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Characterization of Natural Killer cells from patients affected by pleural effusions

Caratterizzazione di cellule Natural Killer in pazienti affetti da versamento pleurico

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INDEX

INDEX **1**

ABBREVIATIONS **3**

SUMMARY **5**

INTRODUCTION **7**

IMMUNE SYSTEM AND TUMOR MICROENVIRONMENT 7

IMMUNE SYSTEM CELLS: FEATURES AND POLARIZATION 12

Macrophages *12*

Neutrophils *14*

Dendritic cells *16*

Myeloid-derived suppressor cells *17*

T and B lymphocytes *18*

Innate Lymphoid Cells (ILCs) and Natural Killer cells *19*

Natural Killer cells *20*

- NK cell activation 21
- NK cell subsets 23
- NK cell receptors 25
- NK cell killing strategies 26
- NK cells and angiogenesis 28
- NK cells and cancer 29

INFLAMMATORY AND NEOPLASTIC PLEURAL EFFUSIONS 31

AIMS OF THE STUDY **34**

PATIENTS, MATERIALS AND METHODS **35**

PATIENT SELECTION 35

NK CELL PHENOTYPE ANALYSIS 35

EVALUATION OF NK CELL CYTOKINE PRODUCTION 36

MORPHOGENESIS OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS 36

EVALUATION OF NK CELL CYTOTOXICITY 37
POLARIZATION OF NK CELLS FROM HEALTHY DONORS WITH PE SUPERNATANTS 38
EVALUATION OF IN VITRO-CONDITIONED NK CELL CYTOKINE PRODUCTION 38
FLOW CYTOMETRY ANALYSES 39
STATISTICAL ANALYSES 39

RESULTS **40**

NK CELLS ISOLATED FROM MALIGNANT PLEURAL EFFUSION ARE ENRICHED IN CD56BRIGHT CD16- NK CELLS 40
NK CELLS FROM PLEURAL EFFUSIONS SHOW AN INCREASED EXPRESSION OF THE DECIDUAL NK CELL MARKER CD49A 41
PLEURAL EFFUSION NK CELLS ARE PREDOMINANTLY IMMATURE AND HIGHLY ACTIVATED 41
INHIBITORY AND ACTIVATING RECEPTOR EXPRESSION ON NK CELLS FROM PLEURAL EFFUSIONS ARE NOT DEREGULATED 42
EVALUATION OF CYTOKINE PRODUCTION: VEGF-PRODUCING NK CELLS ARE INCREASED IN PLEURAL EFFUSIONS 43
PLEURAL EFFUSION FLUIDS FUNCTIONALLY PROMOTE ANGIOGENESIS-ASSOCIATED EFFECTS ON ENDOTHELIAL CELLS 44
NK CELLS FROM TMPE CAN INDUCE CAPILLARY LIKE STRUCTURES FORMATION IN VITRO 44
THE PERCENTAGE OF CYTOTOXIC PERFORIN+ NK CELLS IS STRONGLY DIMINISHED IN PLEURAL EFFUSIONS 45
NK CELLS FROM PLEURAL EFFUSIONS SHOW LOWER CYTOTOXICITY EX VIVO 45
IL-2-TREATEMENT INCREASE CYTOTOXICITY OF PLEURAL EFFUSION-DERIVED NK CELLS BUT IT IS PARTIALLY INHIBITED BY TGFB AND, MORE STRONGLY, BY AUTOLOGOUS PLEURAL FLUID 46
NK CELLS OF HEALTHY DONORS CONDITIONED WITH PLEURAL EFFUSION SUPERNATANTS ACQUIRE PARTIAL DECIDUAL-LIKE FEATURES 48

DISCUSSION AND CONCLUSIONS **50**

LEGENDS OF TABLES AND FIGURES **56**

TABLES AND FIGURES **62**

REFERENCES **77**

APPENDIX **94**

ABBREVIATIONS

ADCC: Antibody-Dependent Cell-mediated Cytotoxicity

APC: Antigen Presenting Cells

cDC: conventional (or myeloid) Dendritic Cell

CXCL12/SDF-1: C-X-C motif chemokine ligand 12 / Stromal Derived Factor 1

dNK: decidual Natural Killer cell

FBS: Fetal Bovine Serum

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

hPB: Peripheral Blood derived from healthy donor

HUVEC: Human Umbilical Vein Endothelial Cell

ICS: Intracellular Cytokine Staining

IFNγ: Interferon gamma

IL-2: Interleukin 2

IL-8: Interleukin 8

IL-15: Interleukin 15

ILC: Innate Lymphoid Cell

iPB: Peripheral Blood derived from patient with a Pleural Effusion caused by inflammatory disease

iPE: Pleural Effusion derived from patient with a Pleural Effusion caused by inflammatory disease

K562: human erythroleukemia tumor cell line

M1: Type 1 Macrophages

M2: Type 2 Macrophages

mAb: monoclonal Antibody

M-CSF: Macrophage Colony-Stimulating Factor

MDSC: Myeloid-Derived Suppressor Cell

MHC: Major Histocompatibility Complex

MMP: metalloproteinase

NK: Natural Killer cell

NSCLC: Non-Small Cell Lung Cancer

OPN: Osteopontin

PBS: Phosphate Buffered Saline

pDC: plasmacytoid Dendritic Cell

PFA: Paraformaldehyde

PlGF: Placental Growth Factor

PMA: Phorbol Myristate Acetate

ptPB: Peripheral Blood derived from patient with a Pleural Effusion caused by primary tumor

ptPE: Pleural Effusion derived from patient with a Pleural Effusion caused by primary tumor

regDC: regulatory Dendritic Cell

TAM: Tumor-Associated Macrophages

TGFβ: Transforming Growth Factor beta

Th1: T Helper 1 Lymphocytes

Th2: T Helper 2 Lymphocytes

tmPB: Peripheral Blood derived from patient with a Pleural Effusion caused by tumor metastasis

tmPE: Pleural Effusion derived from patient with a Pleural Effusion caused by tumor metastasis

TNFα: Tumor Necrosis Factor alpha

Treg: Regulatory T Cell

TUMIC: Tumor Microenvironment

VEGF: Vascular Endothelial Growth Factor

SUMMARY

Natural Killer (NK) cells are lymphoid cells involved in tumor recognition and eradication. NK cell activity is impaired in cancer patients and in non-small cell lung cancer it has been shown that they acquire pro-angiogenic phenotype and functions similar to those of NK cells in the decidua. In this work, we characterized NK cells derived from inflammatory or malignant pleural effusions.

We collected peripheral blood samples from healthy donors (hPB) and from patients with inflammatory (iPB) or malignant pleural effusions [from primary tumors (iPB) or tumor metastasis (tmPB) of different origins], in addition to inflammatory (iPE) and malignant pleural effusion fluids (ptPE and tmPE). We performed *ex vivo* FACS analysis of phenotype and cytokine production of PB and PE-derived NK cells, and analyzed cytotoxic NK cell function by using a CD107a NK cell degranulation assay against K562 target cell line. In addition, we performed a 3-day culture with IL-2, IL-2 and TGFβ and with IL-2 in a conditioned media containing 33% of pleural fluid supernatant, to evaluate cytotoxicity of NK cells after treatment. We also isolated NK cells from buffy coats of healthy donors and cultured them for 7 days with IL-15 and with IL-15 with pleural fluid supernatant to evaluate polarization toward a pro-angiogenic phenotype caused by pleural effusion soluble factors.

We found significantly increased levels of CD56bright CD16- NK cell in iPE (35%), ptPE (40%) and tmPE (60%) if compared with the PB of all samples analyzed (5%). PE-NK cells display an increased expression of CD49a decidual NK cell surface marker, are poorly mature (low expression of CD57) and yet activated (high expression CD69) and exhibit a deregulated expression of activating and inhibitory receptors.

PE-NK cells display a higher amount of intracellular VEGF if compared to healthy and autologous PB-NK cells, especially those found in tmPE; supernatants resulting from NK

cells found in pleural effusions derived from patients with metastatic tumor (tmPE) induce formation of capillary-like structures *in vitro*.

All patient's NK cells analyzed showed a lower cytotoxicity *ex vivo* than hPB-NK cells.

After 3-day culture with IL-2, NK cell cytotoxic function increased. Moreover, addition of TGFβ induced partial inhibition of cytotoxicity, whereas addition of PE supernatants resulted in total inhibition of cytotoxic function.

NK cells Isolated from buffy coats from healthy donors, after 7-day culture with IL-15 + PE, increased CD56bright CD16- NK cell subset and VEGF production. Moreover, PE supernatants could induce a decreased production of IFNγ.

Our data suggest a predominant role of tumor microenvironment in the NK cell polarization towards a pro-angiogenic and pro-tumor state.

INTRODUCTION

IMMUNE SYSTEM AND TUMOR MICROENVIRONMENT

The immune system can be considered a set of lymphoid organs, different cell types, humoral factors and cytokines. The ability to discriminate foreign pathogens and, at the same time, the tolerance to self-antigens is crucial for the achievement of homeostasis. The first line of host defenses consists in mechanical and chemical-physical barriers, after which participate the components of innate immunity that includes phagocytic cells (macrophages, neutrophils and dendritic cells), different types of immune and non-immune cells, which release inflammatory mediators, and Natural Killer cells. The main protagonists of the adaptive immunity are T lymphocytes, responsible for cellular immunity, and B cells, involved in humoral immunity. For a full and effective response, innate and adaptive mechanisms are interconnected into a sophisticated process of communication despite differences in the specificity and timing of activation [1, 2].
In the 19th century initial studies from most types of cancers showed the presence of inflammatory leukocytes in growing cancers and it was hypothesized that a relationship between chronic inflammation and cancer existed. Considering most types of cancer, infiltrating inflammatory cells may be different in terms of number and type of cells, however, this presence has long been interpreted as an attempt to interfere with tumor progression, termed "immune surveillance" [3]. It is demonstrated that there is a connection between a good patient prognosis and the presence of immune cell infiltrates [4, 5, 6, 7]. However, cellular and humoral responses that are generated in tissues because of infection by foreign pathogens, are stronger than those mediated by the tumor. Effectively, most tumor-associated antigens are considered "self" and non-dangerous for the host [8].

Recent studies have explained the mechanisms that connect inflammation and cancer: microbial infections, viral infections and autoimmune disease are considered promoter of chronic inflammation associated with cancer development [9]. Examples of pathologies implicated in this process are gastric cancer and gastric mucosal lymphoma caused by Helicobacter Pylori, hepatocellular carcinoma caused by hepatitis B or C virus and colon cancer correlated with inflammatory bowel disease [10]. Supporting the theory of the pro-tumor role of chronic inflammation there is the evidence that the treatment with nonsteroidal anti-inflammatory agents, such as cycloxygenase-2 (COX-2) inhibitors, reduces the risk of developing colon and breast cancer and the related mortality [11].

It was documented that alterations in different classes of oncogenes and tumor-suppressor genes lead to activate signaling pathways involved in inflammation. Examples are the tyrosine kinase RET, RAS, MYC and PTEN [12, 13, 14, 15].

Cancer-related inflammation can also activate transcription factors which are the key inducers of inflammatory mediators and contributes to tumor development promoting genomic instability, alteration in epigenetic events, enhancing proliferation and resistance to apoptosis of initiated cells, inducing tumor angiogenesis and tissue remodeling with consequent promotion of tumor cells invasion and metastasis [10].

