NK cells acquire PD-1 from the membrane of tumor cells.

Hasim M.S.[1,2], Vulpis E.[3], Sciumè G.[3], Shih H.Y.[4], Scheer A.[5], MacMillan O.[1,2,5], Petrucci M.T.[6], Santoni A.[3,7], Zingoni A.[3], Ardolino M.[1,2,5]

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

2: CI3, University of Ottawa, Ottawa, ON

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

4: National Eye Institute, NIH, Bethesda, MD

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

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

7: IRCCS Neuromed, Pozzilli, Italy

Correspondence:

Michele Ardolino

501 Smyth Road, Cancer Center, 3-328, Ottawa, ON, K1H 8M2

m.ardolino@uottawa.ca

Tel: +1-613-737-8899 ext 77257

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Abstract:

NK cells are key effectors of cancer immunosurveillance and immunotherapy and yet, much is still unknown about how cancer evades NK cell responses. Recent studies showed that checkpoint receptors, including PD-1, inhibit NK cell functions, but the mechanisms underlying the expression of these receptors remains unknown. Here, using two mouse models of leukemia, we show that NK cells, rather than intrinsically expressing the protein, are decorated with exogenous PD-1 by acquisition of membrane fragments from tumor cells. PD-1 acquisition, both ex vivo and in vivo, was a feature not only of NK cells, but also of $CD8^+$ T cells. PD-1 acquisition occurred with a mechanism consistent with trogocytosis and did not require engagement of PD-1-ligands on NK cells. In vivo results were corroborated in humans, where PD-1^+ NK cells from multiple myeloma patients also stained for cancer cell markers. Our results, in addition to shedding light on a previously unappreciated mechanism underlying the presence of PD-1 on NK and T cells, reveal the immuno-regulatory effect of membrane transfer occurring when immune cells contact tumor cells.

38 **Introduction:**

39 NK cells are key effectors of cancer immune-surveillance, and therapeutic strategies to harness
40 their activity against tumors are emerging as a complementation to T cell-based immunotherapies
41 (1, 2). To design more effective immunotherapies, a deep understanding of the pathways that
42 regulate NK cell responses is required. Given the clinical success obtained by immune-checkpoint
43 blockade immunotherapy (3), much effort was invested to address whether NK cells are inhibited
44 by checkpoint receptors, and whether NK cells partake in the therapeutic efficacy of checkpoint
45 blockade. We recently showed that NK cells are suppressed by PD-1 and contribute to the efficacy
46 of PD-1/L1 blockade in mouse models of cancer (4), and several studies revealed that PD-1 is
47 found on the surface of human NK cells in cancer patients, including multiple myeloma (MM) and
48 many solid tumor indications (5-9). Intracellular, but not surface, PD-1 protein was found in resting
49 human NK cells (10), suggesting that PD-1 can be quickly displayed on the surface of NK cells
50 upon stimulation. NK cells from mice infected with mouse cytomegalovirus express PD-1 both at
51 the transcript and at the protein level (11), and a PD-1^{high} NK cell population was identified in
52 human cytomegalovirus-seropositive healthy individuals (12). Therefore, there is now evidence
53 that NK cells, in different scenarios, express PD-1, but the mechanisms underlying PD-1
54 expression are still not clear.

55

56 Contrasting these reports, a recent paper convincingly showed that PD-1 is only minimally
57 expressed, both at the transcript and at the protein level, in NK cells in cancer patients and mouse
58 models of cancer or viral infection (13). Consistent with that study, we show here that, rather than
59 relying on endogenous expression, NK cells acquire PD-1 from tumor cells in two mouse models
60 of leukemia. We also found that in MM patients only NK cells that are positive for tumor cell

61 markers also stained for PD-1, suggesting that PD-1 on NK cells was acquired from MM cells.
62 Altogether, our data shed light on a new mechanism that could regulate NK cell functions via
63 acquisition of PD-1 from tumor cells and clarify why PD-1 protein can be detected on intratumoral
64 NK cells despite the lack of gene expression.

65 **Results and Discussion:**

66 When in contact with neighboring cells, lymphocytes often acquire proteins expressed on the
67 surface of the cells they interact with (14-19). RMA cells, which we extensively used in our last
68 study to show that PD-1 inhibits NK cell anti-tumor functions (4), derive from transformation of
69 murine T cells, and express high levels of PD-1 in culture (Fig. 1A). Given that: *i)* NK cells
70 infiltrating RMA tumors, but not splenic NK cells in the same animals, highly and consistently
71 stained for PD-1 (4); *ii)* RMA tumors express PD-1 (Fig. 1A); and *iii)* NK cells acquire membrane
72 fragments from target cells (20), we hypothesized that, rather than endogenously expressing PD-
73 1, NK cells acquire PD-1 from tumor cells via membrane transfer. To test this hypothesis, we
74 stably transduced RMA cells with a retroviral vector encoding the syngeneic marker Thy-1.1 (not
75 expressed by C57BL/6 mice, which express the Thy-1.2 allelic variant) and then generated a
76 PD-1-deficient RMA line (RMA-*Pdcd1*$^{-/-}$*Thy1.1*) (Fig. 1A). To determine if PD-1 was acquired
77 by NK cells via membrane transfer, we magnetically isolated splenic NK cells from *Pdcd1*$^{+/+}$ or
78 *Pdcd1*$^{-/-}$ mice (purity ~90%) and co-cultured them with either RMA or RMA-*Pdcd1*$^{-/-}$, both
79 expressing Thy1.1. After 3 days, we analyzed PD-1 and Thy-1.1 staining levels on NK cells by
80 flow cytometry. In absence of tumor cells, NK cells did not stain for PD-1 or Thy-1.1. In sharp
81 contrast, NK cells from both *Pdcd1*$^{+/+}$ and *Pdcd1*$^{-/-}$ mice stained positively for PD-1 when
82 incubated with RMA cells, but not RMA-*Pdcd1*$^{-/-}$ cells (Fig. 1B), whereas Thy-1.1 was abundantly
83 detected on the surface of NK cells in all conditions (Fig. 1B). These data indicate that NK cells
84 acquire surface proteins from tumor cells, and that PD-1 is not expressed by NK cells, but rather
85 acquired from tumor cells in these settings. The antibody used for PD-1 staining, clone 29F.1A12,
86 proved to bind to nuclear antigens on dying cells resulting in false-positive PD-1 staining (21).
87 However, we excluded dying cells from our analyses using viability staining and we failed to see

