, as well as staining with both antibodies, for the three immune subsets is shown. While this is the gating strategy employed for most experiments, in few experiments some markers were excluded/added depending on specific experimental needs.

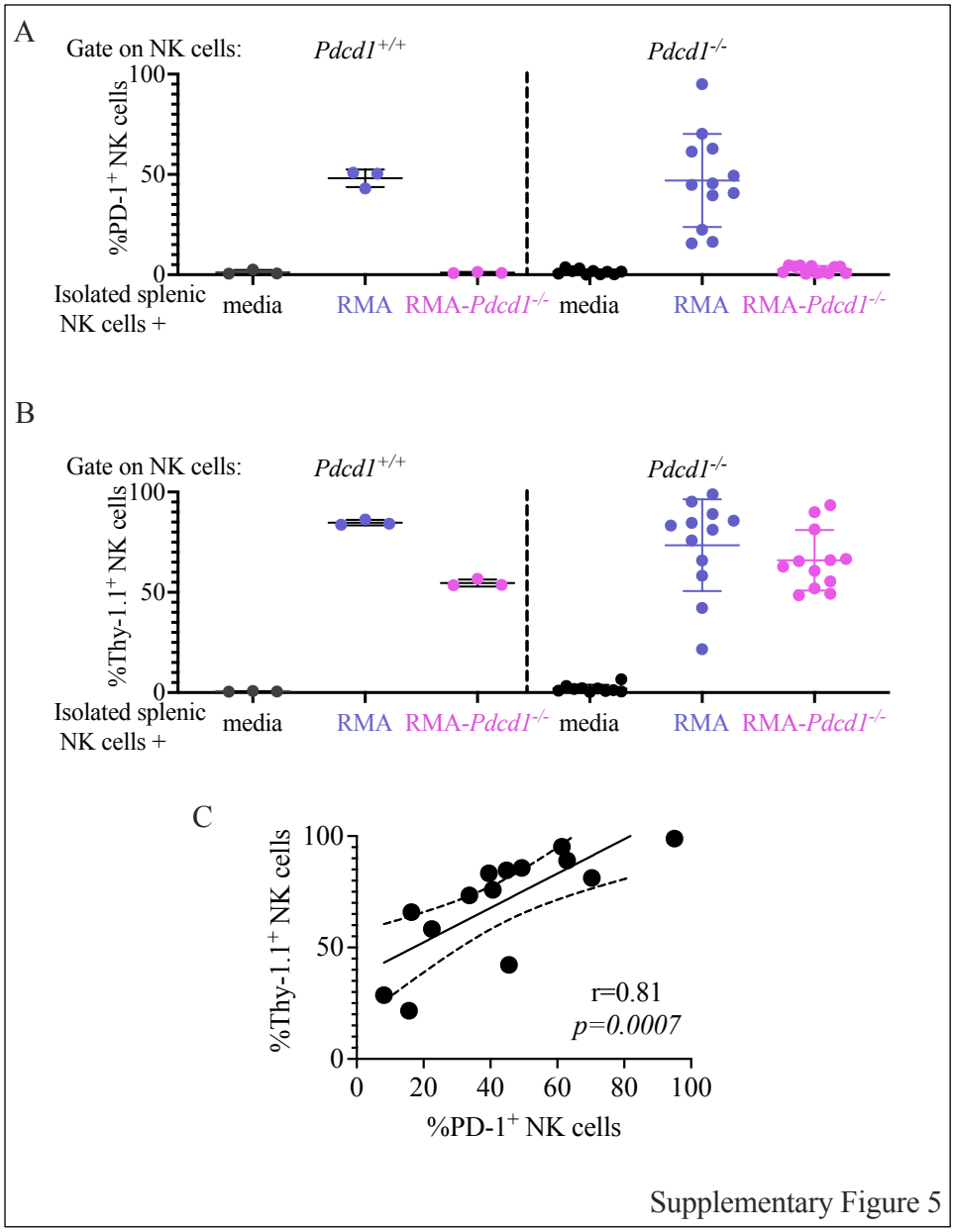



Supplementary Figure 3

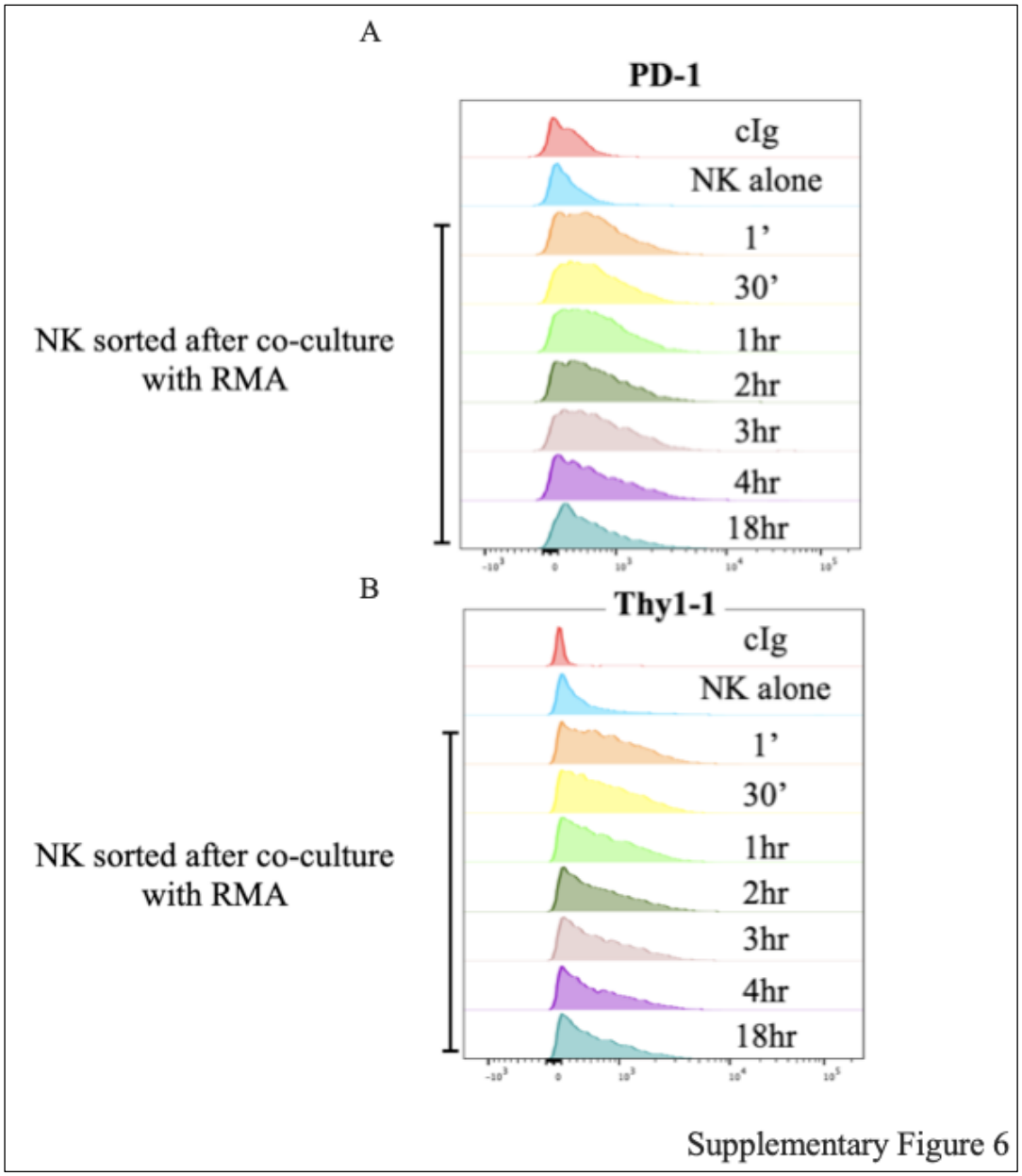
**Supplementary Figure 4: lymphocytes acquire PD-1 and Thy-1.1 from RMA tumor cells.** Splenocytes from *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ littermates were incubated with RMA-*Thy1.1* or RMA-*Pdcd1*$^{-/-}$*Thy1.1*. After 3 days, cells were stained with Thy-1.1 and PD-1 antibodies. A: NK cells; B: CD8$^{+}$ T cells; C: B cells. Results from three independent experiments were pooled, n=3-4.



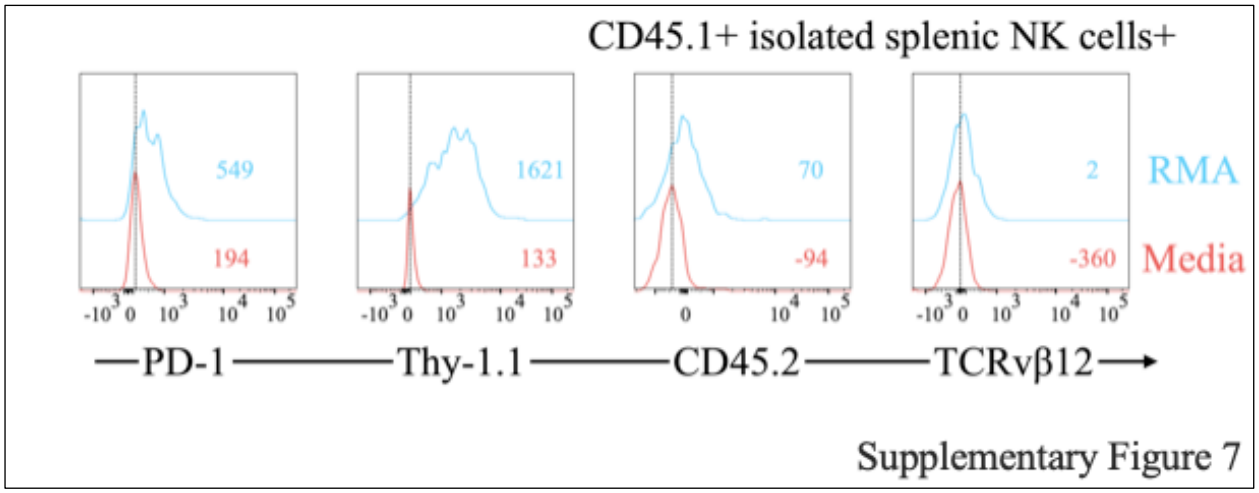
**Supplementary Figure 5: PD-1 and Thy-1.1 are co-acquired by NK cells.** NK cells isolated from the spleens of *Pdcd1*$^{-/-}$ mice were co-cultured with RMA or RMA-*Pdcd1*$^{-/-}$ cells for three days. A and B show the frequency of PD-1$^{+}$ or Thy-1.1$^{+}$ NK cells in the 14 mice analyzed in the 13 experiments performed. In C the correlation between PD-1$^{+}$ and Thy-1.1$^{+}$ NK cells is depicted. 95%-confidence interval is also showed, statistical analysis with Spearman correlation test.



