Identification of a subset of human natural killer cells expressing high levels of programmed death 1: A phenotypic and functional characterization

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Background: Programmed death 1 (PD-1) is an immunologic checkpoint that limits immune responses by delivering potent inhibitory signals to T cells on interaction with specific ligands expressed on tumor/virus-infected cells, thus contributing to immune escape mechanisms. Therapeutic PD-1 blockade has been shown to mediate tumor eradication with impressive clinical results. Little is known about the expression/function of PD-1 on human natural killer (NK) cells.
Objective: We sought to clarify whether human NK cells can express PD-1 and analyze their phenotypic/functional features.
Methods: We performed multiparametric cytofluorimetric analysis of PD-1^{+} NK cells and their functional characterization using degranulation, cytokine production, and proliferation assays.
Results: We provide unequivocal evidence that PD-1 is highly expressed (PD-1^{bright}) on an NK cell subset detectable in the peripheral blood of approximately one fourth of healthy subjects. These donors are always serologically positive for human cytomegalovirus. PD-1 is expressed by CD56^{dim} but not CD56^{bright} NK cells and is confined to fully mature NK cells characterized by the NKG2A^{-}KIR^{+}CD57^{+} phenotype. Proportions of PD-1^{bright} NK cells were higher in the ascites of a cohort of patients with ovarian carcinoma, suggesting their possible induction/expansion in tumor environments. Functional analysis revealed a reduced proliferative capability in response to cytokines, low degranulation, and impaired cytokine production on interaction with tumor targets.
Conclusions: We have identified and characterized a novel subpopulation of human NK cells expressing high levels of PD-1. These cells have the phenotypic characteristics of fully mature NK cells and are increased in patients with ovarian carcinoma. They display low proliferative responses and impaired antitumor activity that can be partially restored by antibody-mediated disruption of PD-1/programmed death ligand interaction. (J Allergy Clin Immunol 2017;139:335-46.)

Key words: *Natural killer cells, programmed death receptor, ovarian carcinoma, tumor escape, immune checkpoint, natural killer cell degranulation, natural killer cell proliferation, natural killer cell cytokine production, CD57^{+} natural killer cells, cytomegalovirus*

The programmed death 1 (PD-1; CD279) gene belongs to the immunoglobulin gene super family and encodes a 55-kDa type I transmembrane protein.[1,2] The protein's structure includes an extracellular IgV domain, followed by a transmembrane region and a cytoplasmic tail. The intracellular tail contains 2 phosphorylation sites, one located in an immunoreceptor tyrosine–based inhibitory motif and the other in an immunoreceptor tyrosine–based switch motif.

PD-1 is involved in peripheral tolerance because of its ability to inhibit cytolytic effector T cells and to prevent their attack on certain normal tissues. In particular, PD-1 functions as an immune checkpoint that, in concert with other checkpoints, prevents overreaction of the immune system and consequent tissue damage.[3]

However, in contrast to this important beneficial role in maintaining peripheral tolerance and T-cell homeostasis, on interaction with PD-1 ligands (PD-L1 [CD274] and PD-L2 [CD273]) expressed on tumor-infected and/or virus-infected cells, PD-1 inhibits T-cell function, contributing to immune escape mechanisms frequently occurring in patients with cancer and chronic viral infections.[4] In particular, in activated T cells the engagement of PD-1 by its ligands expressed on cancer cells prevents the expansion and function of effector T cells (leading to the generation of "exhausted" T cells), thus resulting in severe impairment of antitumor and antiviral T-cell responses.[5-8] Remarkably, IFN-γ is a potent inducer of PD-L1 expression,[9] suggesting that the immune responses mediated by T_H1/natural killer (NK) cells or the IFN-γ therapy itself might favor PD-1–mediated tumor evasion.

PD-1 has unique functional characteristics related to those of cytotoxic T-lymphocyte antigen 4 (CTLA-4; CD152), a major

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Abbreviations used

APC: Allophycocyanin
CFSE: Carboxyfluorescein succinimidyl ester
CTLA-4: Cytotoxic T-lymphocyte antigen 4
DNAM-1: DNAX accessory molecule-1
FITC: Fluorescein isothiocyanate
HCMV: Human cytomegalovirus
HD: Healthy donor
KIR: Killer immunoglobulin-like receptor
LIR-1: Leukocyte immunoglobulin-like receptor 1
NCR: Natural cytotoxicity receptor
NK: Natural killer
PD-1: Programmed death receptor
PB: Peripheral blood
PD-L: Programmed death ligand
PE: Phycoerythrin
PF: Peritoneal fluid/ascites
R-ADCC: Reverse antibody-dependent cellular cytotoxicity
Siglec: Sialic acid–binding immunoglobulin-like lectin

inhibitory coreceptor expressed on T cells. Notably, CTLA-4 has both cell-intrinsic (ie, on CTLA-4$^+$ effector cells) and extrinsic (on forkhead box P3–positive regulatory T cells) activities. As a consequence, deficiency in CTLA-4 function results in severe antigen-nonspecific autoimmune phenotypes.[10] In contrast, the effect of engaging PD-1 is mainly cell intrinsic. The cell-intrinsic function of PD-1, as well as the regulation of PD-1 expression, might be responsible for the chronic and relatively milder pathologic phenotypes resulting from PD-1 blockade through either antibody-masking or genetic manipulation. Antibody-mediated blocking of PD-1 is now being exploited in clinics as a therapeutic tool for boosting immune responses in patients with different diseases, primarily in cancer. Importantly, targeting PD-1/PD-L1 interactions can also improve the efficacy of adoptive cell therapies in tumors or chronic viral infections.[11-14] In some instances PD-1 blockade has been shown to mediate tumor eradication,[15-17] resulting in impressive and highly encouraging clinical results. Indeed, accumulating data indicate that the administration of an mAb to PD-1, used alone or in combination with other drugs, might provide a highly successful therapeutic tool in different types of advanced tumors, including melanoma, lung cancer, and ovarian carcinoma.[18-20]

NK cell function is regulated by an array of germline-encoded surface receptors that, on interaction with their ligands, transmit either inhibitory or activating signals.[21-23] Mature human NK cells express inhibitory receptors specific for HLA class I molecules, including killer immunoglobulin-like receptors (KIRs), which are able to discriminate among different HLA-A, HLA-B, and HLA-C allotypes,[24] and the CD94/NKG2A heterodimer specific for HLA-E.[25,26] These receptors allow NK cells to spare HLA class I$^+$ autologous normal cells and to kill cells in which HLA class I expression is downregulated (eg, by tumor transformation or viral infection) or allogeneic cells expressing nonself HLA class I alleles unable to engage inhibitory KIRs (an event that can occur in the haploidentical hematopoietic stem cell transplantation setting).[27,28] Among the non–HLA-specific triggering receptors, NKp46, NKp30, NKp44 (collectively termed natural cytotoxicity receptors [NCRs]),[22] NKG2D,[29] DNAX accessory molecule-1 (DNAM-1; CD226),[26] and CD16 play a major role in NK cell activation. In addition, human NK cells can express HLA class I–specific activating receptors, including KIR2DS1,[30,31] KIR2DS4,[32,33] and CD94/NKG2C.[26]

Two main NK cell subsets characterized by distinct phenotypic and functional properties have been described, namely the CD56^{bright}CD16$^{-/low}$ and CD56^{dim}CD16$^+$ subsets. CD56^{bright} NK cells express high levels of CD94/NKG2A but virtually no KIRs. Infrequent in peripheral blood (PB; approximately 10%), they predominate in secondary lymphoid compartments. CD56^{bright} NK cells produce high amounts of immunoregulatory cytokines but are poorly cytotoxic. In contrast, the CD56^{dim} subset is largely represented in PB (approximately 90%) and is characterized by high surface expression of KIRs, high cytotoxic activity against tumor- and virus-infected targets, and rapid production of cytokines on receptor-mediated cell activation.[34,35]

A constitutive or inducible expression of PD-1 has been detected in different cell populations, including T, B, and myeloid cells,[14,36] whereas little is known regarding PD-1 expression on NK cells to date. In human subjects it has been reported that NK cells from patients with multiple myeloma[37] or patients with posttransplantation lymphoproliferative disorders[38] can express low PD-1 levels. On the other hand, expression of PD-1 by resting NK cells from immunocompetent healthy subjects has been poorly defined.

In the present study we show that PD-1 is expressed at high levels (PD-1^{bright}) on a discrete subset of mature CD56^{dim}NKG2A$^-$KIR$^+$CD57$^+$ NK cells in approximately one fourth of a large number of donors with no evident disease (thereafter defined as healthy donors [HDs]) and, more frequently, in the PB of a cohort of patients with ovarian carcinoma. Functional analysis revealed that PD-1$^+$ NK cells display poor cytokine-induced proliferation and lower degranulation and cytokine production compared with PD-1$^-$ cells. Antibody-mediated disruption of the PD-1/PD-L interaction could revert, at least in part, the impaired NK cell degranulation against an ovarian carcinoma cell line. Remarkably, a relatively large PD-1^{bright} NK cell subset was detected in peritoneal fluid/ascites (PF) of patients with ovarian carcinoma, suggesting that PD-1$^+$ cells can be induced by the tumor microenvironment or recruited to the tumor site. Also, in this case the impaired NK cell degranulation was reversible with anti–PD-L antibodies.

METHODS

Patients and samples

This study included 200 buffy coats collected from volunteer blood donors admitted to the blood transfusion center of IRCCS S. Martino-IST after obtaining informed consent, and the study was approved by the Ethical Committee of IRCCS S. Martino-IST (39/2012). Thirty patients with seropapillary ovarian carcinoma subjected to primary surgery before chemotherapy (in accordance with a protocol approved by the Spedali Civili of Brescia institutional ethical board) were also enrolled, and informed consent was obtained from all patients according to the Declaration of Helsinki.