88 any staining in *Pdcd1*$^{-/-}$ NK cells incubated with RMA-*Pdcd1*$^{-/-}$ tumor cells (Fig. 1B), indicating
89 the specificity of the detected PD-1 signal.
90
91 Acquisition of PD-1 was not limited to NK cells but was also observed on other immune cells, as
92 shown in experiments using whole splenocytes. In these conditions, PD-1 and Thy1.1 were not
93 only acquired by NK cells, but also by CD8^{+} T and B cells (Fig. 1C). To complement published
94 transcriptomic data revealing limited *Pdcd1* mRNA expression (13) and our flow-cytometric
95 analysis showing high abundance of PD-1 protein on NK cells, we analyzed the *Pdcd1* locus in T
96 cells and NK cells using a published dataset (22). ATAC-seq analysis revealed that the promoter
97 region of the *Pdcd1* locus was not accessible in splenic NK cells, in contrast to the promoter region
98 of the *Tigit* locus which was accessible on both NK cells and CD8^{+} T cells (Fig. 1D).
99
100 Acquisition of PD-1 by NK cells was not limited to NK-RMA interactions. C1498 cells, often used
101 as a leukemia model, derive from transformation of murine NKT cells (23). A fraction of C1498
102 cells (~5%) expressed PD-1 in culture (Fig. 1E). We sorted PD-1^{+}C1498 cells, confirmed that they
103 stably expressed PD-1 upon 2 weeks in culture (Fig. 1F), and then incubated them for one or three
104 days with *Pdcd1*$^{-/-}$ NK cells. After 24 hrs, only NK cells incubated with C1498-PD-1+ cells stained
105 positively for PD-1 (Fig. 1G). PD-1 staining was further increased after 3 days of incubation, when
106 we also observed a modest shift in NK cells incubated with C1498 parental cells (Fig. 1G),
107 suggesting that PD-1 acquisition on NK cells depended on the level of expression on the donor
108 cells and increased over time.
109

110 Given that NK cells acquired proteins expressed by the hematopoietic cell lines RMA and C1498,
111 we next investigated if the same phenomenon could be observed with non-hematopoietic tumors.
112 We transduced 4T1, CT26 and TRAMP-C2, all of non-hematopoietic origin, with a *Thy1.1*
113 encoding vector, sorted the Thy-1.1+ population, and co-cultured them with purified splenic NK
114 cells for 3 days. As expected, Thy-1.1 decorated the surface of NK cells incubated not only with
115 RMA cells, but also with tumor cells of non-hematopoietic origin (Fig. 1H), confirming that NK
116 cells broadly acquire proteins from the surface of tumor cells they interact with. Expression of
117 PD-1 in non-hematopoietic cell lines was not observed, therefore we could not investigate if PD-1
118 was transferred to NK cells in these settings.
119
120 To better understand the nature of protein transfer from tumor to NK cells, we ran kinetic
121 experiments where splenic NK cells isolated from *Pdcd1*$^{-/-}$ mice were incubated with RMA cells
122 for 2, 8, 24 or 48 hrs, before analyzing PD-1 staining on NK cells. NK cells acquired PD-1 as early
123 as 2 hrs, and both the percentage of PD-1^{+} NK cells and the magnitude of PD-1 acquisition
124 increased over time (Fig. 2A), corroborating what observed using C1498 cells (Fig. 1G). Since
125 NK cells acquired PD-1 as early as 2 hrs, we repeated the co-culture including earlier time points.
126 NK cells acquired PD-1 as early as 15 minutes after co-culture with RMA cells was initiated (Fig.
127 2B), confirming previous reports showing that a few minutes of interactions are sufficient for NK
128 cells to acquire proteins from neighboring cells (14).
129
130 Transwell experiments, where NK and RMA cells were co-cultured for 24 hrs separated by a
131 semi-permeable membrane, revealed that cell-cell contact was required for NK cells to acquire
132 PD-1 from tumor cells (Fig. 2C), excluding a role for soluble or exosomal PD-1 in the acquisition

