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

Mohammed S. Hasim[1,2], Marie Marotel[1,2], Jonathan J. Hodgins[1,2,3], Elisabetta Vulpis[4], Han-Yun Shih[5], Amit Scheer[3], Olivia MacMillan[1,2,3], Fernando G. Alonso[1], Kelly P. Burke[6,7], David P. Cook[1], Maria Teresa Petrucci[8], Angela Santoni[4,9], Padraic G. Fallon[10], Arlene H. Sharpe[11,12], Giuseppe Sciumè[4], Andre Veillette[13,14,15], Alessandra Zingoni[4], Arleigh McCurdy[1,16], Michele Ardolino[1,2,3,*]

1: Cancer Therapeutics Program, Ottawa Hospital Research Institute, Ottawa, ON

2: CI3, University of Ottawa, Ottawa, ON

3: Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON

4: Department of Molecular Medicine, Sapienza University of Rome, Laboratory affiliated to Istituto Pasteur Italia – Fondazione Cenci-Bolognetti, Rome, Italy

5: Neuro-Immune Regulome Unit, National Eye Institute, NIH, Bethesda, MD

6: Department of Medical Oncology, Dana Farber Cancer Institute, Boston, MA

7: Harvard Medical School, Boston, MA

8: Department of Cellular Biotechnology and Hematology, "Sapienza" University of Rome, Rome, Italy

9: IRCCS Neuromed, Pozzilli, Italy

10: School of Medicine, Trinity Biomedical Sciences Institute, Trinity College Dublin, Ireland

11: Department of Immunology, Blavatnik Institute, Harvard Medical School, Boston, MA

12: Evergrande Center for Immunologic Diseases, Harvard Medical School and Brigham and Women's Hospital, Boston, MA

24 13: Laboratory of Molecular Oncology, Institut de recherches cliniques de Montréal, Montréal,
25 Québec, Canada
26 14: Department of Medicine, University of Montréal, Montréal, Québec, Canada
27 15: Department of Medicine, McGill University, Montréal, Québec, Canada
28 16: Division of Hematology, Department of Medicine, University of Ottawa, Ottawa, ON
29
30 * Correspondence:
31 Michele Ardolino
32 501 Smyth Road, Cancer Center, 3-328, Ottawa, ON, K1H 8M2
33 m.ardolino@uottawa.ca
34 Tel: +1-613-737-8899 ext 77257

Once sentence summary:

Natural Killer cells are inhibited by PD-1 acquired from the surface of tumor cells via trogocytosis.

Abstract:

Leucocytes often perform trogocytosis, the process by which cells acquire parts of the plasma membrane from interacting cells. Accumulating evidence indicates that trogocytosis modulates immune responses, but the underlying molecular mechanisms are unclear. Here, using two mouse models of leukemia, we found that cytotoxic lymphocytes perform trogocytosis at high rates with tumor cells. While performing trogocytosis, both Natural Killer and $CD8^+$ T cells acquire the checkpoint receptor PD-1 from leukemia cells. In vitro and in vivo investigation revealed that PD-1 protein found on the surface of Natural Killer cells, rather than being endogenously expressed, was derived entirely from leukemia cells. Mechanistically, SLAM receptors were essential for PD-1 trogocytosis. PD-1 acquired via trogocytosis actively suppressed anti-tumor immunity, as revealed by the positive outcome of PD-1 blockade in PD-1-deficient mice. PD-1 trogocytosis was corroborated in patients with clonal plasma cell disorders, where Natural Killer cells that stained for PD-1 also stained for tumor cell markers. Our results, in addition to shedding light on a previously unappreciated mechanism underlying the presence of PD-1 on Natural Killer and cytotoxic T cells, reveal the immune-regulatory effect of membrane transfer occurring when immune cells contact tumor cells.

54 **Introduction:**

55 During trogocytosis immune cells acquire parts of the membrane of cells they interact with (*1, 2*).
56 First characterized in αβ-T cells (*3-8*), it later became clear that virtually all immune cells perform
57 trogocytosis (*7, 9-16*). This intercellular transfer of membranes results in the acquisition of proteins
58 that would otherwise not be endogenously expressed by the cell performing trogocytosis, as in the
59 case of NK cells that acquire viral proteins from infected cells (*17, 18*), or cancer antigens from
60 tumor cells (*19*). Proteins transferred via trogocytosis are functional and influence the response of
61 the accepting cell (*11, 16, 18, 20-24*). The pathophysiological relevance of trogocytosis is
62 underscored by the high extent that immune cells perform it in the context of infections (*25, 26*),
63 autoimmune diseases (*27*), and cancer (*23, 28, 29*). Natural Killer (NK) cells are important
64 mediators of the response against intracellular pathogens and tumors (*30-32*) and have been
65 amongst the first immune cells shown to perform trogocytosis (*10-12*). Trogocytosis has been
66 reported to contribute to the negative regulation of NK cell responses in different contexts. For
67 example, acquisition of m157 or NKG2D ligands results in sustained and unproductive
68 crosslinking of activating receptors leading to NK cell anergy (*18, 33, 34*), but also promotes NK
69 fratricide (*34, 35*). On the other hand, acquisition of MHC molecules from target cells engaged
70 Ly49 receptors in *cis*, sustaining inhibitory signaling that dampened NK cell activation (*11*).
71 Finally, trogocytosis of HLA-G from cancer cells resulted in the generation of NK cells with
72 suppressive properties (*36*).

73

74 We recently reported that NK cells are suppressed by the checkpoint receptor PD-1 and contribute
75 to the therapeutic efficacy of PD-1/L1 blockade in mouse models of cancer (*37*). These results,
76 corroborated by others (*38-43*), were at least partially confuted by findings that murine and human

77 NK cells fail to endogenously express *Pdcd1* mRNA or PD-1 protein (*44*). In light of our results
78 indicating that PD-1 is found on the surface of NK cells, and considering the high trogocytosis
79 activity of NK cells, we propose that NK cells acquire PD-1 directly from tumor cells. Mechanistic
80 experiments corroborated our hypothesis and revealed that SLAM receptors were essential for
81 PD-1 trogocytosis. Functionally, trogocytosed PD-1 suppressed NK cell mediated cancer
82 immunosurveillance. Finally, analysis of NK cells in patients with clonal plasma cell disorders
83 suggests that PD-1 trogocytosis occurs in cancer patients. Altogether, our data shed light on a new
84 mechanism that regulates NK cell function via acquisition of PD-1 from tumor cells.
85

86 **Materials and Methods.**

87 **Mice and in vivo procedures.**

88 Mice were maintained at the University of Ottawa. *Pdcd1* knockout mice
89 (B6.Cg-Pdcd1tm1.1Shr/J)(*45*) were purchased from The Jackson Laboratory and crossed with
90 C57BL/6J mice purchased from The Jackson Laboratory to obtain *Pdcd1* heterozygous mice.
91 Heterozygous mice were bred to obtain $Pdcd1^{+/+}$ and $Pdcd1^{-/-}$ littermates. $Ncr1^{+/Cre}$ mice (*46*) were
92 kindly gifted by Dr. Vivier (INSERM, Marseille, France) and crossed with $Cd274^{fl/fl}$ mice (*47*),
93 kindly gifted by Dr. Fallon (Trinity College, Dublin, Ireland). Mice were then crossed with
94 $Pdcd1^{-/-}$ mice. $Cd274^{-/-}$ mice (*48*) were obtained from Dr. Sharpe (Harvard Medical School,
95 Boston, MA). SLAM-ko mice (*49*) were donated by Dr. Veillette (Institut de recherches cliniques
96 de Montréal, Montréal, QC). $Itgal1^{-/-}$ mice (*50*) were purchased from The Jackson Laboratory.
97 $Klrk1^{-/-}$ mice (*51*) and B6 Cd45.1 mice were kindly gifted by Dr. Raulet (University of California,
98 Berkeley, Berkeley, CA). NCG mice were purchased from Charles Rivers Laboratories. For all

99 experiments, sex-matched (both males and females) and age-matched (7 to 18 weeks old) mice
100 were used.
101
102 For subcutaneous injections, tumor cells were resuspended in 100 µl PBS and injected in the left
103 flank. Tumors were collected when tumor volume was approximately 300 mm^3. In some
104 experiments, $0.5x10^6$ tumor cells were resuspended in 100 µl Growth Factor Reduced Matrigel
105 (BD) and injected in both the left and right flank of the same mouse.
106
107 Tumor outgrowth of parental or PD-1-deficient RMA-S-*Pdl1* cells was assessed in *Pdcd1*$^{-/-}$ or
108 NCG mice injected with $0.1\ x10^6$ tumor cells resuspended in 100 µl Matrigel.
109
110 For immunotherapy experiments, $0.5x10^6$ tumor cells were resuspended in 100 µl Matrigel mixed
111 with 20 µg of anti-PD-1 (RMP1-14) or control antibody (1-1) (both by Leinco). In some
112 experiments, Fc-silent RMP-14 (*52*) was used.
113
114 When indicated, mice were depleted of NK cells with i.p. injection of 200 µg of NKR-P1C
115 antibody (PK136, Leinco).
116
117 **Cell lines.**
118 All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and maintained
119 in RPMI culture medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, 0.2 mg/ml
120 glutamine, 10 µg/ml gentamycin sulfate, 20 mM HEPES, and 5% FCS. Cell line identity was
121 confirmed by flow cytometry when possible, and cells were regularly tested for mycoplasma.

122

123 **Ex vivo experiments.**

124 Murine splenic NK cells were isolated using the EasySep™ Mouse NK Cell Isolation Kit
125 (StemCell Technologies). In all experiments with isolated NK cells, 1000 U/mL rhIL-2 (NIH BRB
126 Preclinical Repository) was added to the culture medium. In most co-culture experiments NK cells
127 were labelled with Cell Trace Violet proliferation dye (BD Bioscience) and tumor cells with CFSE
128 (Biolegend), or vice-versa. 100,000 NK cells were co-cultured with tumor cells at a 1:1 ratio in 24
129 well plates in a final volume of 1 mL. When whole spleens were used, 200,000 splenocytes were
130 co-cultured with tumor cells at a 2:1 ratio in 6 well plates, in a final volume of 3 ml.

131

132 In ex vivo cytokine stimulation experiments, isolated splenic NK cells were cultured with 10ng/ml
133 or 100ng/ml of IL-15 (Peprotech), 1,000U/ml IL-2, 100ng/ml IL-5 (Peprotech), 100 ng/ml IL-6
134 (Peprotech), 20ng/ml IL-12 (Peprotech) + 100 ng/ml IL-18 (Leinco), 10ng/ml TGF-β1
135 (Peprotech), 1,000U/ml Type I IFN (PBL Assay) or 25nM of the glucocorticoid Corticosterone
136 (Sigma) for 3 days.

137

138 For transwell experiments (0.4 μm filter, Millipore), co-culture was set up in 6-well plates with a
139 final volume of 3 mL.

140

141 In sup transfer experiments, RMA cells were seeded at 200,000 cells/ml and cultured for 3 days.
142 Conditioned media was collected, centrifuged, filtered, diluted 1:1 with fresh media and added to
143 NK cells for 24 hrs.

144

145 For membrane dye transfer experiments, NK cells were labelled with CFSE and RMA cells with
146 CellVue Claret FarRed (Sigma-Aldrich). 10,000 NK cells were then co-cultured with RMA cells
147 at a 1:10 ratio in 96-well V-bottom plates with a final volume of 100 µL.
148
149 In experiments where ATP production was pharmacologically blocked, NK cells were pre-treated
150 with 50 mM of Sodium Azide (Sigma) for 2hrs or with 13 µM of Antimycin-A (Sigma) for 1hr,
151 washed and then incubated with RMA cells for one hour.
152
153 In experiments where PD-L1 was blocked, 5 µg of PD-L1 blocking antibody clone 10F.9G2 (or
154 isotype control) was added to the co-culture.
155
156 In experiments where PD-1 was blocked in vitro, tumor cells were incubated with 5 µg of
157 RMP1-14, Fc-silent RMP1-14 or control isotype for 20 minutes, then NK cells were added to the
158 culture. After 2 days, additional 5 µg of antibodies were added to the co-culture and cells were
159 harvested and analyzed after 24 hrs. To check PD-1 saturation, an aliquot of co-culture or tumor
160 cell alone was stained with directly conjugated RMP1-14 or a non-competing PD-1 antibody
161 (29F.1A12).
162
163 **Flow cytometry.**
164 When needed, tumors were excised from mice, cut in pieces, resuspended in serum-free media,
165 and dissociated using a gentle MACS dissociator (Miltenyi). Following dissociation, the single
166 cell suspension was passed through a 40 µm filter and cells were washed and resuspended in PBS
167 for staining. Spleens were harvested, gently dissociated through a 40 µm filter, washed, and red

168 blood cells were lysed using ACK buffer (Sigma), then washed and resuspended in PBS for
169 staining.
170
171 The cellular preparation was stained with the Zombie NIR Fixable Viability Dye (BioLegend) for
172 20 mins in PBS to label dead cells. Cells were then washed with flow buffer (PBS + 0.5% BSA)
173 and incubated for 20 minutes with purified rat anti-mouse CD16/CD32 (Clone 2.4G2) (BD
174 Biosciences) to block FcγRII/III receptors, followed by washing in flow buffer, and then incubated
175 for a further 20 minutes with primary specific antibodies. Cells were washed and resuspended in
176 flow buffer for sample acquisition or fixed in BD Cytofix/Cytoperm and acquired within 7 days.
177 Flow cytometry was performed using an LSRFortessa (BD) or a Celesta (BD), and data were
178 analyzed with FlowJo software (Tree Star Inc.)
179
180 **Antibodies.**
181 For experiments with murine cells, the following antibodies were used: *i)* from BD Biosciences:
182 anti-CD3ε (clone 145-2C11); anti-CD8a (clone 53-6.7); anti-CD11b (clone M1/70); anti-CD11c
183 (clone HL3); anti-CD45.2 (clone 104); anti-CD49b (clone DX5); anti-CD69 (clone H1.2F3); anti-
184 Ly6G (clone 1A8); anti-NKR-P1C (clone PK136); anti-Sca-1 (clone D7); *ii)* from Biolegend: anti-
185 CD4 (clone RM4-5); anti-CD19 (clone 6D5); anti-TCRvβ12 (clone MR11-1); anti-Thy-1.1 (clone
186 OX-7); anti-F4/80 (clone BM8); anti-Ly6c (clone HK1.4); anti-NKp46 (clone 29A1.4); anti-PD-
187 1 (clone 29F.1A12); anti-PD-L1 (clone 10F.9G2); rat IgG2a isotype control; and mouse-IgG1
188 isotype control; *iii)* from Abcam: anti-CD45.1 (clone A20).
189

190 For experiments with MM patients, the following antibodies were used: anti-CD3 (clone SK7),
191 anti-CD7 (clone M-T701), anti-CD16 (clone 3G8), anti-CD38 (clone HIT2), anti CD45 (clone
192 HI30), anti-CD56 (clone NCAM16.2), anti-CD138 (clone MI15) and anti-PD1 (clone EH12.1), all
193 from BD Biosciences.
194
195 **Generation of cell line variants.**
196 RMA, and C1498 cells were transduced with the retroviral expression vector
197 MSCV-IRES-Thy1.1-DEST (Addgene, 17442), by spin infection (800 x g for 2 hours at 37°C)
198 with 8 µg/ml polybrene, and Thy1.1+ cells were sorted.
199
200 Single-guide RNA (sgRNA) targeting the first exon of the *Pdcd1* gene (sequence:
201 TGTGGGTCCGGCAGGTACCC) was cloned into the LentiCRISPR lentiviral backbone vector
202 (Addgene 52961), also containing the *Cas9* gene. Lentiviral expression vectors were generated by
203 transfecting 293T cells with 2 µg vector with 2 µg packaging plus polymerase-encoding plasmids
204 using Lipofectamine 2000. Virus-containing supernatants were used to transduce RMA-*Thy1.1*
205 cells by spin infection and PD-1 negative cells were sorted.
206
207 C1498-PD-1+ cells were obtained by sorting PD-1+ C1498 parental cells.
208
209 Generation of RMA-S-*Pdl1* cells was previously described(*37*).
210
211 All engineered cells were regularly assessed for phenotype maintenance by flow cytometry.
212

213 **ATAC-seq.**

214 Genomic snapshots were generated using IGV software (Broad Institute) using data available on
215 GEO: GSE77695(*53*) and GEO: GSE145299(*54*).

216

217 **Analysis of patients.**

218 BM aspirates were obtained from patients with clonal plasma cell disorder enrolled at the Ottawa
219 Hospital Research Institute and at the Division of Hematology ("Sapienza" University of Rome).
220 BM samples were lysed using a buffer composed of 1.5 M NH_4Cl, 100 mM $NaHCO_3$, and 10 mM
221 EDTA and then stained as described above.

222

223 **Statistical analysis.**

224 Differences in tumor growth curves were analyzed with a Two-way ANOVA. Comparison
225 between two groups were performed with Student's t-test (two tailed, paired or unpaired).
226 Comparison between three groups were performed with ANOVA. $p<0.05$ was considered a
227 statistically significant difference.

228

229 **Study approvals.**

230 Mouse studies were reviewed and approved by Animal Care Veterinary Services at the University
231 of Ottawa in accordance with the guidelines of Canadian Institutes of Health Research. For human
232 studies, informed and written consent in accordance with the Declaration of Helsinki was obtained
233 from all patients, and approval was obtained from the Ethics Committee of the Sapienza University
234 of Rome (RIF.CE: 5191) or of The Ottawa Hospital (REB 20180221-02H).

235

236 **Results:**

237 **Natural Killer cells acquire PD-1 from tumor cells.**

238 Consistent with what has been previously been reported (*37, 44*) murine NK cells stimulated ex
239 vivo with a panel of inflammatory mediators failed to upregulate PD-1 at the protein level (Fig.
240 S1). Lack of PD-1 induction was in line with epigenetic analysis of the *Pdcd1* locus, which was
241 not accessible in splenic NK cells, either before or after cytokine stimulation, in sharp contrast
242 with the promoter of another checkpoint receptor (*Tigit*) in NK cells, or *Pdcd1* locus in $CD8^+$ T
243 cells (Fig. S2).

