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

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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.

Introduction:

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

Contrasting these reports, a recent paper convincingly showed that PD-1 is only minimally expressed, both at the transcript and at the protein level, in NK cells in cancer patients and mouse models of cancer or viral infection (13). Consistent with that study, we show here that, rather than relying on endogenous expression, NK cells acquire PD-1 from tumor cells in two mouse models 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.

Results and Discussion:

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

any staining in *Pdcd1*$^{-/-}$ NK cells incubated with RMA-*Pdcd1*$^{-/-}$ tumor cells (Fig. 1B), indicating the specificity of the detected PD-1 signal.

Acquisition of PD-1 was not limited to NK cells but was also observed on other immune cells, as shown in experiments using whole splenocytes. In these conditions, PD-1 and Thy1.1 were not only acquired by NK cells, but also by CD8^{+} T and B cells (Fig. 1C). To complement published transcriptomic data revealing limited *Pdcd1* mRNA expression (13) and our flow-cytometric analysis showing high abundance of PD-1 protein on NK cells, we analyzed the *Pdcd1* locus in T cells and NK cells using a published dataset (22). ATAC-seq analysis revealed that the promoter region of the *Pdcd1* locus was not accessible in splenic NK cells, in contrast to the promoter region of the *Tigit* locus which was accessible on both NK cells and CD8^{+} T cells (Fig. 1D).

Acquisition of PD-1 by NK cells was not limited to NK-RMA interactions. C1498 cells, often used as a leukemia model, derive from transformation of murine NKT cells (23). A fraction of C1498 cells (~5%) expressed PD-1 in culture (Fig. 1E). We sorted PD-1^{+}C1498 cells, confirmed that they stably expressed PD-1 upon 2 weeks in culture (Fig. 1F), and then incubated them for one or three days with *Pdcd1*$^{-/-}$ NK cells. After 24 hrs, only NK cells incubated with C1498-PD-1+ cells stained positively for PD-1 (Fig. 1G). PD-1 staining was further increased after 3 days of incubation, when we also observed a modest shift in NK cells incubated with C1498 parental cells (Fig. 1G), suggesting that PD-1 acquisition on NK cells depended on the level of expression on the donor cells and increased over time.

Given that NK cells acquired proteins expressed by the hematopoietic cell lines RMA and C1498, we next investigated if the same phenomenon could be observed with non-hematopoietic tumors. We transduced 4T1, CT26 and TRAMP-C2, all of non-hematopoietic origin, with a *Thy1.1* encoding vector, sorted the Thy-1.1+ population, and co-cultured them with purified splenic NK cells for 3 days. As expected, Thy-1.1 decorated the surface of NK cells incubated not only with RMA cells, but also with tumor cells of non-hematopoietic origin (Fig. 1H), confirming that NK cells broadly acquire proteins from the surface of tumor cells they interact with. Expression of PD-1 in non-hematopoietic cell lines was not observed, therefore we could not investigate if PD-1 was transferred to NK cells in these settings.

To better understand the nature of protein transfer from tumor to NK cells, we ran kinetic experiments where splenic NK cells isolated from *Pdcd1*$^{-/-}$ mice were incubated with RMA cells for 2, 8, 24 or 48 hrs, before analyzing PD-1 staining on NK cells. NK cells acquired PD-1 as early as 2 hrs, and both the percentage of PD-1^{+} NK cells and the magnitude of PD-1 acquisition increased over time (Fig. 2A), corroborating what observed using C1498 cells (Fig. 1G). Since NK cells acquired PD-1 as early as 2 hrs, we repeated the co-culture including earlier time points. NK cells acquired PD-1 as early as 15 minutes after co-culture with RMA cells was initiated (Fig. 2B), confirming previous reports showing that a few minutes of interactions are sufficient for NK cells to acquire proteins from neighboring cells (14).