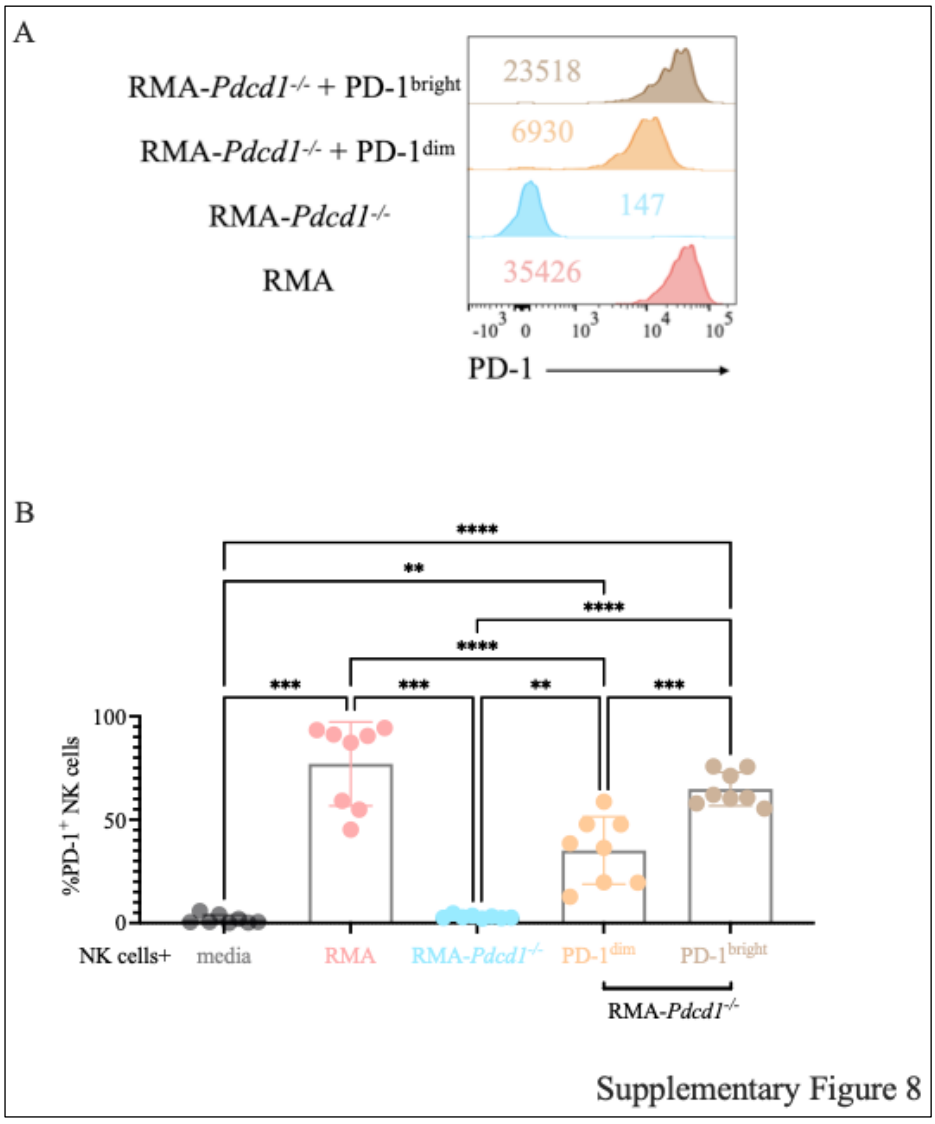
**Supplementary Figure 6: PD-1 and Thy-1.1 are stables hours after they have been acquired by NK cells.** NK cells isolated from the spleens of *Pdcd1*$^{-/-}$ mice were co-cultured with RMA cells for three days and then sorted and stained for PD-1 and Thy-1.1 at the indicated time points. The experiment shown is representative of 3 performed.



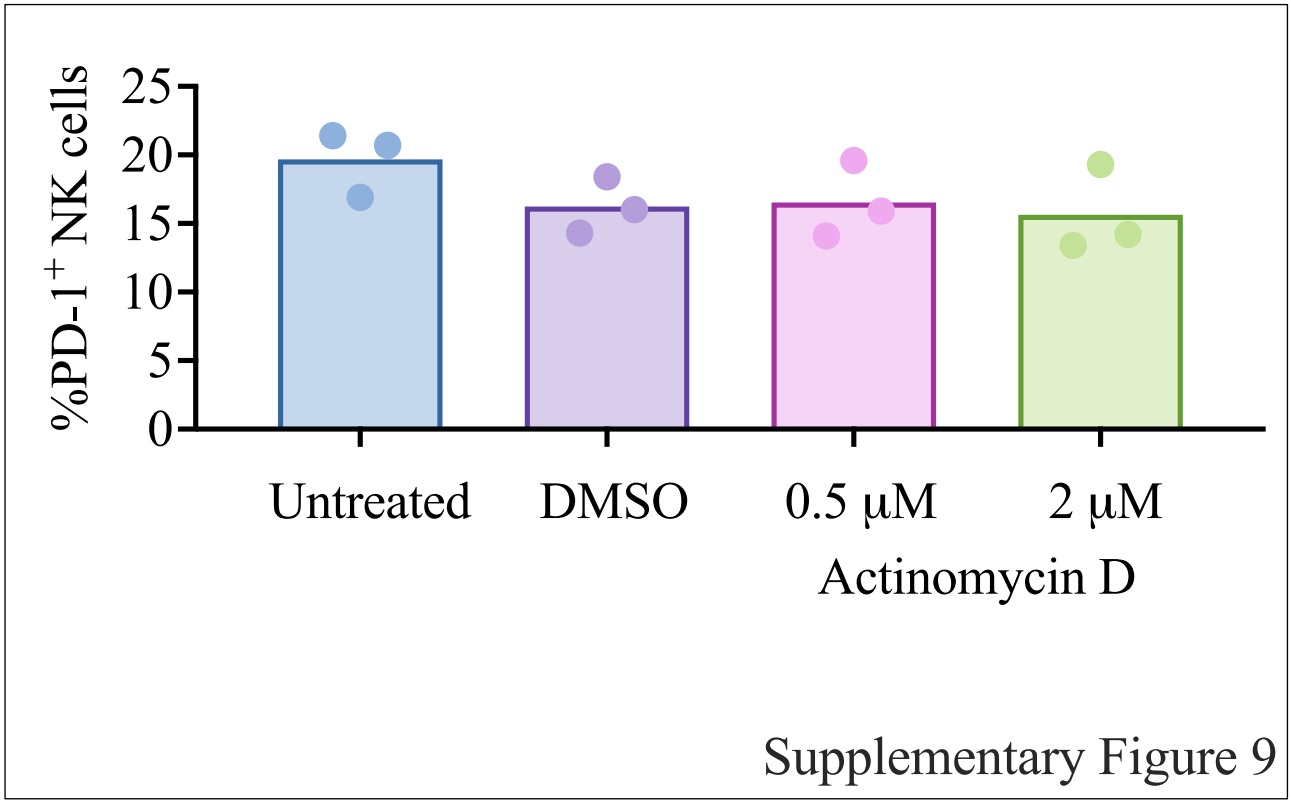
**Supplementary Figure 7: NK cells acquire at least four proteins they do not endogenously express from RMA cells.** CD45.1+ NK cells were co-cultured with RMA cells for three days and then PD-1, Thy-1.1, CD45.2 and TCRvβ12 staining was analyzed by flow cytometry. Representative of three performed with similar results.



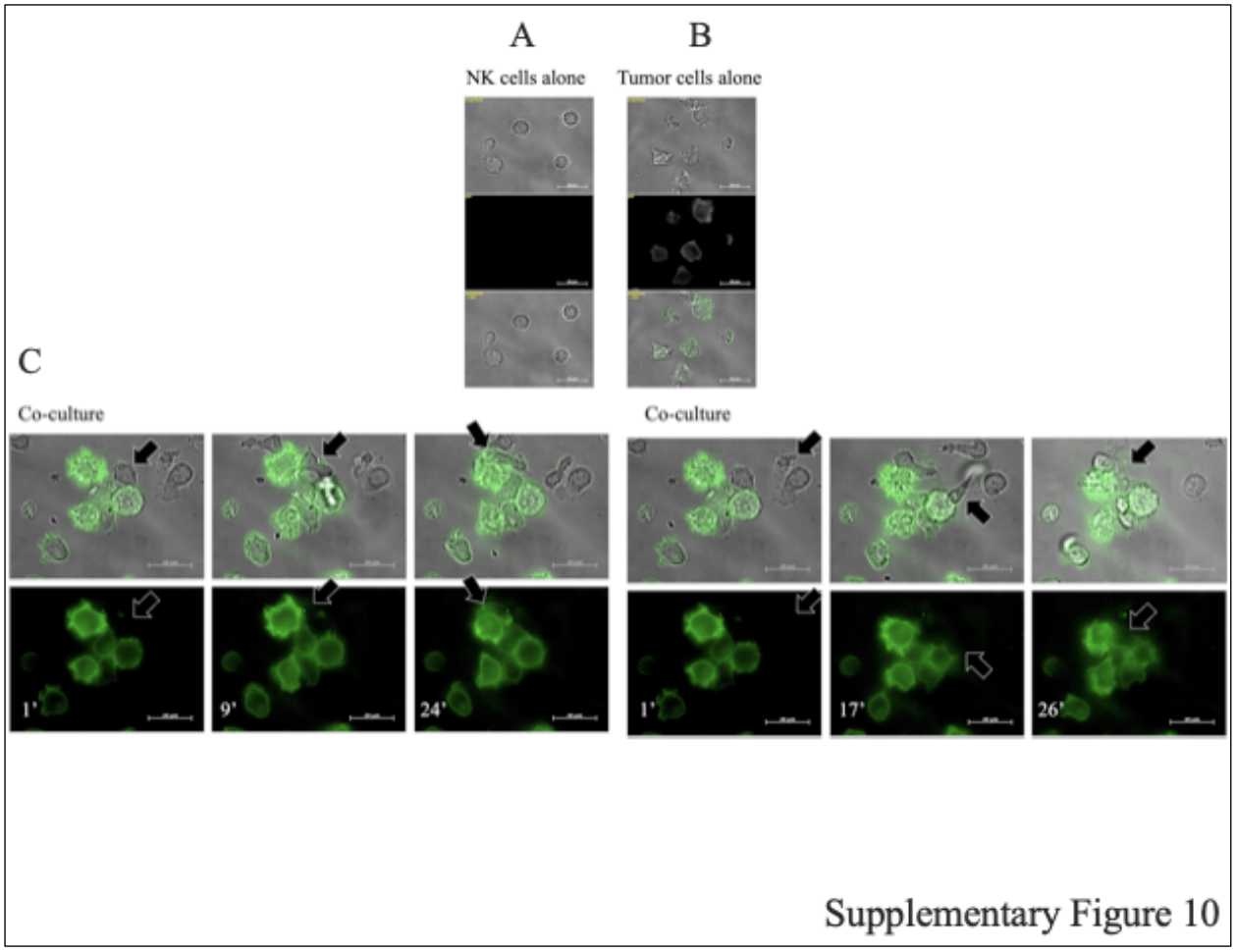
**Supplementary Figure 8: higher PD-1 expression on tumor cells results in higher PD-1 acquisition on NK cells.** (A) PD-1 expression levels on different RMA variants are shown, the numbers in the plot refer to PD-1 geometric MFI. (B) Negatively isolated splenic NK cells from *Pdcd1*$^{-/-}$ mice were cultured with rh-IL-2 for 3 days and then mixed with different RMA variants for 2-3 hrs. PD-1 staining on NK cells is shown. Two independent experiments were pooled, n=8. Statistical analysis with one-way ANOVA with multiple comparisons. **: p<0.01; ***: p<0.001; ****: p<0.0001.