Over the past three decades it has been possible to understand the function of immune cells in promoting tumor progression [16, 17]. Individuals subject to develop chronic inflammatory diseases show an increased risk of developing cancer [18]. Therefore, it was necessary to redefine the classical definition of carcinomas, extending it to the microenvironment in which most cellular components display immunosuppressive properties. Tumor cells not only try to escape from host defense mechanisms, but also acquire benefit from these conditions; cancer is also able to reprogram cells to create a favorable cell microenvironment to improve tumor progression [19].

The tumor microenvironment (TUMIC) generally includes, in addition to proliferating cells, a wide range of inflammatory and immune cells as well as stromal cells and endothelial cells able of develop tumor-promoting functions during all carcinogenesis

stages [20]. The main TUMIC features are hypoxia, deregulated pH (approximately 6,7-6,9 at the extracellular level and 7,4 in tumor cells), different metabolic state and a different vascular architecture that is usually fenestrated [21, 22].

It is important to emphasize that while normal blood vessels are lined by a single layer of interconnected endothelial cells, in the contest of TUMIC, endothelial cells are part of fenestrated vessels highly permeable for soluble blood components [23]. Tumor vessels are chaotically branched and display irregular diameters because of the compression of their walls by tumor and stromal cells [24, 25]. If the tumor growth exceeds over 1 mm, it requires a neo-vascularization to provide the right amount of oxygen and nutrients for proliferating cancer cells. However, during tumor expansion, the distances that must be covered by the vasculature increase and a localized hypoxia occurs [26, 27, 28]. A hypoxic environment is created very quickly during tumor development, resulting, in tumor cell, in an expression of genes that respond to hypoxia [29]; hypoxic stress plays a key role in regulating angiogenesis, and it is required for tumor invasive growth and metastasis formation [30]. This extremely heterogeneous realities, such as the tumor microenvironment, is enriched in immune cells-derived cytokines, chemokines and pro-angiogenic molecules, including: tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF), and Interleukin 1 (IL-1) and 6 (IL-6) [18]. The production of VEGF is a mechanism by which tumor infiltrating leukocytes increase angiogenesis and tumor development [31, 32]. The connection between angiogenesis induced by hypoxia and immune system tolerance has been studied and clarified [33, 34, 35]. The association between angiogenesis and immunosuppression may be justified in part by considering the immunosuppressive activity of angiogenic factors like VEGF, a cytokine secreted by most cancers [36, 37]. Hypoxic zones in the tumor can attract various immune cells in which the expression of hypoxia-induced transcription factors (HIF) is associated with the acquisition of a pro-angiogenic and immunosuppressive phenotype, such as Treg cells or myeloid-derived suppressor cells [38].

Therefore, the immune cells may directly or indirectly destroy cancer cells, but on the other hand can promote tumor growth and progression [1, 39]. This pro-tumor polarization was widely described in macrophages but it can also be extended to other innate immunity cellular components (Figure I) [40].

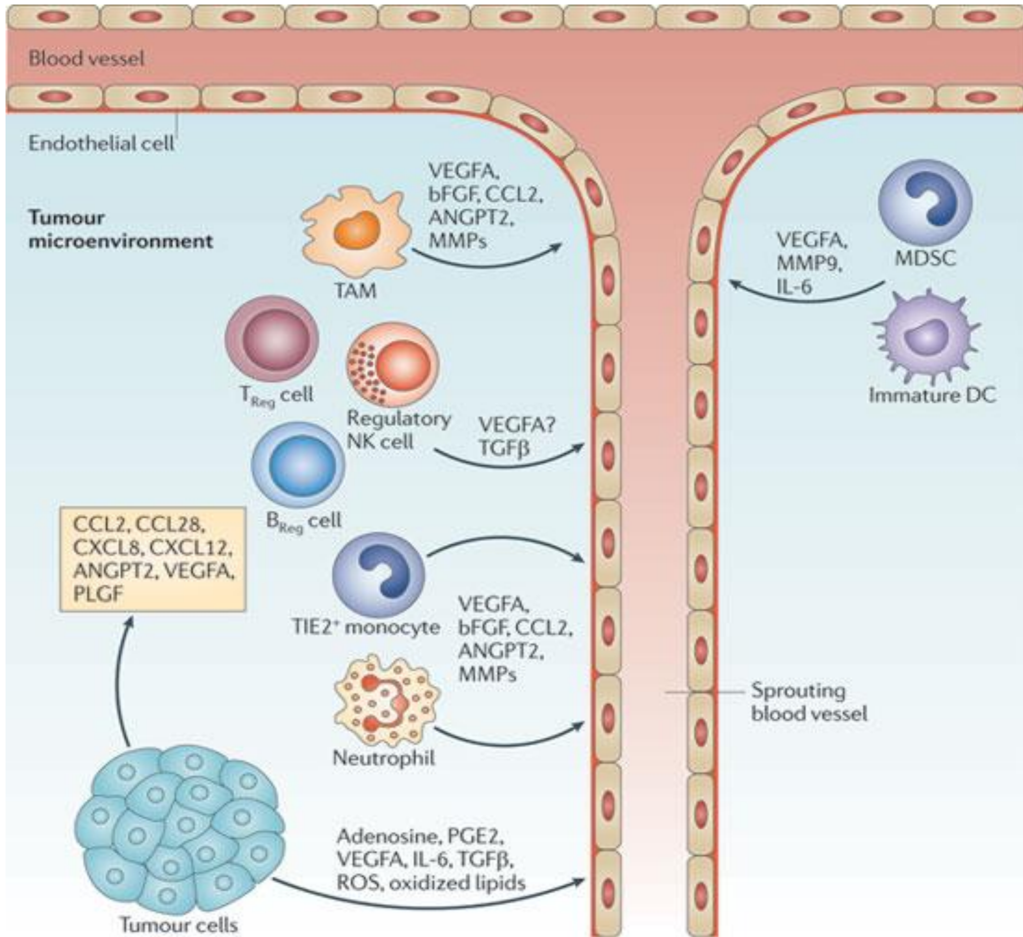


Figure I. Within the tumor microenvironment, multiple cell types with established roles in immunosuppression have been shown to promote angiogenesis, through the production of various growth factors. Tumor cells, either in the steady state or in response to hypoxia, secrete soluble factors that recruit immunosuppressive cells to the tumor site. Recruited immunosuppressive cells include regulatory NK cells. The immunosuppressive cells secrete factors (such as VEGFA) that directly promote endothelial cell proliferation and migration, and/or induce the production of matrix metalloproteinases that act on the extracellular matrix, allowing for the development of new blood vessels. Pro-angiogenic growth factors derived from these cells probably promote angiogenesis in an additive or synergistic manner, together with tumor-derived VEGFA, transforming growth factor-β (TGFβ), adenosine, prostaglandin E2 (PGE2), interleukin-6 (IL-6), reactive oxygen species (ROS) and oxidized lipids. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology. Motz GT, Coukos G. The parallel lives of angiogenesis and immunosuppression: cancer and other tales. Nat Rev Immunol. 2011 Sep 23;11(10):702-11, copyright 2011. [41].

IMMUNE SYSTEM CELLS: FEATURES AND POLARIZATION

Macrophages

Macrophages are immune cells recruited after tissue damage and inflammation, and they are professional phagocytes able to recognize and eliminate parasites and microorganisms. In fact, during bacterial infection, macrophages participate in the acute inflammatory response to eliminate invading pathogens; subsequently can remove tissue waste [42]. Macrophages are versatile cells with pleiotropic functions, even opposite: they can be immune-stimulatory and pro-inflammatory, or immunosuppressive and anti-inflammatory [42, 43]. Microenvironment stimuli can induce in macrophages different states of polarization [18].

Macrophages called M1, or classically activated, are stimulated by bacterial products (such as lipopolysaccharide, LPS) and cytokines produced by T helper 1 (Th1) lymphocytes. They also produce pro-inflammatory cytokines, they can mediate pathogens elimination and to kill cancer cells. Generally, they have an IL-23high, IL-10low, IL-12high phenotype and are also able to produce reactive oxygen species and nitric oxide [42, 44]. On the other hand, M2 macrophages are polarized in T helper 2 (Th2) lymphocyte-rich microenvironments, i.e. in the presence of cytokines such as interleukin 4 (IL-4) and 13 (IL-13) [43, 45]. M2 macrophages also promote tissue remodeling and angiogenesis [9, 46, 47], and suppress adaptive immune responses. Both M1 and M2 macrophages could be recruited in cancers microenvironment such as monocytes because of chemokines produced by tumor cells and stromal cells, but they can also migrate from adjacent tissues [9].

Tumor-associated macrophages (TAMs) are derived from peripheral blood monocytes that are recruited in tumor mass thanks to growth factors like VEGF, chemokines including CCL2 and molecules involved in CXCL12/SDF-1/CXCR4 signaling pathway [9, 48, 49]. In the tumor site, monocytes differentiate into macrophages primarily due to

the presence of the macrophage colony-stimulating factor (M-CSF) produced by neoplastic cells [50, 51].
TAMs show pro-tumor features: they can induce neo-angiogenesis, to promote secretion of soluble factors that support cancer cell resistance to apoptotic stimuli and to stimulate malignant cells proliferation and invasion. Tumor-associated macrophages have also been associated with direct suppression of adaptive immunity by producing interleukin 10 (IL-10) and TGFβ that suppress Th1 lymphocytes; producing chemokines such as CCL17, CCL18 and CCL22 which are attractive for Th2-type cells that lack of cytotoxic activity and for T regulatory cells (Treg) [52]. Under hypoxic conditions, that is a common characteristic of solid tumors *in vivo*, proangiogenic factors are produced by TAMs, including: VEGF, epidermal growth factor (EGF), some members of the family of fibroblast growth factors (FGF) which are able to stimulate the recruitment and the migration of endothelial cells (EC), platelet-derived growth factor (PDGF), also implicated in the recruitment of pericytes, the CXC angiogenic chemokines, (CXCL8/IL-8 and CXCL12/SDF-1), thymidine phosphorylase and TGFβ [9, 49]. Because of hypoxia there is an up-regulation of the pro-tumor cytokine TNFα, [53], of the immunosuppressive cytokine IL-10 and of arginase [54]. The cytokines produced by TAMs can also act indirectly on angiogenesis by autocrine stimulation [55]. The molecular profile of TAMs is characterized by a defective NF-kB and by an inability to up-regulate interleukin 12 (IL-12) and other inflammatory cytokines following inflammation [56]. In addition to producing VEGF, tumor-associated macrophages are also influenced by this angiogenic factor: they produce the receptor VEGFR1 [57] and respond to it by migrating and modulating their biological activity [58, 59]. Cancer cells take advantage of the degradation of the extracellular matrix by macrophages and they can perform local invasion, penetrate the blood vessels and spread and give rise to metastasis [60]. Basement membrane and extracellular matrix components degradation is the result of the release of proteases including different matrix metalloproteinases (MMPs 1, 2, 3, 9, and 12), as well as plasmin and urokinase. These factors also induce the destabilization of the vascular system as well as the migration

and proliferation of endothelial cells [9, 49, 61]. Interestingly, it has been shown that a subset of TAMs may also counteract tumor growth using a nonspecific cytotoxicity activity based on phagocytosis and on cancer cell lysis mediated by factors such as IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [62]. A crucial target for anti-angiogenic therapy could be represented by TEMs (Tie2 expressing macrophages), a subset of TAMs associated with the construction of the pre-metastatic niche, whose deletion inhibits angiogenesis and tumor growth [63, 64, 65].