Isolation, culture of human leukocytes, and gate strategy

Mononuclear cells were obtained from heparinized PB[39] and from PF of patients with ovarian carcinoma (after depletion of epithelial cell adhesion molecule [ESA]$^+$ and CD90$^+$ cells by using Magnetic Dynabeads Goat anti-Mouse IgG)[40] by means of density gradient centrifugation over Ficoll (Sigma, St Louis, Mo) and then resuspended in RPMI 1640 supplemented with 2 mmol/L glutamine, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 10% heat-inactivated FCS (Società Prodotti Antibiotici, Milano, Italy).

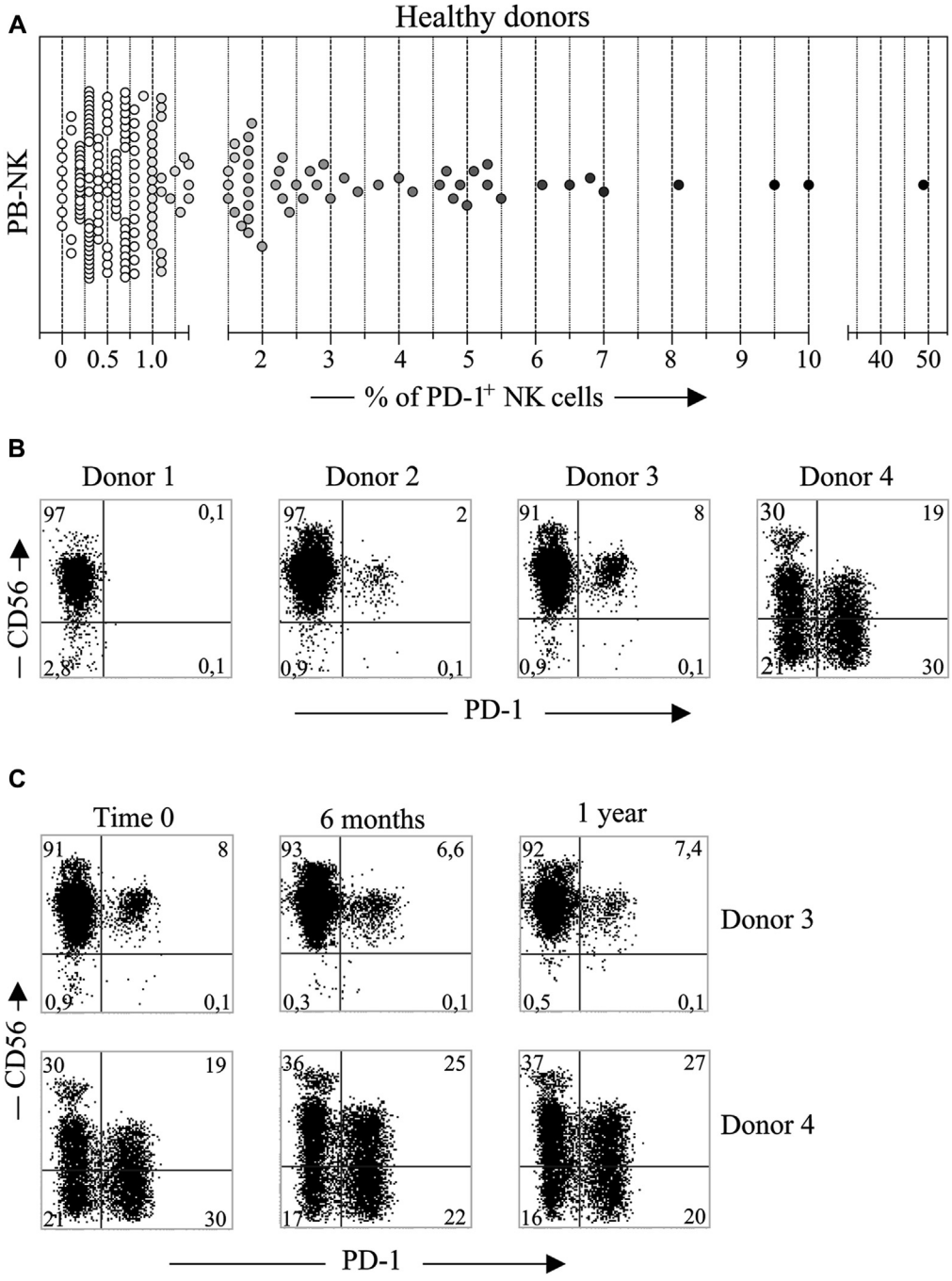

FIG 1. PD-1$^+$ NK cells in the PB of HDs. **A,** The *x-axis* represents the percentage of PD-1$^+$ NK cells of 200 HDs. The *y-axis* represents the various donors with NK cells at different PD-1$^+$ percentages. **B,** Cytofluorimetric analysis of PD-1 expression in PB NK cells of 4 representative donors. **C,** Cytofluorimetric analysis of PD-1 expression in PB NK cells of 2 representative PD-1$^+$ donors at different time points. Gate strategy used: A.

Gate strategy

For more information on gate strategies, see Fig E1 in this article's Online Repository at www.jacionline.org. In Fig 1 analysis of NK cells was made on $CD56^{+/-}CD3^-CD20^-CD16^{+/-}$ gated cells (gate strategy A). In Fig 2 analysis of NK cells was made on $CD56^+CD3^-CD20^-CD16^{+/-}$ gated cells (gate strategy B). In Figs 3 and 4 (panels A and C) and Figs 5, 6, and 7 (panel A) analysis of PD-1$^+$ and PD-1$^-$ NK cell subsets was made on $CD56^{dim}CD3^-CD20^-CD16^{+/-}$ gated cells (gate strategy C). In Figs 4 (panel B) and 7 (panels B and C) analysis of PD-1$^+$ and PD-1$^-$ NK cell subsets was made on $CD56^{dim}CD3^-CD20^-CD16^{+/-}NKG2A^-KIR^+CD57^+$ gated cells (gate strategy D). In Fig 8 analysis of NK cells was made on $CD56^{+/-}CD3^-CD20^-CD16^{+/-}$ gated cells (gate strategy A), as in Fig 1.

Statistical analysis

The independent samples *t* test was used for evaluating quantitative variables. The test is a statistical technique used to analyze the mean comparison of 2 independent groups. The statistical level of significance was preset at .05.

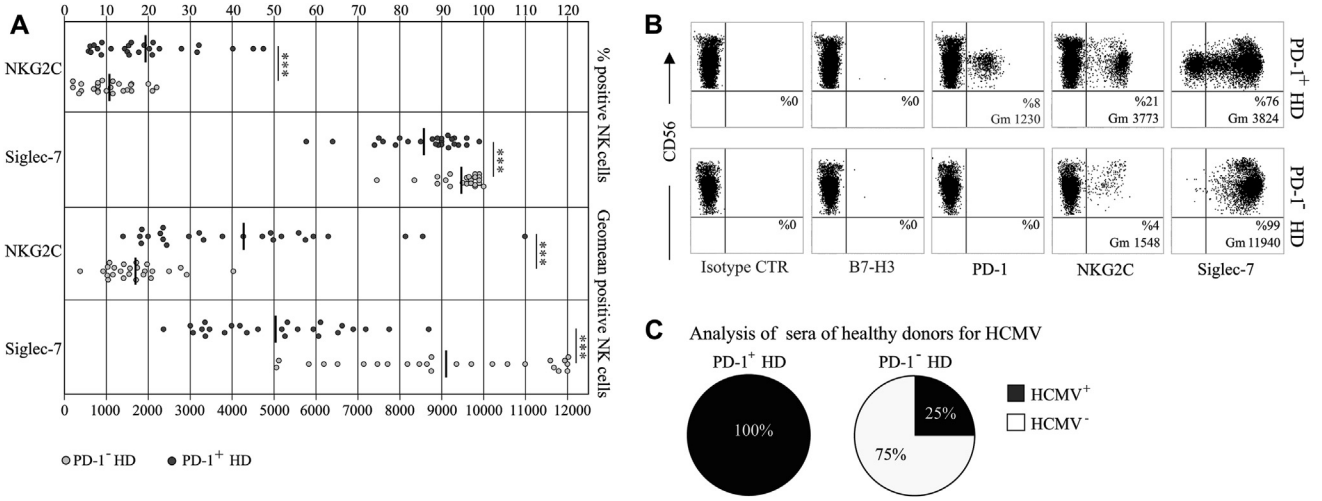

FIG 2. Expression of NKG2C and Siglec-7 in PB NK cells of representative PD-1$^+$ and PD-1$^-$ HDs. **A,** Percentages and geometric means in 50 donors. ***$P < .001$. **B,** Cytofluorimetric analysis of PD-1, NKG2C, and Siglec-7 expression in NK cells of 1 PD-1$^+$ and 1 PD-1$^-$ donors. B7-H3 was used as a negative control. **C,** Percentage of seropositivity for HCMV in sera derived from 40 donors expressing or not expressing PD-1 on NK cells. Gate strategy used: B.