Transwell experiments, where NK and RMA cells were co-cultured for 24 hrs separated by a semi-permeable membrane, revealed that cell-cell contact was required for NK cells to acquire PD-1 from tumor cells (Fig. 2C), excluding a role for soluble or exosomal PD-1 in the acquisition

of PD-1 on NK cells. Rapid cell contact-dependent acquisition of proteins from NK cells to target cells is consistent with trogocytosis, i.e. the acquisition of membrane fragments often performed by immune cells (20). Corroborating this hypothesis, PD-1 transfer was accompanied by acquisition of lipids from tumor cells, as revealed by experiments where NK cells were co-cultured with RMA cells previously labelled with Cell-Vue, a dye that intercalates in the lipid regions of the cellular membrane. Not only NK cells became robustly positive for the dye, but also PD-1 staining was more abundantly detected on NK cells that also acquired Cell-Vue (Fig. 2D). These experiments indicate that PD-1 is acquired contextually with transfer of whole membrane fragments from tumor to NK cells. Acquisition of proteins from donor cells can be facilitated via receptor-ligand engagement, a phenomenon known as trans-endocytosis, which NK cells are known to mediate (19). In culture, NK cells fail to express PD-L2 but express PD-L1 (Fig. 2E and (24)), which could therefore serve as a ligand for trans-endocytosis-driven acquisition of PD-1 from RMA cells. However, when we co-cultured NK cells and RMA cells in the presence of a saturating dose of PD-L1 blocking antibody, PD-1 acquisition was not reduced (Fig. 2F-G) as we would expect if trans-endocytosis was involved. These data suggest that PD-1 trogocytosis does not rely on engagement of PD-L1 expressed on NK cells.

As we showed that PD-1 is abundantly present in NK cells infiltrating RMA tumors (4), and in light of our discovery that NK cells acquire PD-1 from RMA cells ex vivo, we performed in vivo studies to determine if intratumoral NK cells acquired PD-1 from cancer cells. We injected *Pdcd1*$^{+/+}$ or *Pdcd1*$^{-/-}$ mice with RMA or RMA-*Pdcd1*$^{-/-}$, both expressing Thy-1.1, and when tumors reached ~300 mm^3 we analyzed intratumoral NK cells. In all cohorts of mice, NK cells infiltrating 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,

NK cells that stained for CD138 also stained highly for PD-1, at the same levels as tumor cells (Fig. 4B in green), whereas CD138-negative NK cells were PD-1 negative, as were NK cells in the peripheral blood, where no blasts were found (Fig. 4B, in blue and red, respectively). Moreover, the patient with poor PD-1 expression on tumor cells also showed lower PD-1 staining on $CD138^{+}$ NK cells in the BM (Fig. 4B, P2). Therefore, these data are consistent with the idea that NK cells in MM patients acquire PD-1 and cancer cell markers from tumor cells.

In conclusions, this study highlights a new mechanism by PD-1 localizes on the surface not only of NK cells, but also of T cells. We propose that acquisition of PD-1 by membrane transfer is a previously unappreciated immune-modulatory mechanism employed by tumor cells to evade immuno-surveillance. In light of these results, it will be important for future immune-profiling efforts based on transcriptomic analysis to take into account that proteins are acquired, sometimes at surprisingly high levels, by immune cells in the tumor microenvironment. While our study has exclusively focused on tumors of hematopoietic origin, PD-1 acquisition is likely to occur in solid malignancies as well, where NK cells and T cells constantly interact with other immune cells, some of which express PD-1. Finally, pursuant to our previous studies and given its known importance in suppressing anti-cancer responses we focused on PD-1, it is conceivable that other proteins with immunomodulatory potential will be acquired by NK and T cells while interacting with tumor cells. Characterization of the mechanisms underlying membrane transfer and identification of molecules transferred to immune cells is required to elucidate how immune cells 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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Methods.

Mice and in vivo procedures.

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

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

Cell lines.

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

Ex vivo co-culture experiments.

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

Preclinical Repository) was added to the culture medium. For co-culture experiments NK cells were labelled with Cell Trace Violet proliferation dye (BD Bioscience) and tumor cells with CFSE (Biolegend), unless otherwise indicated. 100,000 NK cells were co-cultured with tumor cells at a 1:1 ratio in 24 well plates in a final volume of 1 mL.

