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ORIGINAL RESEARCH

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PD-1 is expressed in cytotoxic granules of NK cells and rapidly mobilized to the cell membrane following recognition of tumor cells

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ABSTRACT

The contribution of the T cell-related inhibitory checkpoint PD-1 to the regulation of NK cell activity is still not clear with contradictory results concerning its expression and role in the modulation of NK cell cytotoxicity. We provide novel key findings on the mechanism involved in the regulation of PD-1 expression on NK cell membrane and its functional consequences for the elimination of cancer cells. In contrast to freshly isolated NK cells from cancer patients, those from healthy donors did not express PD-1 on the cell membrane. However, when healthy NK cells were incubated with tumor target cells, membrane PD-1 expression increased, concurrent with the CD107a surface mobilization. This finding suggested that PD-1 was translocated to the cell membrane during NK cell degranulation after contact with target cells. Indeed, cytosolic PD-1 was expressed in freshly-isolated-NK cells and partly co-localized with CD107a and GzmB, confirming that membrane PD-1 corresponded to a pool of preformed PD-1. Moreover, NK cells that had mobilized PD-1 to the cell membrane presented a significantly reduced anti-tumor activity on PD-L1-expressing-tumor cells *in vitro* and *in vivo*, which was partly reversed by using anti-PD-1 blocking antibodies. Our results indicate that NK cells from healthy individuals express cytotoxic granule-associated PD-1, which is rapidly mobilized to the cell membrane after interaction with tumor target cells. This novel finding helps to understand how PD-1 expression is regulated on NK cell membrane and the functional consequences of this expression during the elimination of tumor cells, which will help to design more efficient NK cell-based cancer immunotherapies.

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Introduction

Natural killer (NK) cells are cytotoxic lymphocytes from the innate immune system that are critically involved in the elimination of viral-infected and tumor cells. NK cell activation as well as NK cell-mediated elimination of target cells are regulated by a balance between positive and negative signals transduced by activating and inhibitory receptors that recognize specific ligands on target cells.[1–3] The main inhibitory ligands are MHC-I/HLA-I proteins that are recognized by the KIR (killer-cell immunoglobulin-like receptor) family (HLA-A/B/C), NKG2A (HLA-E/G), and ILT2 (HLA-G) receptors.[4] Regarding activating signals, several cellular-derived ligands are recognized by activating or costimulatory receptors such as Natural Cytotoxicity Receptors (NKp30, NKp44, and NKp46) NKG2D, NKG2C, or DNAM-1.[4] While some of these receptors are highly specific to NK cells like NKp46, others likeNKp30, NKG2D, or DNAM-1 are also expressed by some T-cell subsets. All these activating and inhibitory receptors have been extensively studied and characterized, and they are considered NK cell-associated immune checkpoints that regulate NK cell activity and tolerance, preventing the elimination of self-healthy cells.[5] In contrast, the role of other immune checkpoints, traditionally associated with the regulation of T cell-mediated responses, is less clear.[5,6] T cell inhibitory immune checkpoints are physiological regulators of peripheral tolerance that prevent T cell activation against self-derived antigens.[7] The first described immune checkpoint receptors were CTLA-4 and PD-1 and since then, several others have been found including TIM3, VISTA, LAG-3, and TIGIT.[7] Tumors have learned to exploit these physiological mechanisms of tolerance to overcome host immunosurveillance, preventing T cell activation and elimination of cancer cells. Monoclonal antibodies (mAb) against CTLA-4 and PD-1 (or against its ligand PD-L1) have shown good clinical efficacy

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against cancers like melanoma or lung carcinoma that present a high tumor mutational burden.[8] Notably, the use of mAb against PD-1 or PD-L1 has been expanded to more cancer types than CTLA-4 mAb, which might be related to a broader expression of PD-1 in immune cells.[9]

PD-1 was originally described as a negative regulator of TCR-mediated signaling in T cells, in which it modulates the activation threshold during antigen recognition mediated by the TCR/CD3 complex. However, in contrast to T cells, activation of NK cells does not depend on TCR-mediated antigen recognition. Thus, the role of PD-1 in the physiological regulation of NK cell function is less characterized than in T cells, and the results are often not clear and/or contradictory.[9–11] At present, PD-1 membrane expression (mPD-1) in healthy donors has been restricted to a subset of adaptive NK cells from CMV chronic infected donors.[12,13] In addition, different studies have shown that NK cells from cancer patients express PD-1.[5,13–16] From a functional point of view, recent findings using *in vivo* mouse models[17,18] indicate that NK cells contribute to the antitumoral activity of PD-1 and/or PD-L1 mAb.[5,18] Despite these findings, the regulation of the expression and function of PD-1 in naive and activated NK cells from healthy donors (HD) is less clearly understood, a question that might influence the efficacy of adoptive NK cell transfer in cancer. Recently, it was shown that freshly isolated as well as activated NK cells expressed a pool of preformed cytosolic PD-1.[17] In this work, two different isoforms corresponding to full PD-1 and a lighter PD-1 isoform were detected. Moreover, secreted PD-1 (sPD-1) has been found to be increased in biological fluids and NK cells in patients with different pathologies.[19,20]

Although few studies have analyzed the role of sPD-1 in cancer, some experimental evidence correlates the level of sPD-1 with cancer stage, prognosis, and response to treatment.[21]

Despite the studies that indicate that NK cells from healthy donors express cytoplasmic PD-1, it is not known what the stimulus is to mobilize this cytoplasmic PD-1 pool to the cell membrane and the functional consequences of this mobilization in NK cell anti-tumoral activity. Here, we hypothesize that NK cell degranulation during target cell elimination leads to the mobilization of cytosolic PD-1 to the cell membrane, which might affect the sensitivity of tumor cells to NK cell-mediated cytotoxicity.

Materials and methods

Cell lines

The HLA-null K562 cell line was cultured in Roswell Park Memorial Institute medium (RPMI; Sigma-Aldrich). SH-SY5Y neuroblastoma and HCT-116 colorectal cancer cell lines expressing Green Fluorescence Protein (GFP) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich). Both culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyCloneTM), penicillin/streptomycin (Sigma-Aldrich), and 2 mM Ultraglutamine (Lonza).

The human primary NK cells were enriched using CD56 microbeads for magnetic positive selection (MACS, Miltenyi) from either freshly isolated Peripheral Blood Mononuclear cells (PBMCs) or overnight PBMC and target cell co-cultures (see "NK cell cytotoxicity assay" below). PBMCs were obtained by Ficoll-Paque gradient centrifugation from blood obtained from Healthy Donors (CEICA, C.I.PI11/006), leukemia, and lung cancer patients (CEICA.PI19/052) or COVID-19 patients (CEICA. PI20/165).

PD-L1 knock out by CRISPR/Cas9

To knock down PDL1 in HCT-116 cells, they were co-transfected using polyethylenimine (PEI, Sigma) transfection reagent with pooled Pdcd-1L1 CRISPR/Cas9 knockout (KO) plasmids (sc-425636) and Pdcd-1L1 homology-directed DNA repair (HDR) plasmids (sc-425636-HDR) (Santa Cruz Biotechnology). After overnight incubation, the medium was replaced with a fresh medium, and 2 days later, transfected cells were selected using 2 µg/mL puromycin (InvivoGen).

Cell transfection was performed as described above using pooled CRISPR-Cas9 KO plasmids (Santa Cruz Biotechnology) and homology-directed DNA repair (HDR) plasmids, corresponding to the cut sites generated by the CRISPR-Cas9 KO plasmids, which are coded for a puromycin resistance cassette and red fluorescent protein (RFP; Santa Cruz Biotechnology).

Flow cytometry analyses

NK cells were analyzed for cell surface marker expression by flow cytometry with a Gallios cytometer (Beckman Coulter). The following fluorophore-conjugated antibodies were used: CD56-PerCP-Vio700 (clone REA196), CD56-PE (clone REA196), PD-1-VioBright FITC (clone PD1.3.1.3), CD335 (NKp46)-APC (clone 9E2), CD159a (NKG2A)-PE-Vio770 (clone REA110), and CD223 (LAG3)-APC (clone REA351) all purchased from Miltenyi; CD337 (NKp30)-PE (clone p30-15) and CD314 (NKG2D)-BV421 (clone 1D11) purchased from BD Bioscience and PD-1-Alexa Fluor700 (clone EH12.2H7) and TIM3-PE/Cy7 (clone F38-2E2) purchased from Biolegend. A dump channel was designed to exclude dead and non-interest cells, consisting of the following fluorophore-conjugated antibodies: CD3-VioGreen (clone REA613), CD19-VioGreen (clone REA675), CD14-VioGreen (clone REA599) (Miltenyi) and LIVE/DEAD™ Fixable Yellow Stain (Thermo Fisher). The gating strategy for NK cells is shown in supplemental figure S1. Antibodies were diluted in phosphate-buffered saline (PBS) supplemented with 5% FBS and Fcγ Block Reagent (BD Biosciences) and incubated for 20 min at 4°C in the dark. Analyses of total (intracellular and membrane) PD-1 expression were performed by consecutive extracellular and intracellular labeling with the isotype or anti-PD-1 antibody. Fixation and permeabilization were performed in FMO, isotype, and total stained samples using FoxP3 Staining Buffer Set (Miltenyi) following the manufacturer's instructions. PD-1 surface expression was also evaluated in NK cells from blood samples of leukemia, lung cancer, and COVID19 patients. PD-L1 expression was assessed in tumor target cells using an anti-PD-L1-APC antibody (clone MIH3) purchased from Biolegend.