244

245 Considering the conflicting evidence regarding PD-1 expression on NK cells (*37, 43, 44*) we
246 hypothesized that rather than endogenously expressing the protein, NK cells acquired PD-1 from
247 other cells. To test this hypothesis, we initially used RMA cells, which derive from transformation
248 of murine T cells (*55*), express high levels of PD-1 (Fig. 1A, in red), and were used extensively in
249 our previous study (*37*). We generated RMA cells expressing the syngeneic marker Thy-1.1 (not
250 expressed by C57BL/6 mice, which express the Thy-1.2 allelic variant) and targeted PD-1 with
251 CRISPR/Cas9 (RMA-*Pdcd1*$^{-/-}$*Thy1.1*) (Fig. 1A, in blue and purple, respectively). We then
252 co-cultured tumor cells with splenocytes from *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ littermates with RMA cells
253 expressing PD-1 or not. In the absence of tumor cells, immune cells did not stain for PD-1 or
254 Thy-1.1. In sharp contrast, NK, T and B cells from both *Pdcd1*$^{+/+}$ and *Pdcd1*$^{-/-}$ mice stained
255 positively for PD-1 when incubated with RMA cells, but not RMA-*Pdcd1*$^{-/-}$ cells (Fig. 1B),
256 indicating that PD-1 was not endogenously expressed by innate and adaptive lymphocytes, but
257 acquired from tumor cells in these settings. Consistent results were obtained by using NK cells
258 isolated from *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ mice (purity ~90%) (Fig. 1C and Fig. S3A). Regardless of PD-1

259 expression on tumor cells, Thy-1.1 was detected in abundance on the surface of immune cells (Fig.
260 1B-C, Fig. S3B). Acquisition of PD-1 and Thy-1.1 by NK cells was tightly correlated (Fig. S3C),
261 suggesting that the two molecules were transferred to NK cells as part of a unique phenomenon.
262 To determine if other proteins endogenously expressed by RMA cells were acquired by NK cells,
263 we co-cultured CD45.1-expressing NK cells with RMA cells (which express CD45.2). In addition
264 to PD-1 and Thy-1.1, NK cells also acquired TCRvβ12 and CD45.2 (Fig. S4), although the staining
265 was weaker than for PD-1 and Thy-1.1. These data indicate that when interacting with RMA cells,
266 NK cells acquire several proteins they would not endogenously express.
267
268 To expand on these results, we next employed C1498 cells, an often-used model of leukemia(*56-*
269 *58*). A fraction of C1498 cells (~5%) endogenously expressed PD-1 in culture (Fig. 2A). We sorted
270 PD-1^{+}C1498 cells, confirmed that they stably expressed PD-1 upon 2 weeks in culture (Fig. 2B),
271 and then incubated them with splenocytes from *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ littermates. In accordance
272 with the results obtained with RMA cells, both NK cells and CD8^{+} T cells from *Pdcd1*$^{-/-}$ mice
273 acquired PD-1 when incubated with C1498 cells, and more so if tumor cells had higher PD-1
274 expression (Fig. 2C). PD-1 staining observed in *Pdcd1*$^{-/-}$ mice was very similar to what observed
275 in the *Pdcd1*$^{+/+}$ littermate controls, suggesting that even in the C1498 model, the most PD-1 was
276 not endogenously expressed by immune cells, but rather came from the tumor cells. Similar
277 experiments were repeated using purified NK cells from *Pdcd1*$^{-/-}$ NK cells. After 24 hours, NK
278 cells incubated with PD-1+C1498 cells stained positively for PD-1 (Fig. 2D). PD-1 staining was
279 further increased at 72 hours, when we also observed a shift in NK cells incubated with C1498
280 parental cells (Fig. 2D). Taken together, these data show that NK cells and CD8 T cells acquire
281 PD-1 from leukemia tumor cell lines in vitro.

282

283 **Trogocytosis is responsible for intercellular transfer of PD-1 from tumor to NK cells.**

284 Once we established that NK cells acquired PD-1 from tumor cells, we next investigated whether
285 trogocytosis was responsible for PD-1 transfer. Cell-cell contact is required for trogocytosis.
286 Consistent with our hypothesis that PD-1 is acquired by trogocytosis, *Pdcd1*$^{-/-}$ NK cells cultured
287 in transwell with tumor cells (where physical interaction between the two cell types is precluded)
288 failed to stain for PD-1 and Thy-1.1 (Fig. 3A). Further, NK cells incubated with supernatant
289 conditioned by RMA cells failed to stain for PD-1 (Fig. 3B). These experiments reveal that cell-cell
290 contact is required for PD-1 acquisition by NK cells, and suggest that soluble or exosomal PD-1
291 is not responsible for PD-1 transfer.

292

293 Blocking ATP synthesis is known to interfere with trogocytosis.(*10*) Consistent with the idea that
294 PD-1 is acquired via trogocytosis by NK cells, pretreatment of NK cells with sodium azide or
295 Antimycin-A, which both prevent ATP synthesis, resulted in a strong reduction of PD-1 and
296 Thy-1.1 acquisition (Fig. 3C).

297

298 Transfer of proteins via trogocytosis is accompanied by transfer of membrane lipids. PD-1 transfer
299 was coupled with acquisition of lipids from tumor cells, as revealed by experiments wherein NK
300 cells were co-cultured with RMA cells previously labelled with Cell-Vue, a dye that intercalates
301 in the lipid regions of the cellular membrane (Fig. 3D). Not only did NK cells become robustly
302 positive for the dye, but PD-1 staining was more abundantly detected on NK cells that also
303 acquired lipids from tumor cells (Fig. 3D). These experiments indicate that PD-1, together with

304 other proteins, is acquired contextually with transfer of whole membrane fragments, which is
305 consistent with trogocytosis.
306
307 **SLAM receptors are required for NK cells to trogocytose PD-1 from tumor cells.**
308 Acquisition of proteins from donor cells can be facilitated by receptor-ligand engagement, a
309 process known as trans-endocytosis, which NK cells are known to mediate (*59*). In culture, NK
310 cells fail to express PD-L2 but express PD-L1 (Fig. 4A and (*60*)), which could therefore serve as
311 a ligand for trans-endocytosis-driven PD-1 acquisition. However, a saturating dose of PD-L1
312 blocking antibody did not reduce PD-1 acquisition (Fig. 4B-C) as we would expect if
313 trans-endocytosis was involved. Similar results were obtained blocking PD-1 on RMA cells with
314 an antibody that prevents binding of PD-L1. Despite the antibodies saturated PD-1 on the
315 membrane of RMA cells (Fig. S5A), PD-1 and Thy-1.1 were still effectively transferred to NK
316 cells (Fig. S5B). These experiments not only suggest that PD-1/PD-L1 binding is not required for
317 PD-1 transfer, but also imply that Fc-receptor engagement by PD-1 antibodies does not facilitate
318 trogocytosis (*61*). Finally, we sought genetic corroboration using PD-L1-deficient NK cells from
319 two different mouse strains: a full body PD-L1 knock-out (*Cd274*$^{-/-}$) (*48*) and an NK cell specific
320 PD-L1 knockout (*Ncr1*$^{+/Cre}$ x *Cd274*$^{fl/fl}$) (*47*) that we crossed with PD-1-deficient mice (*Pdcd1*$^{-/-}$
321 *Ncr1*$^{+/Cre}$ *Cd274*$^{fl/fl}$). PD-L1 deficient NK cells acquired PD-1 and Thy-1.1 at levels similar to NK
322 cells isolated from PD-L1 expressing controls (Fig. 4D-E). PD-L1 was also dispensable for PD-1
323 and Thy-1.1 acquisition in CD8^{+} T cells and B cells (Fig. S6).
324
325 SLAM receptors are important mediators of cell-cell interactions between hematopoietic cells and
326 are abundantly expressed not only by NK cells but also by T and B cells (*62*). Given the broad

327 expression of SLAM family members, their importance in regulating the activation of different
328 immune cells and considering that PD-1 was trogocytosed by both innate and adaptive
329 lymphocytes, we hypothesized that SLAM receptors promoted PD-1 trogocytosis. To test this
330 hypothesis, we cultured splenocytes from mice where the whole *SLAM* locus was deleted (*49*) with
331 tumor cells and then assessed PD-1 and Thy-1.1 staining on NK, T and B cells. Consistent with
332 our hypothesis, NK cells from SLAM-deficient mice failed to acquire PD-1 from RMA cells (Fig.
333 4F-G). Not only was PD-1 acquisition abolished, but more broadly, SLAM-deficient NK cells
334 failed to perform trogocytosis with tumor cells, as revealed by lack of Thy-1.1 transfer (Fig. 4F-G).
335 In addition to NK cells, SLAM-deficient T and B cells also displayed reduced trogocytosis (Fig.
336 S7), confirming that SLAM receptors are key mediators for trogocytosis between immune cells
337 and leukemia cells.
338
339 Given the importance of SLAM receptors in mediating cell-cell interactions, we analyzed if
340 deficiency in other adhesion molecules also interfered with trogocytosis. LFA-1 is a key adhesion
341 molecule, but NK cells lacking expression of CD11a,(*50*) a subunit of LFA-1, did not present a
342 deficit in PD-1 or Thy-1.1 acquisition (Fig. 4H). Similar results were also observed analyzing
343 $CD8^+$ T and B cells (Fig. S8). NKG2D, an activating receptor ubiquitously expressed by NK
344 cells,(*63*) was also not involved in mediating trogocytosis between NK and RMA cells (Fig. S9).
345 Taken together, these results indicate that SLAM receptors, but not other adhesion molecules or
346 activating receptors, mediate PD-1 trogocytosis from tumor to immune cells.
347
348 **Activated NK cells acquire PD-1 from tumor cells in vivo.**

349 Next, we performed in vivo studies to determine if intratumoral NK cells trogocytosed PD-1. We
350 injected *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ littermates with RMA or RMA-*Pdcd1*$^{-/-}$ cells, both expressing
351 Thy-1.1, and when tumors reached ~300 mm^3 we analyzed PD-1 staining on intratumoral
352 lymphocytes. In all cohorts of mice, NK cells infiltrating the tumors stained intensely for Thy-1.1
353 (Fig. 5A-D, Y axis), showing that trogocytosis occurred in vivo. Strikingly, high levels of PD-1
354 were detected on the surface of NK cells only when tumor cells expressed PD-1, not only in
355 *Pdcd1*$^{+/+}$, but also in *Pdcd1*$^{-/-}$ mice (Fig. 5A and C vs B and D). These data show not only that
356 PD-1 is acquired by tumor infiltrating NK cells, but also that trogocytosis is the major mechanism
357 leading to PD-1 presence on the surface of NK cells in the RMA model. Consistent with what was
358 observed on NK cells, CD8$^+$ T cells from *Pdcd1*$^{-/-}$ mice also acquired Thy-1.1 and PD-1 from
359 tumor cells (Fig. 5C). As expected, PD-1 staining in CD8$^+$ T cells was also observed in *Pdcd1*$^{+/+}$
360 mice injected with PD-1-deficient RMA cells, confirming that CD8$^+$ T cells endogenously
361 expressed PD-1 in vivo. In our previous study, we reported that PD-1 staining was higher on
362 activated NK cells (*37*). Analysis of NK and T cells from *Pdcd1*$^{-/-}$ mice infiltrating RMA tumors
363 confirmed that PD-1$^+$ NK and T cells also stained more brightly for activation markers such as
364 Sca-1 and CD69 (Fig. 5E), supporting the idea that the NK cells that are activated by the encounter
365 with tumor cells are also the ones more susceptible to acquiring PD-1 and therefore being inhibited
366 by it.
367
368 To further support our hypothesis that PD-1 was trogocytosed by NK cells in the tumor
369 microenvironment, we injected *Pdcd1*$^{-/-}$ mice with RMA or RMA-*Pdcd1*$^{-/-}$ cells in either flank. As
370 previously reported (*37*), splenic NK cells failed to stain for PD-1 (Fig. 5F). Consistent with what
371 is described in Fig. 5A-D, NK cells in both tumors acquired Thy-1.1 from tumor cells, but only

372 NK cells in PD-1 expressing tumors also stained for PD-1 (Fig. 5F). Taken together, these results
373 highlight how activated NK cells perform trogocytosis and acquire PD-1 in the tumor
374 microenvironment in vivo.
375
376 **PD-1 acquired via trogocytosis inhibits NK cell responses against cancer.**
377 Once we established that NK cells trogocytose PD-1 in vivo, we sought to determine if
378 trogocytosed PD-1 suppressed anti-tumor immunity. For these studies, rather than using RMA
379 cells (which are resistant to both T and NK cell responses (*37, 55, 56, 64*)) we took advantage of
380 RMA-S-*Pdl1* cells we previously generated (*37*). Similar to RMA, these cells express high levels
381 of PD-1, but differently than RMA, they lack MHC class I expression and are therefore susceptible
382 to NK-mediated control (*37*). Using CRISPR/Cas9, we generated an RMA-S-*Pdl1* variant lacking
383 PD-1 expression (Fig. 6A). When injected in *Pdcd1*$^{-/-}$ mice, where the only source of PD-1 on NK
384 cells are tumor cells, we observed a dramatic deceleration in outgrowth of tumor cells lacking
385 PD-1 expression (Fig. 6B). However, lack of PD-1 did not delay cell growth in vitro, nor it
386 prevented RMA-S-*Pdl1-Pdcd1*$^{-/-}$ cells from growing tumors in immunodeficient mice (Fig. 6C).
387 These data indicate that, rather than having cell intrinsic growth defects, PD-1-deficient tumor
388 cells have reduced capacity of forming ectopic tumors as they fail to inhibit NK cells via PD-1
389 transfer.
390
391 Using the RMA-S-*Pdl1* model, we previously showed that PD-1 blockade rescued the ability of
392 NK cells to control tumor growth in vivo (*37*). Considering that PD-1 expression in tumor cells
393 promoted in vivo growth in a cell extrinsic fashion, we reasoned that the therapeutic effect of PD-1
394 blockade should also be observed in *Pdcd1*$^{-/-}$ mice. In accordance with our hypothesis, when we

395 treated PD-1-deficient mice injected with RMA-S-*Pdl1* cells with a PD-1 blocking antibody
396 (RMP1-14) we observed a dramatic reduction in tumor outgrowth (Fig. 6D). On the other hand,
397 when we injected PD-1-deficient mice with PD-1 deficient RMA-S-*Pdl1* cells, PD-1 blockade had
398 no therapeutic effect (Fig. 6E).
399
400 To confirm that NK cells, and not other components of the immune response, were inhibited by
401 tumor derived PD-1, we injected RMA-S-*Pdl1* cells in mice where NK cells were depleted using
402 a monoclonal antibody (PK136) and treated the mice with PD-1 blockade. NK cell depletion was
403 sufficient to abolish the therapeutic effect of the blocking antibody, whereas PD-1 blockade
404 delayed tumor outgrowth in the control group (Fig. 6F). Corroboration of these results came from
405 experiments where PD-1 antibodies failed in immunocompromised mice (Fig. 6G). Moreover, the
406 similar in vivo growth of tumor cells in immunocompromised mice (Fig. 6G) excluded a tumor
407 cell intrinsic effect of PD-1 blocking antibodies.
408
409 Finally, to rule out that the therapeutic effect of PD-1 antibodies was due to Antibody-Dependent
410 Cellular Cytotoxicity (ADCC) potentially mediated by NK cells against cancer cells coated with
411 PD-1 antibodies, we employed an engineered version of anti-PD-1 that lacks the ability to bind to
412 Fc-receptors (Fc-silent RMP1-14) (*52*). Treatment with Fc-silent PD-1 antibodies delayed the
413 outgrowth of PD-1 expressing tumors (Fig. 6H), indicating that the therapeutic effect of PD-1
414 antibodies in *Pdcd1*$^{-/-}$ mice was not due to ADCC. Taken together, these results indicate that
415 trogocytosed PD-1 inhibits the anti-tumor activity of NK cells, which can be rescued by PD-1
416 blocking antibodies.
417

418 **Identification of an NK cells population in multiple myeloma patients staining for tumor cell**
419 **markers and PD-1.**

420 Finally, to determine if NK cells acquire PD-1 from tumor cells in cancer patients, we analyzed
421 PD-1 staining in NK cells from the bone marrow (BM) of patients with clonal plasma cell disorders
422 (Table 1 details patients' information). Pathological analysis showed that of the 28 patients
423 analyzed, 21 were diagnosed with multiple myeloma (MM), 3 with monoclonal gammopathy of
424 undetermined significance (MGUS), 3 with smoldering myeloma, and 1 with solitary
425 plasmacytoma. For these studies, we relied on CD138, a protein frequently expressed by clonal
426 plasma cells but not by NK cells (*65*), as a surrogate trogocytosis marker. Flow cytometry analysis
427 confirmed the presence in several patients of a high scattering $CD138^{+}$ population (Fig. S10), that
428 we identified as clonal plasma cells. In support of our gating strategy, patients diagnosed with MM
429 presented a higher $CD138^{+}$ frequency than MGUS patients. NK cells were instead gated as low
430 scattering events, live, $CD3^{-}CD56^{+}CD16^{+/-}$ and in most samples CD7 was also used (Fig. S10). In
431 the vast majority of samples analyzed, high scattering-$CD138^{+}$ cells expressed PD-1 (Sup. Fig.
432 10). Indication that NK cells performed trogocytosis came from analysis of BM aspirates where a
433 $CD138^{+}$ NK cell population, in both $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cell subsets, was
434 identified (Fig. 7, in pink and green), corroborating the results obtained in murine models. Notably,
435 we found a sizeable and consistent (albeit often small) population of NK cells that stained for both
436 CD138 and PD-1 (Fig. 7, in green), supporting the idea that NK cells in patients with clonal plasma
437 cell disorders acquire PD-1 and cancer cell markers from tumor cells.
438