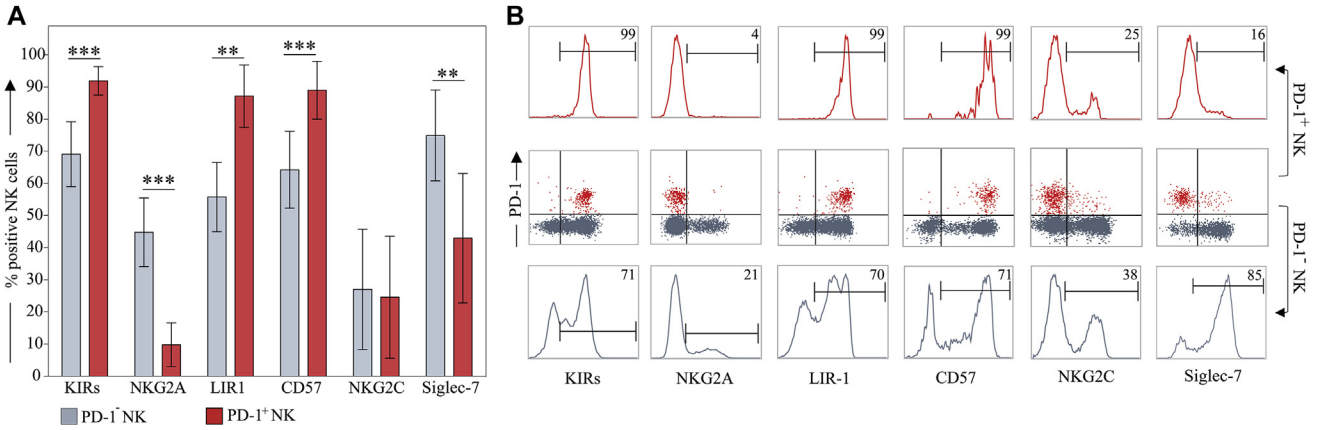

FIG 3. Expression of informative surface receptors in PD-1$^+$ and PD-1$^-$ NK cell subsets from PB of representative PD-1$^+$ HDs. **A,** Percentages and geometric means in 15 donors. **$P < .01$ and ***$P < .001$. **B,** One representative donor. Gate strategy used: C.

Graphic representation and statistical analyses were performed with PASW Statistics, version 18.0, software (formerly SPSS Statistics; IBM, Segrate, Italy) and GraphPad Prism 6 (GraphPad Software, La Jolla, Calif).

RESULTS

PD-1 can be expressed by NK cells of HDs

Freshly isolated PB lymphocytes from a cohort of 200 HDs were assessed for the expression of PD-1 on NK cells by using cytofluorimetric analysis.

A subset characterized by a bright surface PD-1 expression was detected in about 25% of the donors analyzed (Fig 1, *A* and *B*). PD-1$^+$ cells were confined to CD56^{dim} NK cells, whereas the CD56^{bright} subset was consistently PD-1$^-$ (Fig 1, *B*). The percentage of PD-1$^+$ cells was variable among donors, ranging between 1.5% and 10%. In one donor displaying an expansion of the aberrant CD56$^-$CD16$^+$ NK subset,[41,42] PD-1 was found on both CD56^{dim} and CD56$^-$ NK cells, and the percentage of positive cells reached as much as 49% (Fig 1, *B*).

Notably, analysis of the size of the PD-1$^+$ subset at different time points in various subjects revealed substantial stability over time. In Fig 1, *C*, 2 representative HDs are shown in whom the expression of PD-1 was unchanged for at least 1 year.

Because PD-1$^+$ NK cells were present only in some donors, it is possible that the expression of PD-1 might be related to infection. In this regard recent studies indicated that human cytomegalovirus (HCMV) infection leads to NK cell differentiation/maturation and a reconfiguration in the NK cell receptor repertoire, including upregulation of the NKG2C activating receptor[43] and downregulation of the sialic acid–binding immunoglobulin-like lectin (Siglec) 7 (or p75/AIRM-1) inhibitory receptor.[42,44-46] Thus we analyzed PD-1$^+$ and PD-1$^-$ donors to figure out whether the expression of PD-1 correlated with altered expression of these NK cell markers.

We found that PD-1$^+$ subjects frequently display high proportions of NKG2C$^+$ cells, as well as a subset of Siglec-7$^-$ NK cells (Fig 2, *A* and *B*). In addition, in most PD-1$^+$ donors the NKG2C$^+$ cell expansion was characterized by expression of an

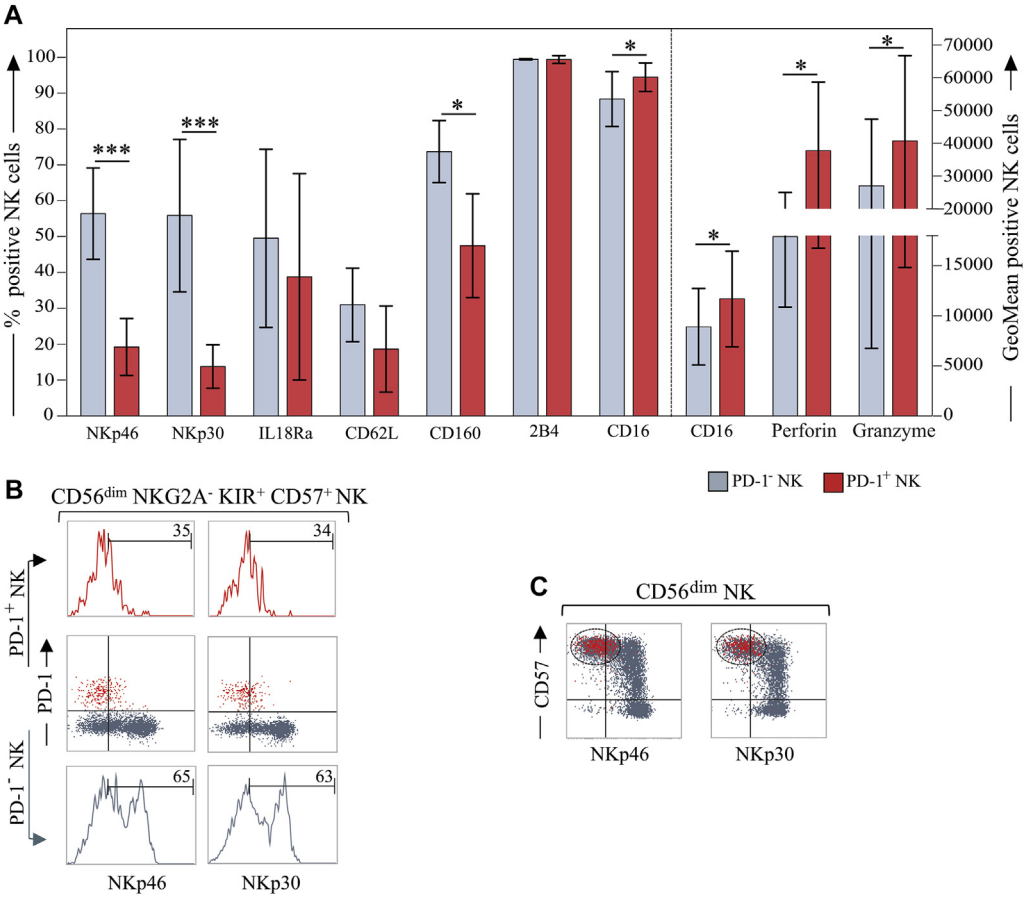

FIG 4. Analysis of additional markers on PD-1$^+$ and PD-1$^-$ PB NK cells in representative PD-1$^+$ HDs. **A,** Percentages of expression of the indicated markers in 8 PD-1$^+$ donors. Gate strategy used: C. *$P < .05$, **$P < .01$, and ***$P < .001$. **B,** Expression of NCRs on PD-1$^+$ and PD-1$^-$ cells in CD56^{dim}NKG2A$^-$KIR$^+$CD57$^+$ gated cells in 1 representative donor. Gate strategy used: D. **C,** PD-1$^+$ NK cells *(black circles)* in relation to NCRs and CD57 surface expression (1 representative donor). Gate strategy used: C.

NKG2C^{bright} (or NKG2C^{high}) phenotype (Fig 2, *B*).[47,48] These results suggested a possible association between PD-1 expression in NK cells and HCMV infection.[42,45,46,49] Thus, when possible, HCMV seropositivity was also analyzed. We found that all the PD-1$^+$ subjects were seropositive for HCMV (Fig 2, *C*).

Previous studies revealed that, among the surface molecules undergoing changes in expression after HCMV infection, an important group is represented by the HLA-specific receptors, including NKG2A, KIR, and leukocyte immunoglobulin-like receptor 1 (LIR-1). In particular, infection by HCMV favors expansion of KIR$^+$ and LIR-1$^+$ NK cells while reducing the fraction of NKG2A$^+$ NK cells.[42,46] In addition, a subset of NKG2A$^-$KIR$^+$LIR-1$^+$ NK cells preferentially acquired CD57.[49] This molecule constitutes a marker of unknown function that is thought to identify mature/terminally differentiated NK cells. CD57$^+$ NK cells appear to be a stable subpopulation, increasing with age and exposure to pathogens (especially HCMV).

By comparing the PD-1$^+$ and PD-1$^-$ NK cell subsets derived from seropositive PD-1$^+$ HDs, we show that the PD-1$^+$ population is primarily composed of mature NKG2A$^-$KIR$^+$LIR-1$^+$ cells expressing CD57 (Fig 3, *A*). In addition, different from PD-1$^-$ NK cells, only a minor fraction of PD-1$^+$ NK cells expressed Siglec-7 (Fig 3, *A* and *B*), whereas NKG2C was expressed on both PD-1$^+$ and PD-1$^-$ NK cells (Fig 3, *A* and *B*). Fig 3, *B*, shows expression of the above markers on PD-1$^+$ and PD-1$^-$ cells of a representative donor.

Further analysis of additional cell-surface markers undergoing variation after HCMV infection in the PD-1$^+$ NK cell subset showed lower levels of NKp46, NKp30, CD160, and CD62 ligand expression (Fig 4, *A*) compared with the PD-1$^-$ subset. On the other hand, perforin/granzyme and CD16 were expressed at higher levels in PD-1$^+$ NK cells. Other receptors/markers, including 2B4 (CD244), IL-18 receptor α (IL-18Rα), NKG2D, and DNAM-1, were expressed at similar levels in PD-1$^+$ and PD-1$^-$ cells (Fig 4, *A*, and data not shown). The activation markers CD69, CD25, and NKp44 were substantially negative on both NK cell subsets (data not shown).