Degranulation assay

NK cell degranulation was studied as previously described.[18] Shortly, CD107a was measured in the membrane using anti-CD107a-APC (Miltenyi) by flow cytometry after 4 hours of incubation of PBMCs with target cells in the presence of BD GolgiStop™ (BD Biosciences). K562 and SH-SY5Y cell lines were used as target cells using a 1:2 PBMC:target ratio. After the incubation, CD3, CD56, CD107a, and PD-1 were extracellularly stained and analyzed by flow cytometry as described above. As a control for specific target cell-mediated degranulation, replicates were made without adding target cells. Results were reported as the percentage of CD107a positive cells in PD-1 positive and negative cells within the $CD3^-$ $CD56^+$ NK cell population.

NK cell cytotoxicity assay

PBMCs from HDs were incubated with IL-15 (5 ng/ml) in the absence or presence of K562 at 1:2 effector:target (E:T) ratio overnight. Subsequently, NK cells were enriched by using MACS technology as described above, and extracellular mPD-1 expression was analyzed by flow cytometry. NK cells were co-cultured with target cells at 1:1 and 6:1 E:T ratio in the presence of IL-15 with or without 50 µg/ml Pembrolizumab (anti-PD-1 antibody) for 24 hours as follows: K562, SH-SY5Y, and HCT-116 wild type and HCT-116.PD-L1.KO cells were used as target cells and their viability was measured by different methods. Fresh K562 were fluorescently labeled with eFluor670 (eBio-science) in free serum media following the manufacturer's protocol, and cell death was analyzed by analyzing phosphatidylserine translocation (Annexin V staining) and membrane integrity (propidium iodide staining) in the eFluor670 positive target cell population.

SH-SY5Y (expressing GFP) cell viability was analyzed by counting the number of green fluorescence cells by fluorescent microscopy as follows. Cells were seeded in flat-bottom 96-multiwell plates overnight, and subsequently, they were incubated with NK cells as indicated above. The wells were scanned with Widefield Multidimensional Microscopy System Leica AF6000 LX. Green fluorescent cell number was measured using ImageJ software. The ratio between live cells after treatment to live cells in non-treated controls was used to determine cell viability. The specific effect of pembrolizumab in NK cell-mediated cytotoxicity was quantified by subtracting the percentage of cell viability without and with 50 µg/ml of Pembrolizumab.

Alternatively, the effect of pembrolizumab on NK cell-mediated cytotoxicity in SH-SY5Y GFP cells was analyzed using IncuCyte S3® Live-Cell Analysis System (Sartorius) by counting viable green cell areas. NK cells isolated from 3 HDs were incubated overnight with IL-15 and subsequently, they were co-cultured with tumor cells with or without pembrolizumab for 72 hours. Phase images and green fluorescence (excitation 440–480 nm, emission 504–544 nm) were acquired every 45 min. Counting viable green cell areas by IncuCyte Cell Analyzer system was also used to evaluate drug sensitivity in HCT-116 WT and KO cells.

Immunofluorescence assays

$CD56^+$ enriched cells resuspended in 1 mL of PBS were added on Poly-L-Lysine (PLL) treated coverslips. After incubation, the attached cells were fixed with 4% paraformaldehyde for 10 min at room temperature (RT). After extensively washing with PBS, cells were incubated with Blocking Solution (1 mg/ml BSA, 3% goat serum, 0.1%Triton X-100 in PBS) for 30 min at RT. Next, the cells were incubated with antibodies in Blocking Solution for 1 hr at 4°C and washed three times with PBS. Immunostainings were performed using the following fluorophore-conjugated antibodies: CD3-VioGreen (clone REA613), CD107a-APC (clone REA792), and PD-1-VioBright FITC (clone PD1.3.1.3) all from Miltenyi, and PD-1-Alexa Fluor 700 (clone EH12.2H7, BioLegend), granzyme B-PE (clone REA226, Miltenyi) and Hoechst (Molecular Probes). Preparations were mounted with Fluoromount-G (EMS), and confocal fluorescence images were acquired using a Zeiss LSM 880 Spectral Confocal Microscope. NK cells and NKT cells were differentiated by the absence/presence of CD3 labeling.

Western blot analysis

Whole-cell extracts were prepared by lysing cells for 15 min on ice in RIPA lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.0% NP-40, 0.1% SDS, and 0.1% Na-deoxycholic acid) supplemented with protease and phosphatase cocktail inhibitors (Complete and PhosSTOP; Roche Applied Science, Mannheim, Germany). Protein concentration in cell lysates was quantified by Bradford (BioRad) in 96 well plates using a Bio-Rad Benchmark Microplate Reader. Whole-cell lysates were separated through SDS-polyacrylamide gels (4–12%) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc.). Membranes were blocked with 5% milk powder in 0.1% Tween20 in PBS (PBS-T) for 1 hr at room temperature, followed by incubation with primary antibodies diluted in 2.5% milk PBS-T. ImmunoCruz Western Blotting Luminol Reagent (Thermoscience) was used to visualize protein levels with light sensitive-films (Phenix Research). The following antibodies were used: PD-1 (D4W2J, 1:250, Cell Signal Technology), and GAPDH and H2AX (1:1000, Santa Cruz Biotechnology)

PD-1 ELISA

PD-1 was measured by enzyme-linked immunosorbent assay (ELISA) using the Human PD-1 ELISA Kit (ab252360, clones EPR22234-42 and EPR22234-127, Abcam) following the manufacturer's protocol. Both total cell lysates and concentrated supernatants were tested, and PD-1 concentration (pg/ml) was calculated using the 4-point-fit calibration curve of standard dilutions. Supernatants were concentrated in Amicon® Ultra 10 kDa Centrifugal Filters. Cell lysates from the MOLT-4 cell line were used as a positive control.

In vivo adoptive NK cell transfer

NK cells for adoptive cell transfer were obtained by selection of 3 out of 10 donors by greater NK/NKT ratio (9,5 ± 2,3, data not shown), and further CD56 positive selection. Enriched NK cells

were co-cultured overnight with K562 target cells and IL-15 (5 ng/ml) and purified again by CD56 positive selection. A pool of NK cells from three selected donors was prepared before administration to avoid perturbation in results depending on HLA-I mismatch with the target cell. B-NDG mice (female, 8-week-old, Envigo) were used which were housed in sterile facilities for immunosuppressed animals at the Center for Biomedical Research of Aragon (CIBA). Protocols were approved by the University of Zaragoza's Advisory Ethics Commission for Animal Research (P.I 47/18). Mice were randomly divided into two groups in different cases: control and NK-cell treated group (n = 5). A mix of HCT-116.GFP wild type and HCT-116.GFP.RFP.PD-L1.KO cell (1:1 ratio) was intraperitoneally injected in all mice (a total of 1×10^6 cells per mouse). In one group, NK cells were injected simultaneously at a 10:1 E:T ratio. Mice in both groups received 10 µg of human recombinant IL-2. After 24 hours, mice were sacrificed, and an intraperitoneal lavage was performed with 10 ml of PBS. Cell suspension obtained was centrifuged and labeled with Annexin-V and SYTOX™ Blue Dead Cell Stain (Thermo Fisher). The red HCT-116.PD-L1.KO cell percentage in total viable green inoculated cells was determined by FACS. A preliminary study (n = 3) was performed following a similar protocol as explained above, but the presence of tumor cells was analyzed for a longer period, 20 days after tumor inoculation and NK cell transfer. Parametric distribution was checked by the Shapiro–Wilk normality test, and differences between both groups were analyzed by unpaired t-test using GraphPad Prism (v7.0) software. Statistical significance was always set at $p < .05$.