133 of PD-1 on NK cells. Rapid cell contact-dependent acquisition of proteins from NK cells to target
134 cells is consistent with trogocytosis, i.e. the acquisition of membrane fragments often performed
135 by immune cells (20). Corroborating this hypothesis, PD-1 transfer was accompanied by
136 acquisition of lipids from tumor cells, as revealed by experiments where NK cells were co-cultured
137 with RMA cells previously labelled with Cell-Vue, a dye that intercalates in the lipid regions of
138 the cellular membrane. Not only NK cells became robustly positive for the dye, but also PD-1
139 staining was more abundantly detected on NK cells that also acquired Cell-Vue (Fig. 2D). These
140 experiments indicate that PD-1 is acquired contextually with transfer of whole membrane
141 fragments from tumor to NK cells. Acquisition of proteins from donor cells can be facilitated via
142 receptor-ligand engagement, a phenomenon known as trans-endocytosis, which NK cells are
143 known to mediate (19). In culture, NK cells fail to express PD-L2 but express PD-L1 (Fig. 2E and
144 (24)), which could therefore serve as a ligand for trans-endocytosis-driven acquisition of PD-1
145 from RMA cells. However, when we co-cultured NK cells and RMA cells in the presence of a
146 saturating dose of PD-L1 blocking antibody, PD-1 acquisition was not reduced (Fig. 2F-G) as we
147 would expect if trans-endocytosis was involved. These data suggest that PD-1 trogocytosis does
148 not rely on engagement of PD-L1 expressed on NK cells.
149
150 As we showed that PD-1 is abundantly present in NK cells infiltrating RMA tumors (4), and in
151 light of our discovery that NK cells acquire PD-1 from RMA cells ex vivo, we performed in vivo
152 studies to determine if intratumoral NK cells acquired PD-1 from cancer cells. We injected
153 *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ mice with RMA or RMA-*Pdcd1*$^{-/-}$, both expressing Thy-1.1, and when tumors
154 reached ~300 mm^3 we analyzed intratumoral NK cells. In all cohorts of mice, NK cells infiltrating
155 the tumors highly stained for Thy-1.1 (Fig. 3A-D, Y axis), showing that membrane-transfer

156 occurred in vivo. Strikingly, high levels of PD-1 were detected on the surface of NK cells only
157 when tumor cells expressed PD-1, not only in *Pdcd1*$^{+/+}$, but also in *Pdcd1*$^{-/-}$ mice (Fig. 3A and C
158 vs B and D). These data not only show that PD-1 is acquired by tumor infiltrating NK cells, but
159 also that membrane-transfer is the major mechanism leading to PD-1 presence on the surface of
160 NK cells in the RMA model. Consistent with what was observed on NK cells, CD8^{+} T cells from
161 *Pdcd1*$^{-/-}$ mice also acquired Thy-1.1 and PD-1 from tumor cells (Fig. 3C), but, as expected, PD-1
162 staining in CD8^{+} T cells was also observed in *Pdcd1*$^{+/+}$ mice injected with PD-1-deficient RMA
163 cells, confirming that CD8^{+} T cells endogenously expressed PD-1. In our previous study, we
164 reported that PD-1 staining was higher on activated NK cells (4). Analysis of NK and T cells from
165 *Pdcd1*$^{-/-}$ mice infiltrating RMA tumors confirmed that PD-1+ NK and T cells also stained more
166 brightly for activation markers such as Sca-1 and CD69 (Fig. 3E). These data show that both T
167 and NK cells acquire fragments of tumor cell membranes in vivo, and that acquisition is higher in
168 activated lymphocytes.
169
170 Finally, to determine if NK cells acquire PD-1 from tumor cells in cancer patients, we analyzed
171 PD-1 staining in NK cells in MM patients, where PD-1 expression in NK cells was originally
172 reported (5). Three out of five patients analyzed had a sizeable (>1%) population of blasts in the
173 bone marrow (BM), whereas in two patients we failed to detect tumor cells. Interestingly, only in
174 the three patients with BM blasts (P1, P2, P3), we found a population of NK cells that stained
175 positive for CD138 (Fig. 4A), a protein that is expressed by MM but not NK cells. The blasts
176 presented heterogeneous expression of PD-1, which was abundant on all tumor cells (in P1), or
177 expressed bimodally by tumor cells (a small fraction - ~5%- in P2 or a larger fraction - %55%- in
178 P3) (Fig. 4B). Strikingly, in the two patients where PD-1 was mostly expressed on the tumor cells,

179 NK cells that stained for CD138 also stained highly for PD-1, at the same levels as tumor cells
180 (Fig. 4B in green), whereas CD138-negative NK cells were PD-1 negative, as were NK cells in
181 the peripheral blood, where no blasts were found (Fig. 4B, in blue and red, respectively).
182 Moreover, the patient with poor PD-1 expression on tumor cells also showed lower PD-1 staining
183 on $CD138^{+}$ NK cells in the BM (Fig. 4B, P2). Therefore, these data are consistent with the idea
184 that NK cells in MM patients acquire PD-1 and cancer cell markers from tumor cells.
185
186 In conclusions, this study highlights a new mechanism by PD-1 localizes on the surface not only
187 of NK cells, but also of T cells. We propose that acquisition of PD-1 by membrane transfer is a
188 previously unappreciated immune-modulatory mechanism employed by tumor cells to evade
189 immuno-surveillance. In light of these results, it will be important for future immune-profiling
190 efforts based on transcriptomic analysis to take into account that proteins are acquired, sometimes
191 at surprisingly high levels, by immune cells in the tumor microenvironment. While our study has
192 exclusively focused on tumors of hematopoietic origin, PD-1 acquisition is likely to occur in solid
193 malignancies as well, where NK cells and T cells constantly interact with other immune cells,
194 some of which express PD-1. Finally, pursuant to our previous studies and given its known
195 importance in suppressing anti-cancer responses we focused on PD-1, it is conceivable that other
196 proteins with immunomodulatory potential will be acquired by NK and T cells while interacting
197 with tumor cells. Characterization of the mechanisms underlying membrane transfer and
198 identification of molecules transferred to immune cells is required to elucidate how immune cells
199 are regulated by checkpoint receptors, and other proteins, in a transcription-independent fashion.

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Author contributions.