Finally, comparison between the PD-1$^+$ and PD-1$^-$ cell fractions of the (highly differentiated) NKG2A$^-$KIR$^+$CD57$^+$ subset revealed that PD-1$^+$ cells display the lowest expression of NKp46 and NKp30 (Fig 4, *B* and *C*). Altogether, these data indicate that expression of PD-1 is confined to NK cells with the phenotypic features of fully mature (terminally differentiated) NK cells.[50-52]

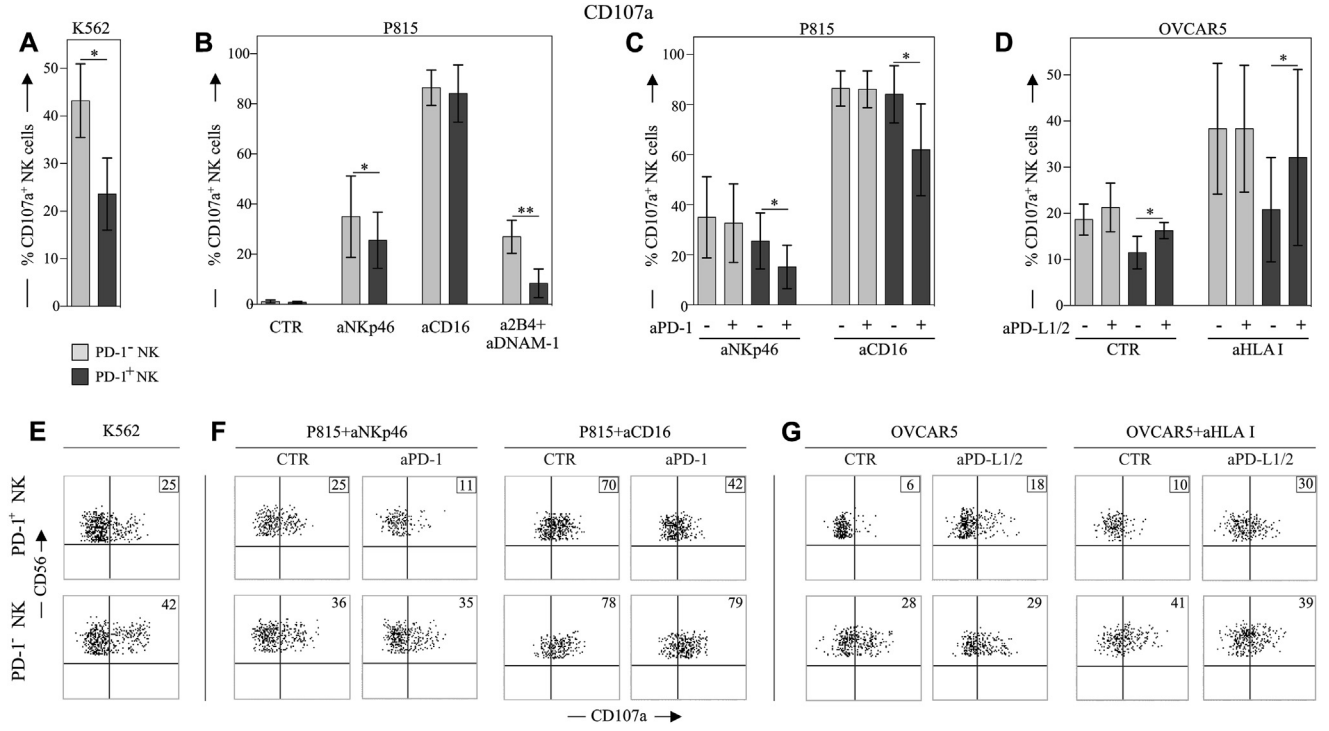

FIG 5. Degranulation (CD107a expression) of PD-1$^+$ and PD-1$^-$ NK cells from representative PD-1$^+$ HDs stimulated with the indicated tumor target cells. **A** and **E,** K562 (6 donors and 1 representative donor, respectively). **B,** P815 alone (CTR) or with the indicated mAbs (6 donors). **C** and **F,** P815 with the indicated mAbs with or without anti–PD-1 mAb (6 donors and 1 representative donor, respectively). **D** and **G,** OVCAR5 alone or in the presence of the indicated mAbs (6 donors and 1 representative donor, respectively). *$P < .05$ and **$P < .01$. Gate strategy used: C.

Functional analysis of PD-1$^+$ NK cells

These experiments were performed on gated PD-1$^+$ or PD-1$^-$ CD56^{dim} NK cell subsets by evaluating NK cell degranulation, as assessed based on surface expression of CD107a on interaction with different target cells. First, PD-1$^+$ and PD-1$^-$ fractions were comparatively analyzed for their ability to undergo degranulation in the presence of K562 erythroleukemia. K562 is a classical NK cell target lacking HLA class I molecules and expressing a series of ligands for activating NK receptors, including B7-H6,[53] which is recognized by NKp30. As shown in Fig 5, *A* and *E*, degranulation in PD-1$^+$ NK cells (NKp30^{low} and NKp46^{low}) was markedly less than in PD-1$^-$ NK cells (NKp30^{high} and NKp46^{high}). Because K562 cells do not express PD-L1/PD-L2, the differences detected could not reflect the interaction between PD-1 and its ligands but rather the low expression of the 2 main NCRs. Next, PD-1$^+$ and PD-1$^-$ NK cell subsets were assessed for degranulation in a reverse antibody-dependent cellular cytotoxicity (R-ADCC) assay against the murine FcγR$^+$ P815 mastocytoma cell line (PD-L1/PD-L2 negative). These experiments were performed in the absence or presence of mAbs specific for triggering NK receptors. The aim was to assess possible additional differences in degranulation after mAb-mediated cross-linking of activating receptors and coreceptors. As shown in Fig 5, *B*, both NK subsets undergo efficient degranulation in the presence of anti-CD16 or anti-NKp46 mAbs. However, in PD-1$^+$ cells the low surface expression of NKp46 resulted in less efficient responses to anti-NKp46 mAb. Interestingly, the 2 subsets displayed different abilities to respond to other triggering stimuli. Thus the combined use of mAbs specific for DNAM-1 and 2B4 coreceptors induced degranulation of PD-1$^-$ but not PD-1$^+$ NK cells (Fig 5, *B*).

Another series of R-ADCC experiments was performed by using anti–PD-1 mAb in combination with anti-NKp46 or anti-CD16 mAbs. The aim of these experiments was to assess whether mAb-mediated cross-linking of PD-1 could inhibit NK cell activation induced by cross-linking of the triggering receptors. As shown in Fig 5, *C* and *F*, mAb-mediated cross-linking of PD-1 resulted in a partial but significant reduction of NK cell responses to triggering receptors.

We further analyzed the effect of engagement of PD-1 by its ligands (PD-L1/PD-L2) expressed on the tumor cell line OVCAR5 (ovarian carcinoma).[54] In these experiments the expression of CD107a on NK cells was analyzed after short-term exposure of NK cells to OVCAR5 in the presence or absence of anti–PD-L1/PD-L2 mAbs, which are capable of blocking the interaction between PD-1 and its ligands (mAb-mediated masking experiments). As shown in Fig 5, *D* and G, PD-1$^-$ NK cells displayed a substantial mobilization of CD107a, both in the absence and presence of anti–PD-L1/PD-L2 mAbs. In contrast, degranulation of PD-1$^+$ NK cells was reduced and could be restored, at least in part, by anti–PD-L1/PD-L2 mAbs. Note that we performed experiments also in the presence of anti–HLA class I mAbs (A6/136)[55] to disrupt the inhibitory interaction between KIRs and HLA class I molecules (expressed at high levels on the surface of OVCAR5; Fig 5, *D* and *G*).