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

For membrane dye transfer experiments, NK cells were labelled with CFSE and RMA cells with CellVue Claret FarRed (Sigma-Aldrich). 10,000 NK cells were then co-cultured with RMA cells at a 1:10 ratio in 96-well V-bottom plates with a final volume of 100 μL.

In the kinetics experiments, NK cells were incubated with RMA cells and an aliquot of cells was removed and fixed with BD Cytofix/perm at the indicated time points. Fixed cells were left at 4°C until all time points were collected. All samples were stained together with the same antibody mix.

In experiments where PD-L1 was blocked, 5 μg of PD-L1 blocking antibody clone 10F.9G2 (or isotype control) was added to the co-culture.

When whole spleens were used, 200,000 splenocytes were co-cultured with tumor cells at a 2:1 ratio in 6 well plates, in a final volume of 3 ml.

Flow cytometry.

When needed, tumors were excised from mice, cut in pieces, resuspended in serum-free media, and dissociated using a gentle MACS dissociator (Miltenyi). Following dissociation, the single cell suspension was passed through a 40 μm filter and cells were washed and resuspended in PBS for staining. Spleens were harvested, gently dissociated through a 40 μm filter, washed, and red blood cells were lysed using ACK buffer (Sigma), then washed and resuspended in PBS for staining.

The cellular preparation was stained with the Zombie NIR Fixable Viability Dye (BioLegend) for 20 mins in PBS to label dead cells. Cells were then washed with flow buffer (PBS + 0.5% BSA) and incubated for 20 minutes with purified rat anti-mouse CD16/CD32 (Clone 2.4G2) (BD Biosciences) to block FcγRII/III receptors, followed by washing in flow buffer, and then incubated for a further 20 minutes with primary specific antibodies. Cells were washed and resuspended in flow buffer for sample acquisition or fixed in BD Cytofix/Cytoperm and acquired within 7 days. Flow cytometry was performed using an LSRFortessa (BD) or a Celesta (BD), and data were analyzed with FlowJo software (Tree Star Inc.)

Antibodies.

For experiments with murine cells, the following antibodies were used: *i)* from BD Biosciences: anti-CD3ε (clone 145-2C11); anti-CD8a (clone 53-6.7); anti-CD11b (clone M1/70); anti-CD11c (clone HL3); anti-CD49b (clone DX5); anti-CD69 (clone H1.2F3); anti-Ly6G (clone 1A8); anti-NK1.1 (clone PK136); anti-Sca-1 (clone D7); *ii)* from Biolegend: anti-CD4 (clone RM4-5); anti-CD19 (clone 6D5); anti-Thy-1.1 (clone OX-7); anti-F4/80 (clone BM8); anti-Ly6c (clone

HK1.4); anti-NKp46 (clone 29A1.4); anti-PD-1 (clone 29F.1A12); anti-PD-L1 (clone 10F.9G2); rat IgG2a isotype control; and mouse-IgG1 isotype control.

For experiments with MM patients, the following antibodies were used: anti-CD138 (clone MI15), anti-CD38 (clone HIT2), anti-CD3 (clone SK7), anti-CD56 (clone NCAM16.2), anti CD45 (clone HI30), anti-CD16 (clone 3G8) and anti-PD1 (clone EH12.1), all from BD Biosciences

Generation of cell line variants.

RMA, 4T1, CT26 and TRAMP-C2 cells were transduced with the retroviral expression vector MSCV-IRES-Thy1.1-DEST (Addgene, 17442), by spin infection (800 x g for 2 hours at 37°C) with 8 μg/ml polybrene, and Thy1.1+ RMA cells were sorted.

Single-guide RNA (sgRNA) targeting the first exon of the *Pdcd1* gene (sequence: TGTGGGTCCGGCAGGTACCC) was cloned into the LentiCRISPR lentiviral backbone vector (Addgene 52961), also containing the *Cas9* gene. Lentiviral expression vectors were generated by transfecting 293T cells with 2 μg vector with 2 μg packaging plus polymerase-encoding plasmids using Lipofectamine 2000. Virus-containing supernatants were used to transduce RMA-*Thy1.1* cells by spin infection and PD-1 negative cells were sorted.