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
Hasim	X			X	X					X
Vulpis				X					X	X
Sciumè	X	X	X				X			X
Shih	X	X	X					X		X
Scheer				X						X
McMillan				X						X
Petrucci						X				
Santoni			X			X	X			X
Zingoni	X		X			X	X			X
Ardolino	X	X	X	X	X		X	X	X	X

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277

278

279 **Methods.**

280 **Mice and in vivo procedures.**

281 Mice were maintained at the University of Ottawa. *Pdcd1* knockout mice
282 (B6.Cg-Pdcd1tm1.1Shr/J) were purchased from The Jackson Laboratory and crossed with
283 C57BL/6J mice purchased from The Jackson Laboratory to obtain *Pdcd1* heterozygous mice.
284 Heterozygous mice were bred to obtain *Pdcd1*$^{+/+}$ and *Pdcd1*$^{-/-}$ littermates, which were used in all
285 experiments. For all experiments, sex-matched (both males and females) and age-matched (7 to
286 18 weeks old) mice were used.

287

288 For subcutaneous injections, tumor cells were resuspended in 100 µl PBS and injected in the left
289 flank. Tumors were collected when tumor volume was approximately 300 mm^3.

290

291 **Cell lines.**

292 All cell lines were cultured at 37ºC in a humidified atmosphere containing 5% CO_2. RMA-Thy1.1,
293 RMA-Thy1.1-*Pdcd1*$^{-/-}$ and C1498 cells were maintained in RPMI culture medium containing 100
294 U/ml penicillin, 100 µg/ml streptomycin, 0.2 mg/ml glutamine, 10 µg/ml gentamycin sulfate, 20
295 mM HEPES, and 5% FCS. 4T1, CT26, and TRAMP-C2 cell lines were cultured in DMEM
296 complemented with the same reagents. Cell line identity was confirmed by flow cytometry when
297 possible, and cells were regularly tested for mycoplasma.

298

299 **Ex vivo co-culture experiments.**

300 Murine splenic NK cells were isolated using the EasySep™ Mouse NK Cell Isolation Kit
301 (StemCell Technologies). In all experiments with isolated NK cells, 1000 U/mL rhIL-2 (NIH BRB

302 Preclinical Repository) was added to the culture medium. For co-culture experiments NK cells
303 were labelled with Cell Trace Violet proliferation dye (BD Bioscience) and tumor cells with CFSE
304 (Biolegend), unless otherwise indicated. 100,000 NK cells were co-cultured with tumor cells at a
305 1:1 ratio in 24 well plates in a final volume of 1 mL.
306
307 For transwell experiments (0.4 µm filter, Millipore), co-culture was set up in 6-well plates with a
308 final volume of 3 mL.
309
310 For membrane dye transfer experiments, NK cells were labelled with CFSE and RMA cells with
311 CellVue Claret FarRed (Sigma-Aldrich). 10,000 NK cells were then co-cultured with RMA cells
312 at a 1:10 ratio in 96-well V-bottom plates with a final volume of 100 µL.
313
314 In the kinetics experiments, NK cells were incubated with RMA cells and an aliquot of cells was
315 removed and fixed with BD Cytofix/perm at the indicated time points. Fixed cells were left at 4°C
316 until all time points were collected. All samples were stained together with the same antibody mix.
317
318 In experiments where PD-L1 was blocked, 5 µg of PD-L1 blocking antibody clone 10F.9G2 (or
319 isotype control) was added to the co-culture.
320
321 When whole spleens were used, 200,000 splenocytes were co-cultured with tumor cells at a 2:1
322 ratio in 6 well plates, in a final volume of 3 ml.
323
324 **Flow cytometry.**

325 When needed, tumors were excised from mice, cut in pieces, resuspended in serum-free media,
326 and dissociated using a gentle MACS dissociator (Miltenyi). Following dissociation, the single
327 cell suspension was passed through a 40 µm filter and cells were washed and resuspended in PBS
328 for staining. Spleens were harvested, gently dissociated through a 40 µm filter, washed, and red
329 blood cells were lysed using ACK buffer (Sigma), then washed and resuspended in PBS for
330 staining.
331
332 The cellular preparation was stained with the Zombie NIR Fixable Viability Dye (BioLegend) for
333 20 mins in PBS to label dead cells. Cells were then washed with flow buffer (PBS + 0.5% BSA)
334 and incubated for 20 minutes with purified rat anti-mouse CD16/CD32 (Clone 2.4G2) (BD
335 Biosciences) to block FcγRII/III receptors, followed by washing in flow buffer, and then incubated
336 for a further 20 minutes with primary specific antibodies. Cells were washed and resuspended in
337 flow buffer for sample acquisition or fixed in BD Cytofix/Cytoperm and acquired within 7 days.
338 Flow cytometry was performed using an LSRFortessa (BD) or a Celesta (BD), and data were
339 analyzed with FlowJo software (Tree Star Inc.)
340
341 **Antibodies.**
342 For experiments with murine cells, the following antibodies were used: *i)* from BD Biosciences:
343 anti-CD3ε (clone 145-2C11); anti-CD8a (clone 53-6.7); anti-CD11b (clone M1/70); anti-CD11c
344 (clone HL3); anti-CD49b (clone DX5); anti-CD69 (clone H1.2F3); anti-Ly6G (clone 1A8);
345 anti-NK1.1 (clone PK136); anti-Sca-1 (clone D7); *ii)* from Biolegend: anti-CD4 (clone RM4-5);
346 anti-CD19 (clone 6D5); anti-Thy-1.1 (clone OX-7); anti-F4/80 (clone BM8); anti-Ly6c (clone

