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).