C1498-PD-1+ cells were obtained by sorting PD-1+ C1498 parental cells.

All engineered cells were regularly assessed for phenotype maintenance by flow cytometry.

ATAC-seq.

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

Analysis of multiple myeloma patients.

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

Study approvals.

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

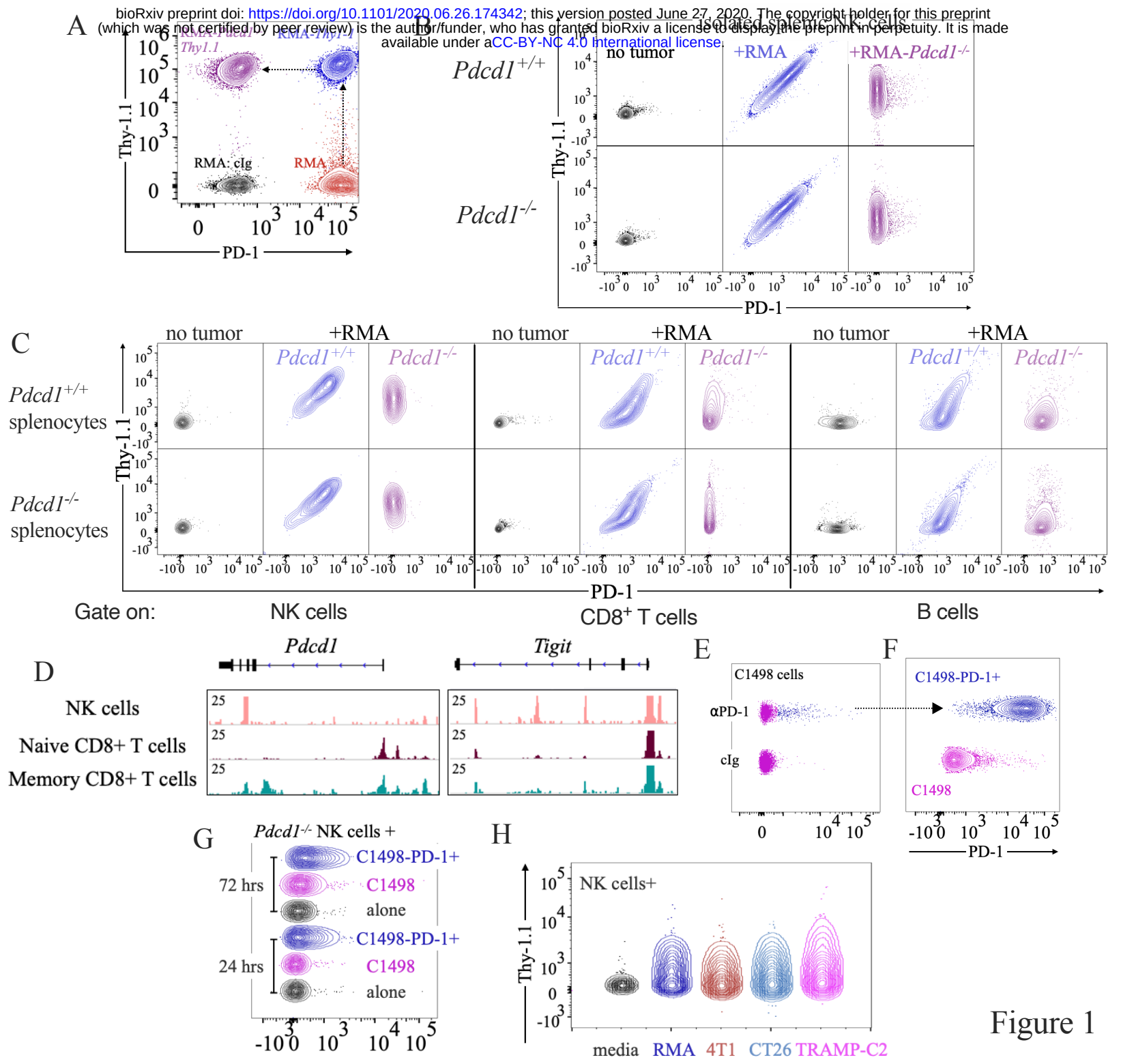

Figure 1

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.