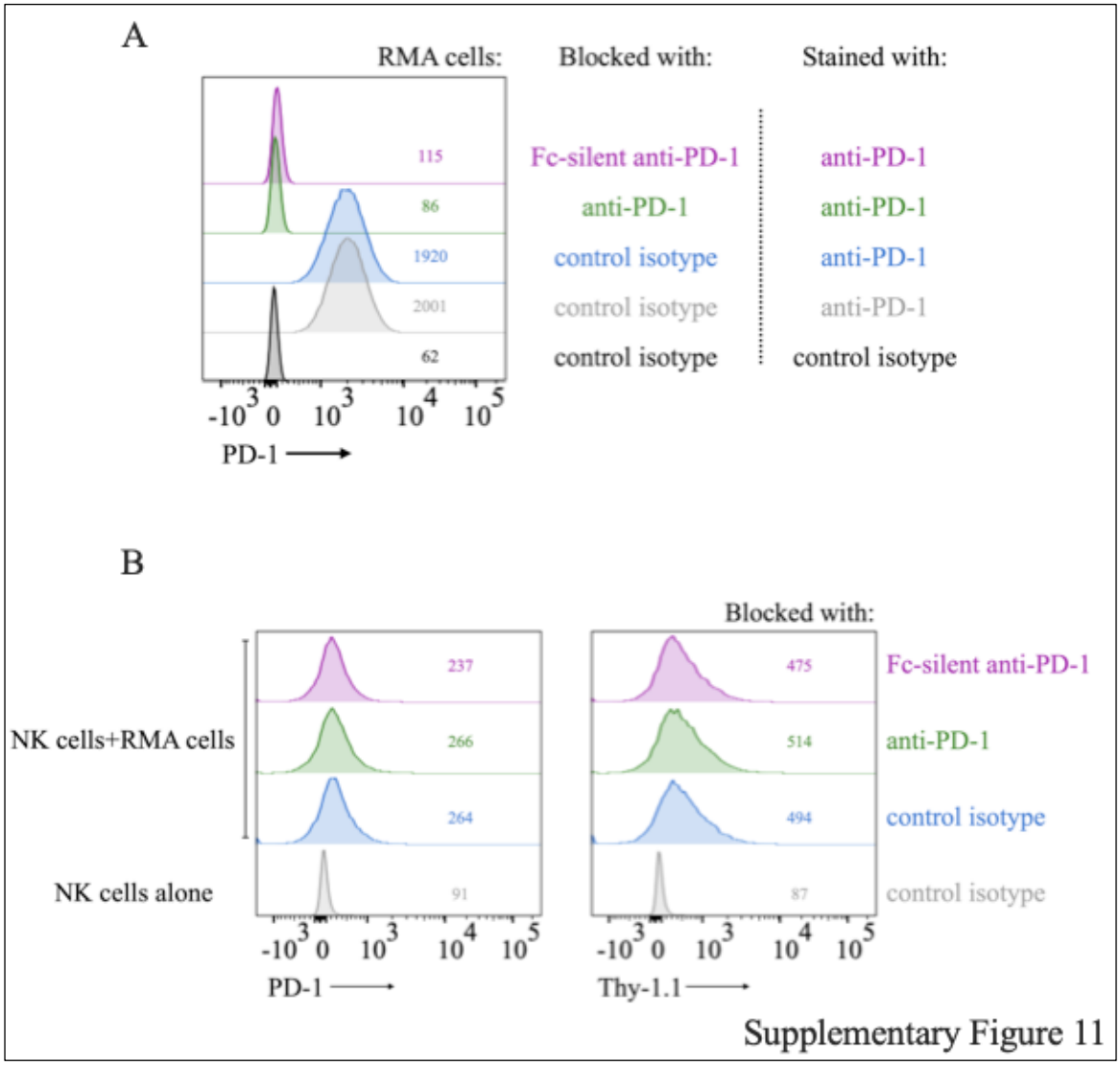
**Supplementary Figure 9: inhibition of transcription in NK cells does not prevent them from acquiring PD-1 from tumor cells.** Negatively isolated splenic NK cells from *Pdcd1*$^{-/-}$ mice were cultured with rh-IL-2 for 3 days, pre-treated with Actinomycin D and then mixed with RMA cells for 2-3 hrs. PD-1 staining on NK cells is shown. Three independent experiments were pooled, n=3. Statistical analysis with one-way ANOVA with multiple comparisons. No statistical differences were observed.



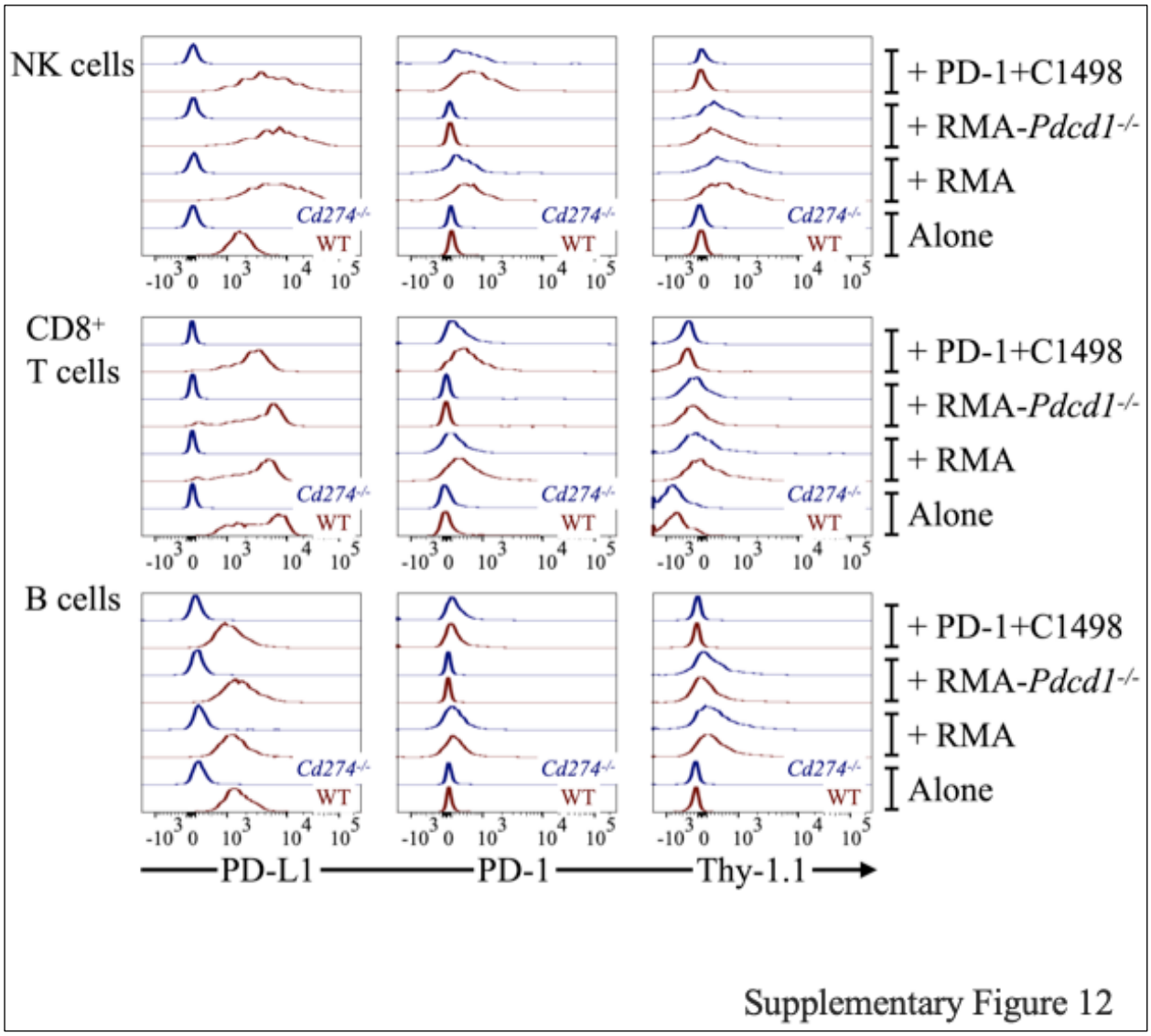
**Supplementary Figure 10: PD-1-GFP fusion protein is acquired by NK cells.** Negatively isolated splenic NK cells from *Pdcd1*$^{-/-}$ mice were cultured with rh-IL-2 for 3 days, mixed with RMA-*Pdcd1*$^{-/-}$ + PD-1-GFP-fusion protein cells and live imaging was performed. In A and B only NK cells and tumor cells were imaged. In C, dynamic acquisition of PD-1-GFP fusion protein is highlighted on two different NK cells. The experiment shown is representative of 2 performed.