347 HK1.4); anti-NKp46 (clone 29A1.4); anti-PD-1 (clone 29F.1A12); anti-PD-L1 (clone 10F.9G2);
348 rat IgG2a isotype control; and mouse-IgG1 isotype control.
349
350 For experiments with MM patients, the following antibodies were used: anti-CD138 (clone MI15),
351 anti-CD38 (clone HIT2), anti-CD3 (clone SK7), anti-CD56 (clone NCAM16.2), anti CD45 (clone
352 HI30), anti-CD16 (clone 3G8) and anti-PD1 (clone EH12.1), all from BD Biosciences
353
354 **Generation of cell line variants.**
355 RMA, 4T1, CT26 and TRAMP-C2 cells were transduced with the retroviral expression vector
356 MSCV-IRES-Thy1.1-DEST (Addgene, 17442), by spin infection (800 x g for 2 hours at 37°C)
357 with 8 μg/ml polybrene, and Thy1.1+ RMA cells were sorted.
358
359 Single-guide RNA (sgRNA) targeting the first exon of the *Pdcd1* gene (sequence:
360 TGTGGGTCCGGCAGGTACCC) was cloned into the LentiCRISPR lentiviral backbone vector
361 (Addgene 52961), also containing the *Cas9* gene. Lentiviral expression vectors were generated by
362 transfecting 293T cells with 2 μg vector with 2 μg packaging plus polymerase-encoding plasmids
363 using Lipofectamine 2000. Virus-containing supernatants were used to transduce RMA-*Thy1.1*
364 cells by spin infection and PD-1 negative cells were sorted.
365
366 C1498-PD-1+ cells were obtained by sorting PD-1+ C1498 parental cells.
367
368 All engineered cells were regularly assessed for phenotype maintenance by flow cytometry.
369

370 **ATAC-seq.**

371 Genomic snapshots were generated using IGV software (Broad Institute) using data available on
372 GEO: GSE77695.

373

374 **Analysis of multiple myeloma patients.**

375 Peripheral blood and BM were obtained from MM patients enrolled at the Division of Hematology
376 ("Sapienza" University of Rome). Both peripheral blood and BM samples were lysed using a
377 buffer composed of 1.5 M NH_4Cl, 100 mM $NaHCO_3$, and 10 mM EDTA and then stained as
378 described above.

379

380 **Study approvals.**

381 Mouse studies were reviewed and approved by Animal Care Veterinary Services at the University
382 of Ottawa in accordance with the guidelines of Canadian Institutes of Health Research. For human
383 studies, informed and written consent in accordance with the Declaration of Helsinki was obtained
384 from all patients, and approval was obtained from the Ethics Committee of the Sapienza University
385 of Rome (RIF.CE: 5191).