Results

PD-1 expression on NK cell surface

Previous studies have shown that freshly isolated NK cells express PD-1 mRNA and cytoplasmic protein and several studies have found that human NK cells from cancer patients express high levels of PD-1 on the cell membrane (mPD-1).[19] In addition, recent evidence using in vivo mouse models indicates the presence of an mPD-1$^+$ NK cell subset in tumor-bearing mice, which contributes to the efficacy of PD-1/PD-L1 blocking mAbs,[17] suggesting relevance of the PD-1/PD-L1 axis in NK cell anti-tumoral responses *in vivo*. However, the role of PD-1 in modulating NK cell anti-tumoral function is still not clear as other studies have reported that NK cells do not express this immune checkpoint.[22,23] Thus, we first decided to analyze if NK cells isolated either from HDs or from patients with different pathologies including cancer and infection expressed mPD-1. As shown in Figure 1(a), only 1,8% of peripheral blood NK cells from HDs showed mPD-1 expression. In contrast, mPD-1 expression was clearly observed in NK cells from patients with lung carcinoma (13,5%) pediatric leukemia (75%), and COVID19 (13,4%), confirming previous studies[13–16,24–26] suggesting that under pathological conditions PD-1 is already expressed on the membrane of freshly isolated NK cells. However, when the intracellular expression of PD-1 was analyzed in NK cells from HD, we found that most NK cells expressed intracellular PD-1 as shown in Figure 1(b), confirming previous findings.[19] In contrast, the PD-1 negative K562 cell line did not show any staining confirming the specificity of PD-1 staining in NK cells. As a positive control, PD-1 expression of T cell leukemia MOLT4 was used, showing a very high PD-1 expression (Figure 1(b)).

Altogether, these results suggest that, under the appropriate conditions, PD-1 is mobilized to the NK cell surface. However, the stimuli that trigger the expression of PD-1 on the cell membrane remain unknown.

To test the hypothesis that the contact with target cells mobilizes cytosolic PD-1 to the cell membrane, PBMCs from HDs were incubated overnight with or without K562 tumor cells. All cell cultures contained IL-15 to keep NK cell viability. Similar to freshly isolated NK cells, few mPD-1$^+$ cells were detected on NK cells incubated without tumor cells (mean 0,8%). However, the level of PD-1 on NK cell surface significantly increased (>15-fold) in cells incubated overnight with K562 tumor cells (Figure 1(c); mean 13,4%), similarly to NK cells freshly isolated from cancer patients. The mPD-1 increase was also confirmed by confocal microscopy (data not shown). These results support the hypothesis that NK cells mobilize PD-1 protein to their surface after tumor cell encountering. Stimulation of PBMC cells with the NK-cell activating cytokine IL-15 alone (NK ON) did not induce PD-1 membrane mobilization (Figure 1(c)) indicating that stimulation by target cells is required to induce mPD-1 expression. This effect seems to be specific for PD-1 since the expression of other receptors/markers either showed no change (LAG3, NKp30, NKG2C, NKG2A, and CD57) or were significantly reduced (NKp46, NKG2D, and TIM3) during incubation with target cells (Figure 1(c)).

NK cell degranulation increases membrane PD-1 expression

Our data indicate that mPD-1 expression on NK cells is increased after incubation with K562 target cells. However, the underlying mechanism is unknown. We wonder whether PD-1 mobilization would be related to granule release involving the direct interaction of NK cells with target cells. To test it, we performed a degranulation assay following CD107a translocation and mPD-1 expression during the incubation of PBMCs with K562 cells. After 4 hours of incubation with two different target cells (K562 or SH-SY5Y), CD107a surface expression was detected on NK cells concomitantly to PD-1 expression (Figures 2(a,b)). In both cell co-cultures, mPD-1 expression was significantly increased in comparison with the control without tumor target cells indicating that this effect is not restricted to a specific target cell. As shown in Figure 2(a,b), the percentage of degranulating CD107a$^+$ cells was significantly increased in the mPD-1$^+$ NK cell subset (31% and 35% in K562 and SH-SY5Y cells, respectively) suggesting an enrichment of mPD-1 in the NK cell population that had degranulated as a consequence of the interaction with target cells. The combination of the PKC activator, PMA plus the calcium ionophore, ionomycin, during a short period (4 h), which has been shown to induce NK cell degranulation,[27] also induced

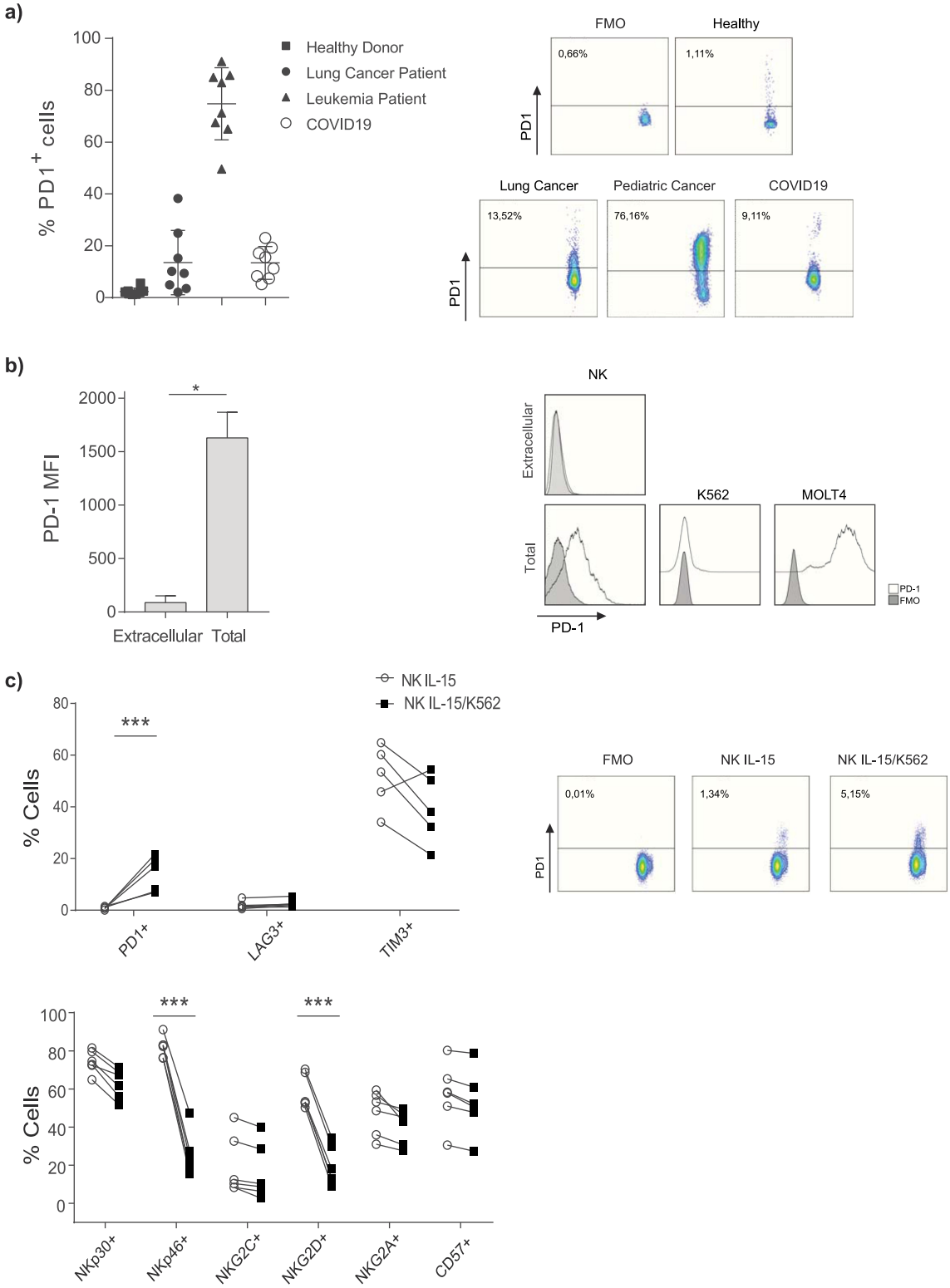

Figure 1. Increase of PD-1 expression on NK cell surface after incubation with target cells. (a) Extracellular mPD-1 expression was analyzed in NK cells from freshly isolated PBMCs from healthy donors (HDs), lung or leukemia cancer and COVID19 patients by flow cytometry. Representative flow cytometry dot plots showing PD-1 expression (clone EH12.2H7) are shown on the right panels and the results from 8 independent donors are shown on the left graph as the mean ±SD. (b) Extracellular (m) and intracellular PD-1 expression were analyzed in NK cells (gated as CD56+, CD3-) from HD-derived freshly isolated PBMCs by flow cytometry. Representative flow cytometry histograms showing extracellular and total PD-1 expression (clone PD.1.3.1.3) are presented on the right panels. K562 and MOLT4 cell lines were used as negative and positive staining controls, respectively. PD-1 labeling is represented in white and FMO (Fluorescence Minus One) control in gray. The results from 4 independent donors are shown on the left graph, displaying the mean ±SD of Mean Fluorescence Intensity (MFI) isotype and PD-1 labeling. (c) NK cells were incubated with IL-15 in the presence or absence of K562, and the indicated extracellular receptors were analyzed by flow cytometry. A representative flow cytometry dot plot showing PD-1 expression (clone EH12.2H7) is shown on the right panels, and the results from 6 independent HDs are shown on the left graph. Numbers in dot plots correspond to the % of mPD-1+ NK cells. NK cells were gated as CD45+ CD56+ CD3-CD14-CD19- excluding dead cells (Cell Viability Marker). Statistical analysis was performed by t-student test comparing the mean values between IL-15 and IL-15/K562 stimulation, ***, $P < .005$.