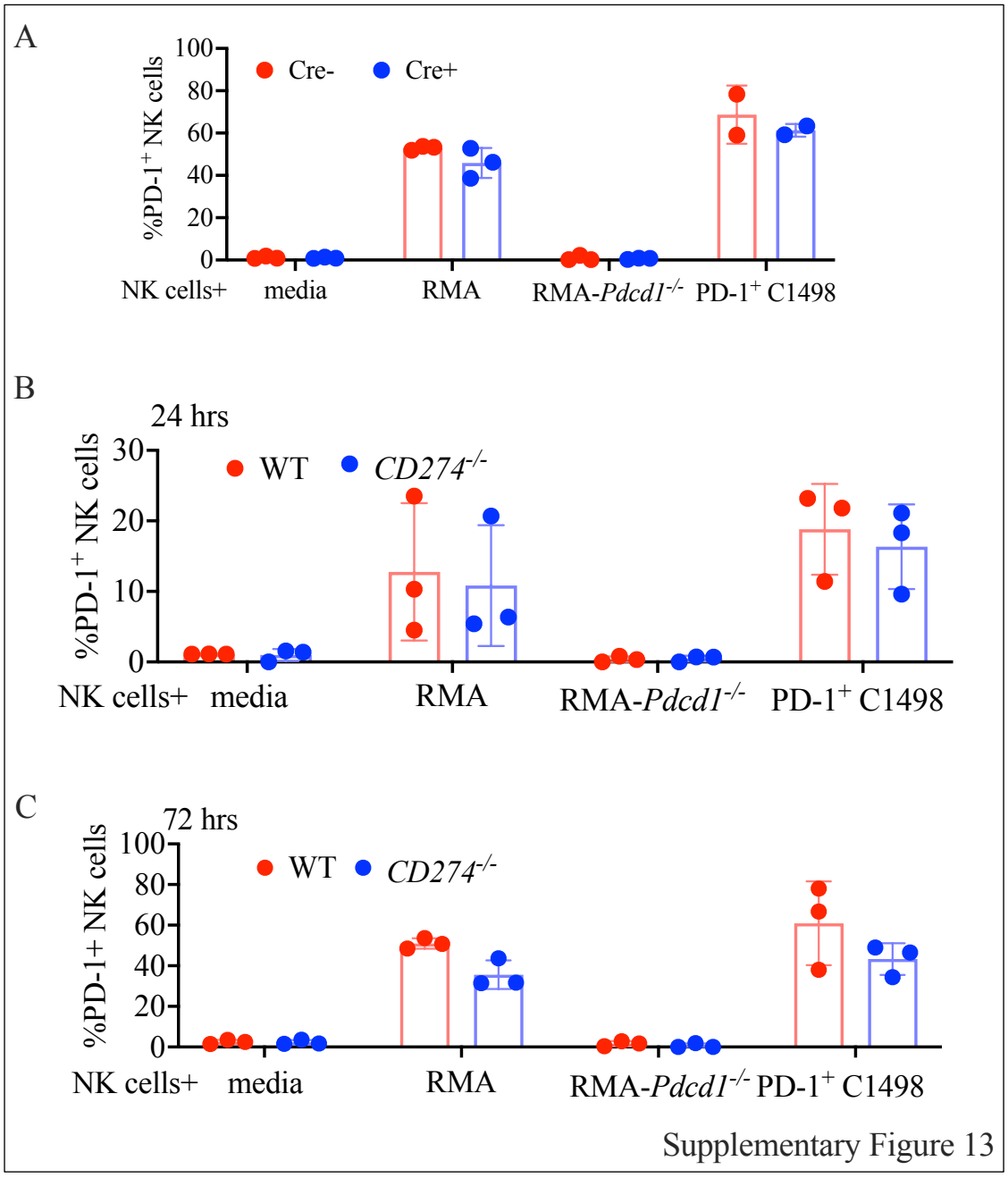
**Supplementary Figure 11: PD-1 antibodies do not affect or promote PD-1 trogocytosis by NK cells.** (A) Saturation of PD-1 sites on RMA cells was assessed by stained with the PD-1 antibody cells that were previously co-incubated with anti-PD-1 or control isotype. (B) NK cells purified from *Pdcd1*$^{-/-}$ mice were incubated with RMA cells in the presence of the indicated blocking antibody and then stained for PD-1 and Thy-1.1. In both A and B, the numbers in the histogram flow plots indicate PD-1 or Thy-1.1 geometric MFI. The experiment depicted is representative of three performed.



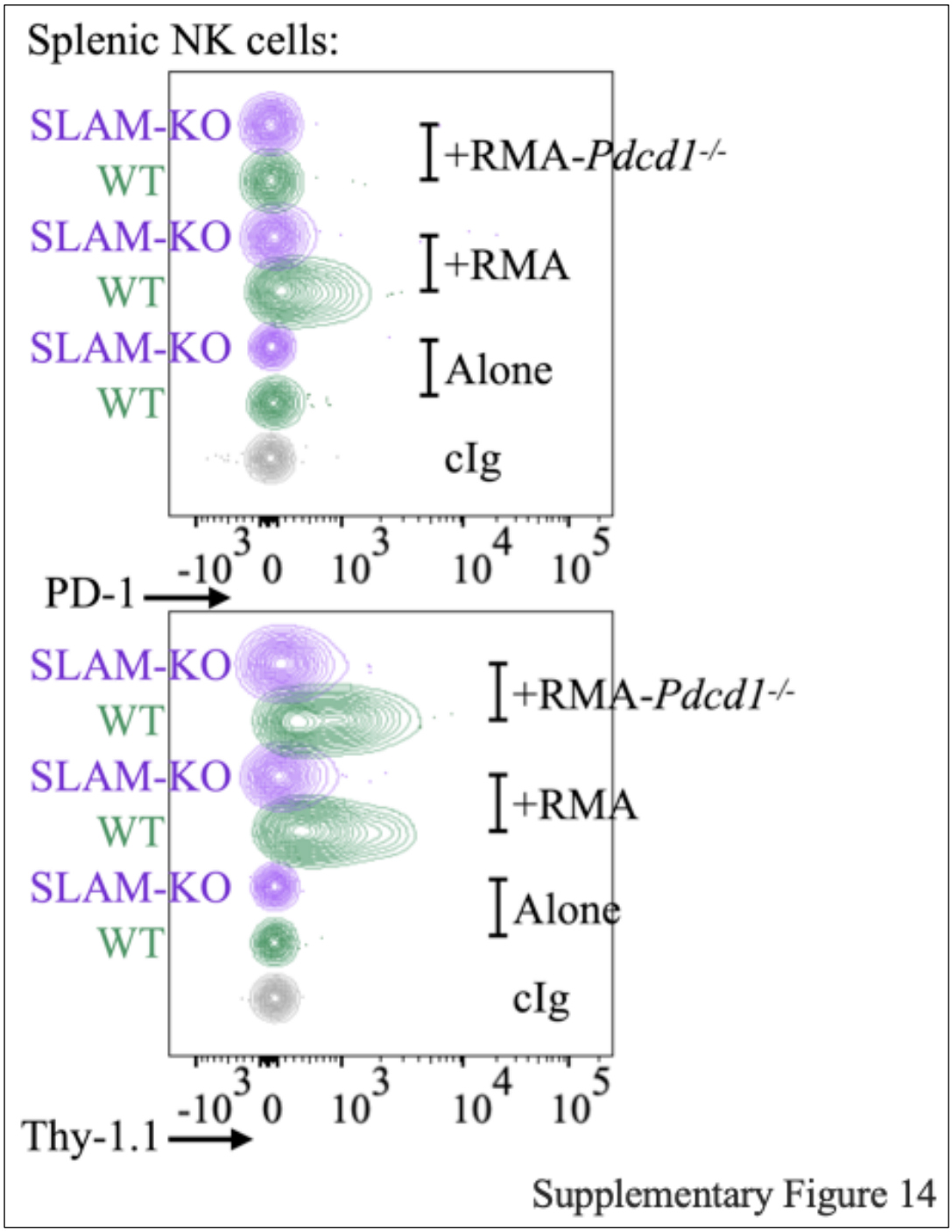
**Supplementary Figure 12: PD-L1 is dispensable for PD-1 trogocytosis in NK, T and B cells.** *Cd274*$^{-/-}$ or wild type splenocytes were cultured with tumor cells for 3 days before PD-1 and Thy-1.1 staining was assessed by flow cytometry. Representative of 3 mice/genotype analyzed.