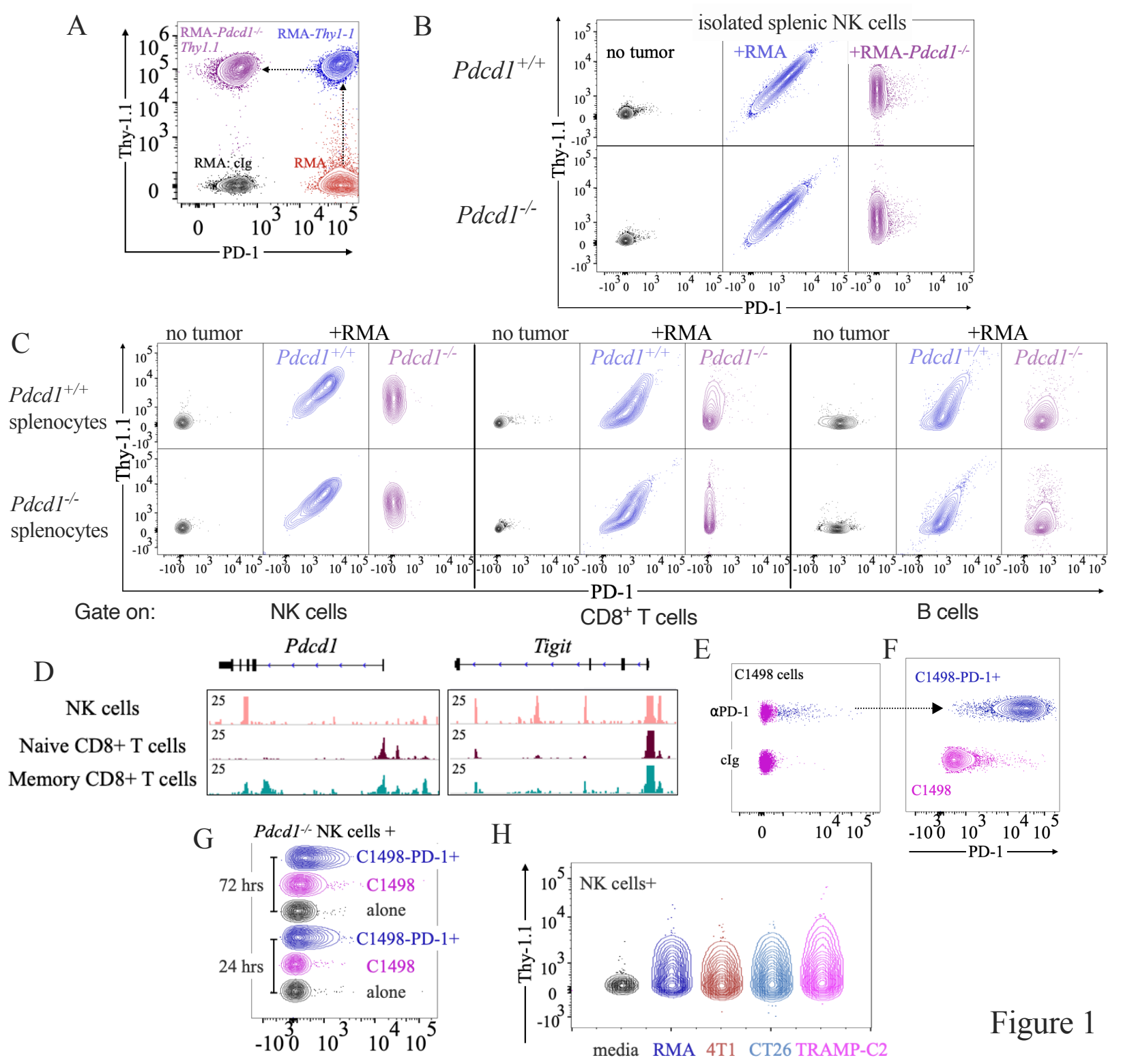

Figure 1: NK cells acquire PD-1 from cancer cells. (A) RMA cells (red) were transduced with a retroviral vector encoding Thy-1.1 to generate RMA-*Thy1.1* (blue) and then PD-1 was knocked-out by CRISPR/Cas9 to generate RMA-*Pdcd1*$^{-/-}$*Thy1.1* (pink). A representative flow-cytometry staining depicting PD-1 and Thy-1.1 expression is shown. (B) NK cells isolated from *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ littermates were incubated with RMA-*Thy1.1* or RMA-*Pdcd1*$^{-/-}$*Thy1.1*. After 3 days, cells were stained with Thy1.1 and PD-1 antibodies. NK cells were gated as singlets/live-NK1.1^{+}NKp46^{+}DX5^{+} events. The experiment depicted is representative of three performed with similar results. (C) Splenocytes from *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ littermates were incubated with RMA-*Thy1.1* or RMA-*Pdcd1*$^{-/-}$*Thy1.1*. After 3 days, cells were stained with Thy1.1 and PD-1 antibodies. CD8^{+} T cells were gated as singlets/live-CD3^{+}CD8^{+} events, B cells as singlets/live-CD19^{+}. The experiment depicted is representative of two three performed with similar results. (D) Genomic snapshots of normalized ATAC-seq signals in NK cells, naïve and memory CD8+ T cells across *Pdcd1* and *Tigit* loci. (E) C1498 cells were stained with PD-1 antibody or isotype control. PD-1+ cells (in blue) were flow-sorted and after 2 weeks in culture stained for PD-1, alongside with parental C1498 cells (F). (G) Splenic NK cells isolated from *Pdcd1*$^{-/-}$ mice were co-cultured with C1498 or C1498-PD-1+ cells, or without tumor cells as a control, for 24 hrs or 72 hrs, and stained for PD-1. The experiment depicted is representative of three performed with similar results. (H) Splenic NK cells isolated from *Pdcd1*$^{-/-}$ mice were co-cultured with RMA, 4T1, CT26 or TRAMP-C2 cells, previously transduced and sorted to stably express Thy-1.1. Thy-1.1 staining was analyzed after 3 days by flow-cytometry. In all panels, NK cells were gated as described in 1B.