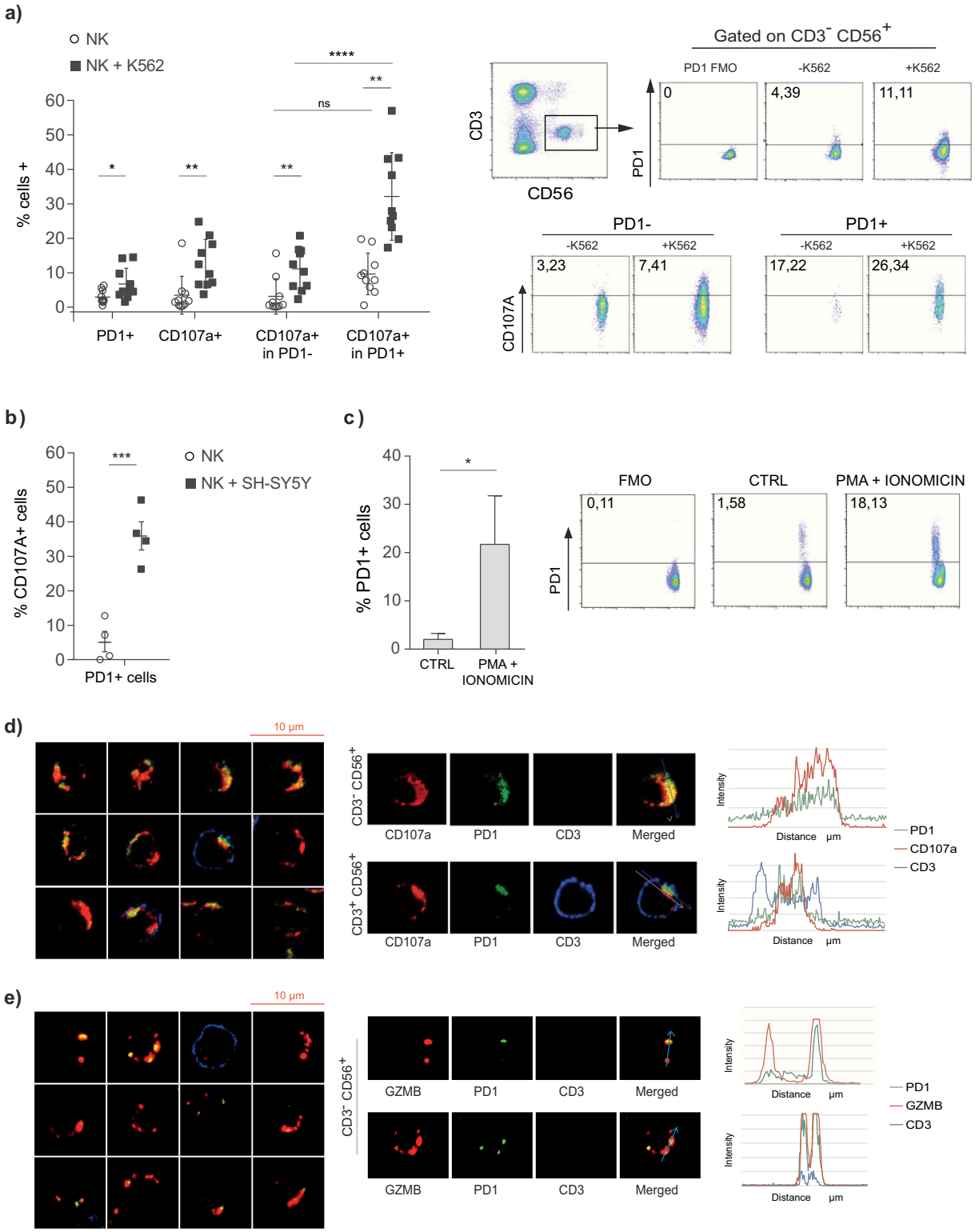

Figure 2. PD-1 is concomitantly translocated to the cell membrane with CD107a during NK cell degranulation and colocalizes with CD107a and granzyme B. a, NK cells were incubated with IL-15 in the presence or absence of K562 cells for 4 h and NK cell degranulation was analyzed by flow cytometry using the CD107a mobilization assay as indicated in methods. A representative flow cytometry dot plot showing PD-1 (clone EH12.2H7, PD.1.3.1.3) and CD107a expression is shown on the right panels. FMO corresponds to the Fluorescence Minus One flow cytometry staining control. The results showing the % of mPD-1 and CD107a expression in total NK cells and the % of CD107a expression in mPD-1^{-} and mPD-1^{+} NK cells from 10 independent HDs are shown on the left graph. Statistical analysis was performed by t-student test comparing the groups indicated by the horizontal lines, *, P < .05; **, P < .01, ****, P < .001. b, A similar experiments as in A was performed, but using the SH-SY5Y target cell line (n = 4 independent HD). c, NK cells were incubated with medium or a combination of PMA and ionomycin for 4 h, and the % of mPD-1+ cells was analyzed by flow cytometry. A representative flow cytometry dot plot is shown on the right panel and the results from 3 independent HDs are shown on the left graph. FMO corresponds to the Fluorescence Minus One flow cytometry staining control. Data in the graphics are represented as the mean ± SD. Statistical analysis was performed by t-student test, *, P < .05. d and e, NK cells were enriched by using anti-CD56 antibodies attached to magnetic beads (MACS, Miltenyi) from HD freshly isolated PBMCs and intracellular CD56, PD-1 (clone EH12.2H7), CD107a, CD3 and granzyme B staining was performed and analyzed by confocal fluorescence microscopy as indicated in methods d, PD-1 (green), CD107a (red) and CD3 (blue). e, PD-1 (green), granzyme B (GZMB) (red), and CD3 (blue). A representative confocal image is shown on the left panels. Colocalization between PD-1 and CD107a or granzyme B is shown as yellow areas and indicated by arrows. The right graphs show the intensity line profiles from the magnified images. Line scans represent variation in marker fluorescence intensity along the line denoted by a blue arrow in NK and NK-T cells.

mPD-1 expression on NK cells (Figure 2(c)) supporting the hypothesis that PD-1 is mobilized to NK cell membrane during cytotoxic granule exocytosis after target cell encountering.

Our results show that a cytoplasmic pool of PD-1 is expressed on freshly isolated NK cells that are mobilized to the cell membrane surface during degranulation. Thus, we proposed that CD107a and PD-1 should share cytoplasmic compartment expression. To explore this possibility, an analysis by immunofluorescence and confocal microscopy was performed in CD56-enriched freshly isolated cells, staining PD-1, CD107a, and CD3. Both NK ($CD3^-$) and NKT ($CD3^+$) cells showed a substantial colocalisation of PD-1 with CD107a (Figure 2(d)). This finding confirms that PD-1 is expressed in some $CD107a^+$ organelles and indicates that PD-1 mobilization was mainly due to the cytoplasmic pool of PD-1 present in these organelles and not due to *de novo* protein synthesis, even when it could take place. Next, we show that PD-1 colocalized with the specific cytotoxic granule marker granzyme B (Figure 2(e)) confirming that PD-1 was mainly present in cytotoxic granules and not in other $CD107a^+$ lysosomal-like compartments. It should be noted that PD-1 seems to be associated with some but not with all $gzmB^+$ granules, indicating that not all cytotoxic granules express PD-1. Thus, this result helps to explain why degranulating CD107a+ positive NK cells (Figure 2(a)) do not express mPD-1, since they would represent cells that mobilized cytotoxic granules that do not contain PD-1.

Here, it should be indicated that PD-1 expression by FACS and confocal microscopy was analyzed using two different mAb (Clones EH12.2H7 and PD1.3.1.3) producing similar results, which together with the result in K562 PD-1 negative cell (Figure 1(b)) discard potential unspecific staining.