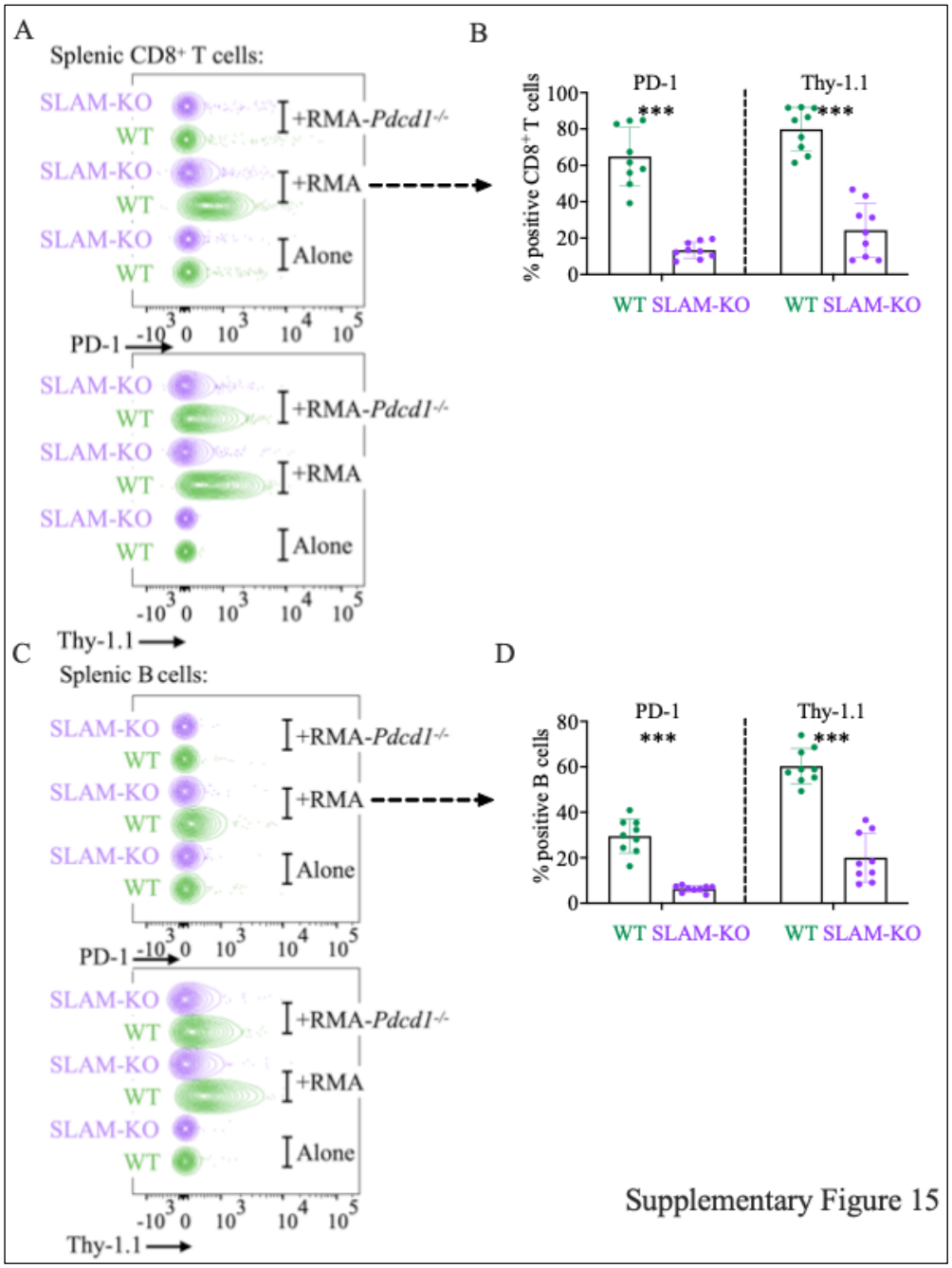
**Supplementary Figure 13: PD-L1 is dispensable for PD-1 trogocytosis in NK, T and B cells.** *Cd274*$^{-/-}$ or wild type splenocytes were cultured with tumor cells for 1 or 3 days before PD-1 and Thy-1.1 staining was assessed by flow cytometry. Three independent experiments were pooled, n=3. No statistical differences were observed.



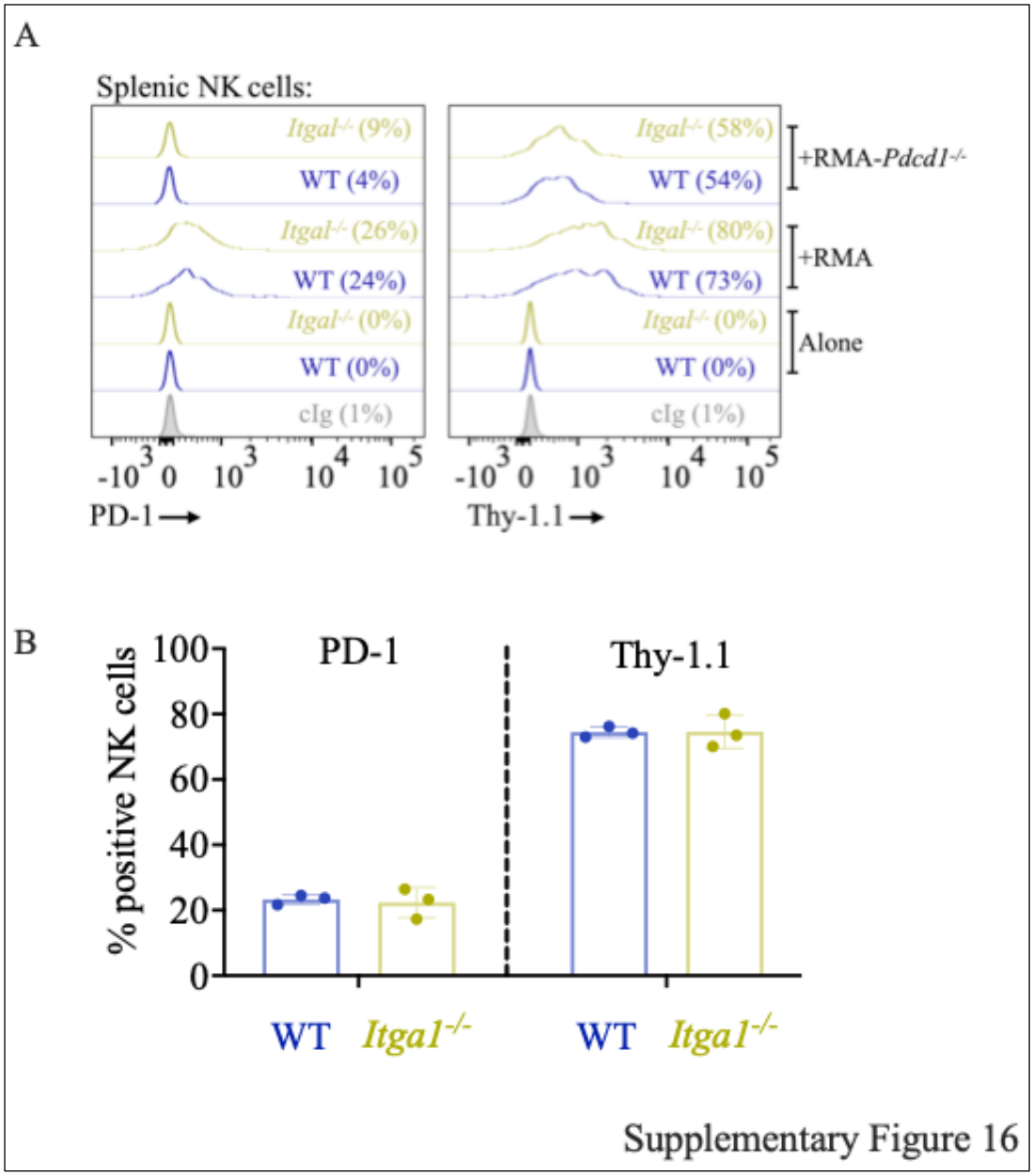
**Supplementary Figure 14: SLAM receptors are required for trogocytosis in NK cells.** SLAM-ko or wild type splenocytes were cultured with tumor cells for three days. Representative stainings of PD-1 or Thy-1.1 on NK cells are depicted.



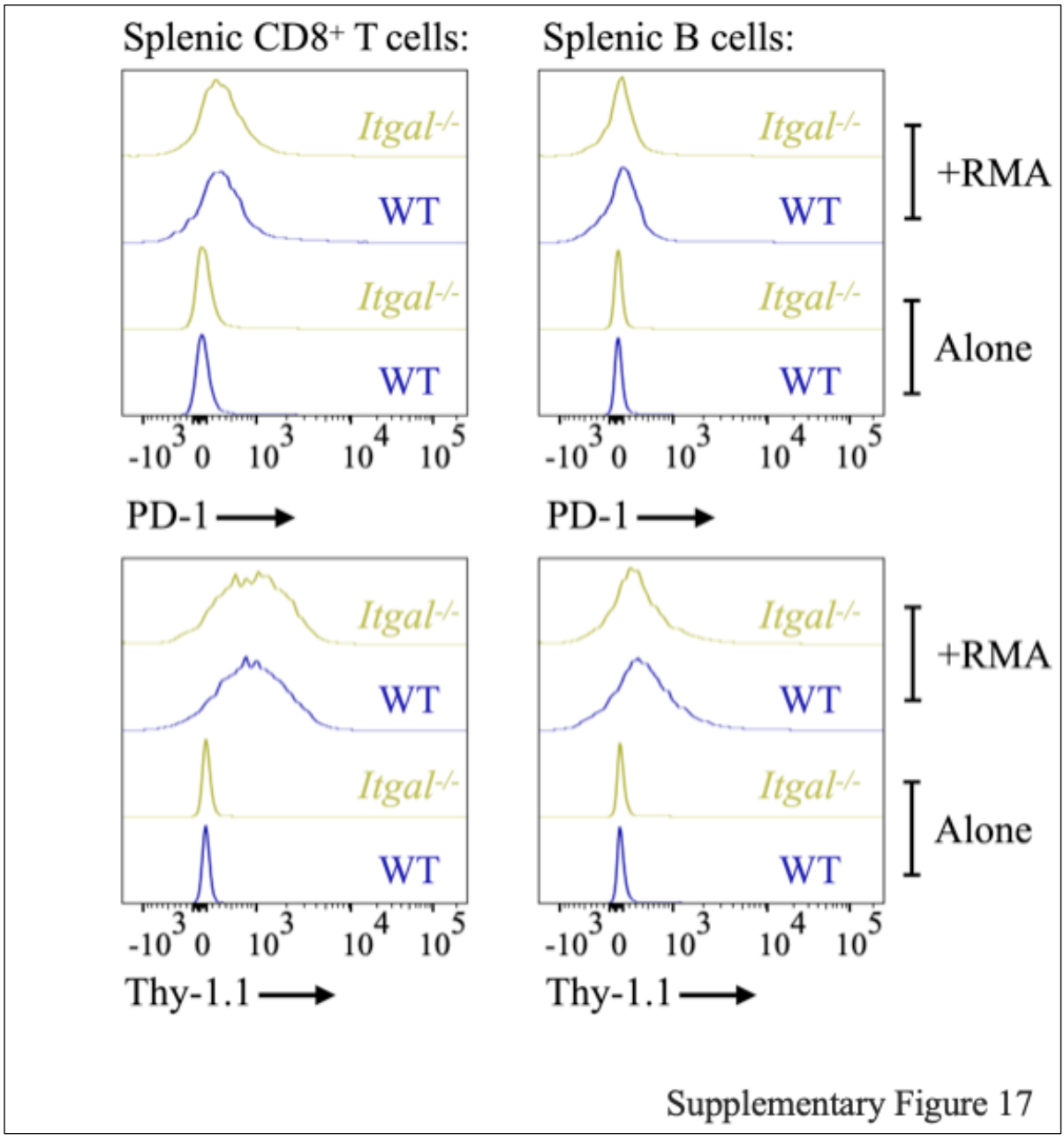
**Supplementary Figure 15: SLAM receptors are required for trogocytosis in CD8$^+$ T and B cells.** SLAM-ko or wild type splenocytes were cultured with tumor cells for three days. Representative staining and cumulative analysis are depicted. Statistical analysis with two-tailed unpaired Student's t-test. ***: $p<0.001$.