439 In conclusion, this study identifies trogocytosis as a new mechanism by which PD-1 is acquired
440 from tumor cells. by NK and T cells. PD-1 trogocytosis strongly relies on SLAM receptors and
441 functionally suppresses the ability of NK cells to eliminate tumors in vivo.
442
443 **Discussion.**
444 The nature of PD-1 expression on NK cells remains fairly elusive, with contrasting
445 evidence indicating that PD-1 is either expressed on not expressed in NK cells (*43, 44*).
446 Considering the importance of PD-1 in suppressing the immune response to cancer (*66*), and given
447 the tremendous interest in the development of NK cell based cancer immunotherapies (*32, 67*),
448 understanding whether PD-1 directly inhibits NK cell function is of the utmost importance. We
449 recently reported that PD-1 suppresses NK cells in several mouse models of cancer (*37*), but
450 previously have not yet deciphered the mechanisms leading to PD-1 upregulation in murine NK
451 cells infiltrating lymphoma mouse models. The lack of PD-1 induction in NK cells following
452 several ex vivo stimulations, combined with the analysis of the *Pdcd1* locus in resting and cytokine
453 stimulated NK cells, prompted us to hypothesize that PD-1 was not endogenously expressed by
454 NK cells but rather be derived from other sources. Several cellular processes have been shown to
455 be responsible for protein transfer. Amongst these processes, trogocytosis, the intercellular
456 exchange of whole membrane fragments, is highly performed by NK cells (*10-12*). Proteins
457 transferred via trogocytosis can have a substantial impact in immune function (*1, 2*). In cancer,
458 trogocytosis has been associated with reduced immune responses and with the failure of
459 immunotherapy. For example, a recent study highlighted how CAR T cells trogocytose antigens
460 from tumor cells and become susceptible to fratricide, greatly limiting the response to cellular
461 therapy (*68*). Trogocytosis triggered by Fc-receptors engaging therapeutic antibodies, performed

462 by myeloid and NK cells, has been a major hurdle limiting the efficacy of monoclonal antibodies
463 against cancer antigens (*15, 61, 69-85*). Despite such evidence and the immense interest in
464 elucidating mechanisms underlying resistance to PD-1 blockade, whether PD-1 is trogocytosed by
465 immune cells has been largely unexplored. Here we show that in some contexts trogocytosis is a
466 major mechanism by which PD-1 becomes localized on the surface of immune cells. This was true
467 not only for NK cells, but also for adaptive lymphocytes. PD-1 acquisition happened in a cell-cell
468 contact dependent fashion, contextualized within the transfer of other proteins and whole
469 membrane fragments and was strongly suppressed by ATP depletion, indicating that PD-1 was
470 trogocytosed by immune cells. Interestingly, PD-1 antibodies did not elicit PD-1 trogocytosis by
471 NK cells, suggesting that PD-1 could be acquired by NK cells even in the absence of Fc-receptor
472 engagement. Mechanistic studies using blocking antibodies and transgenic mice allowed us to
473 exclude a role for PD-L1, abundantly expressed by NK cells, in PD-1 acquisition, ruling out
474 trans-endocytosis as a mechanism of PD-1 transfer. On the other hand, receptors belonging to the
475 SLAM family proved to be essential for intercellular transfer of PD-1 from tumor to immune cells.
476 SLAM receptors are important regulators of immune function and ubiquitously expressed by NK
477 cells (*62*), but also by tumors of hematopoietic origin, including multiple myeloma (*86*). Our
478 finding that SLAMs promote the transfer of PD-1 from tumor to immune cells requires
479 consideration of trogocytosis as an important biological variable when designing mono -or
480 combination therapies targeting these receptors.

481 Trogocytosed PD-1 was functional and suppressed the anti-cancer activity of NK cells. The
482 in vivo studies performed here further expand on our previous findings that NK cells contribute to
483 the therapeutic efficacy of PD-1 blockade (*37*), and explain why checkpoint blockade relies on NK
484 cells despite their lack of PD-1 expression.

485 While more translational studies are required to follow up on this mechanistic data, we
486 successfully identified a subset of NK cells which stained for CD138 in the bone marrow of
487 patients with clonal plasma cell disorders. As CD138 is not expressed by NK cells, we relied on
488 CD138 staining to identify bone marrow NK cells that performed trogocytosis. Consistent with
489 our in vivo results, $CD138^{+}$ NK cells also stained for PD-1, and flow cytometry and bioinformatic
490 analysis of a published dataset indicated that multiple myeloma cells can express PD-1 (*87*). Based
491 on our in vivo results, we propose that PD-1 expression, in addition to benefiting cancer cells with
492 intrinsic signaling (*88*), also promotes immune escape. In fact, tumor cells expressing PD-1 can
493 donate this powerful inhibitory receptor to activated immune cells when they are in direct contact.
494 PD-1 acquisition can however be therapeutically abrogated by checkpoint blockade, potentially
495 rescuing the ability of NK cells to promote anti-cancer immunity.

496 In our current analysis (and differing from our murine results) PD-1 was also found in a
497 fraction of human NK cells that did not stain for CD138. These data are consistent with the idea
498 that human NK cells endogenously express PD-1, as recently corroborated in healthy donors and
499 patients undergoing hematopoietic stem cell transplantation (*43*). Endogenous expression of PD-1
500 does not exclude the possibility that immune cells also rely on trogocytosis to gain further PD-1
501 protein from neighbor cells. This notion is well supported by a recent study that identified
502 trogocytosing NK cells in a broad spectrum of hematopoietic malignancies (*89*). In accordance
503 with our data, NK cells labelled with tumor cell markers also stained for PD-1 (*89*). Whether
504 endogenously expressed by NK cells or acquired from cancer or other immune cells, several
505 reports, including the present one, have highlighted the importance of PD-1 in suppressing NK
506 cells (*37-43*).

507 Finally, in light of these results, it will be important for future immune-profiling efforts
508 based on transcriptomic analysis to take into account that proteins are acquired, sometimes at
509 surprisingly high levels, by immune cells in the tumor microenvironment. Pursuant to our previous
510 studies and given its known importance in suppressing anti-cancer responses we focused on PD-1;
511 however, it is conceivable that other proteins with immunomodulatory potential will be acquired
512 by NK and T cells while interacting with tumor cells. Further characterization of the mechanisms
513 underlying membrane transfer and identification of molecules transferred to immune cells is
514 required to elucidate how immune cells are regulated by checkpoint receptors, and other proteins,
515 in a transcription-independent fashion.
516

517 **Acknowledgment:**
518 We thank members of the Ardolino lab, Drs. Horan, Vanderhyden, Gray, Scott,
519 Bourgeois-Daigneault, Kissov and Piconese for critically reading the manuscript; Fernando Ortiz,
520 Drs. Tang, Stanford, Ito and the CHEO flow-core for support with flow-cytometry; the ACVS
521 facility at the University of Ottawa for support with animal studies. MA is supported by Ride for
522 Dad, CIHR and Myeloma Canada, HYS by the NIH intramural research programs, GS and AS by
523 AIRC, AZ by Sapienza University, PGF by Science Foundation Ireland, AHS by NIH (PO1 grant
524 56299). EV is supported by an AIRC fellowship. MM is the recipient of a CAAIF postdoctoral
525 fellowship. JJH and DC are recipients of a Frederick Banting and Charles Best Canada Graduate
526 Scholarships Doctoral Award from CIHR. We are thankful to Dr. Ravetch (Rockefeller
527 University) for providing us with Fc-silent PD-1 antibodies and to Dr. Raulet, for providing us
528 with mouse lines and continuous support. This manuscript is dedicated to the memory of Dr. Lucas
529 Horan, who loved science and the Bay.

530

531 **Authorship contributions.**

532 Author contributions are detailed according to CRediT criteria.

Author	Conceptualization	Formal analysis	Funding acquisition	Investigation	Methodology	Resources	Supervision	Visualization	Writing - original draft	Writing review - & editing
MSH	X			X	X					X
MM		X		X						X
JJH				X						X
EV				X					X	X
HYS	X	X	X					X		X
AS				X						X
OMM				X						X
FGA				X						X
KPB						X				X
DC		X								X
MTP						X				X
AS			X			X	X			X
PGF						X				X
AS						X				X
GS	X	X	X				X			X
AV						X				X
AZ	X		X			X	X			X
AMC	X		X			X				X
MA	X	X	X	X	X		X	X	X	X

533

534 **Disclosure of conflicts of interests:** MA received monetary compensation from Alloy
535 Therapeutics for consulting. MA is under a contract agreement to perform sponsored research with
536 Actym Therapeutics. Neither consulting nor sponsored research are related to the present article.
537 The other authors have declared that no conflict of interest exists.

538

539 **References:**

540 1. D. Hudrisier, E. Joly, What is trogocytosis and what is its purpose? *Nat Immunol* **4**, 815
541 (2003).
542 2. A. Bettadapur, H. W. Miller, K. S. Ralston, Biting off what can be chewed: Trogocytosis in
543 health, infection and disease. *Infect Immun*, (2020).
544 3. J. F. Huang *et al.*, TCR-Mediated internalization of peptide-MHC complexes acquired by T
545 cells. *Science* **286**, 952-954 (1999).
546 4. I. Hwang *et al.*, T cells can use either T cell receptor or CD28 receptors to absorb and
547 internalize cell surface molecules derived from antigen-presenting cells. *J Exp Med* **191**,
548 1137-1148 (2000).

549 5. D. Hudrisier, J. Riond, H. Mazarguil, J. E. Gairin, E. Joly, Cutting edge: CTLs rapidly capture
550 membrane fragments from target cells in a TCR signaling-dependent manner. *J Immunol*
551 **166**, 3645-3649 (2001).
552 6. D. Hudrisier, J. Riond, L. Garidou, C. Duthoit, E. Joly, T cell activation correlates with an
553 increased proportion of antigen among the materials acquired from target cells. *Eur J*
554 *Immunol* **35**, 2284-2294 (2005).
555 7. D. Hudrisier, A. Aucher, A. L. Puaux, C. Bordier, E. Joly, Capture of target cell membrane
556 components via trogocytosis is triggered by a selected set of surface molecules on T or B
557 cells. *J Immunol* **178**, 3637-3647 (2007).
558 8. D. Hudrisier *et al.*, Ligand binding but undetected functional response of FcR after their
559 capture by T cells via trogocytosis. *J Immunol* **183**, 6102-6113 (2009).
560 9. F. D. Batista, D. Iber, M. S. Neuberger, B cells acquire antigen from target cells after
561 synapse formation. *Nature* **411**, 489-494 (2001).
562 10. L. M. Carlin, K. Eleme, F. E. McCann, D. M. Davis, Intercellular transfer and supramolecular
563 organization of human leukocyte antigen C at inhibitory natural killer cell immune
564 synapses (vol 194, pg 1507, 2001). *Journal of Experimental Medicine* **194**, 1883 (2001).
565 11. A. Sjostrom *et al.*, Acquisition of external major histocompatibility complex class I
566 molecules by natural killer cells expressing inhibitory Ly49 receptors. *J Exp Med* **194**, 1519-
567 1530 (2001).
568 12. J. Tabiasco *et al.*, Active trans-synaptic capture of membrane fragments by natural killer
569 cells. *Eur J Immunol* **32**, 1502-1508 (2002).
570 13. O. B. Herrera *et al.*, A novel pathway of alloantigen presentation by dendritic cells. *J*
571 *Immunol* **173**, 4828-4837 (2004).
572 14. A. Aucher, E. Magdeleine, E. Joly, D. Hudrisier, Capture of plasma membrane fragments
573 from target cells by trogocytosis requires signaling in T cells but not in B cells. *Blood* **111**,
574 5621-5628 (2008).
575 15. S. Daubeuf, M. A. Lindorfer, R. P. Taylor, E. Joly, D. Hudrisier, The direction of plasma
576 membrane exchange between lymphocytes and accessory cells by trogocytosis is
577 influenced by the nature of the accessory cell. *J Immunol* **184**, 1897-1908 (2010).
578 16. K. Miyake *et al.*, Trogocytosis of peptide-MHC class II complexes from dendritic cells
579 confers antigen-presenting ability on basophils. *Proc Natl Acad Sci U S A* **114**, 1111-1116
580 (2017).
581 17. J. Tabiasco *et al.*, Acquisition of viral receptor by NK cells through immunological synapse.
582 *J Immunol* **170**, 5993-5998 (2003).
583 18. C. A. Miner, T. K. Giri, C. E. Meyer, M. Shabsovich, S. K. Tripathy, Acquisition of activation
584 receptor ligand by trogocytosis renders NK cells hyporesponsive. *J Immunol* **194**, 1945-
585 1953 (2015).
586 19. N. Stern-Ginossar *et al.*, Intercellular transfer of carcinoembryonic antigen from tumor
587 cells to NK cells. *J Immunol* **179**, 4424-4434 (2007).
588 20. D. M. Patel, P. Y. Arnold, G. A. White, J. P. Nardella, M. D. Mannie, Class II MHC/peptide
589 complexes are released from APC and are acquired by T cell responders during specific
590 antigen recognition. *J Immunol* **163**, 5201-5210 (1999).

591 21. J. Zimmer, V. Ioannidis, W. Held, H-2D ligand expression by Ly49A+ natural killer (NK) cells
592 precludes ligand uptake from environmental cells: implications for NK cell function. *J Exp*
593 *Med* **194**, 1531-1539 (2001).
594 22. J. LeMaoult *et al.*, Immune regulation by pretenders: cell-to-cell transfers of HLA-G make
595 effector T cells act as regulatory cells. *Blood* **109**, 2040-2048 (2007).
596 23. R. Brown *et al.*, CD86+ or HLA-G+ can be transferred via trogocytosis from myeloma cells
597 to T cells and are associated with poor prognosis. *Blood* **120**, 2055-2063 (2012).
598 24. J. Reed, S. A. Wetzel, Trogocytosis-Mediated Intracellular Signaling in CD4(+) T Cells Drives
599 TH2-Associated Effector Cytokine Production and Differentiation. *J Immunol* **202**, 2873-
600 2887 (2019).
601 25. U. Tomaru *et al.*, Detection of virus-specific T cells and CD8+ T-cell epitopes by acquisition
602 of peptide-HLA-GFP complexes: analysis of T-cell phenotype and function in chronic viral
603 infections. *Nat Med* **9**, 469-476 (2003).
604 26. K. Rosenits, S. J. Keppler, S. Vucikuja, P. Aichele, T cells acquire cell surface determinants
605 of APC via in vivo trogocytosis during viral infections. *Eur J Immunol* **40**, 3450-3457 (2010).
606 27. B. Haastert, R. J. Mellanby, S. M. Anderton, R. A. O'Connor, T cells at the site of
607 autoimmune inflammation show increased potential for trogocytosis. *PLoS One* **8**, e81404
608 (2013).
609 28. R. Brown *et al.*, Trogocytosis generates acquired regulatory T cells adding further
610 complexity to the dysfunctional immune response in multiple myeloma.
611 *Oncoimmunology* **1**, 1658-1660 (2012).
612 29. G. Eisenberg *et al.*, Imprinting of lymphocytes with melanoma antigens acquired by
613 trogocytosis facilitates identification of tumor-reactive T cells. *J Immunol* **190**, 5856-5865
614 (2013).
615 30. E. Vivier *et al.*, Innate or adaptive immunity? The example of natural killer cells. *Science*
616 **331**, 44-49 (2011).
617 31. A. Iannello, T. W. Thompson, M. Ardolino, A. Marcus, D. H. Raulet, Immunosurveillance
618 and immunotherapy of tumors by innate immune cells. *Current opinion in immunology*
619 **38**, 52-58 (2015).
620 32. J. J. Hodgins, S. T. Khan, M. M. Park, R. C. Auer, M. Ardolino, Killers 2.0: NK cell therapies
621 at the forefront of cancer control. *J Clin Invest* **129**, 3499-3510 (2019).
622 33. P. Roda-Navarro, M. Vales-Gomez, S. E. Chisholm, H. T. Reyburn, Transfer of NKG2D and
623 MICB at the cytotoxic NK cell immune synapse correlates with a reduction in NK cell
624 cytotoxic function. *Proc Natl Acad Sci U S A* **103**, 11258-11263 (2006).
625 34. F. E. McCann, P. Eissmann, B. Onfelt, R. Leung, D. M. Davis, The activating NKG2D ligand
626 MHC class I-related chain A transfers from target cells to NK cells in a manner that allows
627 functional consequences. *J Immunol* **178**, 3418-3426 (2007).
628 35. K. Nakamura *et al.*, Fratricide of natural killer cells dressed with tumor-derived NKG2D
629 ligand. *Proc Natl Acad Sci U S A* **110**, 9421-9426 (2013).
630 36. J. Caumartin *et al.*, Trogocytosis-based generation of suppressive NK cells. *Embo J* **26**,
631 1423-1433 (2007).
632 37. J. Hsu *et al.*, Contribution of NK cells to immunotherapy mediated by PD-1/PD-L1
633 blockade. *J Clin Invest* **128**, 4654-4668 (2018).

634 38. D. M. Benson *et al.*, The PD-1/PD-L1 axis modulates the natural killer cell versus multiple
635 myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody.
636 *Blood* **116**, 2286-2294 (2010).
637 39. A. Beldi-Ferchiou *et al.*, PD-1 mediates functional exhaustion of activated NK cells in
638 patients with Kaposi sarcoma. *Oncotarget* **7**, 72961-72977 (2016).
639 40. S. Pesce *et al.*, Identification of a subset of human natural killer cells expressing high levels
640 of programmed death 1: A phenotypic and functional characterization. *J Allergy Clin*
641 *Immunol* **139**, 335-346.e333 (2017).
642 41. Y. Liu *et al.*, Increased expression of programmed cell death protein 1 on NK cells inhibits
643 NK-cell-mediated anti-tumor function and indicates poor prognosis in digestive cancers.
644 *Oncogene* **36**, 6143-6153 (2017).
645 42. F. Vari *et al.*, Immune evasion via PD-1/PD-L1 on NK cells and monocyte/macrophages is
646 more prominent in Hodgkin lymphoma than DLBCL. *Blood* **131**, 1809-1819 (2018).
647 43. Z. Davis *et al.*, Low-density PD-1 expression on resting human natural killer cells is
648 functional and upregulated after transplantation. *Blood Advances* **5**, 1069-1080 (2021).
649 44. S. J. Judge *et al.*, Minimal PD-1 expression in mouse and human NK cells under diverse
650 conditions. *J Clin Invest*, (2020).
651 45. M. E. Keir, G. J. Freeman, A. H. Sharpe, PD-1 regulates self-reactive CD8+ T cell responses
652 to antigen in lymph nodes and tissues. *J Immunol* **179**, 5064-5070 (2007).
653 46. E. Narni-Mancinelli *et al.*, Fate mapping analysis of lymphoid cells expressing the NKp46
654 cell surface receptor. *Proc Natl Acad Sci U S A* **108**, 18324-18329 (2011).
655 47. C. Schwartz *et al.*, ILC2s regulate adaptive Th2 cell functions via PD-L1 checkpoint control.
656 *Journal of Experimental Medicine* **214**, 2507-2521 (2017).
657 48. Y. E. Latchman *et al.*, PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting
658 cells, and host tissues negatively regulates T cells. *Proc Natl Acad Sci U S A* **101**, 10691-
659 10696 (2004).
660 49. H. Guo *et al.*, Deletion of Slam locus in mice reveals inhibitory role of SLAM family in NK
661 cell responses regulated by cytokines and LFA-1. *J Exp Med* **213**, 2187-2207 (2016).
662 50. Z. M. Ding *et al.*, Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and
663 migration. *J Immunol* **163**, 5029-5038 (1999).
664 51. N. Guerra *et al.*, NKG2D-deficient mice are defective in tumor surveillance in models of
665 spontaneous malignancy. *Immunity* **28**, 571-580 (2008).
666 52. R. Dahan *et al.*, FcgammaRs Modulate the Anti-tumor Activity of Antibodies Targeting the
667 PD-1/PD-L1 Axis. *Cancer Cell* **28**, 285-295 (2015).
668 53. H. Y. Shih *et al.*, Developmental Acquisition of Regulomes Underlies Innate Lymphoid Cell
669 Functionality. *Cell* **165**, 1120-1133 (2016).
670 54. G. Sciume *et al.*, Rapid Enhancer Remodeling and Transcription Factor Repurposing
671 Enable High Magnitude Gene Induction upon Acute Activation of NK Cells. *Immunity* **53**,
672 745-758 e744 (2020).
673 55. K. Kärre, H. G. Ljunggren, G. Piontek, R. Kiessling, Selective rejection of H-2-deficient
674 lymphoma variants suggests alternative immune defence strategy. *Nature* **319**, 675-678
675 (1986).
676 56. M. Ardolino *et al.*, Cytokine therapy reverses NK cell anergy in MHC-deficient tumors. *J*
677 *Clin Invest* **124**, 4781-4794 (2014).