NK cell degranulation correlates with the mobilization of membrane PD-1 and the release of PD-1

We decided to analyze if, in addition, to regulate mPD-1 expression, target cell-induced NK cell degranulation was also involved in sPD-1 release. As shown in Figures 3(a,b), by comparing extracellular mPD-1 and total (membrane and cytoplasmic) PD-1 expression, we observed that most NK cells expressed low levels of mPD-1, confirming the previous results (Figure 1). A significant increase in the percentage of $mPD\text{-}1^+$ NK cells was observed after incubation with target cells. Even though the percent of positive cells increases, it is not enough to modify MFI mean of the total NK cell population due to the low percentage of cells that upregulate membrane PD-1. In contrast, the level of total PD-1 (MFI) was significantly reduced in NK cells that had been incubated with target cells in the presence of IL-15 with respect to NK cells incubated alone with IL-15 (1449 to 937 MFI, Figure 3 (b)), suggesting that in addition to promoting mPD-1 exposition, encountering with target cells also induced the release of sPD-1 present in cytotoxic granules. To further confirm this finding, we analyzed the expression of PD-1 in enriched-NK cell lysates by western blot (Figure 3(c)) and quantified cell-associated and sPD-1 by ELISA (Figure 3(d)). Both, western blot and ELISA analyses showed a decrease in PD-1 expression in NK cells incubated with target cells with respect to control NK cells. Supporting the hypothesis that sPD-1 is released during NK cell degranulation, a significant increase of PD-1 in cell supernatants of NK-K562 cell co-cultures was detected by ELISA (Figure 3(d)). PD-1 expression was not detected in K562 by flow cytometry, western blot, and ELISA (Figures 3 (b-d)) confirming the specificity of PD-1 expression in NK cells and that sPD-1 in NK/K562 cell co-cultures is secreted by NK cells. In contrast, the PD-1 positive T cell leukemia MOLT-4 showed a high expression of PD-1 by flow cytometry and western blot.

mPD-1 expression regulates NK cell-mediated elimination of tumor cells *in vitro* and *in vivo*

These findings related to mPD-1 increase with NK cytotoxic effector function, a process that could modulate NK cell activity during tumor cell elimination. Hence, we next wondered whether PD-1 protein mobilization to the cell surface had functional consequences on NK cell-mediated cytotoxicity. Thus, we analyzed the sensitivity of target cells expressing different levels of PD-L1 to NK cell-mediated cell death. K562 cells were selected as $PD\text{-}L1^{low}$ and SH-SY5Y and HCT-116 as $PD\text{-}L1^{high}$ tumor target cells. Additionally, a PD-L1 KO HCT-116 cell line was generated by CRISPR/Casp9 to formally confirm the potential of the PD-1/PD-L1 axis to regulate NK cell cytotoxic function (Figure 4(a)).

To trigger PD-1 expression on NK cell membrane, overnight incubation with K562 was carried out as performed in the previous experiments followed by CD56 MACS enrichment. As a control, we used NK cells incubated only in the presence of IL-15 without target cells. As shown in Figure 4(b), the survival rate of SH-SY5Y cells ($PD\text{-}L1^{high}$) was higher, at both low and high E:T ratios, in the co-cultures with NK cells previously incubated with K562 cells in comparison with control NK cells that had not been pre-incubated with K562 cells (Figure 4(b)). In contrast, when K562 ($PD\text{-}L1^{low}$) were used as target cells, we found that there was no significant difference in the level of cell death induced by NK cells irrespectively of whether they have been pre-incubated with K562 cells or not (Figure 4(c)). The large significant decrease in cytotoxicity between NK cells in both conditions against SH-SY5Y cells, compared with the more modest PD-1 increase in NK membrane, can be related to a recent study[28] that shows that a small population of NK cells, around 6%, will be responsible for target cell killing by a process known as serial killing. The NK cells that have increased PD-1 are precisely the most active ones (cells that have degranulated) and, thus, even if they are just around 10%, a reduction in their activity due to PD1 membrane translocation might be enough to generate this viability gap.

Our data using $PD\text{-}L1^{high/low}$ expressing cells suggest that the low level of mPD-1 expression reached just after overnight incubation with tumor cells, is sufficient to significantly affect NK cell-mediated elimination of cancer cells expressing PD-L1. To confirm that the reduction of NK cell cytotoxic activity was indeed due to mPD-1 expression, we repeated the experiment in SH-SY5Y cells using the PD-1 blocking mAb pembrolizumab. As shown in Figure 4(d), NK cells that had been pre-