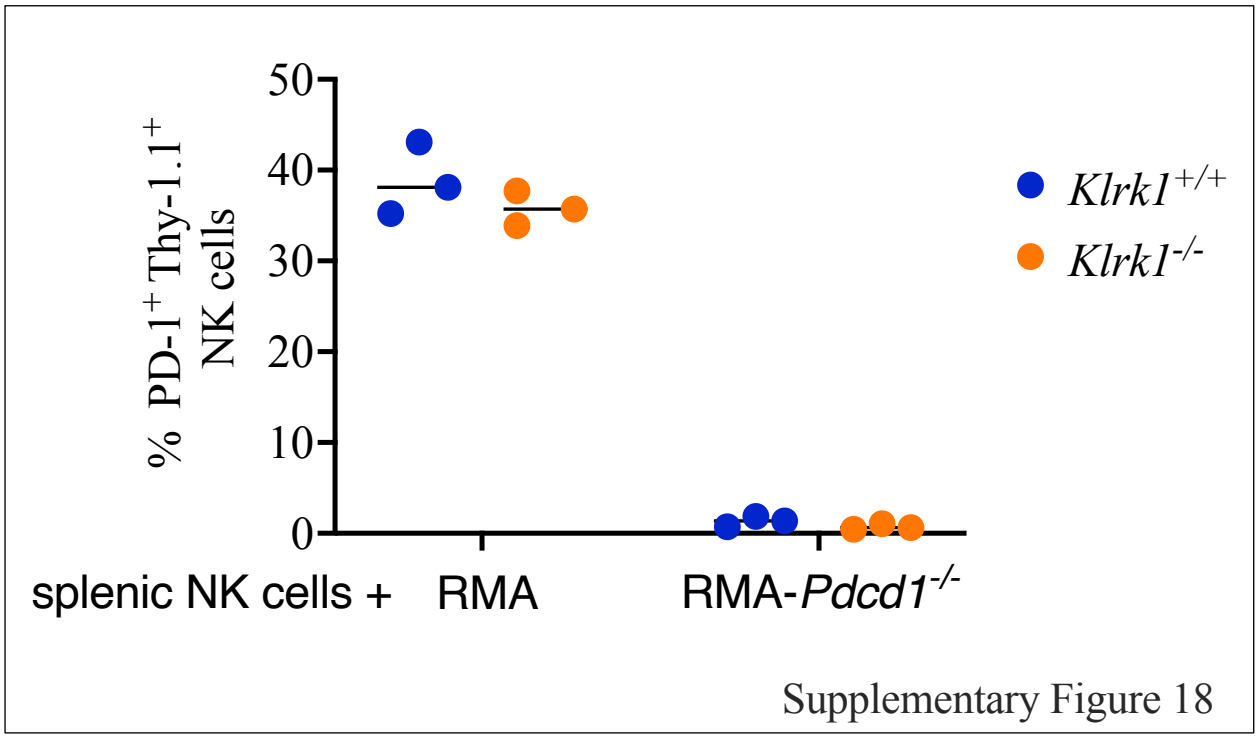
**Supplementary Figure 16: LFA-1 is not necessary for trogocytosis in NK cells.** *Itga1*$^{-/-}$ or wild type splenocytes were cultured with tumor cells for three days when PD-1 and Thy-1.1 staining was assessed on NK cells. A representative staining is shown in panel A, where the frequency of PD-1 or Thy-1.1 is shown in the flow plots, whereas in B three independent experiments were pooled, n=3. No statistical differences were observed.



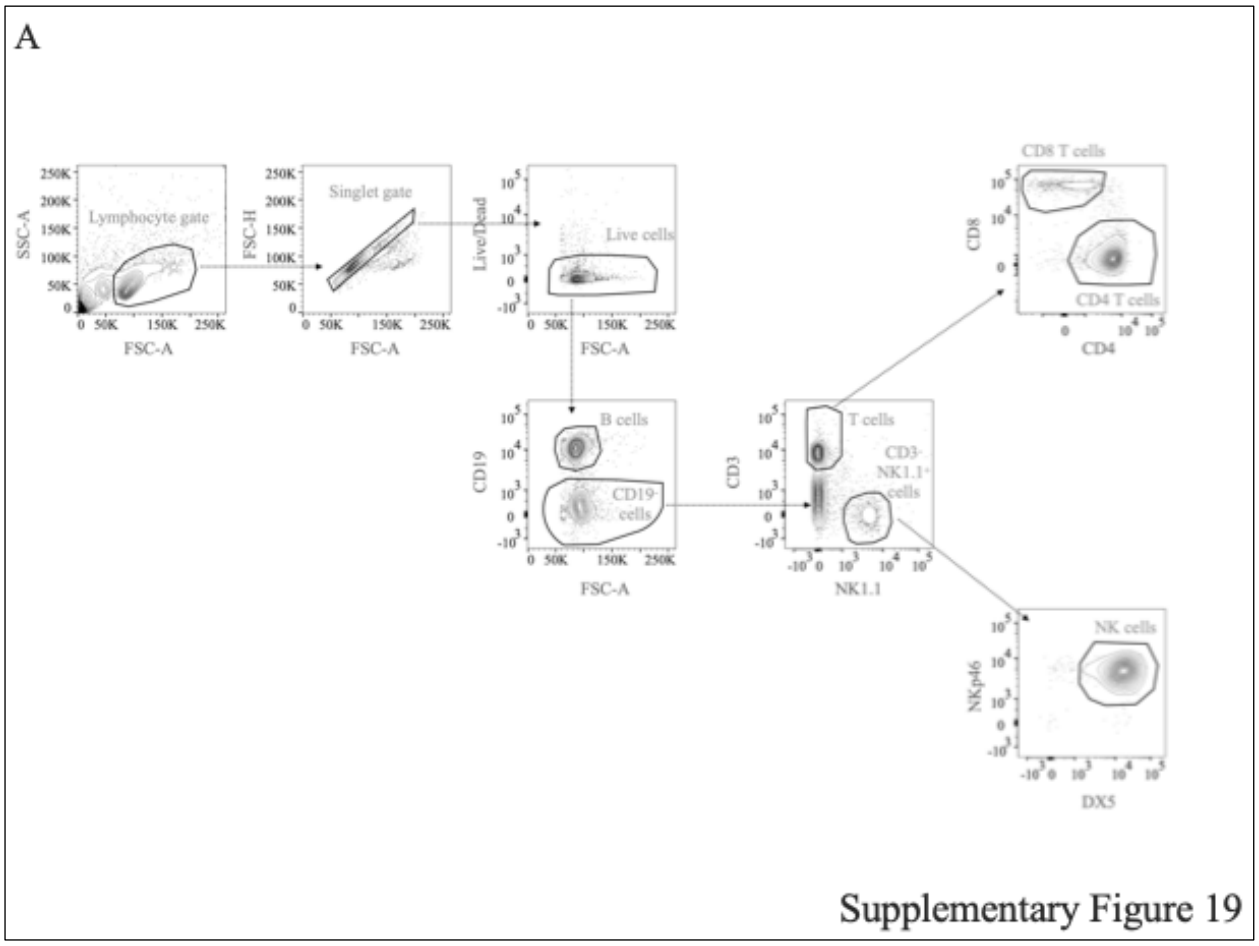
**Supplementary Figure 17: LFA-1 is not necessary for trogocytosis in CD8$^+$ T and B cells.** *Itga1*$^{-/-}$ or wild type splenocytes were cultured with tumor cells for three days when PD-1 and Thy-1.1 staining was assessed on CD8+ T cells and B cells.



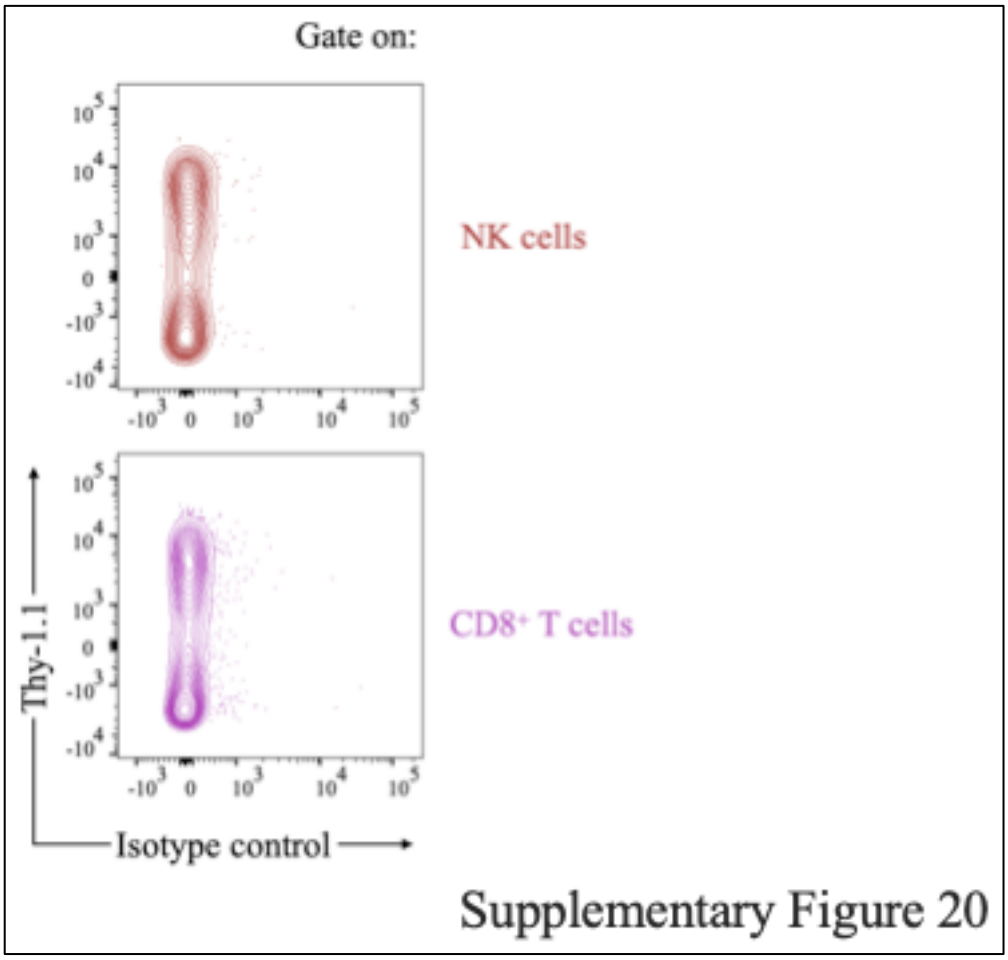
**Supplementary Figure 18: NKG2D is not required for trogocytosis in NK cells.** *Klrk1*$^{-/-}$ or control littermates NK cells were cultured with tumor cells for three days and then stained for PD-1 and Thy-1.1. Three independent experiments were pooled, n=3. No statistical differences were observed.