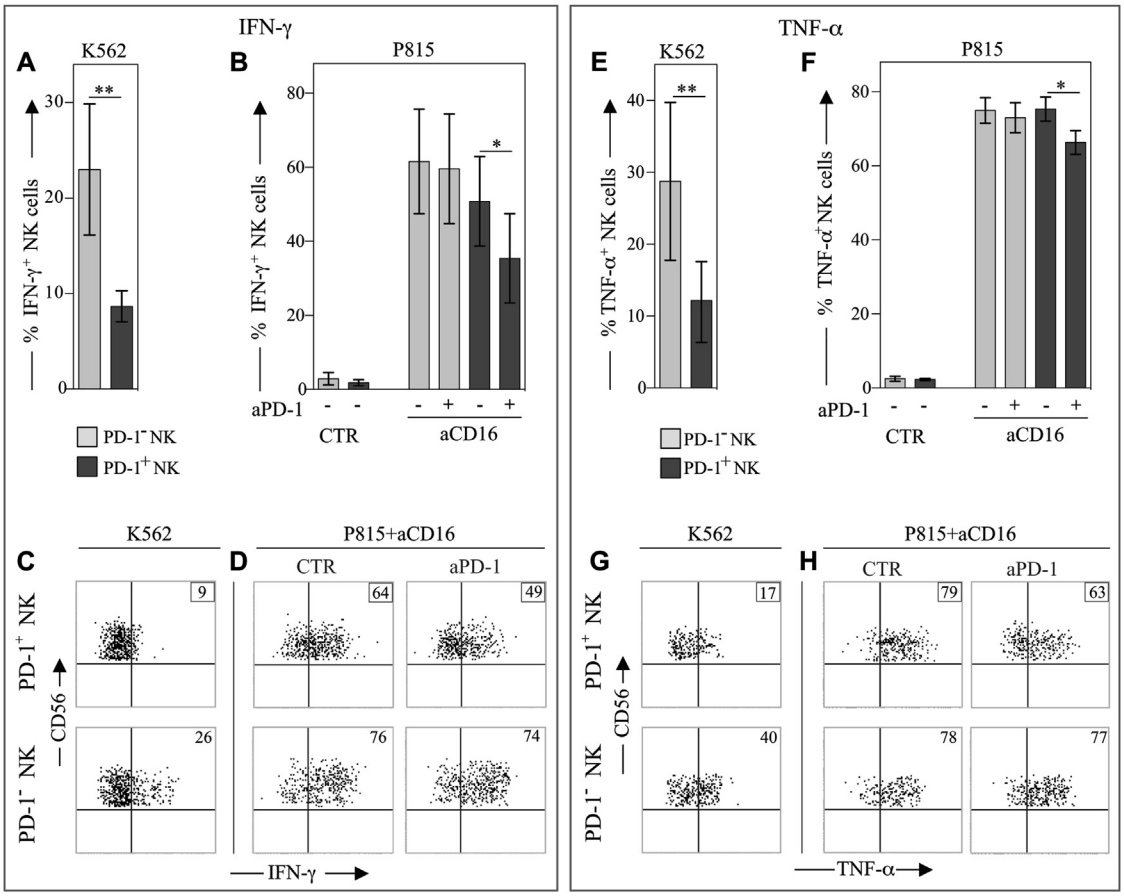

FIG 6. IFN-γ and TNF-α production by PD-1$^+$ and PD-1$^-$ PB NK cells from representative PD-1$^+$ HDs after stimulation with different tumor target cells. **A** and **C,** IFN-γ production after stimulation with K562 (6 donors and 1 representative donor, respectively). **E** and **G,** TNF-α production after stimulation with K562 (6 donors and 1 representative donor, respectively). **B** and **D,** IFN-γ production on stimulation with P815 in the presence or absence of the indicated mAbs (6 donors and 1 representative donor, respectively). **F** and **H,** TNF-α production on stimulation with P815 in the presence or absence of the indicated mAbs (6 donors and 1 representative donor, respectively). *$P < .05$ and **$P < .01$. Gate strategy used: C.

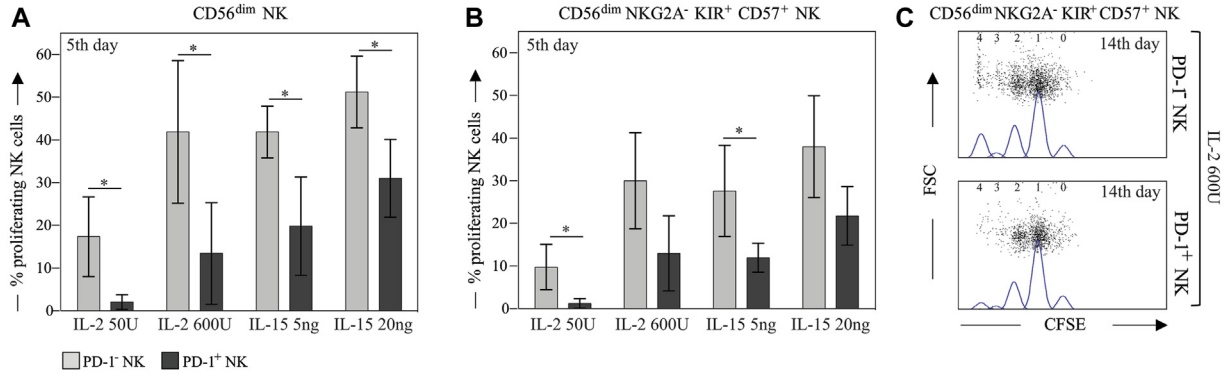

FIG 7. Proliferative responses to cytokines of PD-1$^+$ and PD-1$^-$ NK cells in representative PD-1$^+$ HDs. **A,** Proliferation (CFSE staining) of PD-1$^+$ and PD-1$^-$ CD56^{dim} NK cells. Gate strategy used: C. **B,** Proliferation (CFSE staining) of PD-1$^+$ and PD-1$^-$ CD56^{dim}NKG2A$^-$KIR$^+$CD57$^+$ NK cells. Gate strategy used: D. **C,** Proliferation (CFSE staining) of PD-1$^+$ and PD-1$^-$ CD56^{dim}NKG2A$^-$KIR$^+$CD57$^+$ NK cells. *0-4*, Number of cell generations in CFSE-stained proliferating cells. Gate strategy used: D. *$P < .05$.

Altogether, these experiments indicate that PD-1$^+$ NK cells display a low cytolytic activity against tumor targets, even when these cells lack PD-L1/PD-L2 expression (ie, K562). In addition, the inhibition of NK cell degranulation induced by cross-linking PD-1 and its ligands on OVCAR5 tumor cells was reverted only in part by mAbs specific for PD-L1/PD-L2.

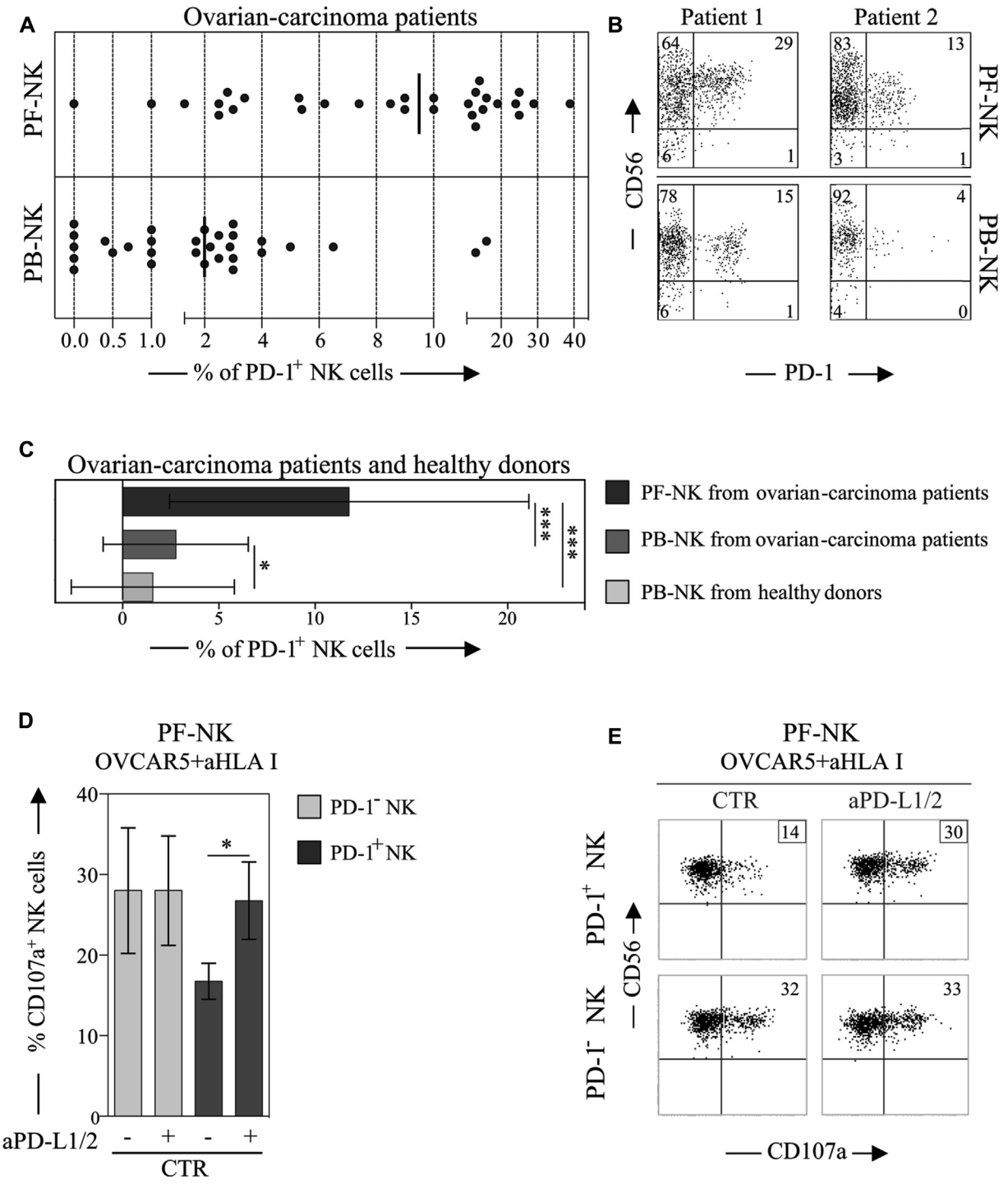

FIG 8. PD-1$^+$ NK cells in PF and PB in representative patients with ovarian carcinoma. **A,** The *x-axis* represents the percentage of PD-1$^+$ NK cells derived from PB or PF of 30 patients. The *y-axis* represents the various donors with NK cells at different PD-1$^+$ percentages. Gate strategy used: A. **B,** PD-1 percentages in 2 representative patients. Gate strategy used: A. **C,** Comparison of the percentages of PD-1$^+$ NK cells between HDs (PB) and patients (PB and PF). Gate strategy used: A. **D** and **E,** Degranulation (CD107a expression) of PD-1$^+$ and PD-1$^-$ NK cells from representative PD-1$^+$ patients stimulated with OVCAR5 tumor target cells in the presence of the indicated mAbs (3 patients and 1 representative patient, respectively). Gate strategy used: C. $^*P < .05$ and $^{***}P < .001$.

Interestingly, the impaired killing/degranulation of PD-1$^+$ (compared with PD-1$^-$) cells could be overcome by strong stimuli, such as cell triggering through CD16 (in R-ADCC). In this case it is likely that the low level of NCR expression by PD-1$^+$ NK cells might be counterbalanced (at least in part) by the expression of high levels of CD16 and perforin/granzyme (Fig 4, *A*).