From circulating monocytes, derived from the bone marrow, originate tissue-resident and inflammatory macrophages [66]. In the same way, TAMs are derived from circulating monocytes or tissue-resident macrophages [67]. Macrophage mobilization into tumor tissues is regulated by multiple microenvironmental signals such as cytokines, chemokines, extracellular matrix components, and hypoxia. Hypoxic areas release higher amount of chemoattractants such as VEGF-A, endothelin and EMAPII that enhance macrophage migration to these hypoxic sites. Hypoxia also imprisons macrophages by decreasing their mobility through the upregulation of MKP-1 enzymes; this terminates the macrophage response to chemoattractants outside the hypoxic areas [68]. There is evidence that both tissue-resident and recruited macrophages may cohabit in tumors and it was demonstrated that TAMs in a murine mammary tumor model are phenotypically and functionally distinct from mammary tissue-resident macrophages and that recruited macrophages may differentiate and form most TAMs [69]

Neutrophils

In the context of circulating white blood cells, the polymorphonuclear leukocytes neutrophils are the most abundant cell population. Foreign microorganisms, captured through phagocytosis, are eliminated by lytic enzymes and anti-microbial molecules contained in their cytoplasmic granules. Neutrophils are involved in the early stages of inflammatory response and they are rapidly recruited to the site of infection. In parallel

to features described for macrophages, neutrophils are sensitive to microenvironment stimuli and modify their behavior, often in diametrically opposed directions, in response to paracrine and endocrine received signals. Different subsets of neutrophils with distinct profiles of cytokine production and surface markers have been described [70, 71].

The neutrophil mechanisms of action consist in degranulation leading the release of lytic enzymes and the production of ROS with antimicrobial potential (O_2-, H_2O_2, HOCl) [72]. Neutrophils are also able to secrete various cytokines such as TNFα, IL-1β, IL-1Rα, IL-12 and VEGF and chemokines such as CXCL1, CXCL8/IL-8, CXCL9, CXCL10, CCL3 and CCL4 [73, 74] directly involved in tissues remodeling and angiogenesis. For example, CXCL1/macrophage inflammatory protein-2 (MIP-2) leads to recruitment of neutrophils that, in turn, release biologically active VEGF-A, resulting in angiogenesis *in vivo* [75]. However, neutrophils are also able of produce a wide range of anti-cancer and anti-angiogenic molecules. Upon stimulation with pro-inflammatory stimuli, neutrophils have enzymatic activities that, *in vitro*, generate biologically active angiostatin-like fragments. Because angiostatin is a potent inhibitor of angiogenesis, tumor growth, and metastasis, the data suggest that activated neutrophils not only act as potent effectors of inflammation, but might also play a critical role in the inhibition of angiogenesis in inflammatory diseases and tumors, by generation of a potent anti-angiogenic molecule [76].

The pro-angiogenic and anti-angiogenic activities of neutrophils, and their role in tissue destruction or reconstruction, suggest that there are different subsets of neutrophils, characterized by different activities: anti-tumorigenic N1 neutrophils were found to be associated with direct tumor cell killing as well as activation of CD8+ T cells and pro-tumorigenic N2 neutrophils were also detected [77].

Tumor-associated neutrophils (TANs) were found in a variety of tumors (such as colon adenocarcinoma, melanoma and gastric carcinoma) suggesting a potential role in tumor progression and angiogenesis (78). In fact, these cells can promote, for example, IL-8-mediated angiogenesis (79). Therefore, TANs can release proteases to degrade and

remodel the extra cellular matrix and TNFα to induce neutrophil degranulation, in addition to VEGF, CXCL8/IL-8 and CXCL1 release [80, 81].

Dendritic cells

The third phagocytic cells of the immune system are dendritic cells (DCs) that are present constitutively in the epithelia of most tissues, as well as in blood and in lymphoid organs. DCs, on the basis of location and morphology, play a critical role in the identification and the stimulation of both innate and acquired immune effector responses that lead to elimination of invading pathogens. Dendritic cells can act as "sentinels" that, once recognized microbial antigens, are able to migrate in secondary lymphoid organs, to process the foreign antigens and to present peptide epitopes to naïve T cells, acting as potent antigen presenting cells (APCs) [82]. APC function and maturation of dendritic cells are promoted by some angiostatic molecules, including the thrombospondin-1 [83] and CXCL4 [84] and CXCL14 chemokines [85]. Moreover, dendritic cells are located at the interface between innate and acquired immunity and for this reason they can establish a crosstalk with NK cells [86] and with neutrophils [87].

Dendritic cell population includes two main cell types: conventional or myeloid dendritic cells (cDCs) and the plasmacytoid DCs (pDCs) [88]. The cDC subset secretes primarily IL-12, while the pDC releases the anti-angiogenic cytokine interferon-alpha (IFNα) [89, 90]; in addition, mature cDCs are able to inhibit angiogenesis releasing angiostatic chemokines (CXCL9, CXCL10 and CCL21) [91].

A third subset of dendritic cells, regulatory dendritic cells (regDCs), is characterized by immune-suppressive features. regDC polarization is promoted by soluble factors such as VEGF, IL-6 and TGFβ, that can contribute to the reduction of mature DCs, to the expansion and accumulation of immature and tolerant DCs and to the possible DCs polarization into a pro-tumor phenotype promoting Th2 or Treg responses [92, 93]. DCs can release two important pro-inflammatory factors, TNFα [94, 95] and osteopontin

[96, 97], which can also function as angiogenic factors. Other cytokines released from DCs and that influence angiogenesis are IL-6 and TGFβ [98].

Different soluble tumor-derived factors, such as VEGF (99), adenosine [100], prostaglandin E2 (PGE2) [101] and TGFβ [102], are crucial for activation, migration of endothelial cell, and for inhibition of DC function that involves suppression of T cells and development of Treg cells. The VEGF-dependent DC inhibition is mediated by VEGFR1 receptor expressed on immature DC [103, 104]. Dendritic cells are also able to secrete different pro-angiogenic chemokines, such as CXCL1, CXCL2, CXCL3, CXCL5, CXCL8/IL-8 and CCL2 [105, 106].

Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) include immature macrophages or granulocytes and myeloid dendritic cell progenitors with high plasticity in their immune-suppressive function. However, these myeloid progenitors may differentiate *in vitro* into mature macrophages, granulocytes or even dendritic cells depending on available cytokines [107]. Two main subsets are described: granulocytic MDSCs and monocytic MDSCs [108, 109]. MDSCs can produce IL-10 and TGFβ, cytokines involved in the generation of tolerogenic DCs (110) and Treg cells [111, 112].

Recruitment and expansion of MDSCs may result from high levels of GM-CSF and IL-1β cytokines in case of chronic inflammation and in some cancer types the link between inflammation and cancer progression has been established [113, 114]. The involvement of myeloid-derived suppressor cells in inhibiting antitumor immunity is also reinforced by the ability to impair Natural Killer cells activity [115, 116], and to down-modulate recirculation of both naïve CD4+ and CD8+ T cells [117]. MDSC's function is also involved in the angiogenesis process through the release of soluble factors such as MMP9 and VEGF, and thanks to the possibility of direct differentiation of these cells into endothelial cells as shown by experimental data obtained from mouse models [61, 118].

T and B lymphocytes

The thymus-derived T-cells are divided into two main subpopulations based on the expression of CD4 or CD8 surface protein. In blood and secondary lymphoid organs, 60-70% of T cells are CD4+ and 30-40% are CD8+ [2]. CD4+ T lymphocytes, also named T helper cells, can activate both cellular and humoral immune responses and they can recognize peptides presented by MHC Class II molecules. Depending on functions and cytokines produced, Th cells can be divided into three major subgroups [119]. The IL-12 cytokine produced by macrophages or dendritic cells promotes the differentiation of Th1 lymphocytes; these cells express T-box transcription factor and produce interleukin 2 (IL-2) and interferon gamma (IFNγ). The IL-4 cytokine produced principally by NK-T and mast cells, induces differentiation of Th2-lymphocytes, expressing the GATA3 transcription factor and producing IL-4, IL-5, IL-9, IL-13 and GM-CSF. Finally, TGFβ and IL-6 allow differentiation of Th17 cells that produce IL-6 and IL-17 [2].
CD8+ T cells are cytotoxic cells (CTLs) that lyse cells through recognition of foreign or mutated antigens bound to MHC Class I molecules, as often happens for intracellular pathogens and for tumor cells.
Instead Treg cells are characterized by CD25 and CD4 surface molecule co-expression and by nuclear transcription factor Foxp3 expression that is essential to their development. The regulatory activity of these cells is mainly due to the secretion of immunomodulatory cytokines, such as IL-10 and TGFβ [120]. Under physiological conditions Treg cells have a beneficial role in preventing autoimmunity [121]. However, in the presence of a tumor, they can expand and migrate into tumor site, to down-regulate the proliferation of effector T cells and to suppress anti-cancer strategy of T helper cells and cytotoxic T cells [122, 123]. CCL22 and CCL28, which produced within the TUMIC, allows the selective recruitment of Treg cells who start to express in a constitutive manner the neuropilin-1 co-receptor [124] and are involved in the process of VEGF production in the tumor site (125). Within the tumor microenvironment, CD8+

T cells are conditioned to acquire a regulatory T cells features with CD8+ CD28- phenotype [126, 127].

A subpopulation of T-lymphocytes consists in NKT cells, that are a relatively newly recognized member of the immune community, with profound effects on the rest of the immune system despite their small numbers. They are true T cells with a T cell receptor (TCR), but unlike conventional T cells that detect peptide antigens presented by conventional major histocompatibility (MHC) molecules, NKT cells recognize lipid antigens presented by CD1d, a non-classical MHC molecule. NKT cells are CD4- CD8- and could have immune-regulatory function through releasing a large amount of cytokines, including IL-4, IFNγ, and TNF [128, 129].

B cells constitute about 15% of peripheral blood leukocytes and can differentiate into plasma cells and, once activated, they produce antibodies against pathogens [130]. The primary immune role of B cells is to produce antibodies, but they can also influence T cell function via antigen presentation and, in some contexts, immune regulation. In many human cancers, the presence of tumor-infiltrating B cells and tumor-reactive antibodies correlates with extended patient survival [131, 132, 133, 134]. On the other hand, T cell responses can be adversely affected by B cell production of immunoregulatory cytokines [135, 136, 137]. The isotype and concentration of tumor-reactive antibodies may also influence tumor progression. Recruitment of B cells into tumors may directly reflect the subtype and strength of the anti-tumor T cell response. As the response becomes chronic, B cells may attenuate T cell responses to decrease host damage, similar to their described role in chronic infection and autoimmunity [138].