678 57. E. Curran *et al.*, STING Pathway Activation Stimulates Potent Immunity against Acute
679 Myeloid Leukemia. *Cell Rep* **15**, 2357-2366 (2016).
680 58. M. Ruzicka *et al.*, RIG-I-based immunotherapy enhances survival in preclinical AML
681 models and sensitizes AML cells to checkpoint blockade. *Leukemia* **34**, 1017-1026 (2020).
682 59. O. M. Anton *et al.*, Trans-endocytosis of intact IL-15Ralpha-IL-15 complex from presenting
683 cells into NK cells favors signaling for proliferation. *Proc Natl Acad Sci U S A* **117**, 522-531
684 (2020).
685 60. W. Dong *et al.*, The Mechanism of Anti-PD-L1 Antibody Efficacy against PD-L1-Negative
686 Tumors Identifies NK Cells Expressing PD-L1 as a Cytolytic Effector. *Cancer discovery* **9**,
687 1422-1437 (2019).
688 61. R. P. Taylor, M. A. Lindorfer, Fcγ-receptor–mediated trogocytosis impacts mAb-based
689 therapies: historical precedence and recent developments. *Blood* **125**, 762-766 (2015).
690 62. N. Wu, A. Veillette, SLAM family receptors in normal immunity and immune pathologies.
691 *Current opinion in immunology* **38**, 45-51 (2016).
692 63. E. O. Long, H. S. Kim, D. Liu, M. E. Peterson, S. Rajagopalan, Controlling natural killer cell
693 responses: integration of signals for activation and inhibition. *Annu Rev Immunol* **31**, 227-
694 258 (2013).
695 64. H. G. Ljunggren, K. Kärre, Host resistance directed selectively against H-2-deficient
696 lymphoma variants. Analysis of the mechanism. *J Exp Med* **162**, 1745-1759 (1985).
697 65. E. A. Stadtmauer *et al.*, Long-term safety and activity of NY-ESO-1 SPEAR T cells after
698 autologous stem cell transplant for myeloma. *Blood Adv* **3**, 2022-2034 (2019).
699 66. A. H. Sharpe, K. E. Pauken, The diverse functions of the PD1 inhibitory pathway. *Nat Rev*
700 *Immunol* **18**, 153-167 (2018).
701 67. J. S. Miller, L. L. Lanier, Natural Killer Cells in Cancer Immunotherapy. *Annual Review of*
702 *Cancer Biology* **3**, 77-103 (2019).
703 68. M. Hamieh *et al.*, CAR T cell trogocytosis and cooperative killing regulate tumour antigen
704 escape. *Nature* **568**, 112-116 (2019).
705 69. A. D. Kennedy *et al.*, Rituximab infusion promotes rapid complement depletion and acute
706 CD20 loss in chronic lymphocytic leukemia. *J Immunol* **172**, 3280-3288 (2004).
707 70. P. V. Beum, A. D. Kennedy, M. E. Williams, M. A. Lindorfer, R. P. Taylor, The shaving
708 reaction: rituximab/CD20 complexes are removed from mantle cell lymphoma and
709 chronic lymphocytic leukemia cells by THP-1 monocytes. *J Immunol* **176**, 2600-2609
710 (2006).
711 71. Y. Li *et al.*, Rituximab-CD20 complexes are shaved from Z138 mantle cell lymphoma cells
712 in intravenous and subcutaneous SCID mouse models. *J Immunol* **179**, 4263-4271 (2007).
713 72. P. V. Beum, D. A. Mack, A. W. Pawluczkowycz, M. A. Lindorfer, R. P. Taylor, Binding of
714 rituximab, trastuzumab, cetuximab, or mAb T101 to cancer cells promotes trogocytosis
715 mediated by THP-1 cells and monocytes. *J Immunol* **181**, 8120-8132 (2008).
716 73. P. V. Beum, M. A. Lindorfer, R. P. Taylor, Within peripheral blood mononuclear cells,
717 antibody-dependent cellular cytotoxicity of rituximab-opsonized Daudi cells is promoted
718 by NK cells and inhibited by monocytes due to shaving. *J Immunol* **181**, 2916-2924 (2008).
719 74. B. A. Nijmeijer *et al.*, A mechanistic rationale for combining alemtuzumab and rituximab
720 in the treatment of ALL. *Blood* **116**, 5930-5940 (2010).

721 75. G. Aue *et al.*, Fractionated subcutaneous rituximab is well-tolerated and preserves CD20
722 expression on tumor cells in patients with chronic lymphocytic leukemia. *Haematologica*
723 **95**, 329-332 (2010).
724 76. T. Pham, P. Mero, J. W. Booth, Dynamics of macrophage trogocytosis of rituximab-coated
725 B cells. *PLoS One* **6**, e14498 (2011).
726 77. A. E. Pedersen, M. B. Jungersen, C. D. Pedersen, Monocytes mediate shaving of B-cell-
727 bound anti-CD20 antibodies. *Immunology* **133**, 239-245 (2011).
728 78. P. V. Beum *et al.*, Loss of CD20 and bound CD20 antibody from opsonized B cells occurs
729 more rapidly because of trogocytosis mediated by Fc receptor-expressing effector cells
730 than direct internalization by the B cells. *J Immunol* **187**, 3438-3447 (2011).
731 79. P. Boross, J. H. Jansen, A. Pastula, C. E. van der Poel, J. H. Leusen, Both activating and
732 inhibitory Fc gamma receptors mediate rituximab-induced trogocytosis of CD20 in mice.
733 *Immunology letters* **143**, 44-52 (2012).
734 80. J. D. Jones, B. J. Hamilton, W. F. Rigby, Rituximab mediates loss of CD19 on B cells in the
735 absence of cell death. *Arthritis and rheumatism* **64**, 3111-3118 (2012).
736 81. F. J. Beurskens *et al.*, Exhaustion of cytotoxic effector systems may limit monoclonal
737 antibody-based immunotherapy in cancer patients. *J Immunol* **188**, 3532-3541 (2012).
738 82. N. A. Baig *et al.*, Induced resistance to ofatumumab-mediated cell clearance mechanisms,
739 including complement-dependent cytotoxicity, in chronic lymphocytic leukemia. *J*
740 *Immunol* **192**, 1620-1629 (2014).
741 83. C. S. Zent *et al.*, Chemoimmunotherapy for relapsed/refractory and progressive 17p13-
742 deleted chronic lymphocytic leukemia (CLL) combining pentostatin, alemtuzumab, and
743 low-dose rituximab is effective and tolerable and limits loss of CD20 expression by
744 circulating CLL cells. *Am J Hematol* **89**, 757-765 (2014).
745 84. M. Carlsten *et al.*, Checkpoint Inhibition of KIR2D with the Monoclonal Antibody IPH2101
746 Induces Contraction and Hyporesponsiveness of NK Cells in Patients with Myeloma.
747 *Clinical cancer research : an official journal of the American Association for Cancer*
748 *Research* **22**, 5211-5222 (2016).
749 85. J. Krejcik *et al.*, Monocytes and Granulocytes Reduce CD38 Expression Levels on Myeloma
750 Cells in Patients Treated with Daratumumab. *Clinical cancer research : an official journal*
751 *of the American Association for Cancer Research* **23**, 7498-7511 (2017).
752 86. S. V. Radhakrishnan, N. Bhardwaj, T. Luetkens, D. Atanackovic, Novel anti-myeloma
753 immunotherapies targeting the SLAM family of receptors. *Oncoimmunology* **6**, e1308618-
754 e1308618 (2017).
755 87. Y. C. Cohen *et al.*, Identification of resistance pathways and therapeutic targets in
756 relapsed multiple myeloma patients through single-cell sequencing. *Nat Med*, (2021).
757 88. H. Zha *et al.*, Non-canonical PD-1 signaling in cancer and its potential implications in clinic.
758 *Journal for ImmunoTherapy of Cancer* **9**, e001230 (2021).
759 89. D. N. Vo *et al.*, Dissecting the NK Cell Population in Hematological Cancers Confirms the
760 Presence of Tumor Cells and Their Impact on NK Population Function. *Vaccines (Basel)* **8**,
761 (2020).
762

763

764 **Table 1**

		Diagnosis			
		Multiple Myeloma	MGUS	Smoldering Myeloma	Solitary Plasmacytoma
	n	21	3	3	1
sex	Male	12	3	2	1
	Female	9	0	1	0
age	Range	50-82	62-72	70-89	77
	Median	68	71	79	77
% plasma cell blasts	Range	0-90	0-9	12-50	0
	Median	21	8	13	0

765

766 **Figure Legends:**

767 **Figure 1: Lymphocytes acquire PD-1 and Thy.1-1 from RMA cancer cells.** (A) RMA cells (red)
768 were transduced with a retroviral vector encoding Thy-1.1 to generate RMA-*Thy1.1* (blue) and then
769 PD-1 was knocked-out by CRISPR/Cas9 to generate RMA-*Pdcd1*$^{-/-}$*Thy1.1* (purple). A representative
770 flow-cytometry staining depicting PD-1 and Thy-1.1 expression is shown. (B) Splenocytes from
771 *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ littermates were incubated with RMA-*Thy1.1* or RMA-*Pdcd1*$^{-/-}$*Thy1.1*. After 3
772 days, cells were stained with Thy1.1 and PD-1 antibodies. NK cells were gated as
773 singlets/live-NK1.1^{+}NKp46^{+}DX5^{+} events. CD8^{+} T cells were gated as singlets/live-CD3^{+}CD8^{+} events,
774 B cells as singlets/live-CD19^{+}. The experiment depicted is representative of three performed with
775 similar results. (C) NK cells isolated from *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ littermates were incubated with
776 RMA-*Thy1.1* or RMA-*Pdcd1*$^{-/-}$*Thy1.1*. After 3 days, cells were stained with Thy1.1 and PD-1
777 antibodies. The experiment depicted is representative of fifteen performed with similar results.

778

779 **Figure 2: NK cells and T cells acquire PD-1 from C1498 cancer cells.** (A) C1498 cells were stained
780 with PD-1 antibody or isotype control. PD-1+ cells (in blue) were flow-sorted and after 2 weeks in
781 culture stained for PD-1, alongside with parental C1498 cells (B). (C) Splenocytes from *Pdcd1*$^{+/+}$ or
782 *Pdcd1*$^{-/-}$ littermates were cultured with C1498 or C1498-PD-1+ cells for 3 days, and then stained with
783 PD-1 antibodies. NK cells and CD8^{+} T cells were gates as described in 1A. The experiment depicted
784 is representative of three performed with similar results. (D) Splenic NK cells isolated from *Pdcd1*$^{-/-}$
785 mice were co-cultured with C1498 or C1498-PD-1+ cells, or without tumor cells as a control, for 24
786 hrs or 72 hrs, and stained for PD-1. The experiment depicted is representative of six performed with
787 similar results.

788

789 **Figure 3: Trogocytosis is responsible for intercellular transfer of PD-1 from tumor to NK cells.**
790 (A) Splenic NK cells isolated from a *Pdcd1*$^{-/-}$ mouse were co-cultured for 24 hrs with RMA cells
791 separated or not by a transwell semi-permeable membrane before staining for PD-1 and Thy-1.1. The
792 experiment depicted is representative of four performed with similar results. (B) Splenic NK cells
793 isolated from a *Pdcd1*$^{-/-}$ mouse were co-cultured for 24 hrs with RMA cells or with media conditioned
794 for three days by RMA cells, then stained for PD-1 and Thy-1.1. The experiment depicted is
795 representative of three performed with similar results. (C) Splenic NK cells isolated from a *Pdcd1*$^{-/-}$
796 mouse were pre-treated with Sodium Azide or Antimycin-A, then co-cultured for one hour with RMA
797 cells and stained for PD-1 and Thy-1.1. Three independent experiments are plotted. Statistical analysis
798 with one-way ANOVA with repeated measurements. (D) NK cells were incubated with RMA cells
799 pre-labelled with Cell-Vue for 24 hrs. Cell-Vue and PD-1 staining on NK cells is depicted, on the left
800 and right respectively.

801

802 **Figure 4: SLAM receptors are essential for trogocytosis.** (A) Splenic NK cells isolated from a
803 *Pdcd1*$^{-/-}$ mouse were cultured for 3 days and then PD-L2 and PD-L1 expression was analyzed by
804 flow-cytometry. Representative of three experiments performed with similar results. (B-C) NK cells
805 were incubated with RMA cells in the presence of a PD-L1 blocking antibody or an isotype control for
806 24 hrs, before being stained for PD-1 and PD-L1. As additional controls, NK cells were: *i)* co-cultured
807 with RMA without adding any antibody; or *ii)* cultured alone without adding tumor cells. The
808 experiment depicted is representative of three performed. (D-E) NK cells were isolated from the spleen
809 of *Pdcd1*$^{-/-}$*Ncr1*$^{+/Cre}$*Cd274*$^{fl/fl}$ (D) or *Cd274*$^{-/-}$ (E) mice and co-cultured for three days with RMA or
810 C1498 tumor cells, when PD-1 and Thy-1.1 staining was assessed by flow cytometry. (F-G)
811 Splenocytes from SLAM-deficient mice or control littermates were co-cultured for three days with
812 RMA or C1498 cells. PD-1 and Thy-1.1 staining on NK cells was then assessed by flow cytometry.
813 The experiment depicted is representative of 3 performed. In G, the frequency of NK cells staining for
814 PD-1 or Thy-1.1 in WT or SLAM-deficient mice analyzed in the three experiments is plotted.
815 Statistical analysis with two-tailed unpaired Student's t-test. ***: $p<0.001$. (H) Splenocytes from
816 LFA-1-deficient or WT mice were co-cultured for three days with RMA cells. Representative of 3
817 experiments performed.

818

819 **Figure 5: Intratumoral lymphocytes acquire PD-1 from tumor cells**. (A-D) *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$
820 mice were injected with RMA or RMA-*Pdcd1*$^{-/-}$ tumors. PD-1 and Thy-1.1 staining was assessed by
821 flow-cytometry on tumor infiltrating NK and T cells (gated as in Fig. 1B and 1C, respectively). The
822 experiment shown is representative of four performed with similar results. (E) Expression of Sca1 and
823 CD69 was analyzed on *Pdcd1*$^{-/-}$ NK and T cells infiltrating RMA tumors, by gating on Thy-1.1^{-}PD-1^{-}

824 (gray), Thy-1.1^{+}PD-1^{-} (red) or Thy-1.1^{+}PD-1^{+} (blue) cells. The mouse depicted is the same depicted in
825 2C. (F) RMA or RMA-Thy.1-1 cells were injected in either flank of a *Pdcd1*$^{-/-}$ mouse. PD-1 and
826 Thy.1-1 staining was analyzed in intratumoral or splenic NK cells. The experiment depicted is
827 representative of two performed.
828
829 **Figure 6: PD-1 blockade is effective in *Pdcd1*$^{-/-}$ mice when NK cells are present and tumor cells**
830 **express PD-1.** (A) PD-1 and PD-L1 expression in RMA-S-*Pdl1* or RMA-S-*Pdl1-Pdcd1*$^{-/-}$ cells was
831 analyzed by flow cytometry. (B-H) In all experiments, the indicated cell lines were injected
832 resuspended in Matrigel, alone or mixed with different PD-1 blocking or control antibody. Tumor
833 growth was assessed over time and data were analyzed with 2-way ANOVA. (B) n=6/group, the
834 experiment depicted is representative of two performed with similar results. (C) n=6/group, the
835 experiment depicted is representative of two performed with similar results. (D) n=4/group, the
836 experiment depicted is representative of two performed with similar results. (E) n=6/group, the
837 experiment depicted is representative of three performed with similar results. (F) n=at least 5/group,
838 the experiment depicted is representative of two performed with similar results. (G) n= 5/group, the
839 experiment depicted is representative of two performed with similar results. (H) n= 5/group, the
840 experiment depicted is representative of two performed with similar results. *: $p<0.05$; **:$p<0.01$;
841 ***:$p<0.001$.
842
843 **Figure 7: NK cells co-stain for CD138 and PD-1 in the bone marrow of patients with clonal**
844 **plasma cell disorders.** The bone marrow aspirates of 28 patients with clonal plasma cell disorders
845 were analyzed by flow cytometry. The frequency of NK cells staining for either, neither or both CD138
846 and PD-1 is depicted.

847 **SUPPLEMENTARY MATERIAL**

848 **Supplementary Figure 1: Inflammatory signals fail to induce PD-1 upregulation in purified NK**
849 **cells in vitro.** Magnetically enriched NK cells from *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ mice were cultured for 72hrs
850 with the inflammatory mediators indicated in the figure. GC=glucocorticoid (Corticosterone). NK
851 cells from 3 mice/genotype where pooled. The experiment depicted is representative of 3 performed.