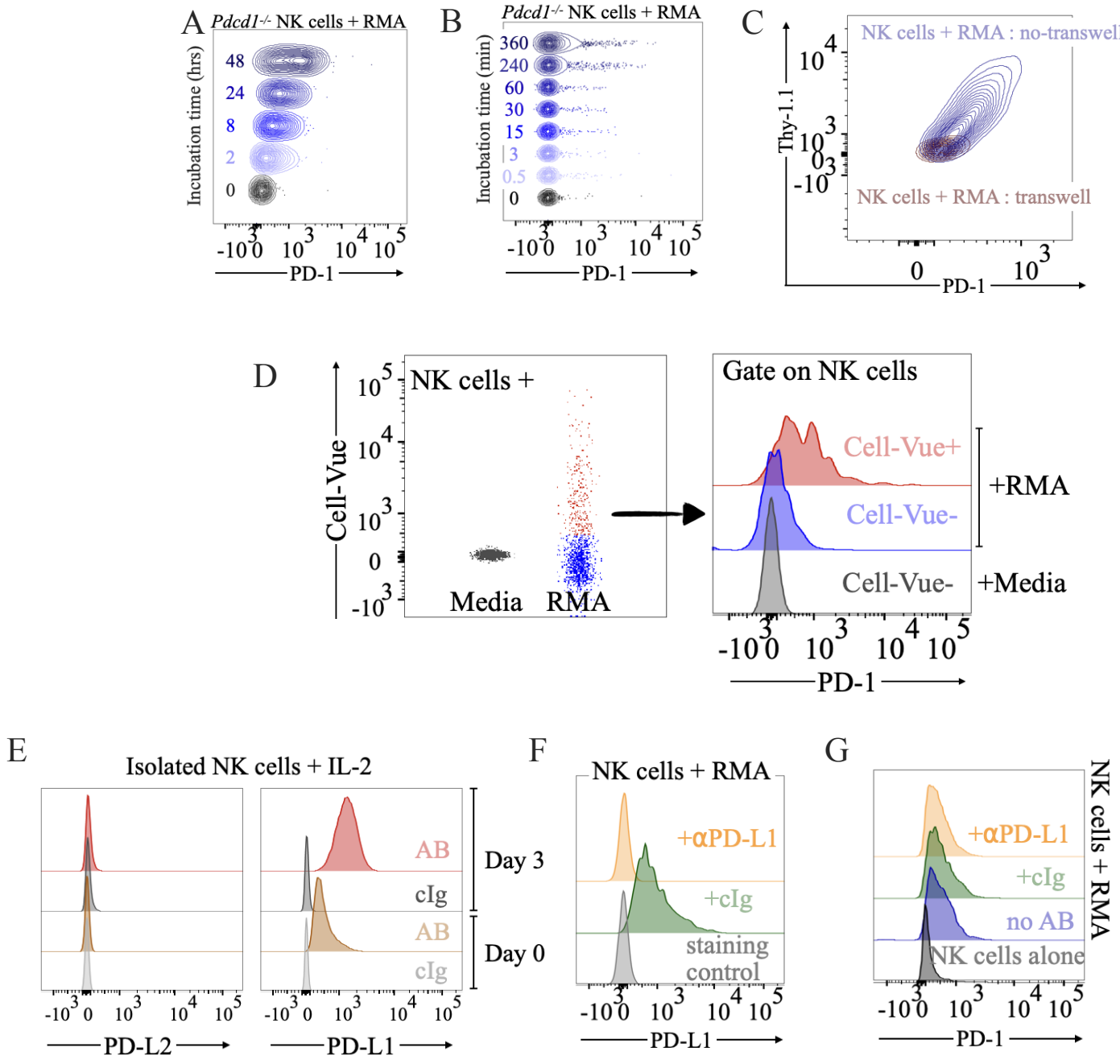

Figure 2

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.