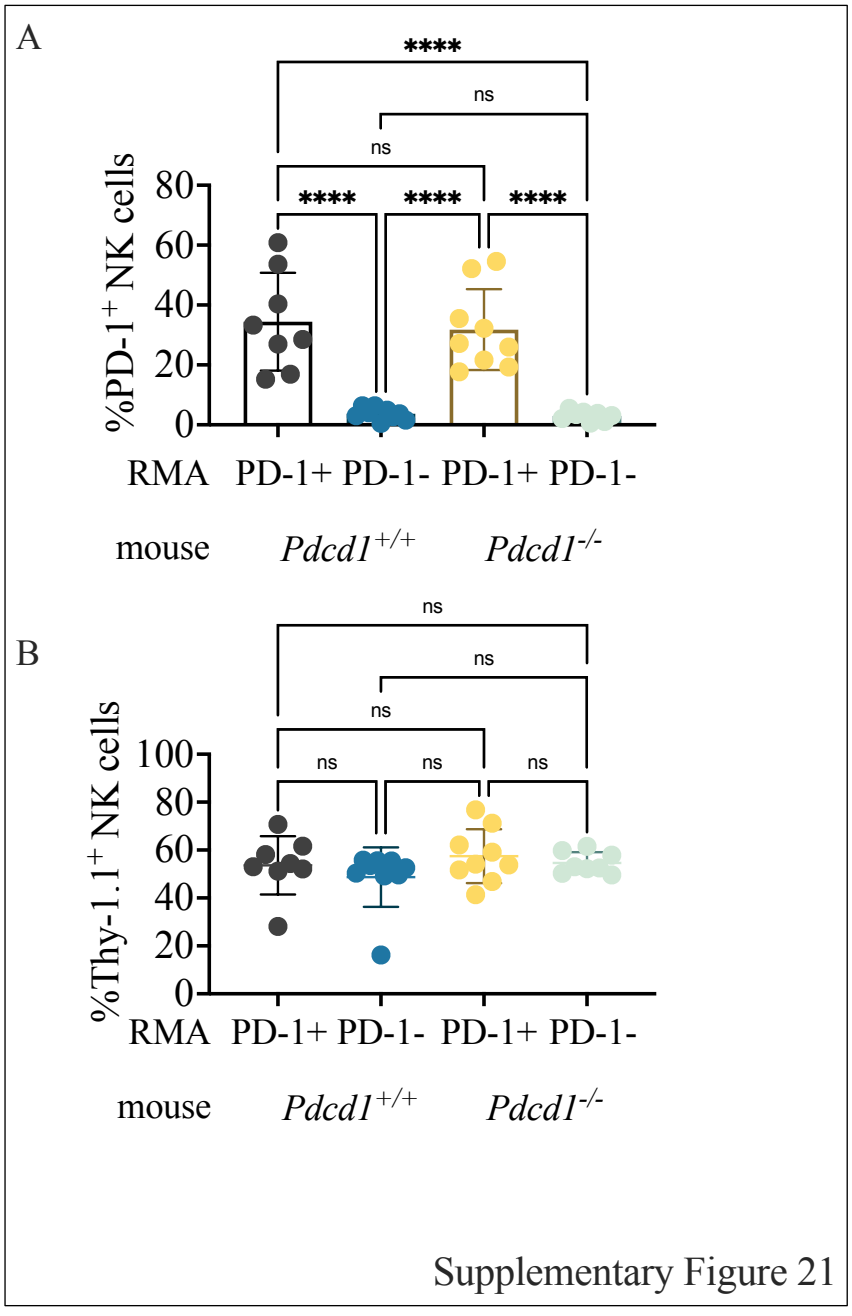
**Supplementary Figure 19: gating strategy in in vivo experiments.** The gating strategy for in vivo experiments is depicted. Lymphocytes were gated using an FSC/SSC gate, then singlets and dead cells were excluded. B cells were identified by CD19 expression. In the CD19- gate, T cells were gated as CD3+, and CD8/CD4 gating was used to identify CD8+ T cells. In the CD3-NK1.1+ gate, cells expressing NKp46 and DX5 were identified as NK cells. While this is the gating strategy employed for most experiments, in few experiments some markers were excluded depending on specific experimental needs.



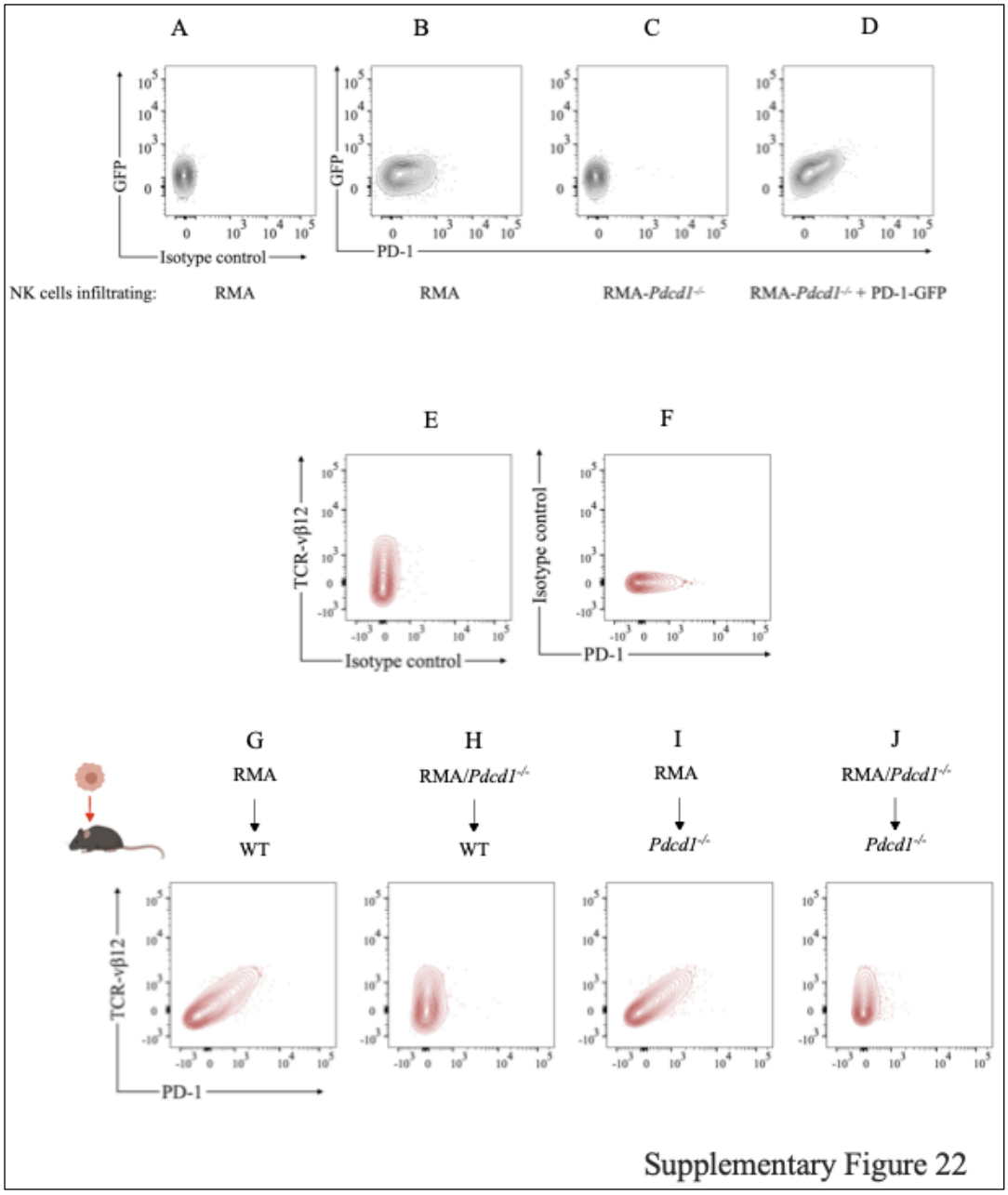
**Supplementary Figure 20: PD-1 staining control in in vivo experiments.** The FMO+control Ig for PD-1 for the in vivo experiments shown in Fig. 5A-D is depicted.



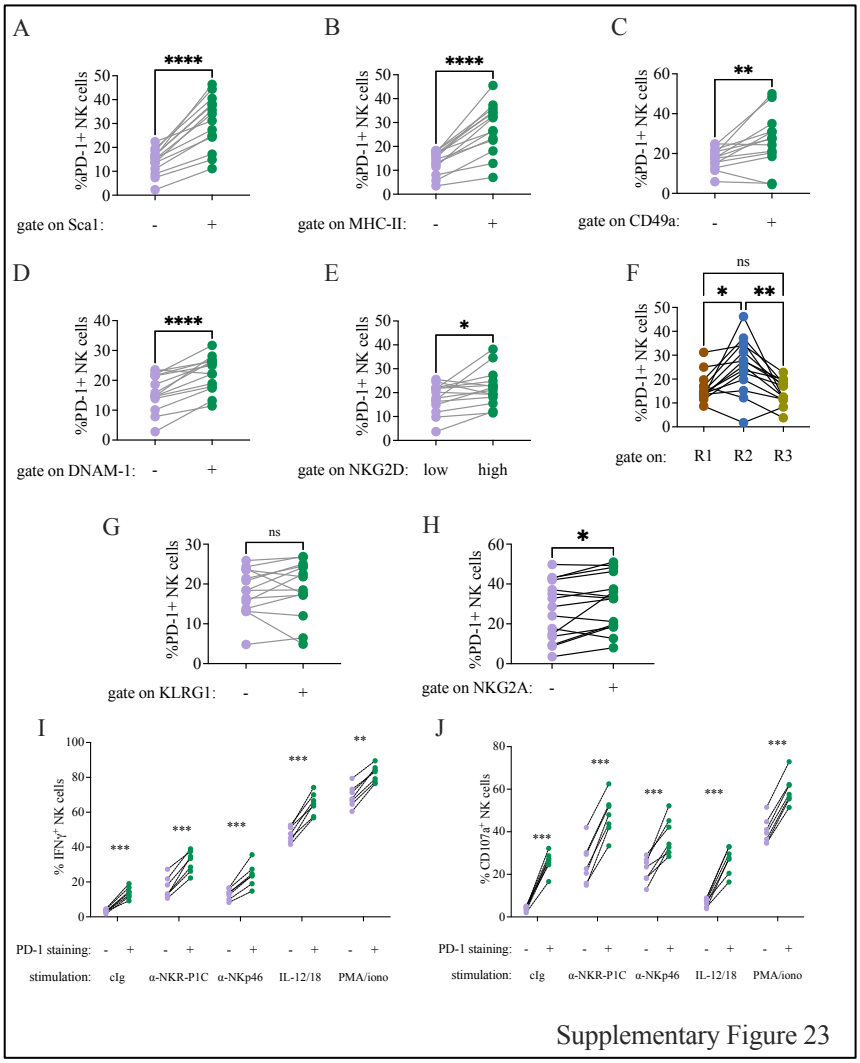
**Supplementary Figure 21: PD-1 and Thy-1.1 are acquired in vivo by NK cells.** PD-1 and Thy-1.1 stainings in tumor infiltrating NK cells are shown. Data from 3 independent experiments were pooled, n=8-9 mice/group. Statistical analysis with one-way ANOVA with multiple comparisons. ****:p<0.0001.