852

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

856

857 **Supplementary Figure 3: PD-1 and Thy-1.1 are co-acquired by NK cells.** NK cells isolated from
858 the spleens of *Pdcd1*$^{-/-}$ mice were co-cultured with RMA or RMA-*Pdcd1*$^{-/-}$ cells for three days. A and
859 B show the frequency of PD-1^{+} or Thy-1.1^{+} NK cells in the 14 mice analyzed in the 13 experiments
860 performed. In A and B Statistical analysis with ANOVA with Dunnet's multiple comparison test. In
861 (C) the correlation between PD-1^{+} and Thy-1.1^{+} NK cells is depicted. 95%-confidence interval is also
862 showed, statistical analysis with Spearman correlation test.

863

864 **Supplementary Figure 4: NK cells acquire at least four proteins they do not endogenously**
865 **express from RMA cells.** CD45.1+ NK cells were co-cultured with RMA cells for three days and then
866 PD-1, Thy-1.1, CD45.2 and TCRvβ12 staining was analyzed by flow cytometry. Representative of
867 three performed with similar results.

868

869

870 **Supplementary Figure 5: PD-1 antibodies do not affect or promote PD-1 trogocytosis by NK**
871 **cells.** (A) Saturation of PD-1 sites on RMA cells was assessed by stained with the PD-1 antibody cells
872 that were previously co-incubated with anti-PD-1 or control isotype. (B) NK cells purified from
873 *Pdcd1*$^{-/-}$ mice were incubated with RMA cells in the presence of the indicated blocking antibody and
874 then stained for PD-1 and Thy-1.1. The experiment depicted is representative of three performed.
875
876 **Supplementary Figure 6: PD-L1 is dispensable for PD-1 trogocytosis in NK, T and B cells.**
877 *Cd274*$^{-/-}$ or wild type splenocytes were cultured with tumor cells for 3 days before PD-1 and Thy-1.1
878 staining was assessed by flow cytometry. Representative of 3 mice/genotype analyzed.
879
880 **Supplementary Figure 7: SLAM receptors are required for trogocytosis in CD8^{+} T and B cells.**
881 SLAM-ko or wild type splenocytes were cultured with tumor cells for three days. Representative
882 staining and cumulative analysis are depicted. Statistical analysis with two-tailed unpaired Student's
883 t-test. ***: $p<0.001$.
884
885 **Supplementary Figure 8: LFA-1 is not necessary for trogocytosis in CD8^{+} T and B cells.** *Itga1*$^{-/-}$ or
886 wild type splenocytes were cultured with tumor cells for three days when PD-1 and Thy-1.1 staining
887 was assessed on CD8+ T cells and B cells.
888
889 **Supplementary Figure 9: NKG2D is not required for trogocytosis in NK cells.** *Klrk1*$^{-/-}$ or control
890 littermates NK cells were cultured with tumor cells for three days and then stained for PD-1 and
891 Thy-1.1.
892

893 **Supplementary Figure 10:** the gating strategy for NK and T cell identification in the bone marrow of

894 patients with clonal plasma cell disorders is displayed.

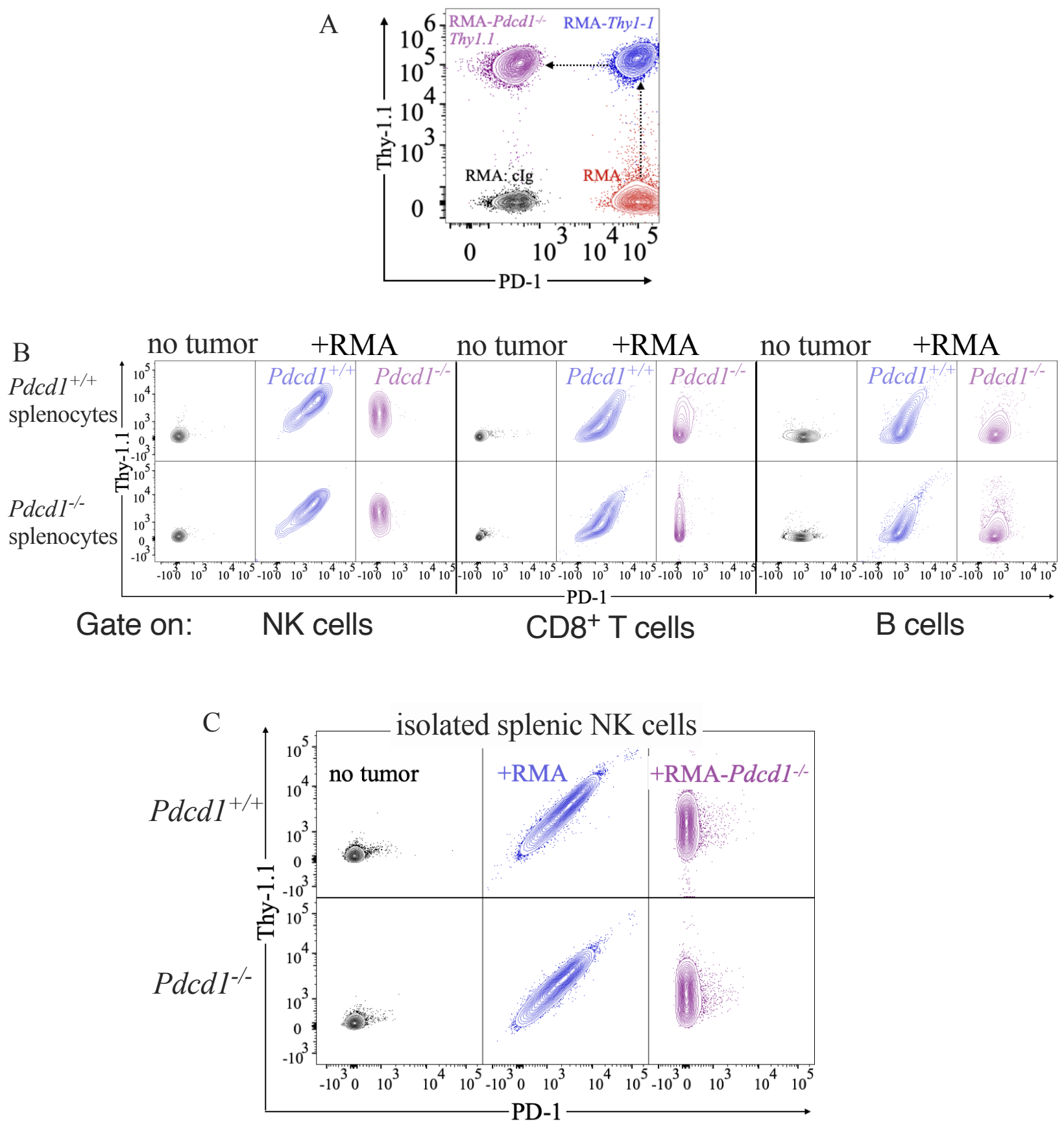

A
RMA-Pdcd1-/- Thy1.1
RMA-Thy1-1
RMA: cIg
RMA
Thy-1.1
PD-1
B
no tumor
+RMA
no tumor
+RMA
no tumor
+RMA
Pdcd1+/+ splenocytes
Pdcd1-/- splenocytes
Pdcd1+/+
Pdcd1-/-
Thy-1.1
PD-1
Gate on:
NK cells
CD8+ T cells
B cells
C
isolated splenic NK cells
no tumor
+RMA
+RMA-Pdcd1-/-
Pdcd1+/+
Pdcd1-/-
Thy-1.1
PD-1

Figure 1

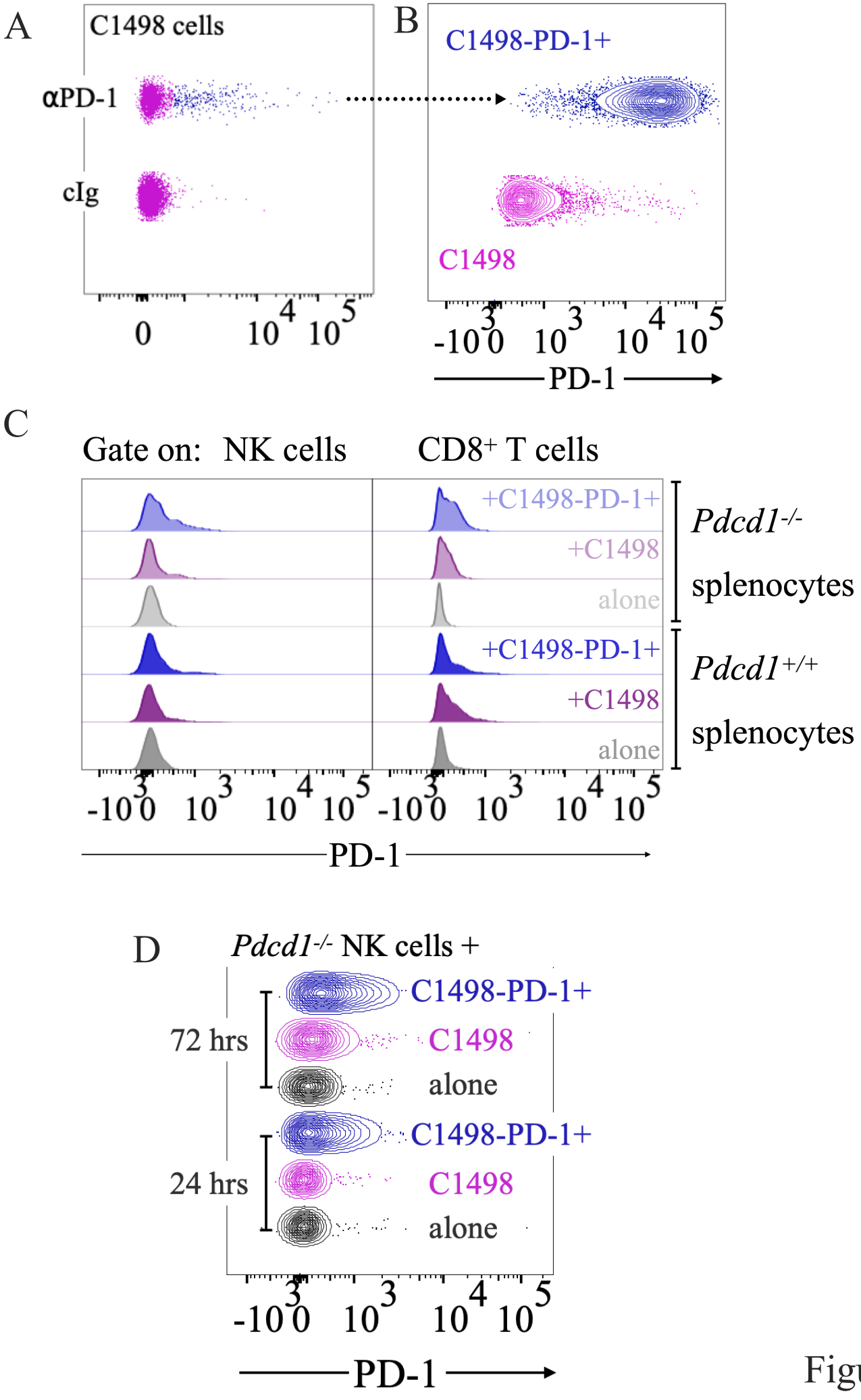

A
C1498 cells
αPD-1
cIg
0
10^4
10^5
B
C1498-PD-1+
C1498
-10
0
10^3
10^4
10^5
PD-1
C
Gate on: NK cells
CD8+ T cells
+C1498-PD-1+
+C1498
alone
+C1498-PD-1+
+C1498
alone
Pdcd1-/- splenocytes
Pdcd1+/+ splenocytes
-10
0
10^3
10^4
10^5
PD-1
D
Pdcd1-/- NK cells +
72 hrs
24 hrs
C1498-PD-1+
C1498
alone
C1498-PD-1+
C1498
alone
-10
0
10^3
10^4
10^5
PD-1

Figure 2

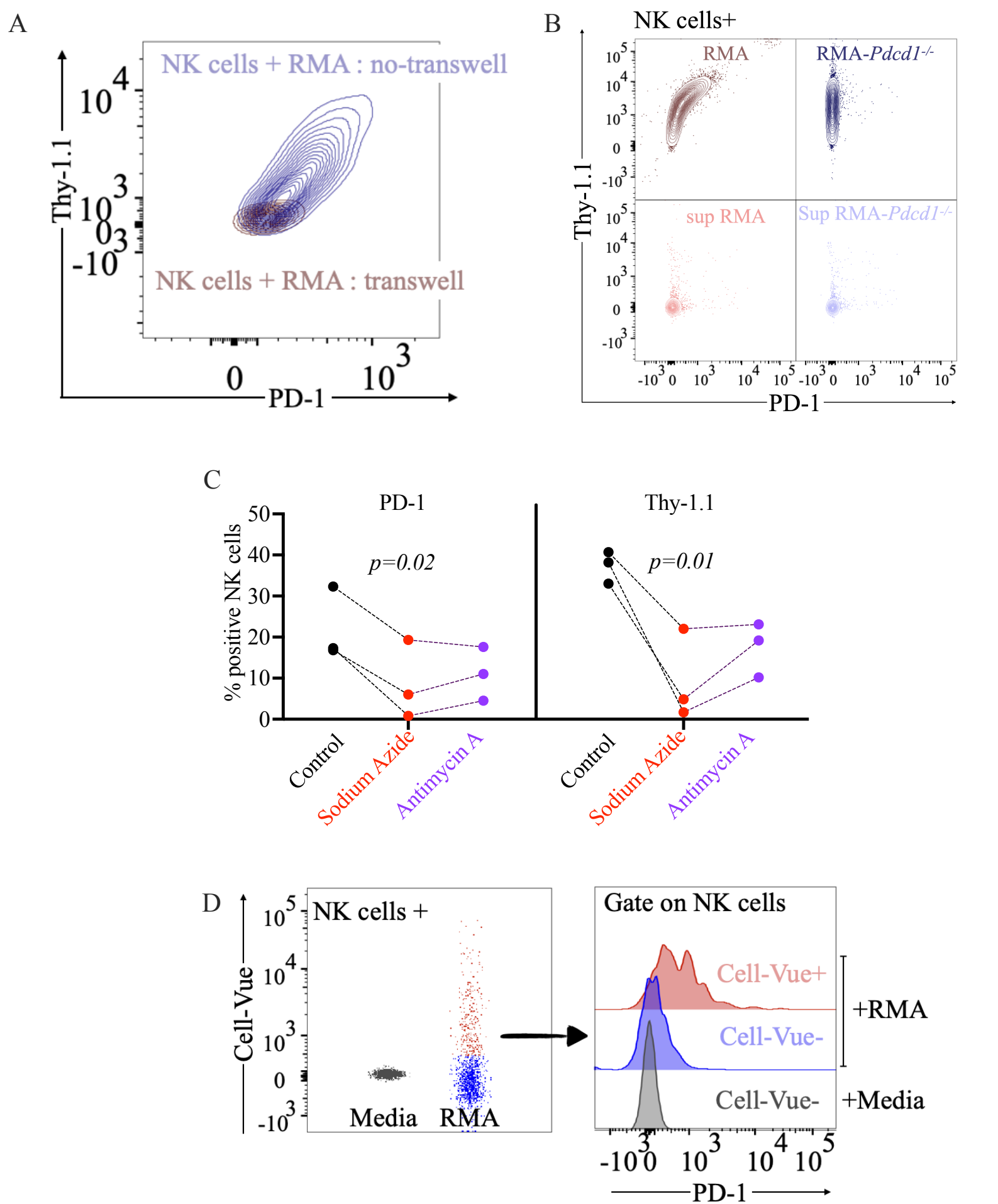

A
NK cells + RMA : no-transwell
NK cells + RMA : transwell
Thy-1.1
PD-1
B
NK cells+
RMA
RMA-Pdcd1-/-
sup RMA
Sup RMA-Pdcd1-/-
Thy-1.1
PD-1
C
PD-1
Thy-1.1
p=0.02
p=0.01
% positive NK cells
Control
Sodium Azide
Antimycin A
D
NK cells +
Cell-Vue
Media
RMA
Gate on NK cells
Cell-Vue+
Cell-Vue-
Cell-Vue-
+RMA
+Media
PD-1