Innate Lymphoid Cells (ILCs) and Natural Killer cells

Innate lymphoid cells (ILCs) represent a family of different innate lymphocytes that, different from T and B lymphocytes, lack recombinant activating genes (RAG-1 or RAG-2) and thus do not express rearranged antigen-specific receptors. ILCs have been

grouped in three major subsets based on their phenotypic and functional features as well as of their dependency on given transcription factors. Briefly, ILC-1 are dependent on T-bet transcription factor and produce interferon IFNγ. Group 2 ILCs (ILC-2) express GATA-3 transcription factor and produce IL-4, IL-5 and IL-13 (Type 2) cytokines, while group 3 ILCs (ILC-3) express RORγt transcription factor and produce IL-17 and IL-22. Natural Killer cells represent the prototypical member of the ILC-1 family [139, 140].

Natural Killer cells

Natural Killer cells are large granular lymphocytes with the ability to lyse various types of cancer cells and virus-infected cells in the absence of a previous activation [141]. NK cells constitute the 5-15% of circulating lymphocytes [142, 143], however this proportion may vary with age of subject [144, 145]. In blood, the turnover of human Natural Killer cells is about two weeks [146] in accordance with data found in mouse models [147]. However, they are also found in tissues such as the spleen, lungs, kidney and liver, as well as in uterus and in slighter amounts in lymph nodes [148, 149, 150, 151].

Upon priming by various soluble factors (for example, IL-15, type I IFN, IL-12, IL-18), NK cells boost the maturation and activation of DCs, macrophages and T cells, through a combination of cell surface receptors and cytokines. Conversely, NK cells can also kill immature DCs, activated CD4+ T cells and hyperactivated macrophages. These NK cell regulatory functions are kept in check by the recognition of constitutively expressed self-molecules (for example, MHC class Ia and MHC class Ib molecules) by means of inhibitory receptors (for example, the inhibitory KIR and the CD94-NKG2A complex), as is represented in Figure II [152].

Natural Killer cells are involved in the crosstalk of DCs; certain NK cell subsets could eliminate immature DCs, while others promote DC maturation, which in turn could induce further activation of NK cells (86); the functions of these innate immune cells are also conditioned by adaptive immunity.

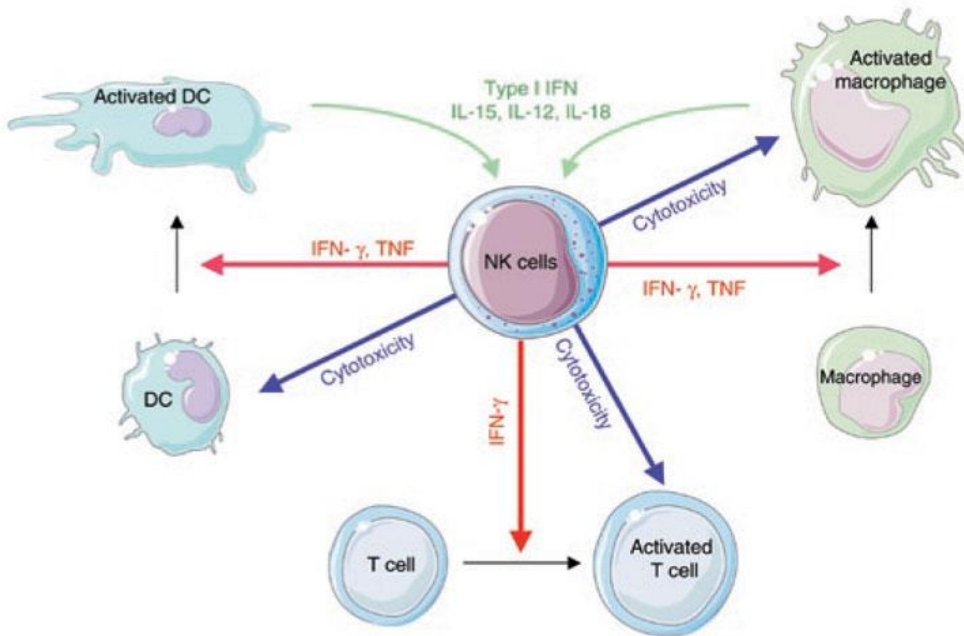


Figure II. Regulation of immune responses by NK cells. Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of Natural Killer cells. Nat Immunol. 2008 May; 9(5):503-10, copyright 2008. [152].

— NK cell activation

Concerning NK cell activation, the main functional hypothesis is related to the "missing self" mechanism (Figure IIIb), since NK cells can detect and lyse cells lacking expression of MHC-I molecules [153]. In 1990, several studies showed the presence of activator and inhibitor surface receptors expressed by NK cells [154], identifying a new recognition model called "induced-self" (Figure IIIc) [155], that complements the previous hypothesis of "missing-self". NK cells are also able to exert Antibody-Dependent Cell-mediated Cytotoxicity (ADCC), a mechanism of cell-mediated immune defense, whereby an effector cell of the immune system actively lyses a target cell, whose membrane-surface antigens have been bound by specific antibodies (Figure IIId) [156].

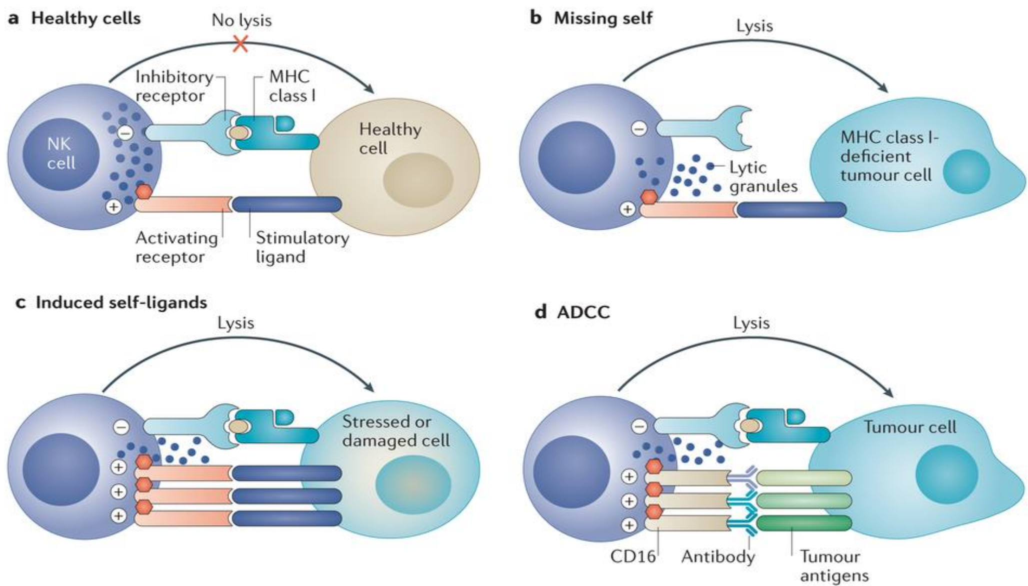


Figure III. a. Balance of signals delivered by activating and inhibitory receptors regulates the recognition of healthy cells by NK cells. **b.** Tumor cells that down-regulate major histocompatibility complex (MHC) class I molecules are detected as 'missing self' and are lysed by NK cells. **c.** Tumor cells can overexpress induced stress ligands recognized by activating NK cell receptors, which override the inhibitory signals and elicit target cell lysis. **d.** Tumor antigen-specific antibodies bind to CD16 and elicit antibody-dependent NK cell-mediated cytotoxicity. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer. Morvan MG, Lanier LL. NK cells and cancer: you can teach innate cells new tricks. Nat Rev Cancer. 2016 Jan;16(1):7-19, copyright 2016. [156].

The NK cell ability to recognize their target is the result of a complex balance between inhibitory and activatory signals and require not only the lack of MHC-I expression on target cells membrane but also the expression of ligands able to trigger activating receptors [157]. The first checkpoint is the lack of MHC-I molecules expression [153, 158]: their down-regulation is observed during cell transformation [159] or viral infection and this phenomenon prevents the binding of NK cells inhibitory receptors to target. Target cell lysis occurs only when the activating signals exceed inhibitory signals [160].

Thus, NK cells can recognize and destroy a wide range of abnormal cells (including cancer cells and virus-infected cells, cells bound by antibodies and allogeneic cells, as well as stressed cells), without damaging healthy cells and normal self-cells [161].

— NK cell subsets

There are several distinct subsets of human Natural Killer cells classified by CD56 surface molecule expression: the main peripheral blood subset consists of the NK cells with low density of CD56 expression (CD56dim) and with high levels of the CD16 (CD16+, the Fc-γ receptor) and represent the 90-95% of NK cells in the blood. These cells kill their targets by releasing cytolytic granules containing perforin and granzyme, and can secrete only precociously high levels of cytokines [162]. *In vitro* interaction with cancer cells results in the production of IFNγ by these innate lymphoid cells [163]. The remaining 5-10% of peripheral blood NK cells are poorly cytotoxic, display lower amounts of perforin [149], but can produce large amounts of cytokines, including IFNγ, TNFα, and GM-CSF, and they show a higher surface density of CD56 (CD56bright) and reduced or negative expression of CD16 (CD16low/-). *In vitro* exposure to specific cytokines, such as IL-2, IL-12 and/or IL-15, induce these cells to acquire a cytotoxic CD56dim CD16+ phenotype with strong killing capacity [164, 165]. CD56high CD16− NK cells are thought to be immature precursors of CD56low CD16+ mature NK cells [142], although some studies have suggested that the two are separate lineages [143].
At the level of the decidua [166] (the hormone-responsive glandular layer of endometrium that sloughs off at each menstrual flow (decidua menstrualis) or at the termination of pregnancy) a third subset of NK cells was found, and called decidual Natural Killer cells (dNK). This subpopulation is characterized by a CD56superbright CD16- phenotype and can release significant amounts of pro-angiogenic factors, especially VEGF, placental growth factor (PlGF) and interleukin 8 (IL-8). They are necessary for the formation of the spiral arteries during endometrial differentiation [166, 167]. The low cytolytic activity of dNK cell seems to be involved in the

implantation of the embryo to avoid non-self-rejection. Compared to the two subtypes found in peripheral blood, dNK cells display specific surface markers, in particular CD9 and CD49a [168].

— NK cell receptors

Among the most important activating receptors there are NKp30, NKp44 and NKp46, normally named "natural cytotoxicity receptors" (NCRs) [169], member of the C-type lectins family NKG2D, 2B4, NTBA, CD69, NKp80 and DNAM-1, as it is shown in Figure IV, together with the most representative human NK cell receptors.