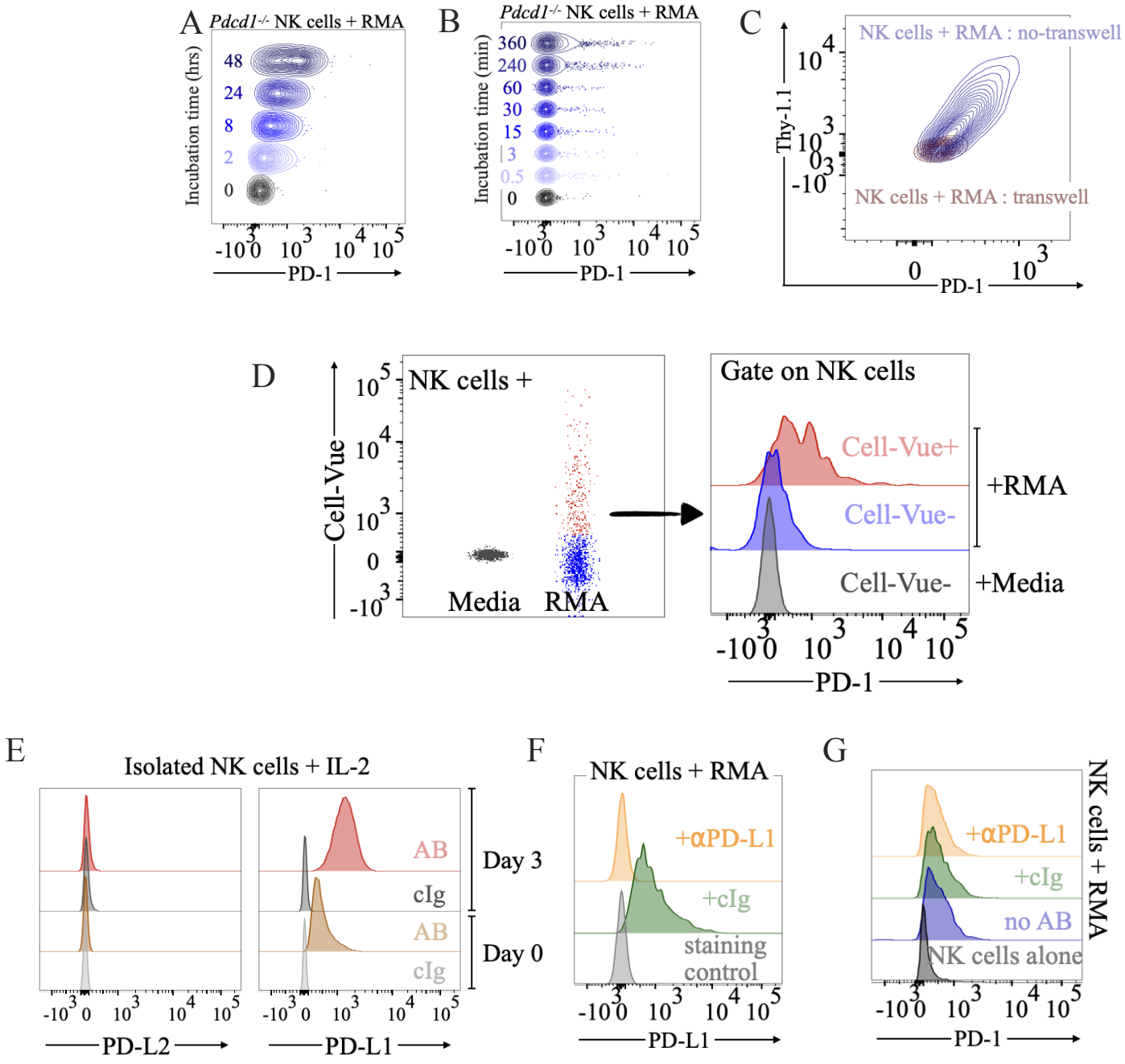

Figure 2

Figure 2. NK cells acquire PD-1 via trogocytosis. Splenic NK cells isolated from a *Pdcd1*$^{-/-}$ mouse were co-cultured with RMA cells for 2, 8, 24 or 48 hrs (A) or 0.5, 3, 15, 30, 60, 240, 360 mins (B) before staining for PD-1. (C) Splenic NK cells isolated from a *Pdcd1*$^{-/-}$ mouse were co-cultured for 24 hrs with RMA cells separated or not by a transwell semi-permeable membrane before staining for PD-1 and Thy-1.1. The experiment depicted is representative of two performed with similar results. (D) NK cells were incubated with RMA cells pre-labelled with Cell-Vue for 24 hrs. Cell-Vue and PD-1 staining on NK cells is depicted, on the left and right respectively. (E) Splenic NK cells isolated from a *Pdcd1*$^{-/-}$ mouse were cultured for 3 days and then PD-L2 and PD-L1 expression was analyzed by flow-cytometry. Representative of three experiments performed with similar results. (F-G) NK cells were incubated with RMA cells in the presence of a PD-L1 blocking antibody or an isotype control for 24 hrs, before being stained for PD-1 and PD-L1. As additional controls, NK cells were: *i)* co-cultured with RMA without adding any antibody; or *ii)* cultured alone without adding tumor cells. In all panels, NK cells were gated as in 1B.