**Supplementary Figure 22: PD-1-GFP and TCR-vβ12 are acquired in vivo by NK cells.** (A-D) PD-1 staining on NK cells infiltrating tumors expressing PD-1 or PD-1-GFP fusion protein, or not expressing PD-1, are shown. The experiment shown is representative of 3 performed. (E-J) PD-1 and TCR-vβ12 stainings in tumor infiltrating NK cells are shown. The experiment shown is representative of 3 performed.

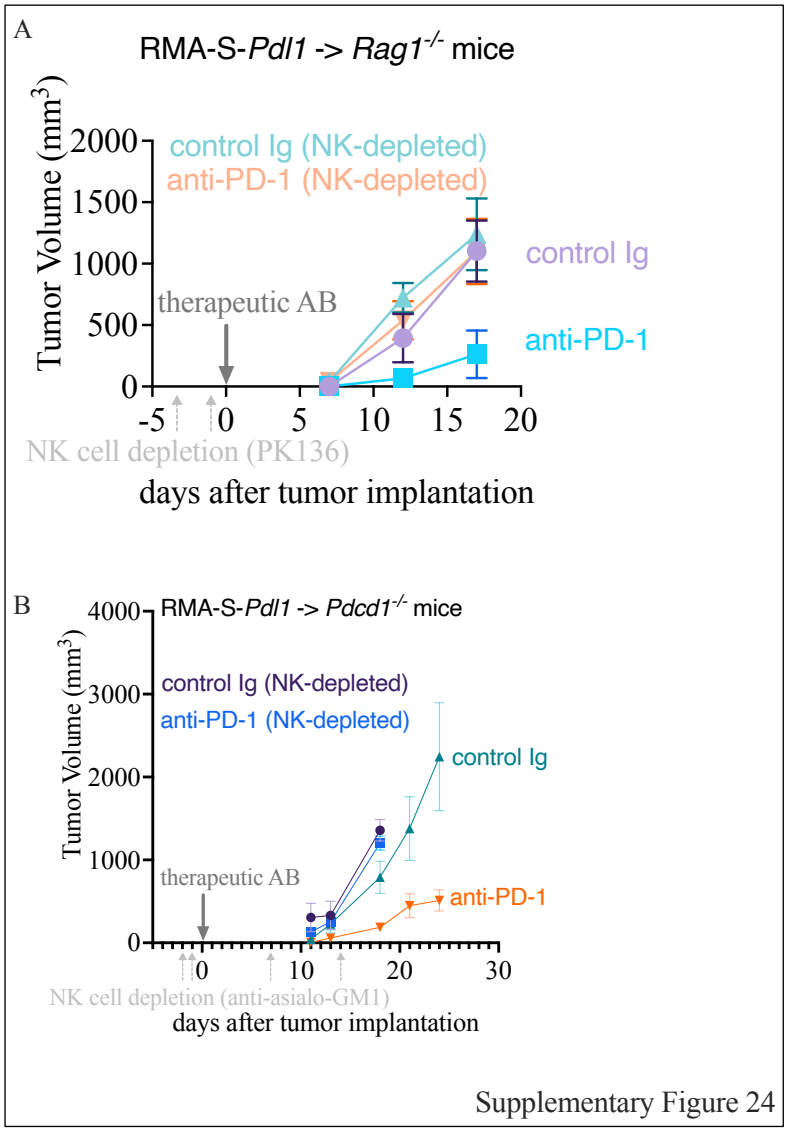


**Supplementary Figure 23: NK cells acquiring PD-1 are activated and functional.** NK cells infiltrating RMA-S-*Pdl1* tumors were stained for PD-1 and co-expression with activation markers (A-C), activating receptors (D-E), maturation markers (F), inhibitory receptors (G-H) was assessed. The experiment shown is representative of 3 performed, n=13-14. Statistical analysis with two-tailed paired Student's t-test. *: $p<0.05$; **: $p<0.01$; ****:$p<0.0001$. (I-J) NK cells infiltrating RMA-S-Pdl1 tumors were stimulated ex vivo and stained for PD-1. IFN-γ expression and CD107a externalization are shown. One experiment representative of three performed is shown, n=7. Statistical analysis with two-tailed paired Student's t-test. **: $p<0.01$; ***:$p<0.001$.

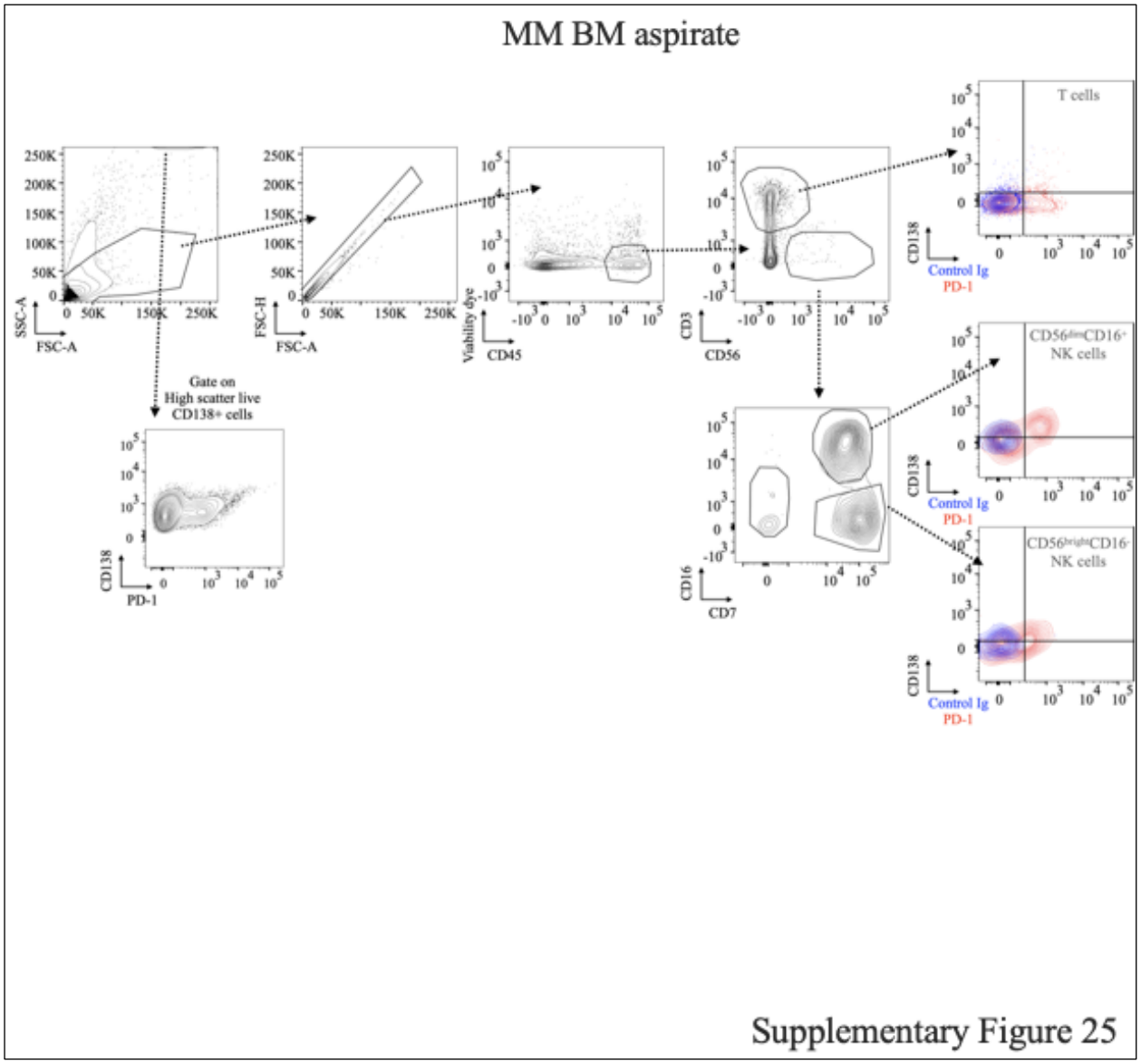



Supplementary Figure 23

**Supplementary Figure 24: Trogocytosed PD-1 inhibits NK cell anti-cancer functions.** (A) *Rag1*$^{-/-}$ mice were depleted or not of NK cells with PK136 antibody and then injected with 0.1x10$^6$ RMA-S-*Pdl1* cells in Matrigel with 20 µg of RMP1-14 (left flank) or isotype control (right flank) antibody. Tumor volumes were then assessed. Representative of 2 performed, n=4. (B) *Pdcd1*$^{-/-}$ mice were depleted or not of NK cells with anti-asialo-GM1 and then injected with 0.1x10$^6$ RMA-S-*Pdl1* cells in Matrigel with 20 µg of RMP1-14 (left flank) or isotype control (right flank) antibody. Tumor volumes were then assessed. Representative of 2 performed, n=3-4.



**Supplementary Figure 25:** the gating strategy for NK and T cell identification in the bone marrow of patients with clonal plasma cell disorders is displayed.



**Supplementary Video S1: PD-1-GFP fusion protein is acquired by NK cells.** Negatively isolated splenic NK cells from *Pdcd1*$^{-/-}$ mice were cultured with rh-IL-2 for 3 days, mixed with RMA-*Pdcd1*$^{-/-}$ + PD-1-GFP-fusion protein cells and live imaging was performed.