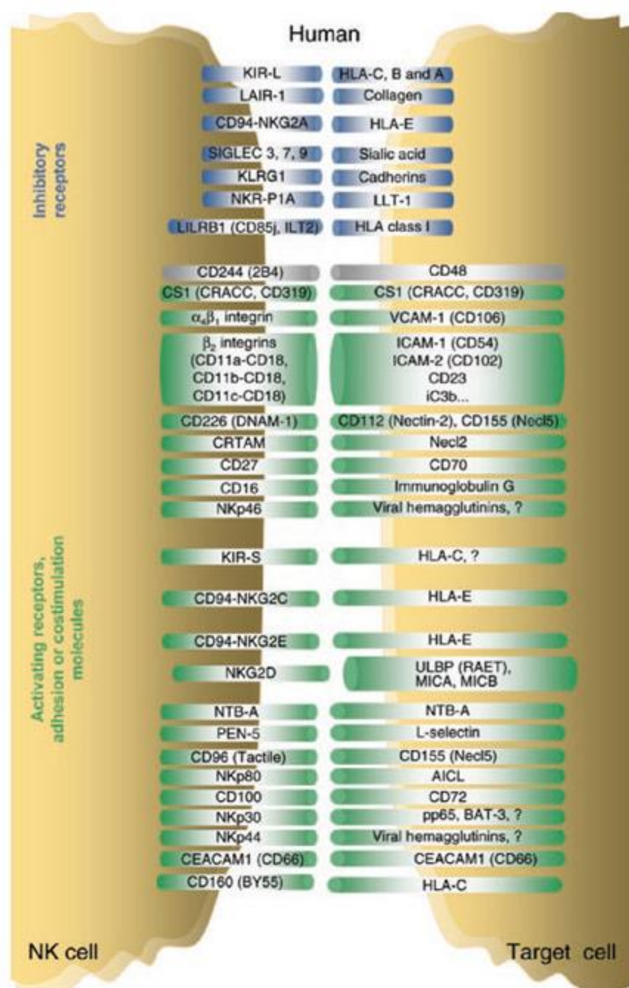


Figure IV. NK cell activation programs result from the integration of multiple activating and inhibitory signals. Inhibitory receptors are in blue; 2B4, which can act as an activating or an inhibitory molecule, is in grey; other receptors are in green. Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of Natural Killer cells. Nat Immunol. 2008 May; 9(5):503-10, copyright 2008. [152].

Another important molecule is the CD16 receptor (Fc-γ-RIIIA) that mediates recognition of the Fc portion of IgG1 and IgG3 antibodies allowing the identification and the attack of opsonized target cells through antibody-dependent cell-mediated cytotoxicity mechanism (ADCC). Many activating receptors are characterized by a common structural motif in the cytoplasm, called ITAM (Immunoreceptor Tyrosine-based Activation Motif) [170].

Specific inhibitory receptors for MHC Class I molecules include KIRs (Killer immunoglobulin-like receptors) in humans, the lectin-like dimers Ly49 in mice, and the lectin-like heterodimers CD94-NKG2A in both species [171, 172]. A conserved peculiarity in these inhibitory receptors is the presence of one or two intra-cytoplasmic inhibitors domains called ITIMs (Immunoreceptor Tyrosine-based Inhibition Motifs) that promote the signal transduction pathway maintained both in human and mouse [173]. Interacting with MHC Class I molecules, constitutively expressed by most healthy cells in conditions of "dynamic equilibrium" but that might be lost because of cellular stress, viral infection or neoplastic transformation, the inhibitory receptors enable NK cells to be educated to recognize the "missing-self" and, at the same time, tolerate self-antigens [174]. However, there are also other inhibitory receptors, including p75/AIRM1 and IRP60, which can recognize molecules outside the family of MHC Class I molecules [175].

— NK cell killing strategies

The target elimination by NK cells can be performed through three main strategies. The first mechanism, shared by T cytotoxic lymphocytes, is faster and more effective to kill cells and consists in releasing perforin and granzymes (including the granzyme B) that are stored in secretory granules [176]. This strategy is implemented either when activatory signals exceed inhibitory signals, or because of ADCC. When Natural Killer cells are activated, the granule exocytosis leads to the release of these molecules against target cells; perforin perforates the target cell membrane forming

homopolymers and creating a hole that facilitates the entry of granzyme in the cell cytoplasm. These serine-proteases act on different target molecules, proteins of the family of caspases, that induce programmed cell death (apoptosis). This leads to chromatin condensation, membrane blebbing and nuclear DNA fragmentation [177, 178]. The whole process is strictly regulated: there are the formation of an "immunological synapse", at the point of contact between Natural Killer cell and the target cell, and there is a rearrangement of the actin cytoskeleton; then NK cell microtubular organizing center and secretory lysosomes polarize toward the immunological synapse. At this point the secretory lysosomes are anchored to the plasma membrane, and then merge with it and release their cytotoxic molecules [179]. Alternatively, there is a slower mechanism that requires the expression of tumor necrosis factor ligand superfamily (FasL/TNF) on the NK cell surface and a Fas death receptor on target cell [180]. An intracellular region, called death domain, characterizes these receptors that recruit an adaptive protein through homophilic interactions, which is required for cytotoxic signal transduction [181]. This initiates the start of enzymatic caspase cascade leading to apoptosis.

Finally, Natural Killer cells can produce IFNγ a cytokine that inhibits cancer cell proliferation *in vitro* and tumor growth *in vivo* by inducing the anti-angiogenic CXCL10 chemokine [182]. IFNγ is also able to potentiate the NK cells cytotoxicity inducing an over-expression of adhesion molecules or an increase of cancer cells susceptibility to cytotoxicity mediated by the release of granules or by death receptor [183]. In addition, the IFNγ produced by Natural Killer cells plays an important role in the production of IL-12 by dendritic cells [184]. In this way, NK cells contribute indirectly at the tumor control starting and maintaining a T cells-mediated effective antitumor response through a crosstalk with dendritic cells [185].

— NK cells and angiogenesis

Physiological angiogenesis is a complex process finely controlled by numerous pro-angiogenic cytokines as well as inhibitory factors. Therefore, to induce angiogenesis, the balance between pro and anti-angiogenic factors near tumor should lean in favor of angiogenic stimuli, either through down-regulation of angiogenesis inhibitors or up-regulation of pro-angiogenic cytokines [186].

The most important inducer of angiogenesis is vascular endothelial growth factor that is part of a gene family that contains 5 related genes (VEGF-A also known as VEGF, VEGF-B, VEGF-C, VEGF-D and PlGF) that are regulators of angiogenesis, lympho-angiogenesis or both. Probably this is the most important inducer of angiogenesis [187]. Specifically, hypoxia regulates the production of VEGF via transcription factor HIF-1α, which also promotes invasiveness by transcriptional activation of the met protooncogene [188]. HIF-1α levels are regulated by prolyl hydroxylase whose activity is directly related to the availability of oxygen [189, 190]. The central regions of solid tumors become hypoxic when the tumor grows, thereby VEGF is produced and consequently the tumor angiogenesis induction occurs [191]. VEGF acts by binding and activating tyrosine kinase receptors VEGFR-1 and VEGFR-2 [192, 193]. It has been shown that activation of these receptors contributes to angiogenesis since antibodies that inhibit their function also inhibit tumor development [194].

Angiogenesis is also induced by CXC chemokines, such as IL-8 and CXCL12/SDF-1 (C-X-C motif chemokine ligand 12 / Stromal Derived Factor 1). The IL-8 was originally discovered as a pro-angiogenic factor derived from macrophages [195]. Some microvascular endothelial cells express CXCR1 and CXCR2 receptors which are activated by IL-8 that induce changes in the cytoskeleton organization, causing cell movement and contributing to increased permeability observed in tumor-associated blood vessels [196]. In addition, IL-8 receptors on endothelial cells promote proliferation and angiogenesis induction. IL-8 also increases the production of MMP2 and MMP9 metalloprotease in endothelial cells [197, 198]. These observations suggest that IL-8

has an important role in the induction of angiogenesis. In fact, various data indicates that the increase in the expression level of IL-8 in various tumors is associated with increased malignancy and ability to metastasize [199, 200]. The neutrophil role is essential in the response to IL-8, responding to this chemokine through CXCR2 chemokine receptor agonists. IL-8 induces intense angiogenic reactions *in vivo*, but no angiogenic response to these factors was observed in neutropenic mice, demonstrating an essential role for neutrophils [201].

CXCL12/SDF-1 is a potent chemoattractant molecule for resting lymphocytes, monocytes and CD34+ hematopoietic progenitor cells [202, 203]. Only CXCL12/SDF-1 binds to CXCR4 receptor, which has only this chemokine known as a ligand [204]. It has been reported that CXCL12/SDF-1 recruits bone marrow-derived endothelial cells precursors to neo-forming blood vessels [205, 206], thus antibodies directed against CXCL12/SDF-1 inhibit angiogenesis [207]. Moreover, CXCL12/SDF-1 stimulates tumor progression through direct effects on CXCR4+ cancer cells, and it has been observed a direct correlation between poor prognosis and CXCR4 presence on cancer cells in different types of malignancies [208, 209].

Osteopontin (OPN) is a glycoprotein containing arginine, glycine, aspartate (RGD) and exists as a component of the extracellular matrix and immobilized as a soluble pro-inflammatory cytokine [210, 211]; it is also able to play important roles in tissue remodeling, fibrosis (210) and angiogenesis [212]. Many of these effects are mediated by OPN binding to Integrin αvβ3 or CD44 receptors [213].

— NK cells and cancer

Studies conducted in both human and mice have helped us to understand that Natural Killer cells play an important role in immune defense against solid cancers and hematological tumors [214, 215, 216, 217]. Nevertheless, as emerged from functional analysis, the cytotoxic activity of tumor infiltrating NK cells (TINKs) is often modulated and inhibited by several factors derived from the tumor milieu [218, 219, 220]. Like

other immune cells, Natural Killer cells may also infiltrate the tumor mass. The TUMIC can affect NK cell functionality through a variety of cytokines and soluble factors, both inhibiting their cytotoxic function and promoting a pro-neoplastic/pro-angiogenic phenotype [221].

Among various solid tumors, lung cancer is the leading cause of cancer-related mortality in men. About 80% of all lung cancers are non-small cell lung cancer (NSCLC) that can be divided at the phenotypic level into two main sub-types: squamous cell carcinoma (SCC) and adenocarcinoma (ADC). Interestingly, lung tissues are particularly enriched in Natural Killer cells [222, 223].

Natural Killer cell infiltrating NSCLC were characterized predominantly by CD56bright CD16- subtype. Moreover, these cells show reduced perforin production if compared to autologous peripheral blood NK cells. In addition, based on the expression of CD107a surface marker, they display reduced cytotoxic capacity following incubation with human erythroleukemic cell line K562 *in vitro* [216, 224].

CD56+ CD16- dNK cells are able to produce pro-angiogenic factors [225] and NSCLC NK cells have the ability to produce proangiogenic factors like VEGF, PlGF and IL-8 [226] A further demonstration of the angiogenic potential of these cells was obtained by testing *ex vivo* the chemotactic capacity of NK cell-derived supernatants on human umbilical vein endothelial cells (HUVECs). Furthermore, it has been observed that the supernatants of tumor infiltrating NK cells can induce a capillary-like organization of HUVECs *in vitro*. It has also been reported that an *in vitro* exposure to the immunosuppressive cytokine TGFβ induces NK cells from peripheral blood of healthy donors to produce angiogenic factors such as VEGF and PlGF, suggesting that this cytokine is involved in the TINK angiogenic switch [226]. This has been added to previous knowledge concerning the ability of TGFβ, very often present in solid tumor microenvironment, to polarize the cytotoxic CD56dim CD16+ NK cell subset into a CD56bright CD16- phenotype with some characteristic similarity to dNK cells [227, 228].

It is of interest to further characterize the NK cell phenotype and function in the context

of malignancies, related to pleural effusions (PA) caused by primary or metastatic pleura tumors [219, 229].