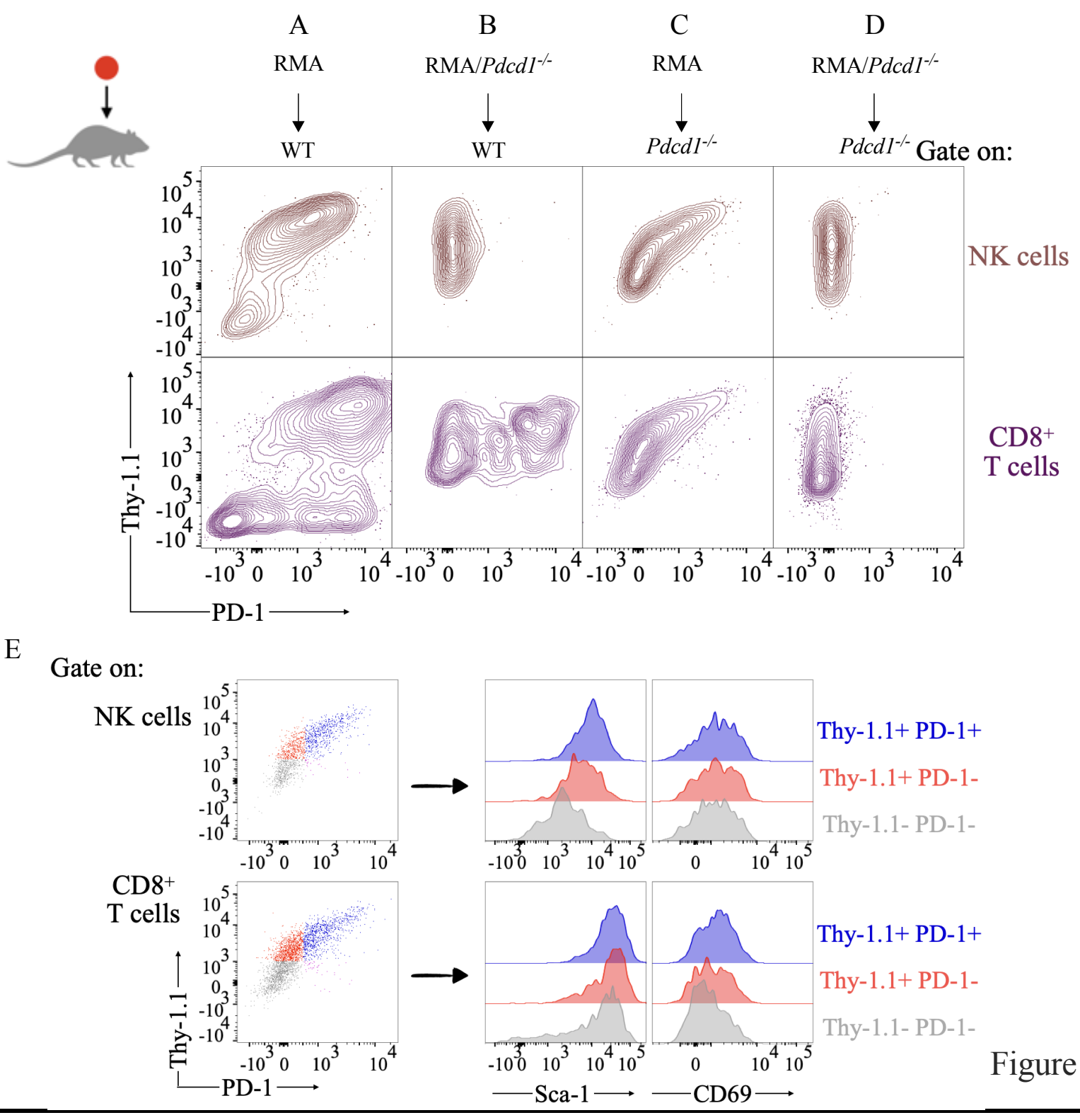

Figure 3

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.