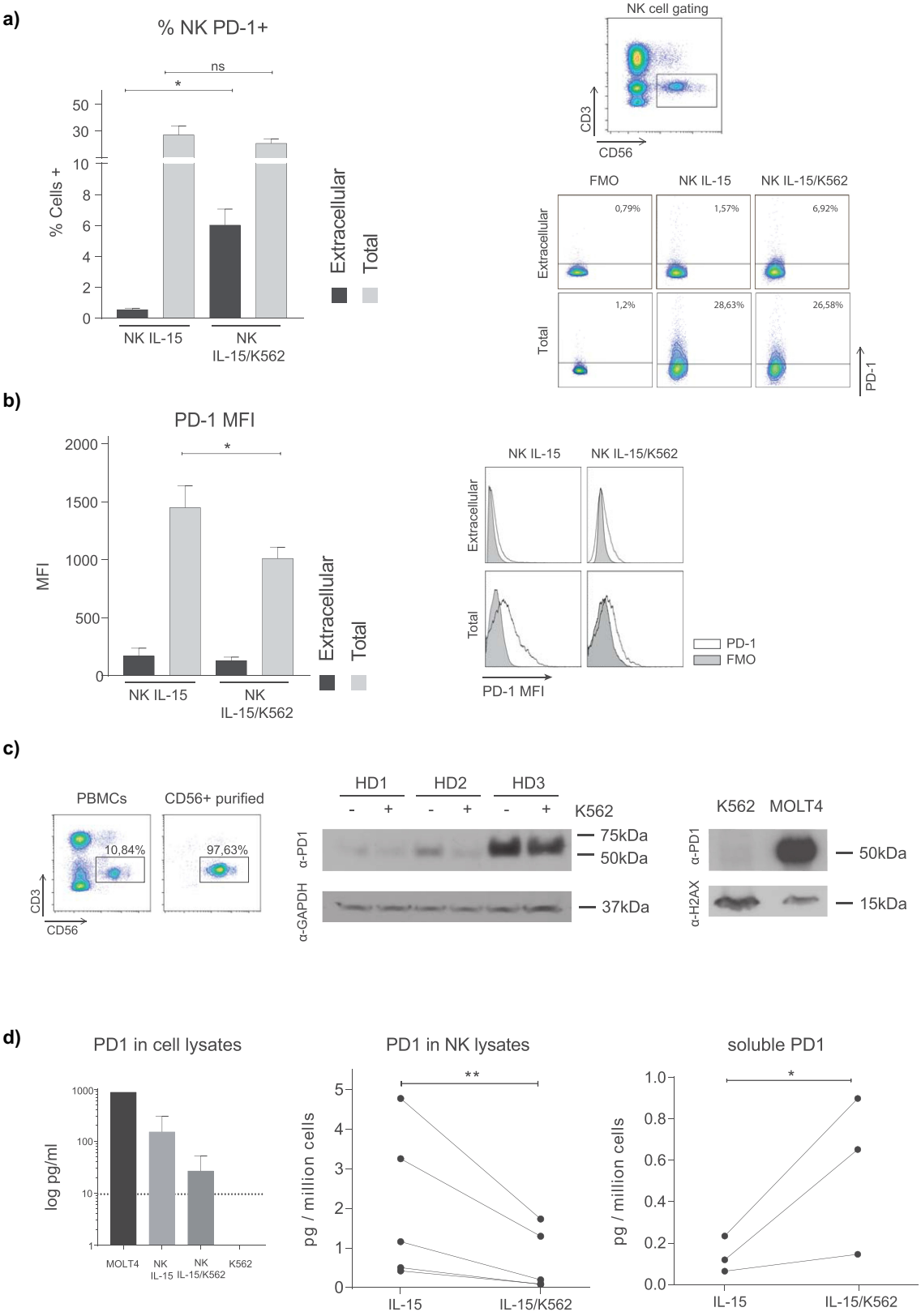

Figure 3. Overnight incubation of NK cells with tumor target cells alters PD-1 protein level in NK cells. NK cells were incubated with IL-15 in the presence or absence of K562 cells, and PD-1 expression was analyzed by flow cytometry (a and b), western blot (c) and ELISA (d). A and B, PD-1 extracellular, and total (extracellular + intracellular staining) expression were analyzed in NK cells (CD56+ CD3- cells), and the results from 5 independent donors are shown. The % of PD1+ NK cells is shown in panel A, and MFI is shown in panel B. Representative flow cytometry dot plots or histograms (a and b, respectively) showing PD-1 expression (clone PD.1.3.1.3) are shown on the right panels, and the results from 5 independent donors are shown on the left graph. Data in the graphics are represented as the mean ± SD. Statistical analysis was performed by ANOVA test comparing the mean, *, P < .05. c, CD56+ selection was performed prior to western blotting NK cells lysate, shown in left dot plots. PD-1 expression of three healthy donors (HD) is shown (clone D4W2J). GAPDH was used as a loading control. PD-1 protein expression in K562 and MOLT-4 (positive control) cell line lysates was also analyzed. H2AX was used as loading control. d, PD-1 concentration (pg/ml) in MOLT4, NK cell, and K562 cells lysates was analyzed. The dotted line represents the detection limit. PD-1 pg per million of NK cells was quantified by ELISA (clones EPR22234-42 and EPR22234-127) in cell lysates (n = 5; middle) and cell culture media (n = 3; right). Statistical analysis was performed by ratio paired t-student test, *, P < .05; **, P < .01.

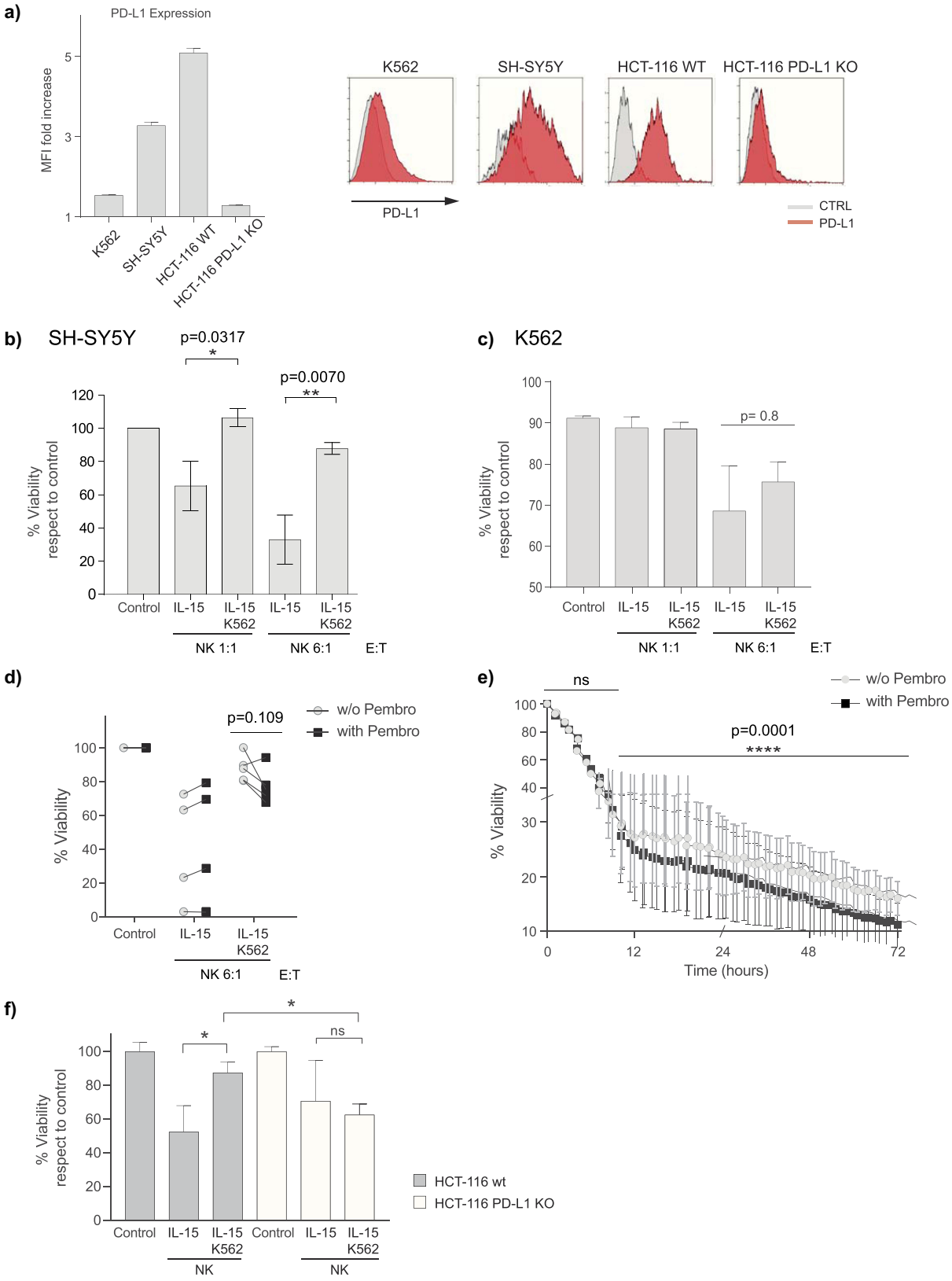

Figure 4. Membrane translocation of PD-1 affects NK-cell mediated cytotoxicity against PD-L1 positive target cells. a, PD-L1 expression was analyzed in K562, SH-SY5Y, HCT-116.wt, and HCT-116.PD-L1.KO cell lines, n = 3. Representative histograms showing PD-L1 expression (clone MIH3) are shown on the right panels. b and c, NK cells were incubated overnight with IL-15 alone or plus K562 cells at 1:2 E:T ratio (IL-15 ± K562) and, subsequently, cell cytotoxicity was analyzed in co-cultures with PD-L1^{high} SH-SY5Y (b) and PD-L1^{low} K562 (c) target cells. Target cell viability is represented as bar graphs from 5 and 3 independent HDs respectively. d, the same experiment as B, but including the blocking PD-1 antibody, Pembrolizumab, was performed (n = 5 independent HD). Statistical analysis was performed by t-student test, *, $P < .05$; **, $P < .01$. e, SH-SY5Y viability was evaluated in real-time for 72 hours in co-cultures with freshly isolated NK cells with/without Pembrolizumab by IncuCyte cell analysis system. The first section of the curve was fitted to the asymmetric sigmoidal and the second part to a line. In both sections, the extra sum-of-squares F test was performed to test if one curve adequately fit both data sets, with or without Pembrolizumab. ns, not significance, ****, $P < .0001$. f, The same experiment as in B was performed but using HCT-116.wt and HCT-116.PD-L1.KO cell lines as target cells. The bar graph shows 4 independent HDs. Data in graphics are represented as the mean ± SD. One-way ANOVA test was performed, ns, not significance, *, $P < .05$.

incubated with K562 cells induced less cell killing than control NK cells, an effect that was partly reversed by pembrolizumab. Cytotoxic activity of control NK cells that had not been pre-incubated with K562 and, thus, did not express mPD-1 was not affected by pembrolizumab, confirming that this effect was restricted to NK cells that had mobilized PD-1 to the cell membrane.

To further support the role of mPD-1 in NK cell activity *in vitro*, we analyzed the effect of pembrolizumab in NK cell cytotoxic dynamics by co-culturing freshly isolated NK cells with tumor target cells (SH-SY5Y) in the presence or absence of pembrolizumab. Tumor cell viability was followed over 72 hours using the IncuCyte Live-Cell Analysis System. As shown in Figure 4(e), during the first 10 hours, NK cells showed a high cytotoxic activity irrespectively of the presence of the PD-1 blocking mAb. After that, when the cytotoxic activity of NK cells slowed down, the curves for both conditions separated, and the cytotoxic activity of NK cells was significantly increased in the presence of Pembrolizumab, confirming the role of mPD-1 in modulating NK cell activity. NK cell viability remained high and constant independently of Pembrolizumab presence throughout the experiment (Supplementary figure S2) discarding a potential contribution of NK cell loss to the results. This result suggests that PD-1 blockage has a positive effect on NK cell cytotoxicity once they have already intensively degranulated and PD-1 is mobilized to the cell membrane. This suggestion is further supported by the results using NK cells in which mPD-1 had been previously induced by pre-incubation with target (K562) cells (Figure 4(b–d)).