Figure 3

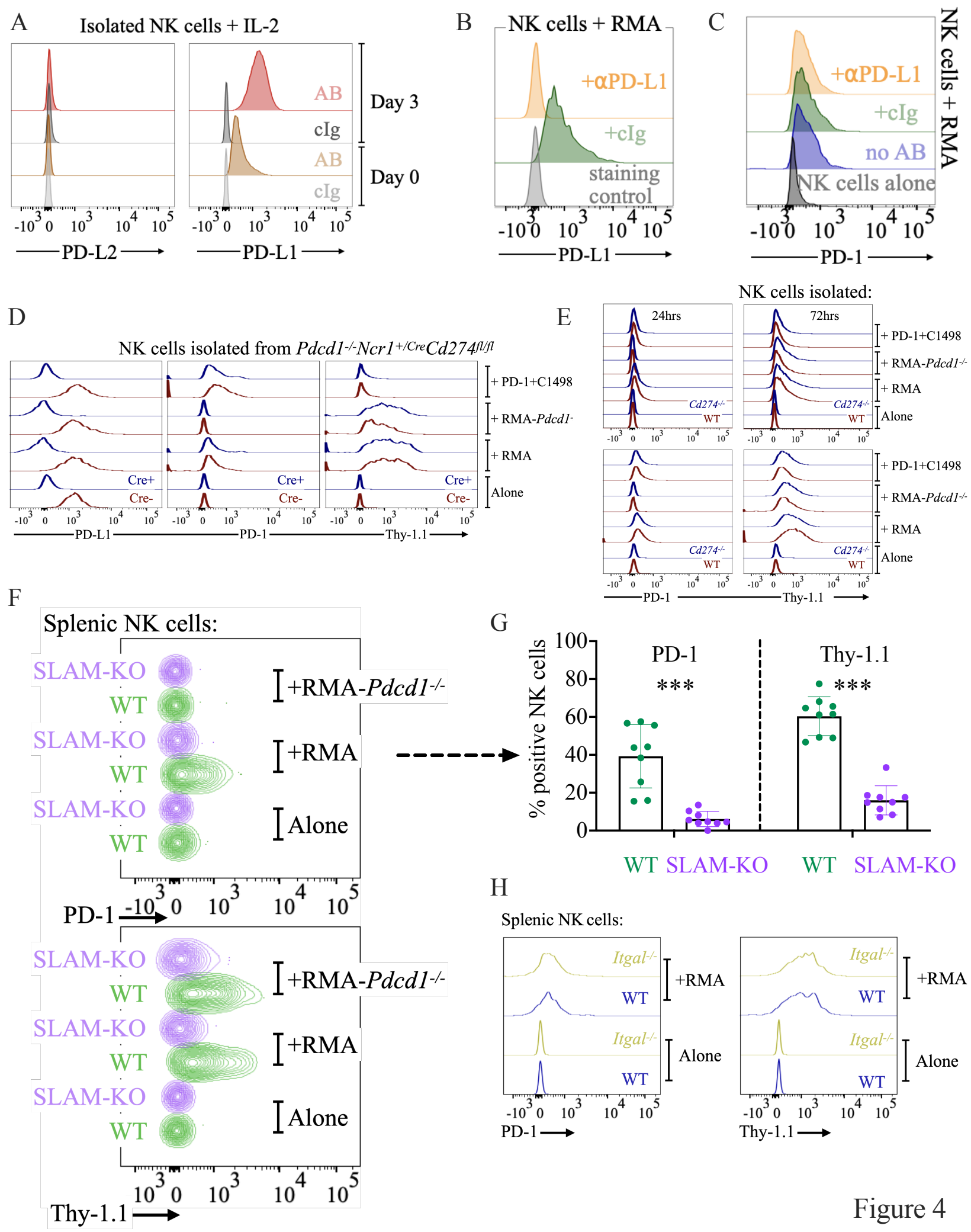

A
Isolated NK cells + IL-2
AB
cIg
AB
cIg
Day 3
Day 0
PD-L2
PD-L1
B
NK cells + RMA
+αPD-L1
+cIg
staining control
PD-L1
C
NK cells + RMA
+αPD-L1
+cIg
no AB
NK cells alone
PD-1
D
NK cells isolated from Pdcd1-/-Ncr1+/CreCd274fl/fl
+ PD-1+C1498
+ RMA-Pdcd1-
+ RMA
Alone
Cre+
Cre-
PD-L1
PD-1
Thy-1.1
E
NK cells isolated:
24hrs
72hrs
+ PD-1+C1498
+ RMA-Pdcd1-/-
+ RMA
Alone
Cd274-/-
WT
PD-1
Thy-1.1
F
Splenic NK cells:
SLAM-KO
WT
+RMA-Pdcd1-/-
+RMA
Alone
PD-1
Thy-1.1
G
% positive NK cells
PD-1
Thy-1.1
***
WT
SLAM-KO
H
Splenic NK cells:
Itgal-/-
WT
+RMA
Alone
PD-1
Thy-1.1

Figure 4

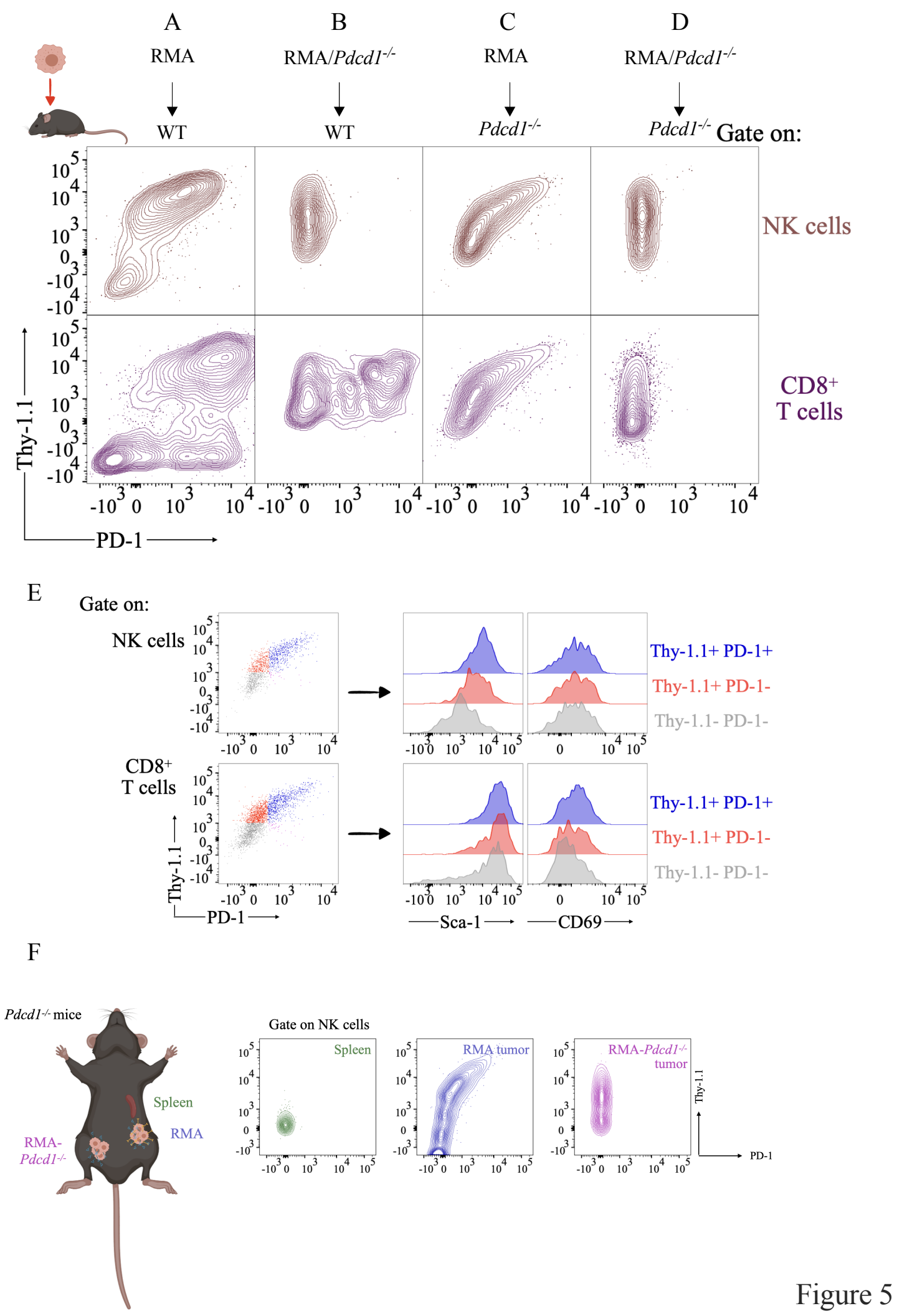

A
B
C
D
RMA
RMA/Pdcd1-/-
RMA
RMA/Pdcd1-/-
WT
WT
Pdcd1-/-
Pdcd1-/-
Gate on:
NK cells
CD8+ T cells
Thy-1.1
PD-1
E
Gate on:
NK cells
CD8+ T cells
Thy-1.1+ PD-1+
Thy-1.1+ PD-1-
Thy-1.1- PD-1-
Sca-1
CD69
F
Pdcd1-/- mice
Spleen
RMA
RMA-Pdcd1-/-
Gate on NK cells
Spleen
RMA tumor
RMA-Pdcd1-/- tumor
Thy-1.1
PD-1

Figure 5

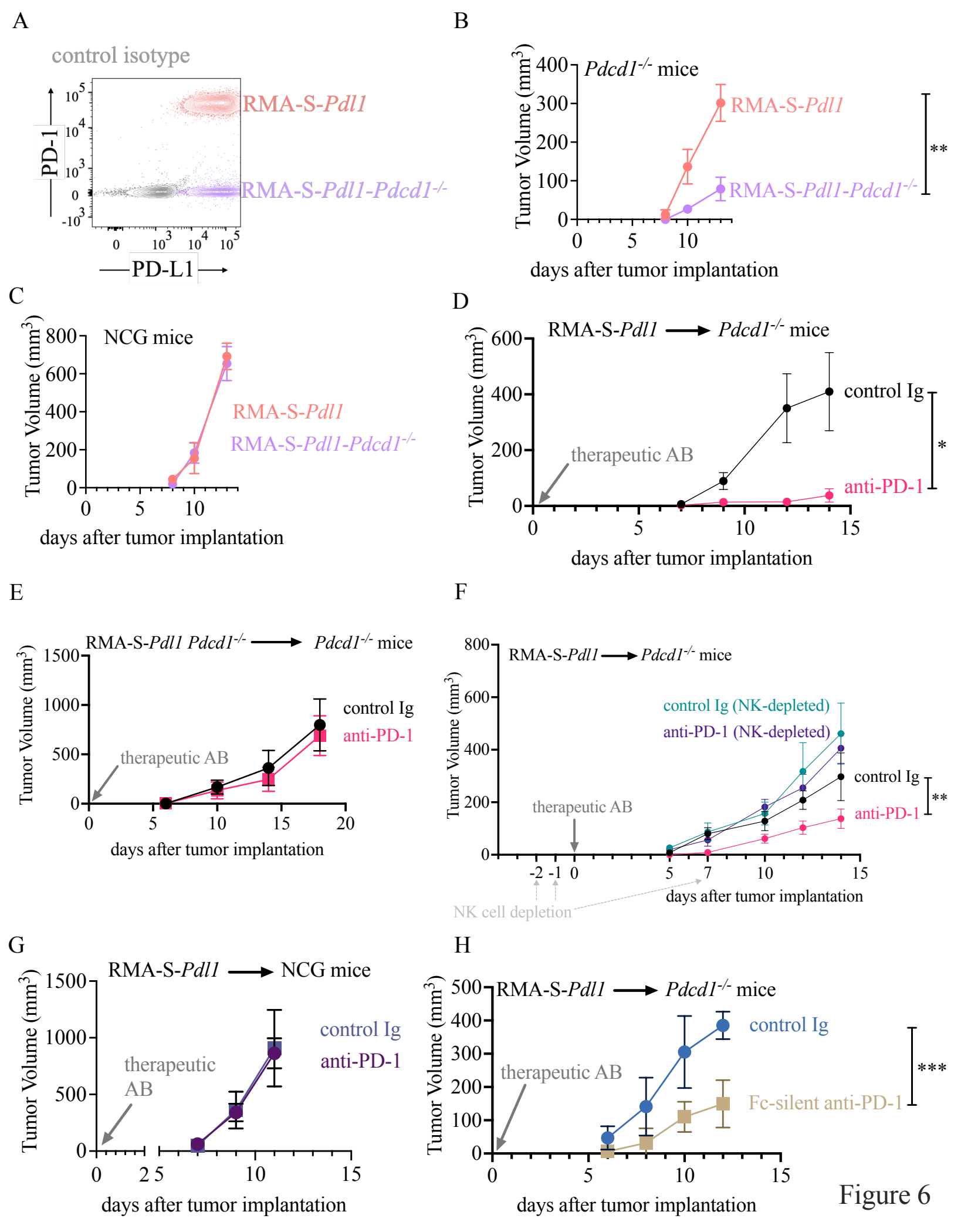

A
control isotype
RMA-S-Pdl1
RMA-S-Pdl1-Pdcd1-/-
PD-1
PD-L1
B
Pdcd1-/- mice
RMA-S-Pdl1
RMA-S-Pdl1-Pdcd1-/-
Tumor Volume (mm3)
days after tumor implantation
**
C
NCG mice
RMA-S-Pdl1
RMA-S-Pdl1-Pdcd1-/-
Tumor Volume (mm3)
days after tumor implantation
D
RMA-S-Pdl1 → Pdcd1-/- mice
control Ig
anti-PD-1
therapeutic AB
Tumor Volume (mm3)
days after tumor implantation
*
E
RMA-S-Pdl1 Pdcd1-/- → Pdcd1-/- mice
control Ig
anti-PD-1
therapeutic AB
Tumor Volume (mm3)
days after tumor implantation
F
RMA-S-Pdl1 → Pdcd1-/- mice
control Ig (NK-depleted)
anti-PD-1 (NK-depleted)
control Ig
anti-PD-1
therapeutic AB
NK cell depletion
Tumor Volume (mm3)
days after tumor implantation
**
G
RMA-S-Pdl1 → NCG mice
control Ig
anti-PD-1
therapeutic AB
Tumor Volume (mm3)
days after tumor implantation
H
RMA-S-Pdl1 → Pdcd1-/- mice
control Ig
Fc-silent anti-PD-1
therapeutic AB
Tumor Volume (mm3)
days after tumor implantation
***

Figure 6

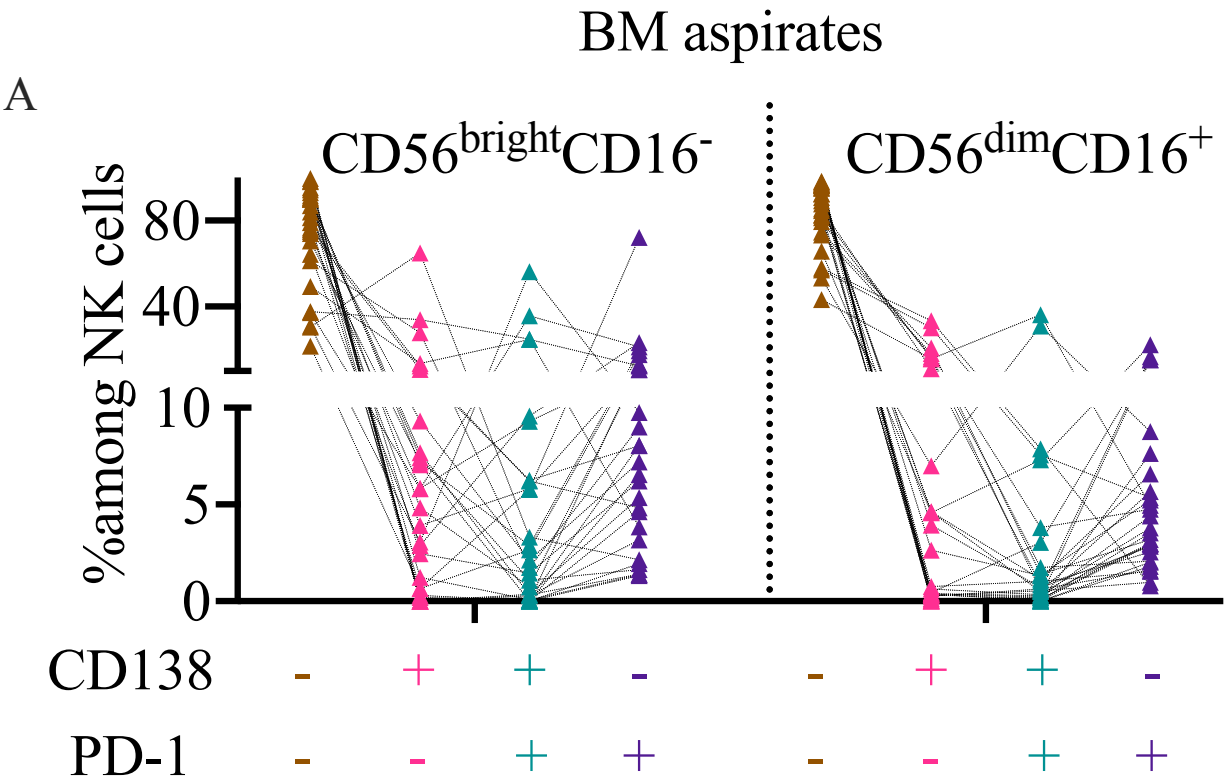
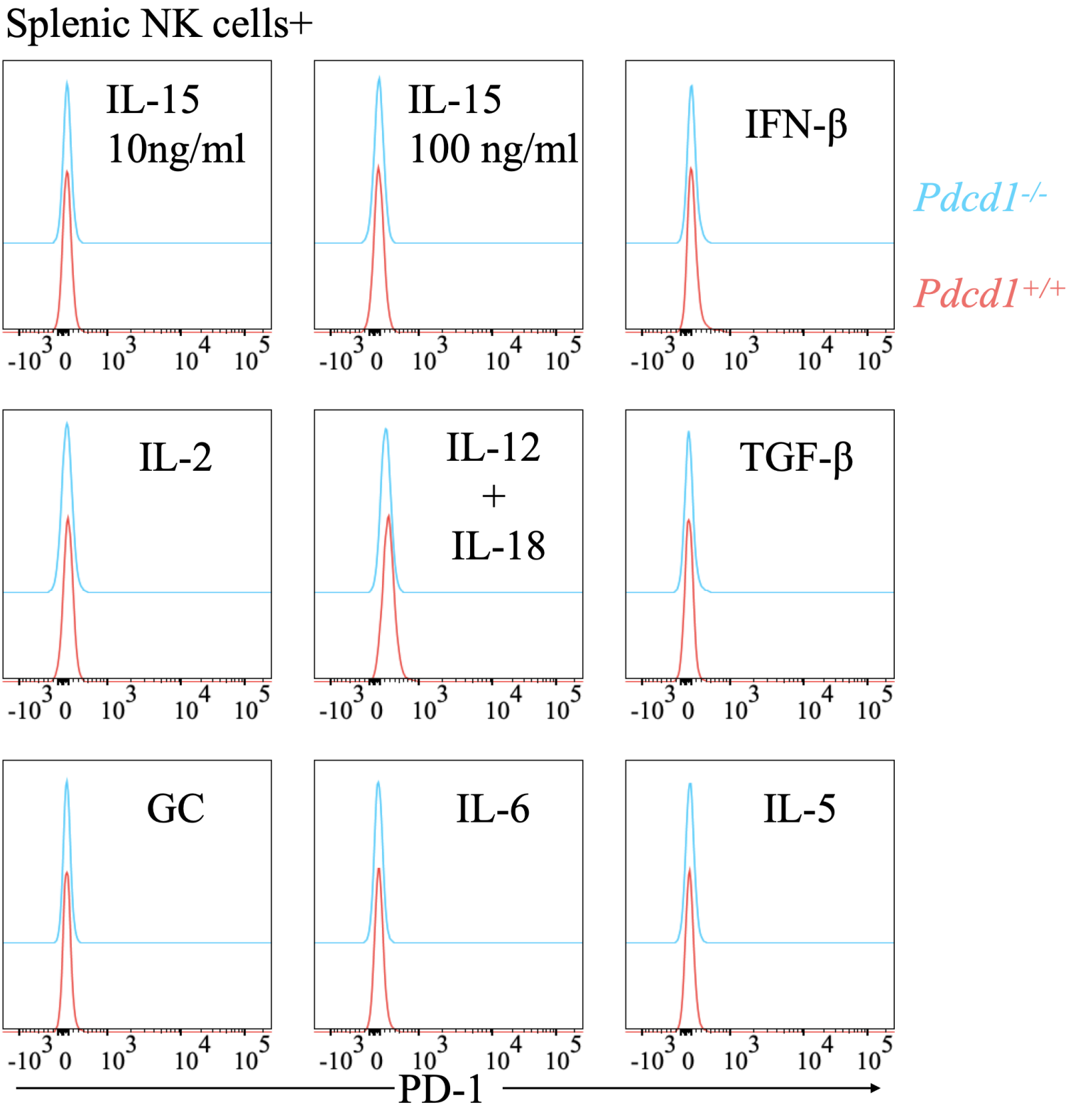
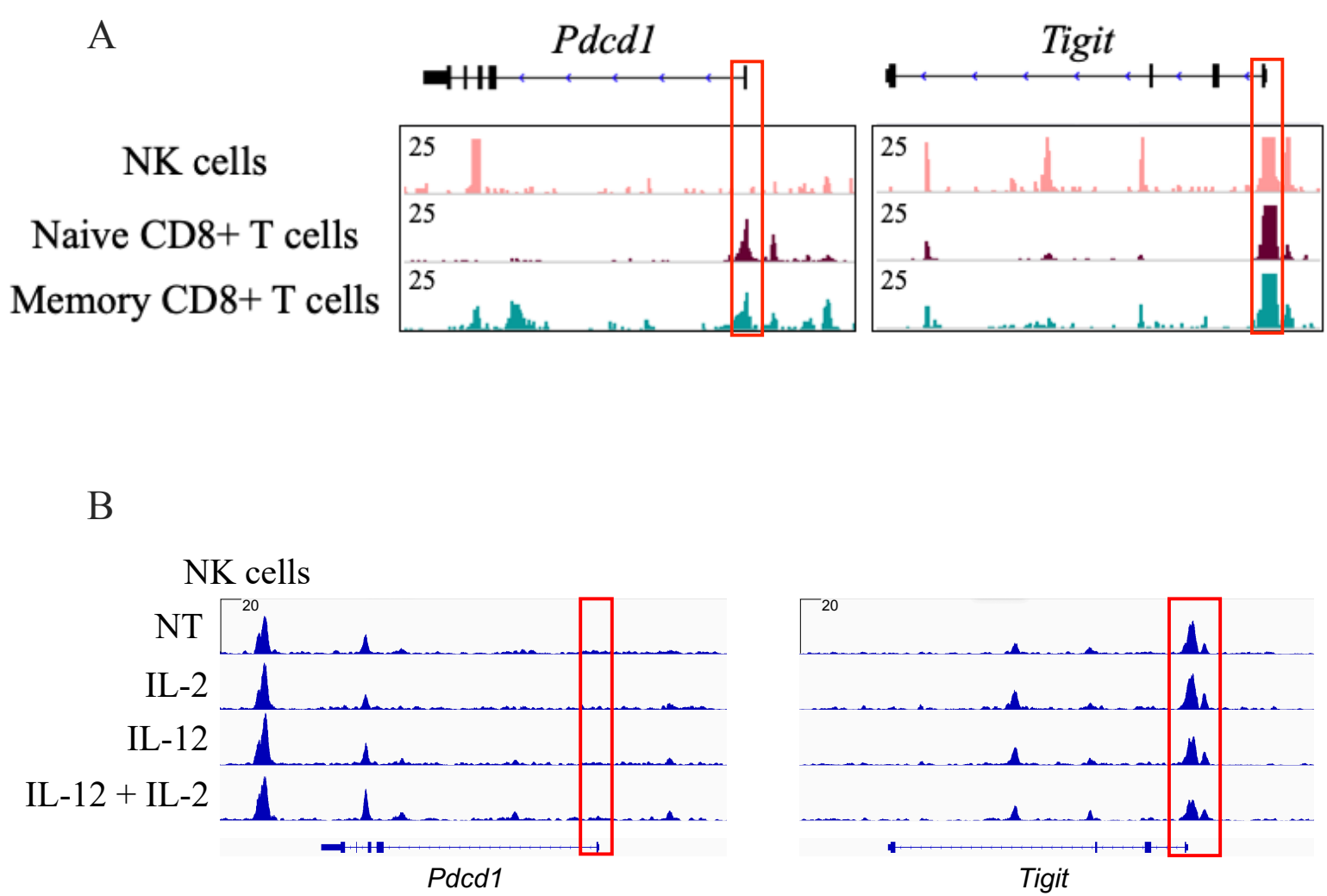