Cells were stimulated with K562 or P815 tumor target cells and assessed for production of IFN-γ and TNF-α in a flow cytometric

assay using intracellular staining to determine whether PD-1$^+$ NK cells would also display an altered capability of releasing cytokines. As shown in Fig 6, K562-induced NK cell activation resulted in production of IFN-γ (Fig 6, *A* and *C*) and TNF-α (Fig 6, *E* and *G*) by most PD-1$^-$ but only a small fraction of PD-1$^+$ NK cells. Remarkably, similar to degranulation, IFN-γ and TNF-α production could be induced in PD-1$^+$ NK cells by anti-CD16 mAbs in a redirected-antibody dependent stimulation assay (P815 cells plus mAb). On the other hand, when anti-CD16 was combined with anti–PD-1 mAb, a significantly lower fraction of PD-1$^+$ NK cells produced cytokines compared with PD-1$^-$ NK cells (Fig 6, *B*, *D*, *F*, and *H*).

Next, the PD-1$^+$ and PD-1$^-$ subsets were analyzed for their capacity to proliferate (by using the carboxyfluorescein succinimidyl ester [CFSE] dilution assay measured at 5 days of culture) in response to rhIL-2 or rhIL-15. As shown in Fig 7, *A*, PD-1$^+$ NK cells are poorly responsive to low doses of rhIL-2 or rhIL-15, whereas they undergo proliferation at higher cytokine doses, although at lower rates than PD-1$^-$ NK cells. A similar difference in the capacity to respond to cytokines was observed also between the PD-1$^+$ and PD-1$^-$ cell fractions of the NKG2A$^-$KIR$^+$CD57$^+$ NK cell population (Fig 7, *B*). Thus proliferation of PD-1$^+$ NK cells is impaired in the presence of low cytokine concentrations, a condition that can occur in certain tumor microenvironments. Fig 7, *C*, shows comparison of the peaks of CFSE dilution in PD-1$^+$ and PD-1$^-$ cells in the presence of rhIL-2. In this case the culture time was extended to 14 days instead of 5 (standard time frame) to better visualize the proliferation peaks.

Expression and function of PD-1 on NK cells from patients with tumors

In these experiments we analyzed whether PD-1$^+$ NK cells could be found also in PB and PF of patients affected by seropapillary ovarian carcinoma.[40] PD-1$^+$ cells could be detected in the PB of the majority of these patients. In addition, in given patients they were present in higher proportions in PF than in PB of the same patients (Fig 8, *A* and *C*). Although, as previously reported,[40] the size of the CD56^{bright} NK cell subsets was usually larger in PF NK cells compared with PB NK cells of the same patients, PD-1$^+$ NK cells were confined to the CD56^{dim} NK cell subset (Fig 8, *B*). Notably, the size of the PD-1$^+$ NK cell subsets detected in PB of the patients was slightly larger than in PBs of HDs (Fig 8, *C*).

Altogether, these data indicate that PD-1$^+$ NK cells might be significantly enriched at the tumor site. As shown in Fig 8, *D* and *E* (and not shown), and in agreement with data obtained in HDs, the ability of PD-1$^+$ PF NK cells to degranulate was lower than that of PD-1$^-$ PF NK cells. Note that the use of mAbs suitable for disrupting the interaction between PD-1 and its ligands might restore the altered functional capability of PD-1$^+$ NK cells toward PD-L1/PD-L2$^+$ tumor target cells (OVCAR5, ovarian carcinoma) (Fig 8, *D* and *E*). This suggest that in patients with ovarian carcinoma, the impaired functional capability of PD-1$^+$ NK cells can be rescued *in vivo* by mAbs targeting the PD-1/PD-L axis of the NK/tumor crosstalk.

DISCUSSION

In the present study we show that PD-1 is brightly expressed by a subset of PB NK cells in a fraction of otherwise healthy subjects. PD-1 expression is restricted to the CD56^{dim} NK cell subset and varies in size among different subjects. The surface phenotype of PD-1$^+$ NK cells is typical of terminally differentiated (NKG2A$^-$KIR$^+$CD57$^+$) NK cells, which are characterized by low NCR and high CD16, perforin, and granzyme expression. As to their functional capabilities, PD-1$^+$ NK cells show impaired proliferative responses to exogenous rhIL-2 and rhIL-15. In addition, degranulation/cytotoxic activity and cytokine production in response to tumor cells are significantly lower in PD-1$^+$ than PD-1$^-$ NK cells.

The expression and function of PD-1 have been analyzed mainly in normal peripheral T cells and tumor-infiltrating lymphocytes,[56-58] whereas only a few studies have been focused on NK cells. In these studies the expression of PD-1 was detected in PB NK cells derived from patients[37,38,59,60] but not from HDs. In addition, the level of expression reported was very low, thus rendering it difficult to discriminate between PD-1$^+$ and PD-1$^-$ cells and to identify a distinct subset of PD-1$^+$ cells. Because of these limitations, no attempts have been made to characterize the PD-1$^+$ NK cell subset for phenotypic and functional properties.

Here we show that some HDs express sizeable levels of PD-1 on CD56^{dim} NK cells and that the size of the PD-1$^+$ NK cell subset varied among donors. Notably, analysis of the size of the PD-1$^+$ subset in given subjects at different time points revealed a substantial stability (Fig 1, *C*). The fact that a PD-1$^+$ NK cell subset could be detected in only some of the subjects analyzed might be a result of latent chronic infections affecting these subjects (in this context it is of note that an increase in PD-1$^+$ lymphocyte numbers has been associated with hepatitis C virus, hepatitis B virus, and HIV infections).[6,7,61-63] Although a correlation exists between the presence of PD-1$^+$ NK cells and HCMV infection, phenotypic analysis of PD-1$^+$ NK cells showed that NKG2C (the expression of which is associated to HCMV infection) is not preferentially expressed by PD-1$^+$ cells. However, similar to NKG2C$^+$ NK cells accumulating in response to HCMV infection, the PD-1$^+$ subset was almost exclusively composed of highly differentiated NKG2A$^-$KIR$^+$CD57$^+$ NK cells. In line with this phenotypic feature, PD-1$^+$ cells expressed very low levels of NCRs (the lowest among CD57$^+$ NK cells) but high levels of CD16. Conceivably, the impaired degranulation and cytokine production in response to tumor target cells (K562) is consequent to the defective expression of NCRs. In this context it is of note that B7-H6, a major surface ligand of NKp30, is expressed by K562 cells.[53] On the other hand, in line with their high content of perforin/granzyme and their high expression of CD16, PD-1$^+$ NK cells could degranulate to levels comparable with PD-1$^-$ cells in response to anti-CD16 mAbs in R-ADCC.[50-52] Thus under this experimental condition, the impaired degranulation of PD-1$^+$ NK cells caused by downregulation of NCRs (NKp30 and NKp46) can be bypassed by a potent stimulus delivered by CD16. Based on the recovery of different functions (cytokine release and cytotoxicity) on cell triggering through CD16 in R-ADCC, it is unlikely that PD-1$^+$ NK cells merely represent a population of exhausted cells with an intrinsic functional defect. Indeed, their functional impairment appears to be mainly consequent to a profound imbalance between inhibitory and triggering signals, reflecting the upregulation/downregulation of different receptors. Notably, in line with this concept, the reduced degranulation of PD-1$^+$ cells after interaction with OVCAR5 reflects not only the poor NCR-mediated cell activation but also the inhibitory

signal mediated by PD-1 on interaction with PD-L1/PD-L2 expressed on these target cells. In this case disruption of the PD-1/PD-L interaction mediated by anti–PD-L mAbs could partially revert functional inhibition. Thus cross-linking of PD-1 mediated by either anti–PD-1 mAbs in R-ADCC (Fig 5, *C* and *F*) or by the engagement of PD-L expressed on target cells (Fig 5, *D* and *G*) can lead to impaired PD-1$^+$ NK cell function.

The sharply reduced proliferative responses of PD-1$^+$ NK cells to low concentrations of exogenous rhIL-2 and rhIL-15 is in line with the finding in mice that highly differentiated memory NK cells display reduced responses to soluble microenvironmental cytokines.[64] Thus it is conceivable that also human CD57$^+$ NK cells can be poorly responsive because they express low amounts of CD122 (IL-2 receptor β, a subunit of the shared receptor for IL-2 and IL-15).[50-52,65] In addition, the PD-1$^+$ NK cell subset is characterized by the lowest CD122 expression among CD57$^+$ NK cells (data not shown). Moreover, IFN-γ production by PD-1$^+$ NK cells in response to exogenous rhIL-12 plus rhIL-18 was highly defective (data not shown).[50-52] Thus it is conceivable that PD-1$^+$ NK cells might represent a population of poorly proliferating cells that could be induced to divide only by high concentrations of microenvironmental cytokines, as suggested by our present data.

PD-1$^+$ NK cells were detected more frequently in the PB of a cohort of patients with seropapillary ovarian carcinoma than in HDs. More importantly, PD-1$^+$ cells could be recovered from the PF of most of these patients.[40] Remarkably, the size of such PF PD-1$^+$ subsets was much larger than in PB from the same subjects. In some patients PD-1$^+$ NK cells were present in PF but not in PB. It is possible that in these cases the size of the PB PD-1$^+$ subset could be less than the limit of detectability. Altogether, these data suggest that PD-1$^+$ NK cells are present more frequently in the PB of patients with certain tumors. In addition, this PD-1$^+$ subset might be greatly increased in size in tumor-associated NK cells, as in the case of PF. Functionally, PD-1$^+$ NK cells isolated from PF display frequently a markedly compromised capability of degranulating on interaction with PD-L1/PD-L2$^+$ tumor target cells. Remarkably, mAb-mediated disruption of PD-1/PD-L interaction could partially restore the NK cell function.

Because PD-1$^+$ cells have been detected in some HDs, it is possible that latent chronic diseases (eg, viral infections) might be responsible for PD-1 induction in NK cells. Indeed, a correlation has been established between HCMV seropositivity and the presence of PB PD-1$^+$ NK cells (Fig 2, *C*). One might speculate that both virus-driven signals and the tumor microenvironment can contribute to PD-1 expression on NK cells in the case of patients with ovarian carcinoma.