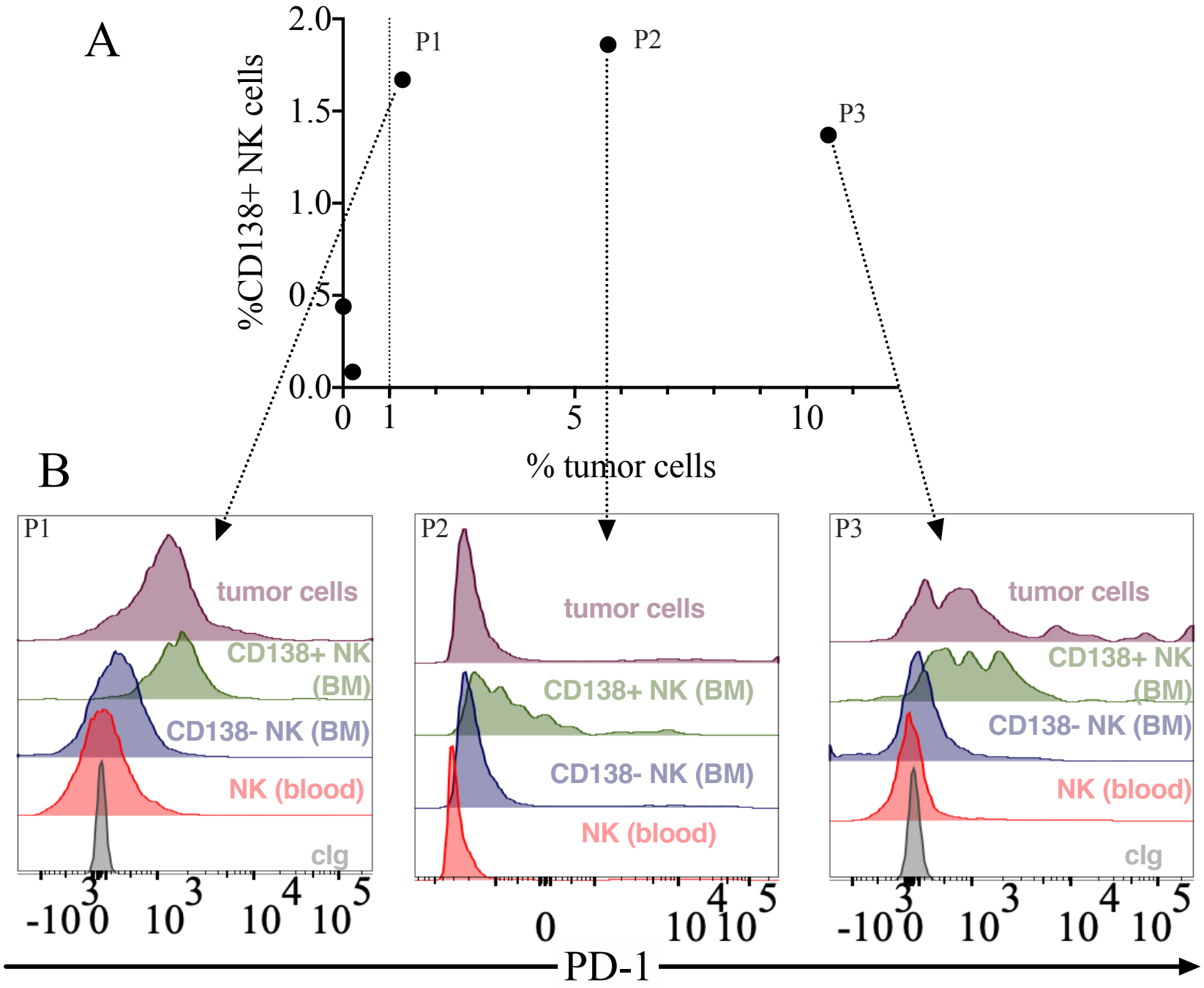

Figure 4

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.