INFLAMMATORY AND NEOPLASTIC PLEURAL EFFUSIONS

The pleura is a thin membrane that consists of five layers: an external fibro elastic layer, an under-pleural highly vascularized layer of loose connective tissue, an elastic surface layer tissue, a submesothelial layer of loose connective tissue and finally a mesothelium layer. The pleura directly adjacent to the lungs is the visceral pleura, the pleura in contact with the rib cage and diaphragm is the parietal pleura. Once fully developed, the pleural space typically contains 0,5-2,0 ml of pleural fluid that allows the parietal and visceral pleura to slide over each other during breathing. The pleural fluid, which normally has a similar composition to plasma with a lower concentration of proteins (< 1,5 g/dl), is derived from the capillaries and exits from the parietal pleural pore and from the lymphatic vessels. The multipotent mesothelial layer located into the pleural space, is very active concerning metabolism, along with humoral and cellular immune components that also plays an active role in the production and absorption of pleural fluid [230].
The abnormal fluid accumulation in the pleural space, because of an imbalance between excessive production and absorption, can lead to a medical condition known as pleural effusion [231]. Pleural lesions could generate from a neoplastic or inflammatory conditions. About the first aspect, the pleura can be the site of primary tumors, such as mesothelioma, or secondary tumors. Secondary metastatic involvement is more common than primary tumors. Approximately 50% of patients with metastatic cancer develop a malignant pleural effusion. At the epidemiological level, more than 75% of all cases of malignant pleural effusion are caused by lung, breast, ovary cancers or lymphomas [232]. In males, the main cause of cancer development is lung cancer, while in females the most common cause is breast cancer [233, 234]. In most metastases cases, it forms a serous or serosanguineous pleural

effusion that often contains cancer cells. In general, the presence of pleural fluid is accompanied by poor prognosis and the survival for patients with metastatic cancer that develop a malignant pleural effusion is around three months [235].

Other causes of pleural effusions are: inflammation secondary to viral or bacterial infection with pleural localization; pleural inflammation in presence of tuberculosis; extension to the pleura, by contiguity, of lung inflammation (pneumonitis) developed into a portion of the lung near the pleural surface (metapneumonic pleurisy); and finally, this can be a pleural manifestation of autoimmune disease [231].

The mechanisms involving the pleural fluid accumulation include: increase of interstitial fluid in the lungs as a result of increased pulmonary capillary pressure (such as heart failure) or permeability (e.g. pneumonia); decreased intrapleural pressure (such as atelectasis, the absence of ventilation of pulmonary alveoli); decreased oncotic pressure of plasma (for example hypoalbuminemia); increase in pleural membrane permeability and lymphatic flow obstructed (e.g. pleural neoplasm or infection) or rupture of the thoracic duct (for example chylothorax). An important distinction is the fact that the pleural effusion may be unilateral or bilateral; it can also occupy the large pleural cavity or be localized to a circumscribed pleural space portion [236, 237].

There are two types pleural effusions based on biochemical characteristics of the fluid: transudative PE and exudative PE. The transudative PEs are the result of an imbalance between hydrostatic and oncotic forces and are caused by a limited number of medical conditions such as heart failure and cirrhosis; less common causes include nephrotic syndrome, atelectasis, peritoneal dialysis, constrictive pericarditis and superior vena cava obstruction. In contrast, the exudative PE occurs when there is an alteration of local factors that influence the accumulation of pleural fluid that induces an excessive liquid production. Causes of this second type of plural effusion may be cancer or inflammatory processes [238].

In 2013, Vacca P. et al. have demonstrated that a preponderance of NK cells in malignant pleural effusions display a CD56bright CD16low phenotype, in contrast with the predominant subtype in the blood. The main activating receptors are not down-

regulated and inhibitory receptors are not up-regulated, contrary to what happens for TINK in solid tumors. Functionally, it is found that these cells, according with their own phenotype, quickly produce large amounts of cytokines, such as IFNγ and TNFα, following short-term stimulation with IL-2. An important aspect that has emerged following *in vitro* IL-2 treatment for 72 hours is the rapid and efficient cytotoxic ability against classical cancer target cells such as K562. The fact that Natural Killer cells examined are not anergic but actively functional was explained as the result of the fact that, at the pleural effusion level, NK cells are not in contact with cancer cells. Therefore, the cytokines and tumor-derived inhibitory factors may be diluted and do not reach the adequate concentration to demonstrate an inhibitory effect. The authors suggest that their findings could have relevant impact concerning *in vivo* therapeutic treatment for patients with primary or metastatic pleural tumors. NK cells cytotoxic activity *in vivo* may be induced or reinforced through local infusions of IL-2. Another possibility would be to expand and activate Natural Killer cells *in vitro* with IL-2 before their reintroduction into the patient.

Analyzing pleural effusion soluble factors, it was demonstrated that malignant PEs were found to have 77-fold higher VEGF-A levels compared to inflammatory PEs in addition with elevated levels of pro-angiogenic factors VEGF-A, CXCL4 and MMP-8, and low levels of pro-inflammatory cytokines IL-8, MCP-1, and TGFβ1 in malignant PEs [239]. In another work, in contrast, it was found that the amount of TGFβ was high in malignant PE. All these molecules have a relevant role in the polarization of immune system cells and could influence also Natural Killer cell functions [240].

These results are very promising and have been the basis for the realization of this project of thesis, for which in addition to malignant pleural effusion was considered another medical condition, i.e. the inflammatory pleural effusion.

AIMS OF THE STUDY

It is extensively demonstrated that the tumor microenvironment (TUMIC) plays a major role in cancer and in tumor progression. Different cell types are present in the TUMIC, such as the malignant-transformed cells (that acquire tumor-promoting functions during carcinogenesis), the stromal cells, the endothelial cells and a wide assortment of immune cells [55].

Immune cells infiltrating tumors typically show a tumor-induced polarization associated with attenuation of antitumor functions and generation of pro-tumor activities, among these the induction of angiogenesis. The immune cells better characterized are macrophages, neutrophils, dendritic cells and the myeloid-derived suppressor cells. Recently, also Natural Killer cells obtain the scientist attention and it was demonstrated that a subset of these cells could contribute, in NSCLC, in the promotion of angiogenesis during cancer [226].

The current investigation aims to determine whether NK cells residing in pleural effusion derived from patients with inflammatory diseases, primary or metastatic tumors of various origins, are characterized by decidual features and pro-angiogenic and pro-tumor characteristics, similar to those found in NSCLC.

It was already demonstrated that IL-2 treatment can promote the restoration of cytotoxicity in NK cells from patients with pleural effusions and our goal is to evaluate if IL-2 could be counteracted by TGFβ, often present in TUMIC, or directly by pleural effusion soluble factors.

The aim of this work is also to evaluate if molecules contained in pleural effusion fluids can induce a switch in healthy NK cells phenotype and functions. We think that a better characterization of Pleural Effusion NK cells may lead in the future to an appropriate therapy able to improve NK cell cytotoxic function contrasting with tumor progression and the angiogensis associated phenomenon.

PATIENTS, MATERIALS AND METHODS

PATIENT SELECTION

Subjects included in this study were healthy donors and patients with pleural effusions caused by inflammatory disease or primary or metastatic tumors of different origin. Healthy Controls (h): n=14 (M: n=8; F: n=6); age: *mean±SEM*=58,1±3,4 (Table 1A). Inflammatory Pleural Effusions (iPE): n=18 (M: n=16; F: n=2); age: *mean±SEM*=72,2±2,2 (Table 1B). Malignant Pleural Effusions from Primary Tumors (ptPE): n=18 (M: n=9; F: n=9); age: mean±SEM=72,8±2,5 (Table 1C). Malignant Pleural Effusions from Tumor Metastasis (tmPE): n=27 (M: n=11; F: n=16); age: *mean±SEM*=68,7±2,4 (Table 1D).
Samples analyzed include peripheral blood of healthy subjects (hPB) and patients with inflammatory pathologies (iPB), primary tumors (ptPB) or tumor metastasis (tmPB) and the respective pleural effusions: iPE, ptPE and tmPE, respectively. All the experiments were performed on fresh samples.
Patients with diabetes, human immunodeficiency virus (HIV)/hepatitis C virus (HCV)/hepatitis B virus (HBV) infection, overt chronic inflammatory conditions, previously treated with chemotherapy or radiotherapy, or those iatrogenically immunosuppressed or having undergone myeloablative therapies were excluded.
All samples were collected from healthy donors and patients at the Ospedale di Circolo of Varese, following informed consent, with local Ethics Committee approval.

NK CELL PHENOTYPE ANALYSIS

Total mononuclear cell suspension derived from peripheral blood and pleural effusion samples was obtained by ficoll hystopaque (LONZA, Basel, Switzerland) gradient stratification. To identify NK cell subsets, cells obtained were subsequently stained with

monoclonal antibodies against surface markers (CD14-PE and CD45-FITC, CD3-PerCP, CD56-APC, CD16-FITC, CD9-PE, CD49a-PE, CD57-PE, CD69-PE, NKp30-PE, NKG2D-PE and NKG2A-PE) all purchased from Miltenyi Biotech (Auburn, CA). After 10 minutes fixation with PFA 1% in PBS, samples were stored at 4°C until a BD FACS Canto II analysis was conducted. Briefly, after physical parameters analysis (FSC/SSC), CD45+ CD14- lymphocytes were gated and assessed for NK cell markers. NK cells were gated on CD45+ CD3- CD56+ total lymphocytes.

Evaluation of NK cell cytokine production

NK cells were subjected to intracellular cytokine staining (ICS) assay after 1h incubation with monensin (2 mM, BD), or, to evaluate the production of IFNγ, after an overnight stimulation with PMA (10 ng/ml, Sigma-Aldrich, St Louis, MO) and Ionomycin (500 ng/ml, Sigma-Aldrich) plus monensin (2 mM, BD). Briefly, following staining with anti-human mAbs CD3-PerCP, CD56-APC, CD16-FITC (Miltenyi Biotec), cells were permeabilized and fixed using the Cytofix/Cytoperm fixation kit (BD), according to the manufacture instructions and finally stained with different anti-cytokine PE-conjugated mAbs (VEGF, CXCL12/SDF-1, perforin, osteopontin, IL-8 and IFNγ, Miltenyi Biotec) and analyzed with a BD FACS Canto II.

Morphogenesis of Human Umbilical Vein Endothelial Cells

The capacity to induce formation of capillary-like networks by endothelial cells seeded on matrigel (BD) was tested using HUVECs at the concentration of 20×10^3 cells/well in a 96-well plate. Negative and positive controls (CTRL- and CTRL+) were obtained using a serum-free medium and a medium containing 10% of FBS, respectively [226].
HUVECs were resuspended in 0,2 ml of a medium containing pleural fluids (1/3 of the medium) and transferred to the matrigel-coated wells for 6-hour incubation.

HUVECs were resuspended in 0,2 ml of a medium containing or containing NK cell supernatants (protein concentration: 15 µg/µl) and transferred to the matrigel-coated wells for 6-hour incubation). NK cell supernatants were obtained with the procedure described below: NK cells were purified from peripheral blood and pleural effusion samples using two steps of MicroBeads-coated with mAbs: CD3 MicroBeads (Miltenyi Biotec): CD3 positive cells were eliminated and the CD3- fraction was purified using CD56 MicroBeads (Miltenyi Biotec) to isolate CD3-/CD56+ NK cells. Purified NK cells were incubated 6 hours in serum-free RPMI medium. Supernatants were collected; residual cells and debris were discarded by centrifugation and supernatants were concentrated with Concentricon devices (Millipore, Temecula, CA) with a 5-kDa membrane pore cutoff.