It is evident that the study of other tumors and infectious diseases is needed to further confirm a correlation with the presence/induction of PD-1$^+$ cells. Remarkably, in a recent study, the clinical efficacy of an anti–PD-1 mAb has been reported in patients with ovarian carcinoma.[66] It is possible that the beneficial effects documented in this study might reflect not only a blockade of PD-1/PD-L on tumor-infiltrating lymphocytes but also on PD-1$^+$ NK cells.

In conclusion, because NK cells are potent effectors with strong cytolytic activity against tumor cells, the finding that PD-1 can be expressed on these cells, particularly in patients with cancer, suggests a possible involvement of the PD-1–mediated inhibitory pathway in the impaired NK cell responses against tumors. Our present finding further supports the notion that PD-1 signaling might downregulate not only cytotoxic T lymphocyte–mediated adaptive responses but also innate responses. Therefore it is conceivable that therapies targeting the PD-1/PD-L axis might rescue not only cytotoxic T-lymphocyte activity but also NK-mediated function. In this context it should be stressed that although in conventional NK cells the effector function is primarily regulated by HLA class I–specific inhibitory receptors (KIR family and CD94/NKG2A)/HLA class I molecule interactions, in the case of PD-1$^+$ NK cells, an additional potent inhibitory pathway might contribute to limit NK cell function. In this case downregulation of HLA class I molecules on tumor cells can be offset by the expression of PD-1 ligands. It is possible that the simultaneous expression of PD-1 together with given inhibitory HLA-specific receptors might provide an additional level of suppression of NK cell antitumor responses and that (mAb-mediated) blocking of different inhibitory checkpoints, including PD-1, might be required to obtain optimal NK cell responses.

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Key messages

- **Unequivocal evidence that PD-1 is highly expressed by a subset of NK cells detectable in a fraction of healthy subjects.**
- **PD-1$^+$ NK cells are CD56^{dim}NKG2A$^-$KIR$^+$CD57$^+$, a phenotypic feature typical of terminally differentiated NK cells. On interactions with tumor cells, they display impaired degranulation/cytotoxic activity and cytokine production and elicit poor proliferation in response to exogenous cytokines. The impaired degranulation against an ovarian carcinoma cell line can be reversed by anti–PD-L antibodies.**
- **PD-1$^+$ NK cells are present in higher proportions in the ascites of a cohort of patients with ovarian carcinoma, suggesting their possible induction/enrichment in tumor microenvironment. Also in this case, PD-1 expression is associated with impaired NK cell degranulation that is reversible with anti–PD-L antibodies.**

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METHODS

mAbs

The following mAbs were isolated in our laboratory, licensed to the indicated companies, and validated for their specificity: anti-2B4 (PP35, IgG_1; eBioscience Affymetrix, San Diego, Calif); anti–DNAM-1 (KRA236, IgG_1), anti-NKp30 (AZ20, IgG_1), anti-NKp44 (Z231, IgG_1), anti-NKp46 (BAB281, IgG_1; Beckman Coulter/Immunotech, Marseille, France), anti–Siglec-7 (QA79, IgG_1; R&D Systems, Abingdon, United Kingdom), anti–Siglec-7 (Z176, IgG_{2b}; Beckman Coulter/Immunotech, Marseille, France). For the following mAbs, the specificity has been validated in the corresponding patents or assigned to a cluster in CD workshop (see the indicated link): anti–LIR-1 (F278, IgG_1; www.hcdm.org/index.php?option=com_molecule&cdnumber=CD85J), KIR3DL1/L2-S1 (AZ158, IgG_{2a}; patents/WO2010081890A1?cl=en), and CD160 (PAX71, IgG_1; www.sciencegateway.org/resources/prow/guide/1660590458_g.htm).

The purified anti–PD-1 mAb (PD1.3.1.3 clone, IgG_{2b}) was originally isolated at the Laboratoire Immunologie des Tumeurs, CRCM, Marseille-Luminy (France),[E1] whereas the anti–PD-1–phycoerythrin (PE) or anti–PD-1–allophycocyanin (APC; clone PD1.3.1.3, IgG_{2b}) was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

Additional mAbs used in this study were as follows: anti–B7-H3 (D-8, IgG_{2b}; Santa Cruz Biotechnology, Dallas, Tex), anti–IL-18Rα (B-E43, IgG_1; Abcam, Cambridge, United Kingdom), anti-ESA (IgG_1; Novocastra Laboratories, Newcastle upon Tyne, United Kingdom), anti-CD90 (IgG_1; BD Biosciences PharMingen, San Jose, Calif), anti-KIR2DL1–PE or anti-KIR2DL1–APC (143211 clone), anti-CD122 (27302 clone), anti-NKG2C–APC (134591 clone), anti-NKG2C (134522 clone), anti–PD-L2 (176611 clone IgG_{2b}, R&D Systems), anti–PD-L2 (326.35 clone, IgG_1),[E1] anti–PD-L1 (27A2, IgG_{2b}; MBL, Woburn, Mass), anti–PD-L1 (PDL1.3.1 clone, IgG_1),[E1] mixture of fluorescein isothiocyanate (FITC)–labeled CD3 plus PC5-labeled CD56, anti-CD56–PC7 (N901 clone), anti-KIR3DL1/S1–APC (Z27 clone), and anti-NKG2A–APC (Z199 clone) were purchased from Beckman Coulter/Immunotech; anti-CD16–PerCP-Cy5.5 (clone 3G8), anti–KIR2DL2/L3-S2–FITC (CH-L clone), anti–CD62 ligand (DREG-5 clone), anti-CD107a–APCH7 (anti-LAMP1), and anti–IFN-γ–PE were from BD Biosciences; and anti-KIR3DL1–FITC (DX9 clone), anti–KIR2DL2/L3-S2–APC (DX27 clone), anti–KIR2DL1-S1 biotin-conjugated (11PB6 clone), anti-CD3–VioGreen (BW264/56 clone), anti-CD20–VioGreen (LT20 clone), anti-CD57–VioBlue (TB03 clone), anti–TNF-α–PE, and anti-biotin VioBlue mAbs were purchased from Miltenyi Biotec. Anti-CD25 (clone BC96, IgG_1) and anti-CD69 (clone FN50, IgG_1) were purchased from BioLegend (San Diego, Calif).

Perforin and granzyme B expression analysis in NK cells was performed with purified anti-perforin mAb from Ancell Corporation (Bayport, Minn) and purified APC-labeled anti-granzyme B mAb from Invitrogen, respectively, after cells were fixed and permeabilized with BD Cytofix/Cytoperm kit from BD Biosciences PharMingen.

Isotype-specific goat anti-mouse secondary antibodies were purchased from Southern Biotechnology (Birmingham, Ala) and, in the case of APC IgG_{2b}, by Jackson ImmunoResearch (Newmarket, United Kingdom).

For R-ADCC and masking experiments, additional mAbs isolated in our laboratory and validated for their specificity were used: anti–HLA class I (A6/136, IgM)[E2] and anti-CD16 (c127, IgG_1; Antibodies-Online, Aachen, Germany).

The staining panels for every experiment are shown in Fig E2.

Cytofluorimetric analyses were performed on a FACSVerse (Becton Dickinson, Mountain View, Calif), and data were analyzed with FACSuite software version 1.0.3.

Analysis of NK cell degranulation and IFN-γ and TNF-α production

For the degranulation assay, PB lymphocytes were cultured overnight in the presence of low doses of rhIL-15 (0.2 ng/mL) and then coincubated with one of the following targets: (1) HLA class I K562 erythroleukemia target cells (a classical target for natural cytotoxicity); (2) the $Fc\gamma R^+$ P815 murine mastocytoma cell line in the presence or absence of different mAbs specific for triggering receptors in combination or not with PD-1, as indicated in the appropriate figure (R-ADCC); and (3) the OVCAR5 ovarian carcinoma cell line in the presence or absence of anti–PD-L-1/2 IgG_1 mAbs (mAb-mediated masking experiments) and anti-HLA I (A6/136) mAbs. Cocultures were performed at an Effector/Target (E/T) ratio of 1:1 in a final volume of 200 μL in round-bottom 96-well plates at 37°C and 5% CO_2 for 4 hours in culture medium supplemented with anti-CD107a–PE mAb. After 1 hour of coincubation, GolgiStop (BD Biosciences PharMingen) was added at a 1:100 dilution. Surface staining was done by incubating the cells with anti-CD3, anti-CD56, anti-CD20, and anti–PD-1 mAbs for 30 minutes at 4°C. The cells were washed and analyzed by using flow cytometry (FACSVerse, Becton Dickinson). Analysis of NK cells was made on $CD56^+CD3^-CD20^-$ $PD\text{-}1^+$ or $PD\text{-}1^-$ gated cells.[E3] The same experimental procedure was used to analyze PB and PF lymphocytes from patients with ovarian carcinoma. The target used in this case was the OVCAR5 cell line in the presence of anti–HLA class I (A6/136) mAbs in combination or not with anti–PDL-1/2 IgG_1 mAbs, as indicated in the appropriate figure.

PB lymphocytes were incubated with low doses of rhIL-15 (0.2 ng/mL) overnight to detect intracellular production of IFN-γ/TNF-α. Then lymphocytes were washed and coincubated with target cells (K562 or P815 plus aCD16 in the presence or in the absence of aPD-1, as indicated in the figures) at an E/T ratio of 1:1 in a final volume of 200 μL in round-botton 96-well plates at 37°C and 5% CO_2 for 4 hours in the presence of GolgiStop. Thereafter, cells were washed, stained as described above for CD107a assays, and then fixed and permeabilized with BD Cytofix/Cytoperm kit from BD Biosciences PharMingen.[E3] IFN-γ and TNF-α production was detected by mean of subsequent intracellular staining with anti–IFN-γ–PE or anti–TNF-α–PE and cytofluorimetric analysis. The percentage of positive cells was calculated, subtracting the baseline CD107a/IFN-γ/TNF-α expression in control cultures without stimuli from targets.