BM aspirates
A
CD56brightCD16-
CD56dimCD16+
%among NK cells
80
40
10
5
0
CD138
PD-1

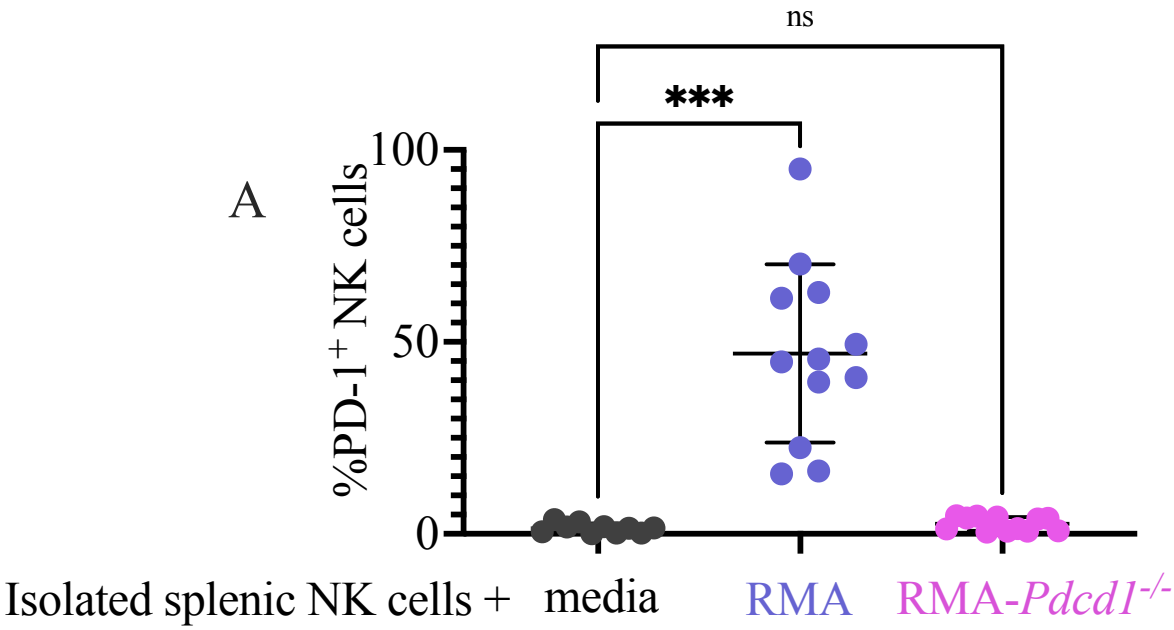
Figure 7

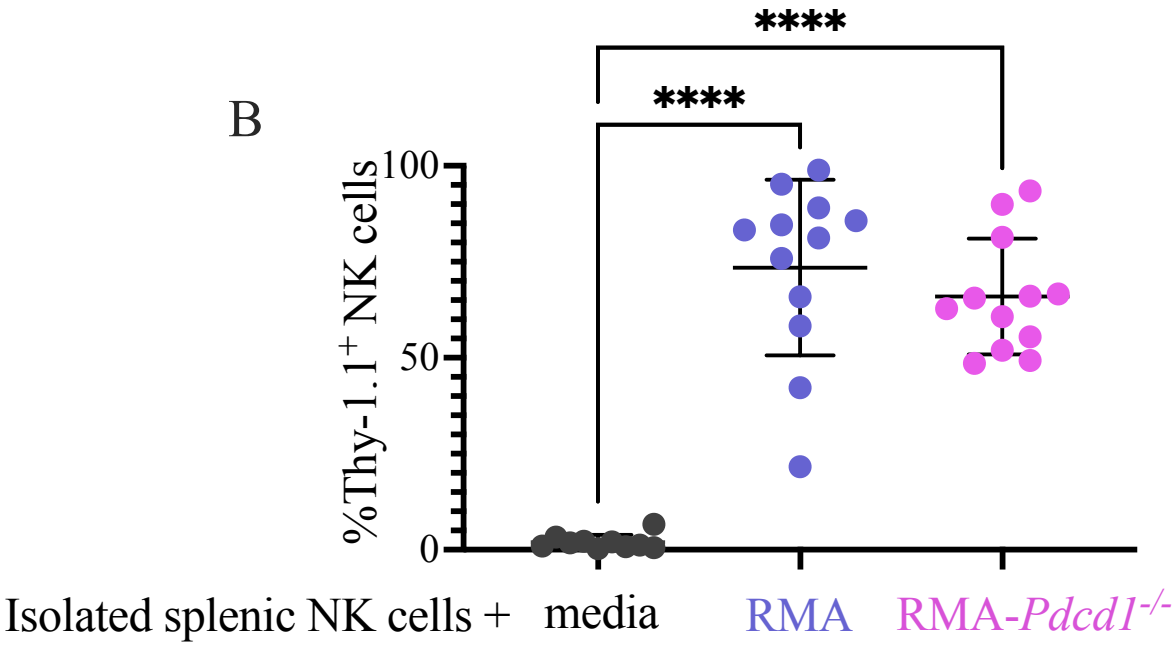


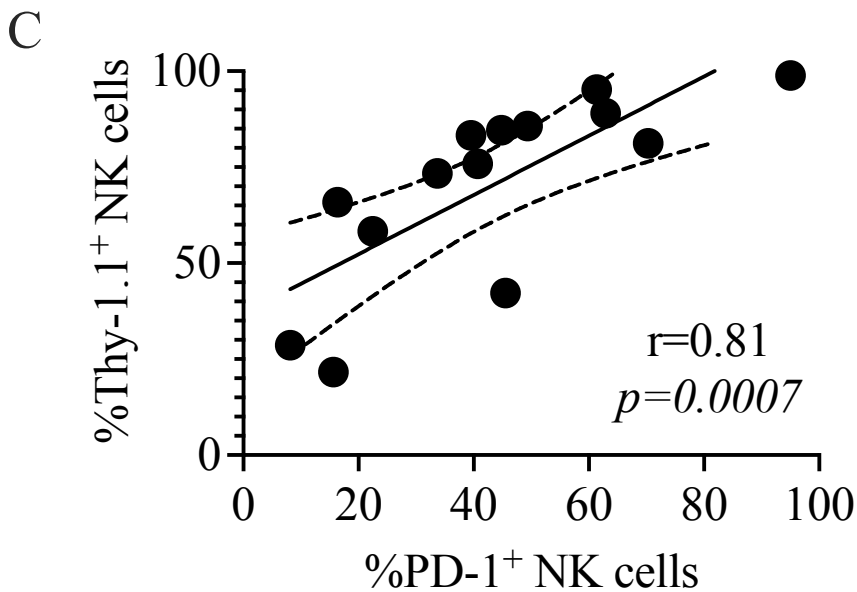
Supplementary Figure 1



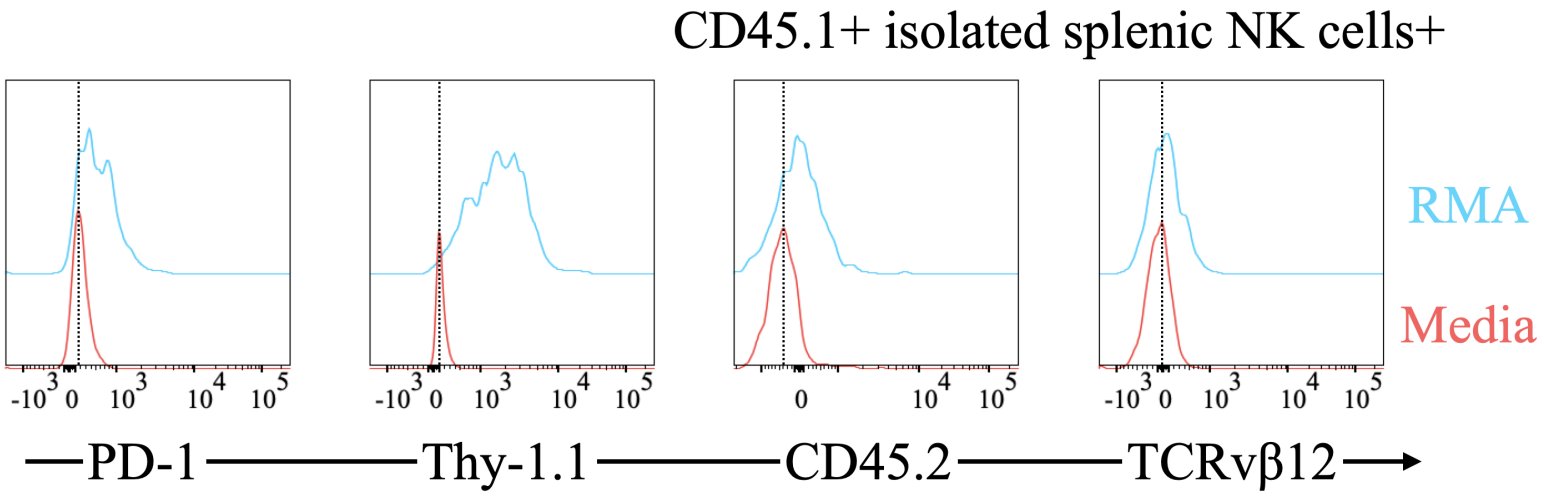
Supplementary Figure 2





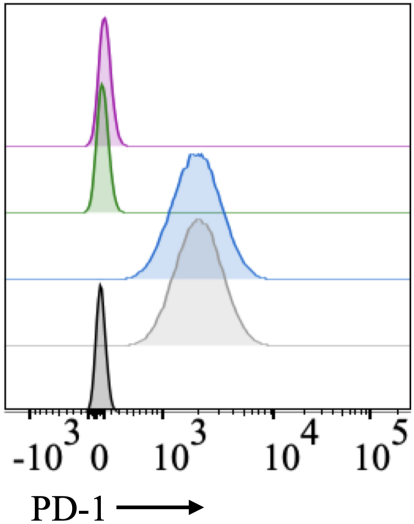


Supplementary Figure 3



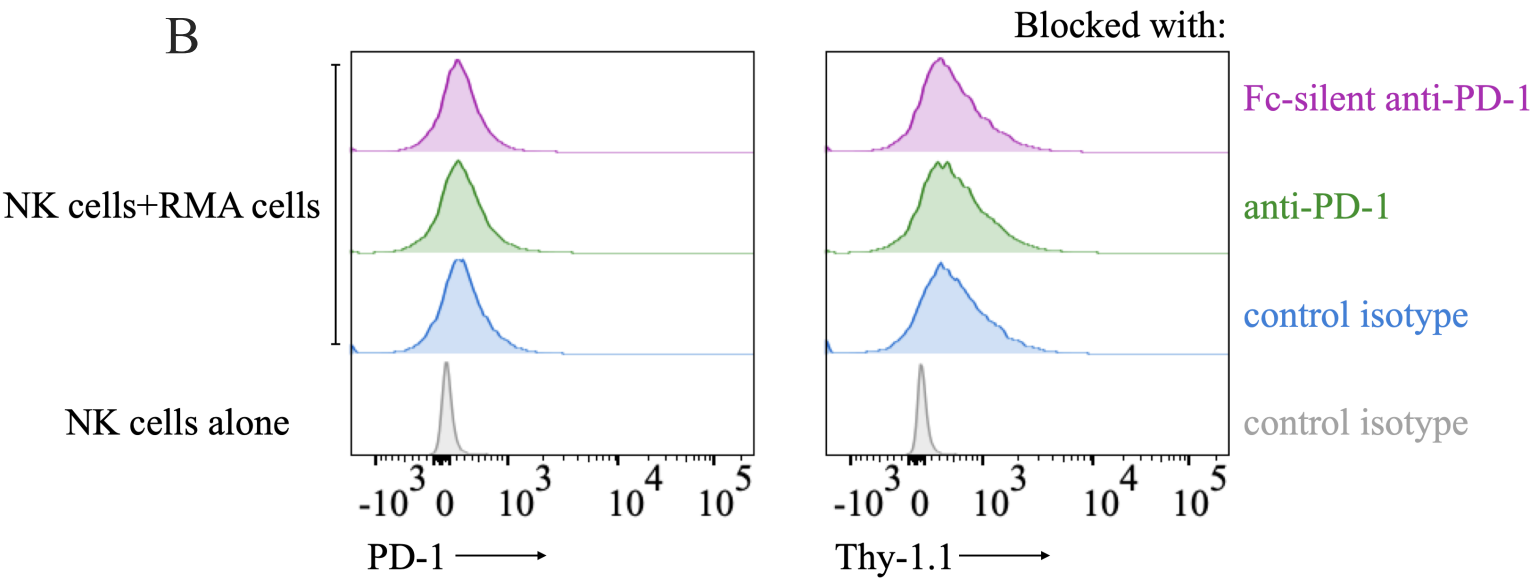
Supplementary Figure 4

A

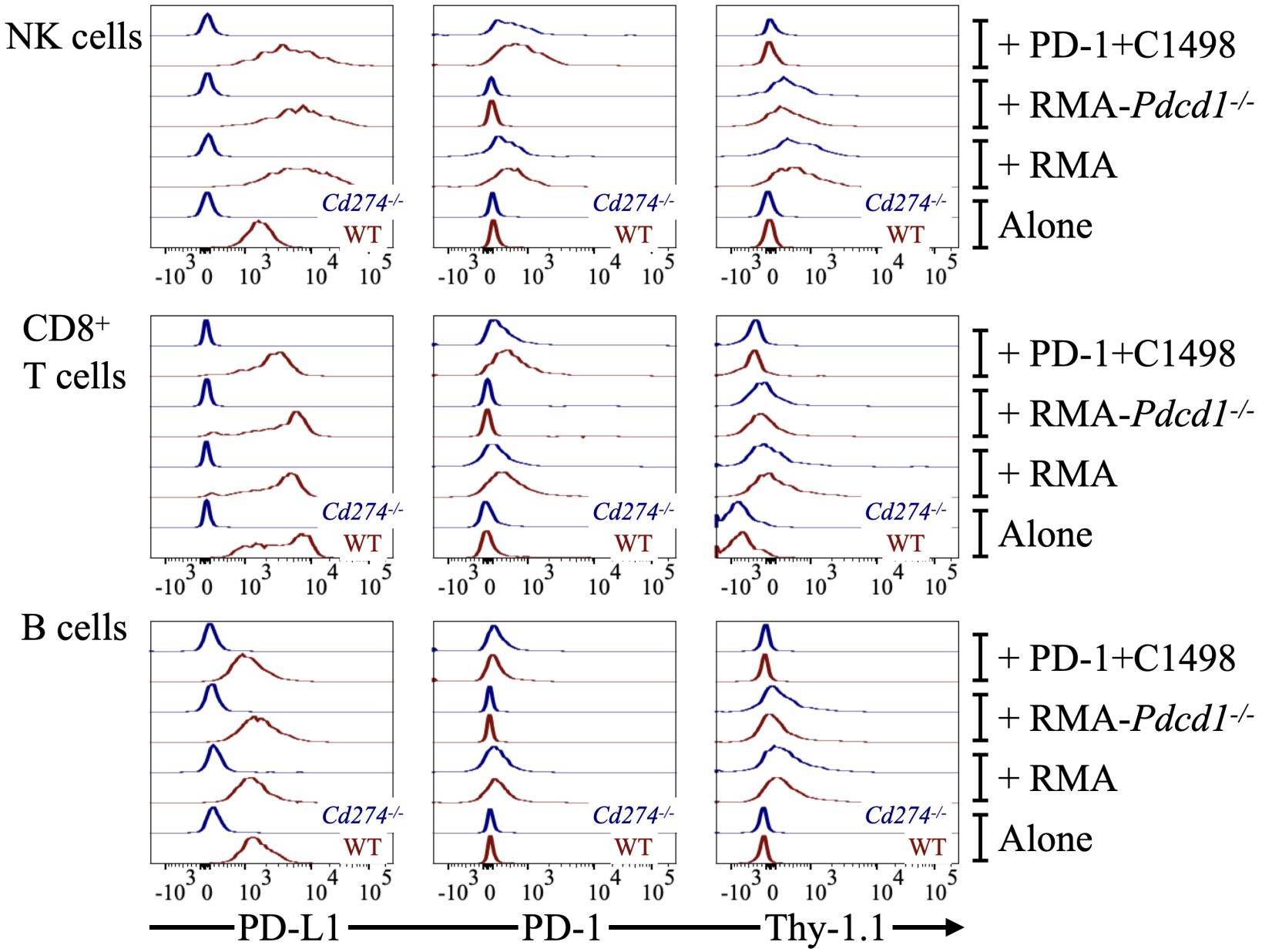


Blocked with:	Stained with:
Fc-silent anti-PD-1	anti-PD-1
anti-PD-1	anti-PD-1
control isotype	anti-PD-1