The morphologic organization was documented with an inverted microscope (Zeiss) and angiogenesis was evaluated with ImageJ software and an Angiogenesis Analyzer Tool.

EVALUATION OF NK CELL CYTOTOXICITY

The NK cell degranulation activity assay was assessed on total mononuclear cells from PB and PE after *in vitro* 4h incubation with the human erythroleukemia K562 tumor cell line target in presence of anti-CD107a mAb (Miltenyi Biotec) and monensin (2 mM, BD) at a NK cell:Target ratio of 1:1. Cells were then stained with anti-CD3 and anti-CD56 mAbs (Miltenyi Biotec), fixed for 10 minutes with PFA 1% in PBS and stored at 4°C until BD FACS Canto II analysis. This assay was performed on fresh PB of healthy donors and on fresh PB and PE of patients with iPE, ptPE and tmPE; *ex vivo* and after a 3-day cell culture with IL-2 (100 U/ml), IL-2 plus TGFβ (10 ng/ml) (Miltenyi Biotec) and IL-2 in a culture medium containing 33% of cell-free supernatants from iPE, ptPE or tmPE. Cell-free supernatants were obtained after pleural fluids centrifugation at 300xg for 15 minutes. Concentrations of IL-2 and TGFβ were used according to the protocol of Bruno et al. [226]. Viability of NK cells incubated with culture media with 33% of PE

supernatants was evaluated counting NK cells in Burker Chamber using Trypan Blue reagent.

POLARIZATION OF NK CELLS FROM HEALTHY DONORS WITH PE SUPERNATANTS

NK cells from 13 buffy coats were isolated with Kit RosetteSep (StemCell Technologies), according to the protocol of the manufacturer. Briefly, after ficoll separation, mononuclear cells and Red Blood Cells (RBC) are incubated in proportion 1:100 with RosetteSep kit in the concentration of 50 µl/ml (reaction volume: $50x10^6$ mononuclear cell/ml). After 20 minutes of incubation at room temperature the suspension was stratified on ficoll to obtain purified NK cells that were isolated through negative selection procedure. The conditioning of the purified NK cells with supernatant of pleural effusion was performed to determine whether the supernatant could polarize NK cells towards a pro-angiogenic phenotype. NK cells purified from each buffy coat are cultured for 7 days in RPMI medium $2x10^6$ NK cells/ml, with 10% of FBS, 1% of Penicillin/Streptomycin and IL-15 (10 ng/ml) or IL-15 in a culture medium containing 33% of iPE, ptPE or tmPE supernatants.
Concentration of IL-15 and culture timing are based on the protocol of Cerdeira and colleagues [241]. Each kind of PE supernatant used was a pool of 10 supernatants derived from 10 different patients. During incubation, every two days, 33% of culture media containing PE was removed and replaced by fresh PE and IL-15 was added to the culture media of all samples. Viability of NK cells incubated with culture media with 33% of PE supernatants was evaluated every two days counting NK cells in Burker Chamber using Trypan Blue reagent.

EVALUATION OF *IN VITRO*-CONDITIONED NK CELL CYTOKINE PRODUCTION

After 7-day culture, NK cells were subjected to ICS assay after 1h incubation with monensin (2 mM, BD), or, to evaluate the production of INFγ, after an overnight

stimulation with PMA (10 ng/ml, Sigma-Aldrich, St Louis, MO) and ionomycin (500 ng/ml, Sigma-Aldrich) plus monensin (2 mM, BD). Briefly, following staining with anti-human mAbs CD3-PerCP, CD56-APC, CD16-FITC (Miltenyi Biotec), cells were permeabilized/fixed using the Cytofix/Cytoperm fixation kit (BD), according to the manufacture instructions and finally stained with different anti-cytokine antibodies and analyzed with a BD FACS Canto II. PE-conjugated mAbs (VEGF, CXCL12/SDF-1, IFNγ, perforin, osteopontin and IL-8) were all from Miltenyi Biotec.

Flow Cytometry Analyses

Flow cytometric analyses were performed using BD FACS Canto II and BD FACSDiva Software (v8.0.1).

Statistical Analyses

Statistical analyses were performed using the GraphPad Prism statistics and graphing program (GraphPad Software, San Diego, CA). One-way ANOVA with Bonferroni post-test were used to evaluate the statistical significance of all performed tests.

RESULTS

NK CELLS ISOLATED FROM MALIGNANT PLEURAL EFFUSION ARE ENRICHED IN CD56BRIGHT CD16- NK CELLS

It was demonstrated that there is an unbalanced distribution of CD56bright CD16- NK cell subsets between peripheral blood of healthy subjects (h), peripheral blood of patient with inflammatory (iPB) and malignant pleural effusions (ptPB and tmPB) and their respective pleural effusion samples (iPE, ptPE and tmPE): the percentages of CD56bright CD16- NK cells are higher in all PE samples, as shown in the representative dot plots (Figure 1).

The analysis of the phenotype performed by flow cytometry demonstrated that the NK cell percentage found in all samples analyzed were very similar with healthy controls (about 10% to 20% of total lymphocytes) as reported in Figure 2A. It is however interesting to consider that the volume of starting material of peripheral blood samples and pleural effusions is very different: to analyze PB-NK cells we used 15 ml of peripheral blood while, for PE-NK cells, pleural effusion volume varies from 1 to 2 liters, hence the number of NK cells obtained from the latter was higher and this situation allows us to performed more experiments with a single sample.

Regarding the two major subpopulations of NK cells it was demonstrated that the percentage of NK cells represented by CD56bright CD16- subset is increased in all pleural effusions analyzed if compared with autologous peripheral blood samples and PB of healthy donors (iPE=35%; ptPE: 40%; tmPE=60%) (Figure 2B). In Figures 2C-D are represented the CD56bright and CD16- NK cells populations, separately.

NK CELLS FROM PLEURAL EFFUSIONS SHOW AN INCREASED EXPRESSION OF THE DECIDUAL NK CELL MARKER CD49A

Evaluating the presence of surface markers, typical of dNK cells, such as CD9 and CD49a, it was observed a partial decidual differentiation of Natural Killer cells isolated from inflammatory and malignant pleural effusions.
Regarding CD9 we did not find differences in CD9+ NK cells percentage between all samples analyzed (Figure 3A) and the Mean Fluorescence Intensity parameter (MFI) was not statistically significant (Figure 3B).
CD49a analysis reveals that the percentage of CD49a+ NK cells found in pleural effusion (especially in ptPE and in tmPE) are significantly higher if compared to PB-NK cell samples (Figure 3C). The same trend could be noticed in the analysis of MFI: the value is higher for NK cells derived from ptPE and tmPE if related to hPB and autologous ptPB and tmPB (Figure 3D).
Therefore, in all types of pleural effusion there was observed an augmented percentage of CD49a+ Natural Killer cells, but not that of CD9+, revealing the acquisition of a partial decidual-like phenotype. Decidual NK cells are characterized by pro-angiogenic features and could help tumor growth. On the other hand, within inflammatory context these data could be interpreted in a positive manner: the growth of new blood vessels may be the preferred way to recruit other immune cells needed to maintain the inflammatory process.

PLEURAL EFFUSION NK CELLS ARE PREDOMINANTLY IMMATURE AND HIGHLY ACTIVATED

The differentiation molecule CD57, which identifies mature Natural Killer cells with a potent cytotoxic potential but a decreased sensitivity to cytokines and a reduced replicative ability [242], was examined. The NK cell percentage that displays this marker is lower in all pleural effusion analyzed, especially in ptPE and tmPE where it reaches

statistically significant differences (Figure 4A). The same trend was noticed in the MFI parameter analysis (Figure 4B). Poorly mature Natural Killer cells can be interpreted in a twofold manner: there may be cells that have not completed the maturation process or they may be fully differentiated NK cells de-differentiated because of stimulation of the microenvironment.

We have also analyzed the percentage of CD56bright CD57+ NK cells, showing that mature CD56bright NK cells were very low in this specific subset: CD56bright CD57+ NK cells were about 20% in ptPB and 1% in ptPE and 10% in tmPB and 0,5% in tmPE (data not shown).

We have observed a partial activation of Natural Killer cells: a high percentage of NK cells display CD69 surface molecule, a marker of NK cell early activation and present also on decidual NK cells [243]. We have demonstrated that CD69 is up-regulated on the NK cell membrane in pleural effusion samples, predominantly in those derived from neoplastic patients (Figure 4C). NK cells positive for this marker display a higher density expression of CD69 molecule on their surface if compared with PB samples, especially in ptPB/PE, as it is represented in the MFI graph (Figure 4D).

Thus, in the context of inflammatory and neoplastic pleural effusions, Natural Killer cells are mainly immature but partially activated.

INHIBITORY AND ACTIVATING RECEPTOR EXPRESSION ON NK CELLS FROM PLEURAL EFFUSIONS ARE NOT DEREGULATED

Natural Killer cell activation is the result of a sophisticated detection system based on the balance between signals transduced by activating receptors, including natural cytotoxic receptors (NKp30, NKp44, NKp46) and NKG2D, and inhibitory receptors such as NKG2A. Regarding NKp30 and NKG2D surface receptor we did not observe relevant differences between all samples (Figures 5A-B). We have also evaluated the percentage of NK cells positive for NKG2A, an inhibitory receptor of NK cells, which seems to be higher in PE samples if compared to hPB, iPB, ptPB and tmPB (Figure 5C) but these data

are not statistically significant and it is necessary to evaluate more samples. The signals from the microenvironment in which Natural Killer cells are located did not affect the expression of the membrane proteins analyzed. It will be interesting to examine the expression of other NK cell receptors.

EVALUATION OF CYTOKINE PRODUCTION: VEGF-PRODUCING NK CELLS ARE INCREASED IN PLEURAL EFFUSIONS

We have focused our attention on NK cell spontaneous cytokine production, angiogenic potential and cytotoxic function. NK cells can secrete cytokines capable of stimulating, guiding and influencing the other immune system cell types responses. In the present study, we investigated Natural Killer cell ability to produce some pro-angiogenic cytokines, including VEGF, CXCL12/SDF-1, osteopontin, IL-8 and the pro-inflammatory molecule IFNγ. Our data suggest that NK cells derived from pleural effusions can produce the pro-angiogenic cytokine VEGF: there is a trend for an increase in percentage of VEGF+ NK cells in all PE analyzed if compared to NK cells derived from autologous peripheral blood and healthy donors peripheral blood. Statistically significant was only the difference between hPB and tmPE (Figure 6A).
We studied the production by NK cells of CXCL12/SDF-1 that in tumors is demonstrated to be a pro-tumor molecule and found no significant differences (Figure 6B). These data seem to exclude CXCL12/SDF-1 as a determinant factor playing a role in the pro-tumor and pro-angiogenic process in the neoplastic pleural effusion disease. Osteopontin+, IL-8+ and IFNγ+ NK cells percentages do not display any differences between samples analyzed (data not shown).