The role of PD-L1 in modulating the cytotoxic activity of NK cells that had mobilized mPD-1 expression was formally confirmed in HCT-116 colorectal cancer cells by using HCT-116.wt and PD-L1.KO cells. As shown in figure 4(f), mPD-1 expressing NK cells showed a significantly higher cytotoxic activity against PD-L1.KO cells than against their wt counterparts (figure 4(f)). In contrast, cell death induced by NK cells that had been incubated with IL15 without K562 cells was similar in both wt and PDL1.KO cells, confirming the relevance of mPD-1 mobilization to NK cell cytotoxic activity *in vitro*. Both wt and PD-L1.KO cells had the same proliferation rate and were equally sensitive to chemotherapy drugs commonly used in colorectal cancer treatment, 5-fluorouracil and oxaliplatin, confirming that PD-L1 knockdown did not confer any survival advantage and/or general sensitivity to other cytotoxic stimulus (Supplementary figure S3).

Finally, the relevance of PD-1 in the regulation of NK anti-tumoral cytotoxic activity was confirmed *in vivo* using a model of adoptive NK cell transfer in immunocompromised b-NDG mice inoculated with HCT-116 colorectal cancer cells. A schematic representation of the model is shown in Figure 5 (a). Mice were injected intraperitoneally with a 1:1 mixture of HCT-116.wt (GFP^+) and HCT-116.PD-L1.KO (GFP^+RFP^+) cells (inoculated samples contained 51,48% HCT-116.PD-L1. KO cells as analyzed by flow cytometry, data not shown) and treated with IL2 alone or in combination with NK cells pre-incubated with K562 cells. As shown in Figure 5(b), NK cell treatment produced a modest but a significant decrease in the proportion of PD-L1.KO/WT cells, indicating a better control of PD-L1 KO tumor development. These differences were not observed when mice were treated with NK cells that had not been pre-incubated with K562 target cells. This result supports the *in vivo* relevance of tumor-induced mPD-1 mobilization on the anti-tumoral activity of NK cells.

As a proof of concept, a preliminary study was performed over a small animal population (n = 3), Supplementary figure 4. The protocol is shown in Figure 5(a), except that inoculated tumor cells were collected after 20 days to find out whether an enrichment of PD-L1 expressing HCT-116 cells was observed once tumor had developed. As shown in Supplementary figure S4, tumors were smaller in mice treated with NK cells than in control mice and the % HCT-116.PD-L1.KO cells were significantly smaller, suggesting a selection of $PDL1^+$ cells during treatment with NK cells similar to the results obtained in short *in vivo* treatments (Figure 5).

Discussion

PD-1 is a well-known inhibitory immune checkpoint involved in the regulation of T cell responses including peripheral tolerance and anti-tumoral activity. In contrast, the role of PD-1 in NK cell function still presents many open questions, which need to be clarified to optimize the therapeutic potential of different immunotherapy approaches including anti-PD-1/L1 antibodies and those based on NK cells. Here, we show that NK cells freshly isolated from HD express very low amounts of the membrane (m) PD-1 but express a pre-synthesized cytoplasmic pool contained in granzyme B^+ cytotoxic granules that are rapidly mobilized to the cell membrane surface during NK cell degranulation after a short interaction with target cells. Thus, our results indicate that the mechanism involved in the mobilization of PD-1 from the cytosol to the cell membrane is granule exocytosis, a novel mechanism that had not been previously described for this immune checkpoint. Our finding contributes to understanding the mechanisms involved in the regulation of NK cell tumor immunity and agrees with the results of Mariotti et al. showing that PD-1 mRNA and protein are present in the cytosol of freshly isolated NK cells from HDs. In addition, it explains the absence of PD-1 on the membrane of those NK cells that have not been recently exposed to target cells.[19] These findings are compatible with a rapid surface expression of PD-1 on NK cells in response to appropriate stimuli like target cell-induced degranulation, as shown here. Our results indicate that, in contrast to T cells, the expression of PD-1 on the cell membrane seems not to be related to NK cell activation as freshly isolated NK cells already express cytoplasmic PD-1, which is rapidly mobilized to the cell membrane in the presence of target cells. In addition, incubation with IL-15 alone was not sufficient to increase mPD-1 expression on NK cells. Supporting our results, a recent study has also shown that NK cell activation with cytokines alone does not increase PD-1 membrane expression at short times.[19] In contrast, during long-term stimulations (6 days), a combination of several cytokines (IL-12, -15, and-18) and glucocorticoids was shown to promote the transcriptional upregulation of PD-1 as well as mPD-1 expression independently of target cell

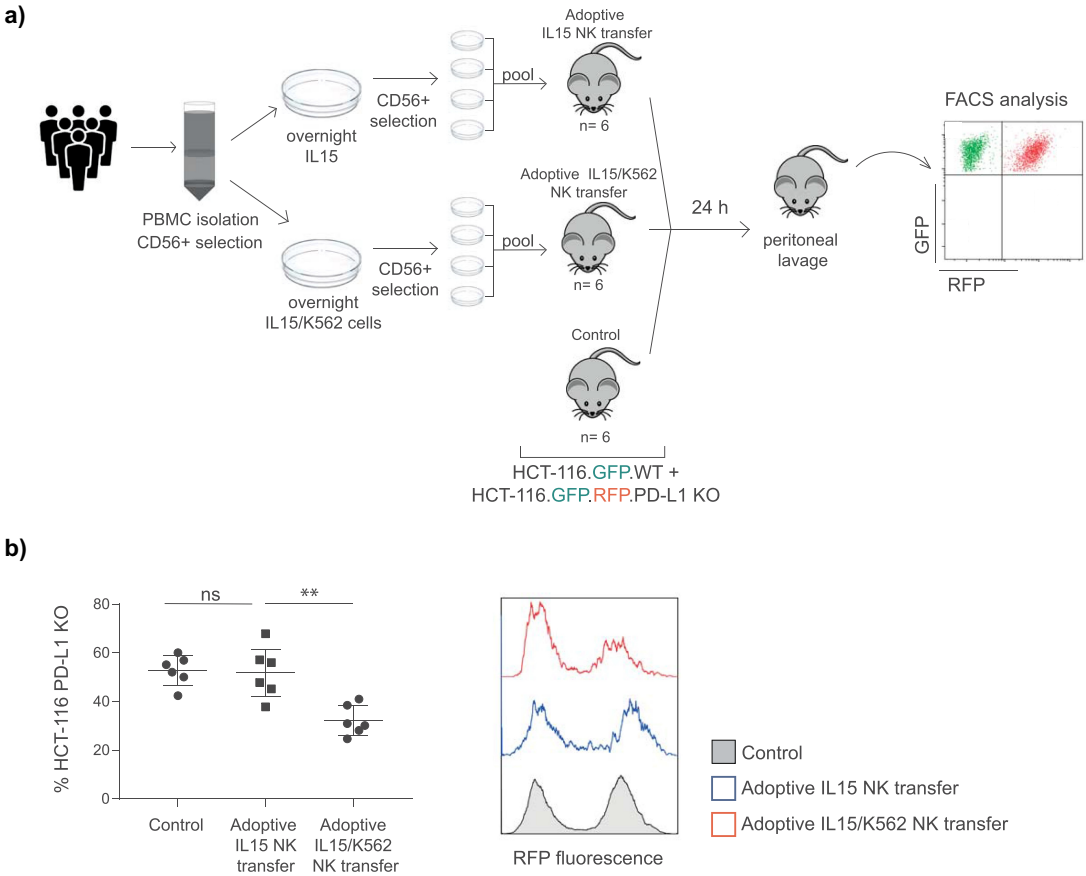

Figure 5. PD-L1 knocking favors *in vivo* control of tumor development by mPD1-expressing NK cells. a, a 1:1 mixture of GFP^+ WT and GFP^+.RFP^+.PD-L1 KO HCT-116 cells was injected i.p. into immunosuppressed mice (B-NDG) and, subsequently, NK cells were inoculated i.p. as indicated in the scheme. Inoculated NK cells were generated from a pool of 4 HDs that had been previously incubated overnight with IL-15 alone or together with K562 cells. After 24 h, intraperitoneal lavage was performed and analyzed by flow cytometry. b, the right panel shows a representative histogram of RFP expression in GFP+ peritoneal cells corresponding RFP^+ to PD-L1 KO HCT-116 cells and RFP^- to WT HCT-116 cells. The left graph shows the percentage of HCT-116 PD-L1 KO cells (GFP^+RFP^+) with respect to total viable HCT-116 (GFP^+) cells (n = 6). Statistical analysis was performed by t-student test, **, $P < .01$, after checking normality with the Shapiro–Wilk test.

stimulation.[29] Similar to our results, an anti-PD-1 blocking mAb also enhanced the cytotoxic activity of NK cells in which mPD-1 had been induced with the indicated stimulus, confirming the functional role of PD-1 in regulating NK cell-mediated cytotoxicity. Thus, it is plausible that two different mechanisms are involved in mPD-1 expression, one dependent on target cell-induced NK cell degranulation and another one independent of target cell stimulation, which might be regulated by cytokines at longer times. Indeed, our results indicate that not all NK cells that express mPD-1 have degranulated (CD107a+), suggesting that mPD-1 expression might be controlled by different mechanisms.