control isotype	anti-PD-1
control isotype	control isotype

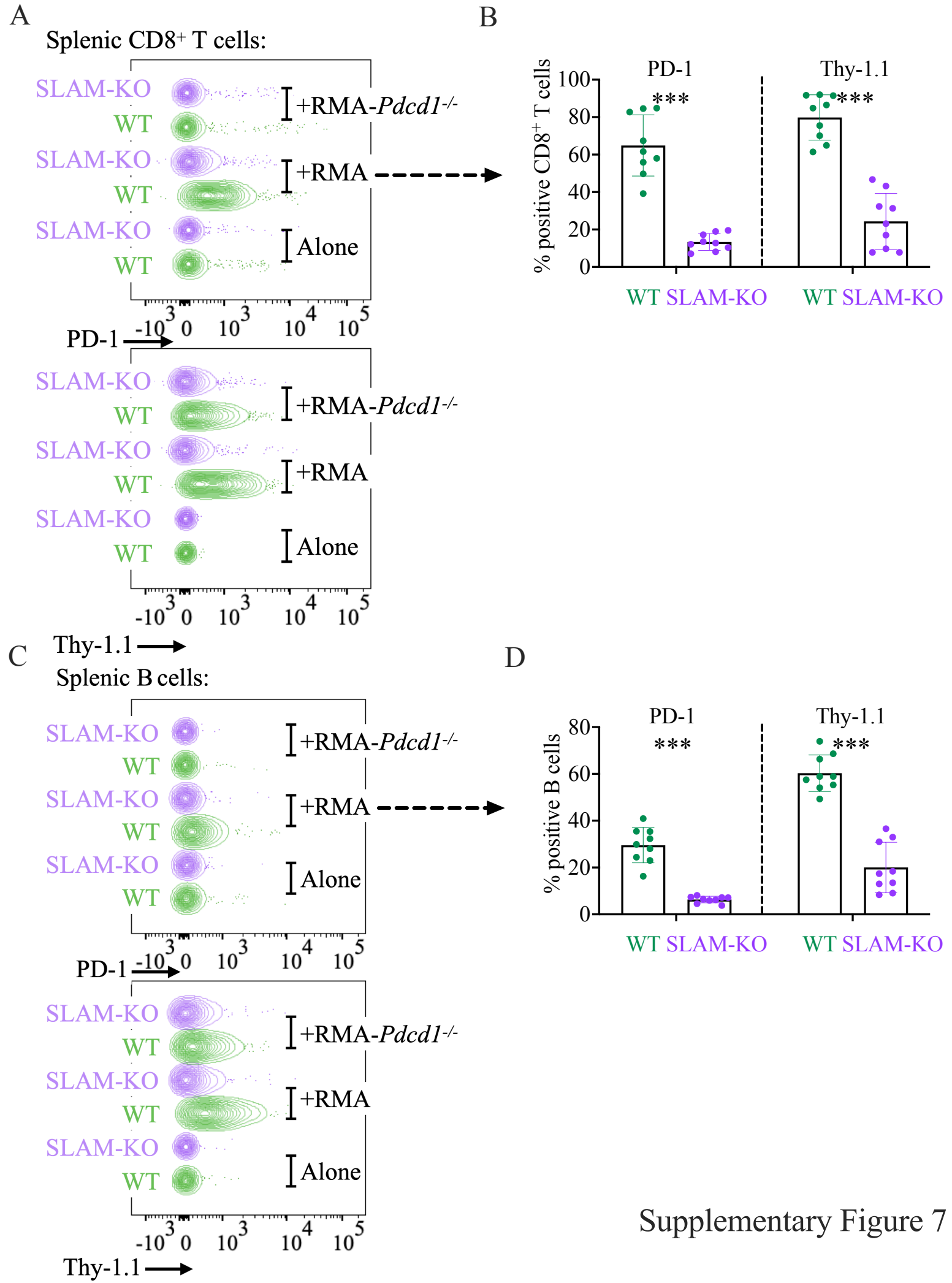
B

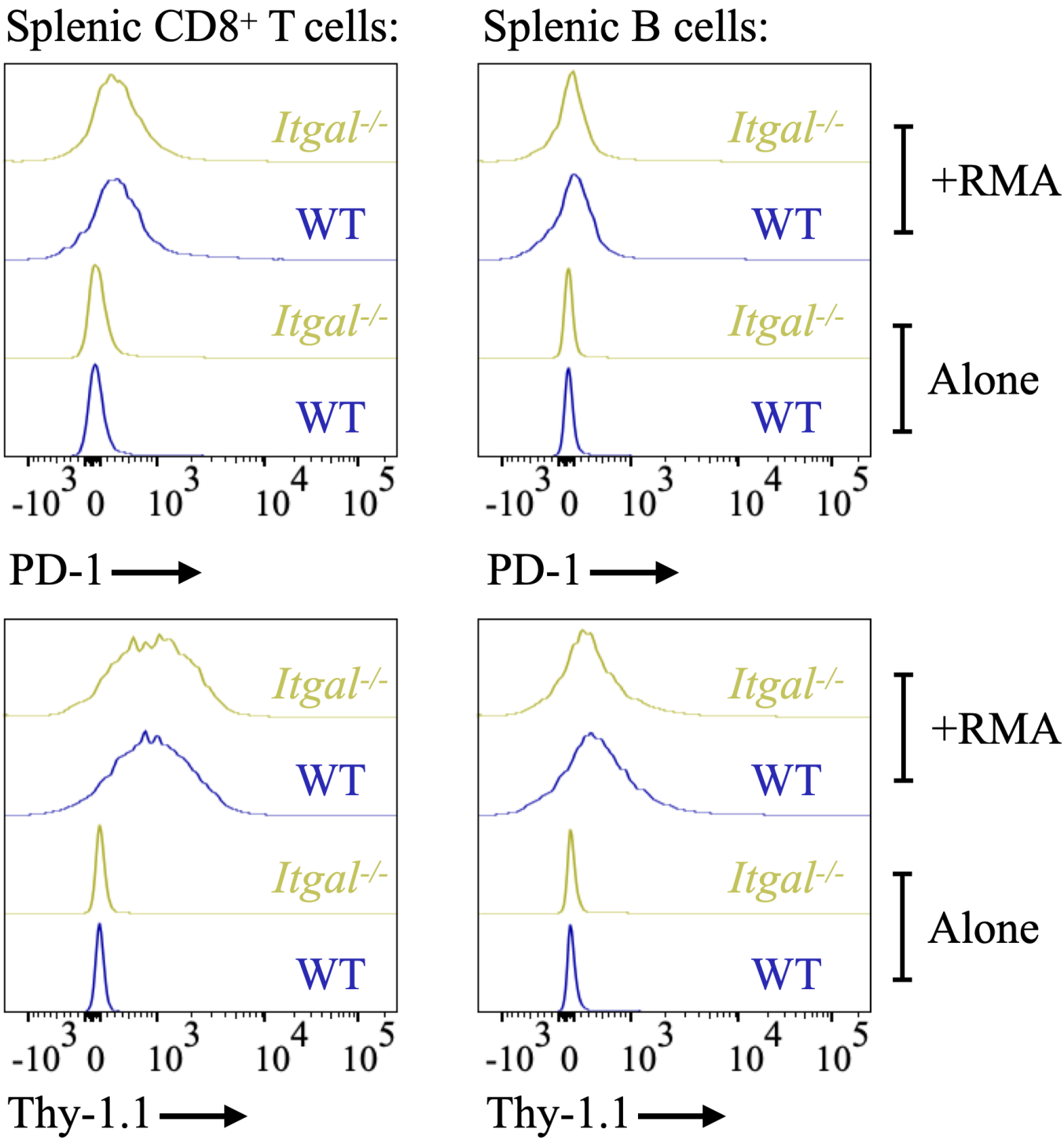


Supplementary Figure 5

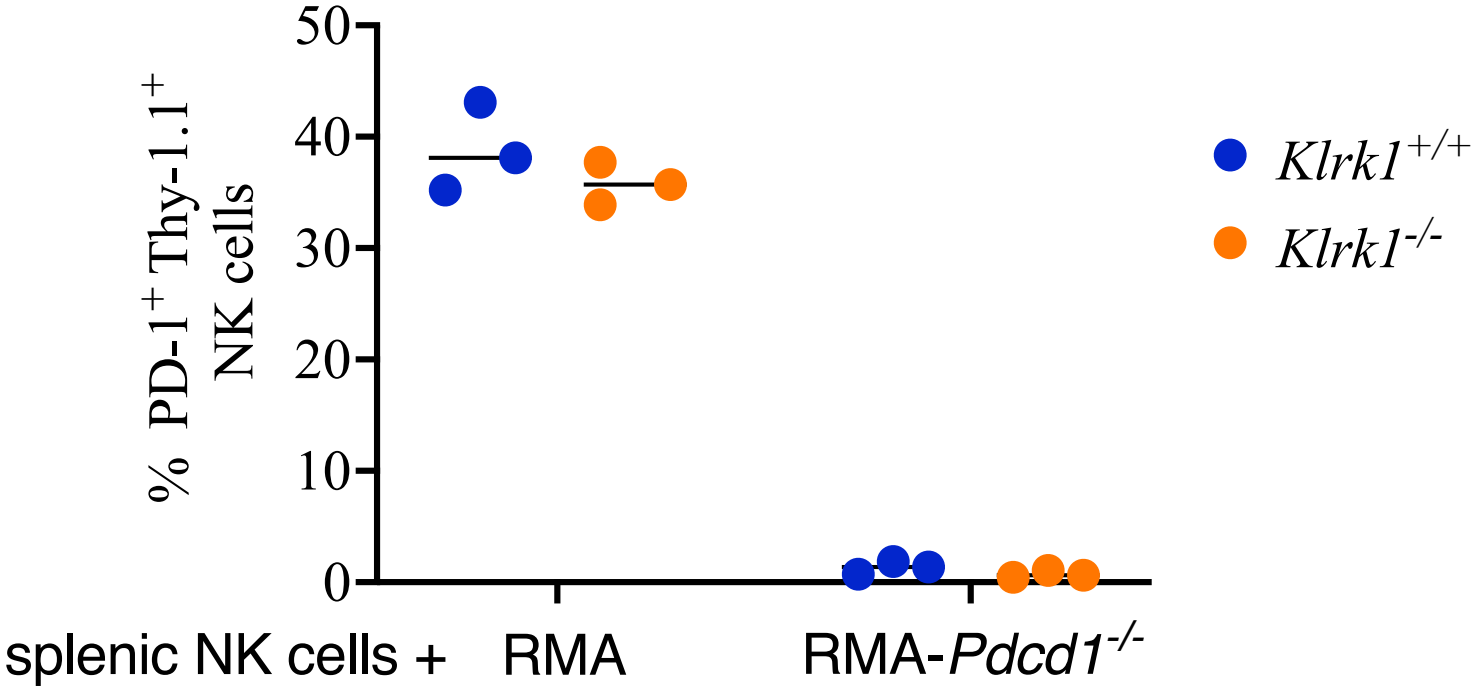


Supplementary Figure 6


A
Splenic CD8+ T cells:
SLAM-KO
WT
SLAM-KO
WT
SLAM-KO
WT
+RMA-Pdcd1-/-
+RMA
Alone
PD-1
Thy-1.1
B
PD-1
Thy-1.1
% positive CD8+ T cells
***
***
WT SLAM-KO
WT SLAM-KO
C
Splenic B cells:
D
% positive B cells
Supplementary Figure 7

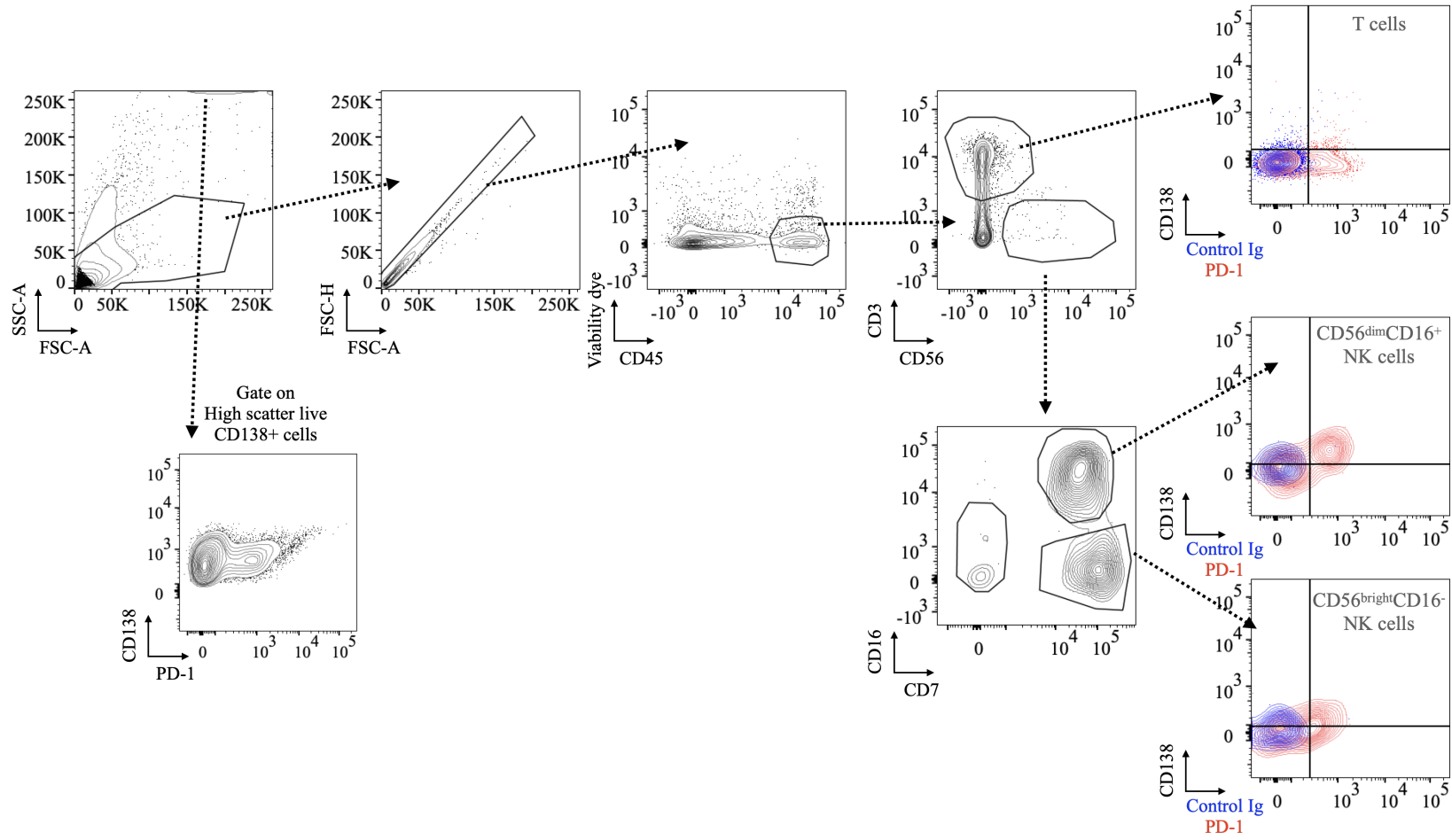


Supplementary Figure 8



Supplementary Figure 9

MM BM aspirate



Supplementary Figure 10