CFSE analysis

The proliferative capability of $PD\text{-}1^+$ cell populations compared with $PD\text{-}1^-$ NK cells was assessed by staining PB NK cells derived from $PD\text{-}1^+$ HDs with CFSE before stimulation with rhIL-15 and rhIL-2 at different doses, as indicated in Fig 7. In Fig 7, *A*, the analysis of $PD\text{-}1^+$ and $PD\text{-}1^-$ NK cell subsets was made on $CD56^{dim}CD3^-CD20^-$ gated cells.

In Fig 7, *B* and *C*, the analysis of NK cells was made on $CD56^{dim}$ NK cells gated on $NKG2A^-KIR^+CD57^+$ cells, as indicated.

In Fig 7, *C*, proliferation analysis was performed with FlowJo analysis software, version 9.4.11 (TreeStar, Ashland, Ore).

ELISA

The seropositivity for HCMV in the HDs analyzed in this study was detected by using Vironostika anti-CMV III (bioMérieux, Grenoble, France), an ELISA for the detection of total antibody to CMV in human serum.

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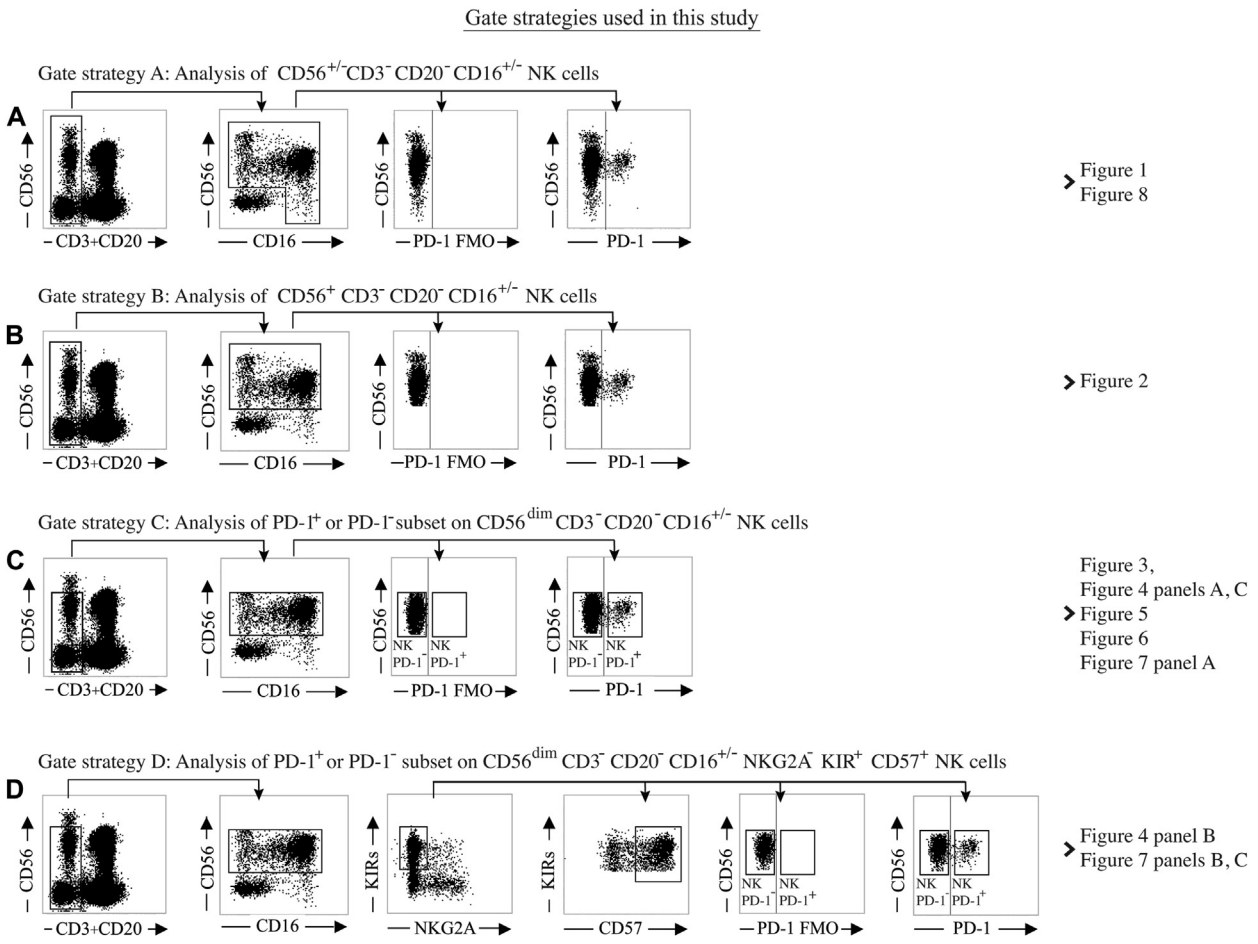

FIG E1. A-D, Illustration of the gating strategies used in this study. In each square the inner frames indicate the gated NK cell populations. *FMO*, Fluorescence minus one control. *Right*, Figures in which a given gate strategy was used.

Staining Panels of mAbs used in the various experiments

FLUOROCHROME
FITC/CFSE
PE
PerCp5.5
Pe-Cy7
APC
APC-Cy7/APC-H7
VioBlue
VioGreen

Strategy gate A/B		
Panel Donors Screening		
mAb	clone	conjugation
X	see "Appendix for X"	Purified
CD16	3G8 IgG1 (C)	PerCp5.5
CD56	N901 IgG1 (C)	Pe-Cy7
CD3+CD20	BW264/56 IgG2a + LT20 IgG1 (C)	VioGreen

Strategy gate C/D		
Panel Phenotype		
mAb	clone	conjugation
KIRs	AZ158 IgG2a (L) +11PB6 +CHLEO IgG1 (C)	FITC (AZ158 purified)
PD-1	PD1.3.1.3 IgG2b (L)	Purified
CD16	3G8 IgG1 (C)	PerCp5.5
CD56	N901 IgG1 (C)	Pe-Cy7
NKG2A	Z199 IgG2b (C)	APC
Y	see "Appendix for Y"	Purified
CD57	TB03 IgM (C)	VioBlue
CD3+CD20	BW264/56 IgG2a + LT20 IgG1 (C)	VioGreen

Strategy gate C		
Panel Perforin/Granzyme		
mAb	clone	conjugation
PD-1	PD1.3.1.3 IgG2b (C)	PE
CD16	3G8 IgG1 (C)	PerCp5.5
CD56	N901 IgG1 (C)	Pe-Cy7
Z	see "Appendix for Z"	
CD3+CD20	BW264/56 IgG2a + LT20 IgG1 (C)	VioGreen

Appendix for X	
mAb (X)	clone
PD-1	PD1.3.1.3 IgG2b (L)
B7-H3	D-8 IgG2b (C)
NKG2C	134522 IgG2b (C)
Siglec-7	Z176 IgG2b (L)

Appendix for Y	
mAb (Y)	clone
NKp46	BAB281 IgG1 (L)
NKp30	AZ20 IgG1 (L)
NKp44	Z231 IgG1 (L)
Siglec-7	QA79 IgG1 (L)
LIR-1	F278 IgG1 (L)
IL18Rα	B-E43 IgG1 (L)
CD122	27302 IgG1 (C)
CD62L	DREG-5 IgG1 (C)
CD160	PAX71 IgG1 (L)
2B4	PP35 IgG1 (L)
DNAM-1	KRA236 IgG1 (L)
NKG2C	134591 IgG1 (C)
CD69	FN50 IgG1 (C)
CD25	BC96 IgG1 (C)

Appendix for Z		
mAb (Z)	clone	conjugation
Perforin	deltaG9 IgG2b (C)	Purified
Granzyme B	GB12 IgG1 (C)	APC

Appendix for W	
mAb (W)	clone
IFN-γ	B27 IgG1 (C)
TNF-α	cA2 IgG1 (C)

FLUOROCHROME
FITC/CFSE
PE
PerCp5.5
Pe-Cy7
APC
APC-Cy7/APC-H7
VioBlue
VioGreen

Strategy gate C		
Panel CD107a		
mAb	clone	conjugation
PD-1	PD1.3.1.3 IgG2b (L)	Purified
CD16	3G8 IgG1 (C)	PerCp5.5
CD56	N901 IgG1 (C)	Pe-Cy7
CD107a	H4A3 IgG1 (C)	APC-H7
CD3+CD20	BW264/56 IgG2a + LT20 IgG1 (C)	VioGreen

Strategy gate C		
Panel IFN-γ/TNF-α		
mAb	clone	conjugation
W	see "Appendix for W"	PE
CD16	3G8 IgG1 (C)	PerCp5.5
CD56	N901 IgG1 (C)	Pe-Cy7
PD-1	PD1.3.1.3 IgG2b (L)	
CD3+CD20	BW264/56 IgG2a + LT20 IgG1 (C)	VioGreen

Strategy gate C		
Panel CFSE		
mAb	clone	conjugation
CFSE		
PD-1	PD1.3.1.3 IgG2b (L)	Purified
CD16	3G8 IgG1 (C)	PerCp5.5
CD56	N901 IgG1 (C)	Pe-Cy7
KIRs	Z27+ 11PB6 + DX27 IgG1 (C)	APC
NKG2A	Z270 IgG1 (L)	Purified
CD57	TB03 IgM (C)	VioBlue
CD3+CD20	BW264/56 IgG2a + LT20 IgG1 (C)	VioGreen

C = Commercial mAbs
L= mAbs Isolated in our Laboratory

Note: In the APC-Cy7/APC-H7 channel, the fluorochrome used is always APC-Cy7, except in the CD107a degranulation experiments.
Further details on mAbs are included in the section "Monoclonal antibodies (mAbs)" in the Online Repository Material and Methods.

FIG E2. Staining panels of mAbs used in the various experiments (functional and cell-surface/intra phenotypic analysis).