Albeit this was not the main objective of our study, we have also found that sPD-1 is released to the cell supernatant during NK-target cell co-cultures, suggesting that granule exocytosis would also regulate the secretion of PD-1. Secreted PD-1, either as an alternative splicing isoform lacking intermembrane exon (Δexon3) or on exosomes, was previously shown to be present in NK cell culture supernatants[19,30] and could present regulatory effects by blocking PD-L1 in different cell types including tumoral and non-tumoral. Indeed, we have been able to detect an increase in PD-1+ NKp30+ exosomes in cell supernatants during HD-derived NK cell degranulation (data not shown). Further studies will be required to analyze if release of sPD-1 by NK cells might contribute to, for example, the modulation of T cell activation in cancer and/or infection.

The expression and function of PD-1 on NK cells is still a matter of controversy. Different studies have shown that specific NK cell subsets express PD-1 under different pathological conditions including cancer and infection,[13,31–33] which has been related to poor prognosis.[14,16,24,34] In addition, a significant role of NK cells in the antitumoral efficacy of antiPD-1 mAbs in cancer was recently established in animal models.[17] Further supporting the role of PD-1 in regulating NK cell function, cytosolic but not membrane expression of PD-1 was found in NK cells from healthy donors.[19] Our results provide additional evidence confirming that NK cells express PD-1 under both physiological and pathological conditions, albeit with a notorious difference. HD-derived NK cells express PD-1 in the cytoplasm, which is rapidly mobilized to the cell membrane after interaction with target cells, while PD-1 is already present on the cell membrane in patient-derived NK cells, a process that could be a signature of a recent target cell encountering.

In contrast to these results, it has been recently reported that PD-1 expression is minimal on the cell membrane of NK cells from HD as well as from different pathological situations.[22] It was

suggested that the contradictory results obtained regarding the expression of PD-1 in NK cells might be influenced by the use of specific antibodies and methods that might produce unspecific staining and a false-positive PD1 expression result.[22] Our results convincingly show that NK cells express PD-1 since different antibody clones (EH12.2H7, PD1.3.1.3, D4W2J, EPR22234-42, and EPR22234-127) and methods have been used including flow cytometry, confocal microscopy, immunoblot, and ELISA. The use of this multimethodological approach discards potential false positives that might be caused by antigens released from dying cells when using specific anti-PD-1 antibody clones.[35] Although it has recently been shown that NK cells might acquire mPD-1 expression from target cells by trogocytosis,[36] the target cells employed in our study (K562, HCT-116, and SH-SY5Y) do not express PD-1 on the cell membrane (Supplementary figure S5) excluding that this mechanism contributes to PD-1 expression in NK cell membrane in our study.

Although cytotoxic granule morphogenesis, composition, and release have been studied for almost 40 years in Tc and NK cells,[37,38] still novel results indicate that the regulation of the cytotoxic activity of these effector cells is more complex than previously expected. For example, a recent study has shown that the main cytotoxic proteins contained in the granules are present as supramolecular attack particles (SMAPs) formed by granzyme B, perforin, and other structural proteins, which are released and transferred from effector to target cells and responsible for target cell killing.[39,40] Thus, our observation that PD-1 is expressed in cytotoxic granules provides a novel potential regulatory mechanism that might control NK cell activity including the release of cytotoxic granules and SMAPs. Indeed, it was previously shown that PD-1 controls T cell degranulation during tuberculosis.[41] Thus, it could be hypothesized that the mobilization of PD-1 to the cell membrane during cytotoxic granule secretion would serve as a regulatory mechanism to prevent effector cell over-reactivity once the offending cell has been eliminated. Supporting this hypothesis, it has been previously found other immunoregulatory molecules in the cytotoxic granules like galectin-1[42] that also form part of the SMAPs.[39]

The biological relevance of mPD-1 mobilization is further indicated by our results using Pembrolizumab, an antiPD-1 blocking Ab and a PD-L1 KO target cell, showing that blocking the interaction between PD-1 and its ligand, PD-L1, significantly increases the antitumoral activity of NK cells *in vitro* and *in vivo*. This increase, albeit modest, is solidly supported by the results obtained in the different experimental models *in vitro* and *in vivo* and might be biologically relevant as it would affect the optimal elimination of cancer cells during cancer immunotherapy, increasing the risk of recurrence. Indeed, our results *in vivo* indicate that treatment of a heterogeneous tumor cell population comprising PD-L1 positive and negative cells with mPD-1$^+$ NK cells induces immune edition and enrichment of the PD-L1 positive tumor cell population. Thus, the expression of PD-1 on NK cells might acquire a relevant role in NK cell mediated anti-tumor response against PD-L1/PD-L2$^+$ tumors, especially, in tumors with down-regulated HLA-I expression, which escape T cell control, and become potentially susceptible to NK cell-mediated killing. Consequently, within this context, the expression of PD-1 protein on NK cell membrane would be a key fact that would cause cancer immune evasion. Therefore, it is plausible that immunotherapies targeting the PD-1/PD-L1 axis might rescue not only cytotoxic T lymphocyte activity but also NK cell-mediated function as suggested by some recent evidence.[17,24,33] On the other hand, our findings would be relevant in the context of treatments based on the use of NK cells from HD-like allogeneic NK cell transfer.[15,43] The strong cytotoxic potential of expanded NK cells against different tumors is well known[15,44–46] and has achieved good clinical outcomes in hematological malignancies.[6,43,47,48] Nevertheless, clinical results in solid tumors have been poor.[49] Among the different aspects contributing to these bad results, the expression of PD-L1 in the tumor microenvironment might play an important role. In this context, the mobilization of PD-1 to the NK cell surface once they interact with tumor target cells could be relevant during allogeneic NK cell therapy and should be considered. Our data show, in line with previous findings,[17] that mPD-1 expression affects the efficient killing of tumor cells by NK cells, which is partly restored by a PD-1 blocking mAb or in cells in which PD-L1 expression has been genetically deleted. This result suggests that the combination of allogeneic NK cells with protocols to counteract the PD-1/PD-L1 axis might enhance NK cell anti-tumor activity.

Albeit beyond the scope of this study, it is tempting to speculate on the different possibilities to explain the role of granule exocytosis-mediated regulation of mPD-1 expression. It could act as a safeguarding mechanism to prevent unfettered cytotoxicity of NK cells *in vivo* as an inhibitory receptor that rapidly accumulates at immunological NK-target cell synapses as described for other inhibitory receptors.[50] Although we have not analyzed CMV infection in our HDs, our results would help to explain the expression of PD-1 in fully mature NK cells isolated from CMV seropositive HDs[13] as well as from cancer patients and the possible enrichment of NK PD-1$^+$cells in tumor microenvironment shown in other studies.[13,24] In the first case, a previous/continuous exposition to CMV antigens or CMV-induced activating ligands would lead to PD-1 membrane expression on the subset of adaptive NK cells. In cancer patients, CMV infection and/or continuous presence of tumor target cells would lead to the expression of PD-1 on the NK cell membrane. All these hypotheses will require further experimental validation.

In conclusion, we provide new evidence indicating that cytotoxic granule exocytosis regulates membrane PD-1 expression on human NK cells, which affects optimal cancer cell killing by NK cells. This finding contributes to understanding the regulation of PD-1 expression on NK cells during the elimination of tumor target cells, which will help to design more efficient protocols to expand and improve the use of adoptive NK cell therapy in cancer.

List of abbreviations

PD-1	Programmed cell death protein 1
NK cell	*Natural killer* cells
PD-L1	programmed cell death ligand 1
mAb	Monoclonal antibody
HD	Healthy patient
wt	wildtype
KO	knockout

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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C ompeting interests

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Data availability statement

The data that support the findings of this study are available from the corresponding author, ARL, upon reasonable request.

Ethics approval and consent to participate

All samples from patients were used after they signed the corresponding informed consent to participate. All samples were provided by the Aragon Biobank (IACS) after the study was approved by the local ethics committee (CEICA).
All animal procedures were approved by the CIBA Animal Experimentation Ethics Committee (number: PI47/18). The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Authors' contributions

CP and ARL designed and performed experiments and wrote the first draft of the MS; SH, LA, and MA designed and performed experiments; CC, MOD, MJA, EMG, and DI selected patients and wrote the MS; ARL and JP conceived and designed the original study and wrote MS; All authors revised and approved the last version of the MS.

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