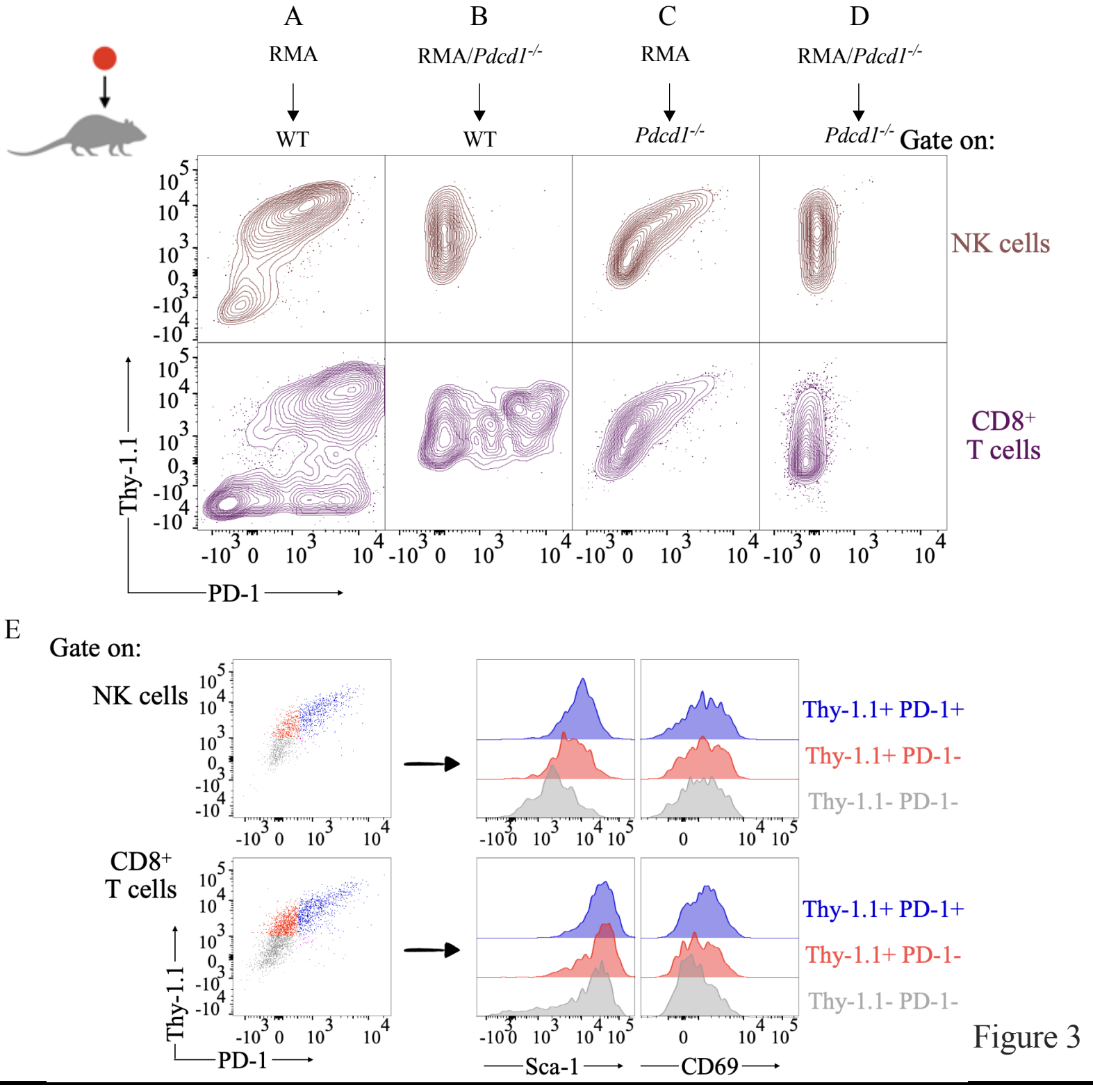

Figure 3: Intratumoral NK and T cells acquire PD-1 from tumor cells. (A-D) *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ mice were injected with RMA or RMA-*Pdcd1*$^{-/-}$ tumors. PD-1 and Thy-1.1 staining was assessed by flow-cytometry on tumor infiltrating NK and T cells (gated as in Fig. 1B and 1C, respectively). Expression of Sca1 and CD69 was analyzed on *Pdcd1*$^{-/-}$ NK and T cells infiltrating RMA tumors, by gating on Thy-1.1^{-}PD-1^{-} (gray), Thy-1.1^{+}PD-1^{-} (red) or Thy-1.1^{+}PD-1^{+} (blue) cells. The mouse depicted in 4E is the same depicted in 4C. The experiment shown is representative of two performed with similar results. At least 4 mice/group were analyzed.

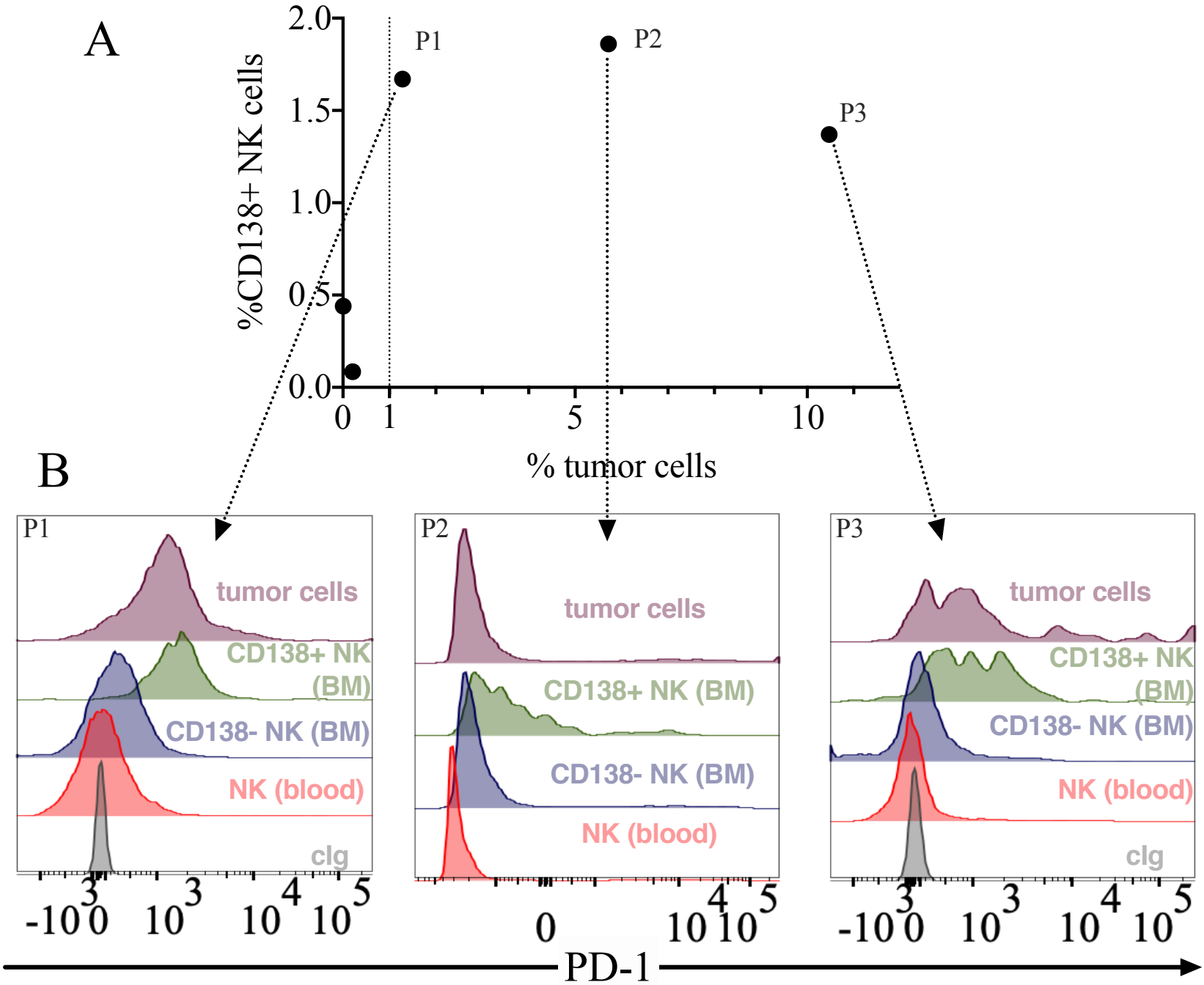

Figure 4

Figure 4: NK cells labelled with MM markers also stain for PD-1. (A) The presence of blasts was correlated with the frequency of $CD138^{+}$ NK cells in BM aspirates from MM patients. Tumor cells were gated as viable-$CD45^{low}CD138^{+}$ events, NK cells were gated as viable-$CD45^{+}CD3^{-}CD56^{+}$ events. (B) PD-1 staining was analyzed by flow-cytometry in MM cells (purple), NK cells in the blood (red), or in the BM ($CD138^{-}$, blue vs $CD138^{+}$, green) of the patients showed in A.