PLEURAL EFFUSION FLUIDS FUNCTIONALLY PROMOTE ANGIOGENESIS-ASSOCIATED EFFECTS ON ENDOTHELIAL CELLS

We evaluated the ability of pleural effusions fluid from patients with inflammatory disease, primary tumor or tumor metastasis, to induce capillary like structure formation *in vitro*. The assay revealed that all pleural effusion fluid analyzed contains pro-angiogenic molecules able to promote morphogenic effect on HUVECs, like it is represented in Figure 7. In Figure 7A capillary like network structures formed by HUVECs can be seen. Parameter analyzed with ImageJ software and Angiogenesis Tool (Figure 7B) display that iPE and ptPE angiogenic properties are like those in positive controls (CTRL+) and that tmPE have the highest proangiogenic potential, confirming the hypothesis that tmPE could be associated with the most pro-angiogenic tumor microenvironment.

NK CELLS FROM TMPE CAN INDUCE CAPILLARY LIKE STRUCTURES FORMATION *IN VITRO*

We evaluated the ability of NK cells derived from peripheral blood and pleural effusions from patients with inflammatory disease, primary tumor or tumor metastasis, to secrete pro-angiogenic molecules that can induce capillary-like structure formation *in vitro* (Figure 8).

NK cells from patients with inflammatory pathologies or primary tumors, both from peripheral blood and from pleural effusions, are not able to induce angiogenesis on HUVECs. On the contrary, capillary-like structure formation is significant in the case of NK cells isolated from tmPE, demonstrating that a more pro-angiogenic TUMIC can induce NK cells with pro-angiogenic properties (Figures 8A-B-C).

THE PERCENTAGE OF CYTOTOXIC PERFORIN+ NK CELLS IS STRONGLY DIMINISHED IN PLEURAL EFFUSIONS

Another crucial characteristic in Natural Killer cell biology is represented by their spontaneous cytotoxic capacity culminating in the induction of an apoptotic process in their cellular targets. A hallmark of NK cell activation, and especially of CD56dim CD16+ NK cells, is the degranulation against target cells, that consist in the releasing of lytic granules containing perforin and granzymes [162]. Therefore, for these set of experiments we have considered the CD56dim CD16+ NK cell subset, known to be the most cytotoxic, to better individuate differences between measured samples. Analyzing NK cells able to produce perforin and consequently to exert their cytotoxic functions we have found a decreased percentage of CD56dim CD16+ perforin+ NK cells in all pleural effusions considered (Figure 9A). Interesting, MFI data also suggest that CD56dim CD16+ NK cells from all patient samples can produce a lower amount of perforin if compared to healthy controls peripheral blood (Figure 9B), suggesting a systemic effect on a crucial NK cell function. In both inflammatory and malignant pleural effusion, the percentage of CD56dim CD16+ perforin-producing NK cells was diminished in as compared to controls.

NK CELLS FROM PLEURAL EFFUSIONS SHOW LOWER CYTOTOXICITY *EX VIVO*

The cytotoxic potential of NK cells, which is the result of the involvement of activating and inhibitory receptors expressed on the membrane surface, was examined in a twofold manner: considering directly the production of perforin and evaluating the exposure, following activation, of the membrane glycoprotein CD107a on the NK cells surface.

We have evaluated the NK cell cytotoxic potential against a classical target, K562 cell line. The choice of these targets depends on their characteristic to be easily killed by

Natural Killer cells as they lack the MHC class I complex, required to inhibit NK activity. The degranulation assay underline the presence of CD107a surface marker, which presence on NK cell membrane correlate with granules exocytosis, is strictly associated with cytotoxicity. CD107a surface exposure is a marker of degranulation and NK cell cytotoxicity [244], as well as CD8+ T cell [245] and CD4+ T cells cytotoxicity [246].
We have analyzed the CD56dim CD16+ NK cell subset, characterized by a cytotoxic potential. CD56dim CD16+ NK cells response is weak in both peripheral blood and malignant pleural effusions from patients, on day zero *ex vivo*, compared to CD56dim CD16+ NK cells from peripheral blood of healthy donors and the same trend occurs also evaluating MFI (Figures 10A-B).
In conclusion, the functional assays conducted in this work showed a diminished percentage of CD56dim CD16+ Natural Killer cells expressing CD107a marker, measured *ex vivo*, in all types of pleural effusions and peripheral blood of patients if compared with CD56dim CD16+ NK cells from healthy subjects.

IL-2-TREATEMENT INCREASE CYTOTOXICITY OF PLEURAL EFFUSION-DERIVED NK CELLS BUT IT IS PARTIALLY INHIBITED BY TGFB AND, MORE STRONGLY, BY AUTOLOGOUS PLEURAL FLUID

As reported in the literature, among the most important cytokines known for their ability to activate NK cell functions there are the interleukins IL-2, IL-12 and IL-15. Specifically, IL-2 and IL-15 induce proliferation and reinforce cytotoxicity of Natural Killer cells, while the IL-12 alone increases the cytolytic activity but has a modest proliferative effect [247]. The binding of each of these cytokines to their respective receptors activates various intracellular kinase signaling pathways [248, 247, 249].
In this study, we wanted to understand whether the 3-day *in vitro* stimulation with the exogenous cytokine IL-2 could make changes in the cytolytic potential of Natural Killer cells isolated from pleural effusions, in particular CD56dim CD16+ NK cells subset.

We investigated NK cell cytotoxicity on the most cytotoxic NK cell subset, CD56dim CD16+, after 3-day *in vitro* stimulation with IL-2, IL-2+TGFβ and IL-2+autologous PE, to evaluate if TGFβ, often present in tumor microenvironment, or other soluble factors residing in pleural effusion could cause a loss of function of IL-2 treatment.

In NK cells from all pleural effusions analyzed, the NK cell effector function increased considerably because of the presence of IL-2 in the culture medium (Figures 11A-B-C). The observation of this reaction may suggest that the CD56dim CD16+ Natural Killer cells in pleural effusions are not fully inhibited: probably within the pleural fluid compartment Natural Killer cells do not show a feature completely compromised for the dilution of immunosuppressive factors and for the lack of close contact with tumor cells or with suppressor cells associated with the tumor.

Hence, the ability of pleural effusion Natural Killer cells to respond quickly to IL-2 *in vitro*, suggests the possibility to induce or enhance the antitumor effects of NK cells by local intra-pleural administration of this cytokine or by a re-infusion of NK cells derived from pleural effusions and activated with IL-2 *ex vivo* [274]. As reported in the literature, also in the case of renal cell carcinoma it was found that intra-tumor NK cells could lyse target cells only after *in vitro* stimulation with IL-2 [250].

For the better understanding of the behavior of Natural Killer cells in the TUMIC we have conditioned NK cells with a typical cytokine often found in the tumor microenvironment: TGFβ. We know that in cancer, TGFβ acts as a "Janus-like" cytokine: in the early stage of tumor formation, it acts as a tumor suppressor, inhibiting the replication of cancer cells and promoting apoptosis. On the contrary, in the later stages of tumor progression, TGFβ exercises a pro-tumor role, promoting cell survival, epithelial-mesenchymal transition and tissue invasion, as well as acting in the tumor microenvironment as an immuno-suppressive and angiogenic agent [251, 252]. After stimulation with IL-2, at day 3, the degranulation activity was increased in CD56dim CD16+ NK cells from all patient’s samples but the addition of TGFβ at the medium culture partially inhibits the IL-2 stimulation (Figures 11A-B-C).

The CD56dim CD16+ NK cells cytotoxicity after 3-day culture with IL-2 plus pleural effusion supernatants are very like those revealed *ex vivo* suggesting that the action of IL-2 *in vivo* could be counteracted by soluble factors present in the fluid microenvironment. Interestingly, NK cells that respond stronger to IL-2 treatment are CD56dim CD16+ NK cells derived from patients with metastatic tumors (Figure 11C). In Figure 11D are shown CD56dim CD16+ CD107a+ NK cells from healthy subjects, *ex vivo* and after 3-day treatment with IL-2 or IL-2 plus iPE, ptPE and tmPE. Cytotoxic CD56dim CD16+ NK cells percentage remains high (about 40%) also after IL-2 treatment and diminishes in presence of TGFβ and, mostly, in the culture conditions with all types of pleural effusion supernatants (Figure 11D). These data necessarily lead to think that the examined pleural effusion supernatants may contain various soluble factors capable of deregulate and then inhibit the cytotoxic potential of Natural Killer cells.

NK CELLS OF HEALTHY DONORS CONDITIONED WITH PLEURAL EFFUSION SUPERNATANTS ACQUIRE PARTIAL DECIDUAL-LIKE FEATURES

The presence in the pleural effusions of factors that can promote a pro-angiogenic phenotype of Natural Killer cells was examined through a polarization of NK cells purified from buffy coat of healthy volunteers. We have examined phenotype and cytokines/chemokines production by NK cells isolated from buffy coat and conditioned for 7 days with IL-15 or IL-15 with 33% of pleural effusion supernatant (IL-15, IL-15+iPE, IL-15+ptPE and IL-15+tmPE).

Regarding the CD56bright CD16- NK cell subset we found that supernatants of both malignant pleural effusions can induce an expansion of this population (Figure 12A). Also, total CD56bright NK cells and CD16- NK cells display the same behavior trend (Figures 12B-C).

Moreover, we have analyzed the production of pro-angiogenic factors, such as VEGF, CXCL12/SDF-1, osteopontin and IL-8. We have demonstrated that there is a trend of increase in the VEGF production by NK cells conditioned with both the treatment

containing malignant pleural effusion supernatants (Figure 13A). Regarding CXCL12/SDF-1+ NK cells, they are present in lower percentage in the condition with IL-15+ptPE and tmPE if compared to other conditions, reflecting what we have already discovered for NK cells *ex vivo* (Figure 13B). The percentages of osteopontin+ and IL8+ NK cells do not display differences between samples analyzed (data not shown)

Finally, we have evaluated the IFNγ pro-inflammatory and anti-tumor cytokine production after 7-day treatment: interestingly, we found that all pleural effusion supernatants used for cell polarization could drastically diminished the percentage of IFNγ-producing NK cells, suggesting a potent inhibitory effect on this important NK cell function exerted by soluble factors inside pleural effusions (Figure 13C).

DISCUSSION AND CONCLUSIONS

Natural Killer cells are an important component of innate immune responses displaying the ability to destroy neoplastic cells or virus-infected cells and, on the other hand, to tolerate self, healthy cells. Natural Killer cell responses are not only restricted to cytotoxic effector mechanisms, but this class of lymphocytes also plays a crucial role in the production of immunoregulatory cytokines and chemokines: these soluble factors affect recruitment and function of other hematopoietic cells, reinforcing the role of NK cells as protagonists at the interface between innate and adaptive immunity [253].

However, the concept of "immune surveillance” exerted by the immune system could be subverted in the context of the tumor microenvironment [3]. The immunosuppressive TUMIC is involved in tumor evasion from NK cell-mediated killing through several cellular and metabolic factors. Immune and stromal cells, as well as the hypoxic stress inside the tumor microenvironment, are known to be negative regulators of NK cell cytotoxicity [254]. Tumor cells themselves develop several strategies to evade NK cell-mediated killing. In this regard, hypoxic stress through its ability to induce tumor resistance and to regulate the differentiation and function of immune-suppressive cells plays a determinant role in shaping the NK cell phenotype and function [255].

Within the tumor tissue, macrophages and other myeloid cells constitute a major component of the immune cell infiltrate [256]. Macrophages can recognize and kill cancer cells through the secretion of various cytokines and factors (M1 phenotype), but also, after phenotypic and functional modification by the tumor, can act in pro-angiogenic and pro-tumor manner (M2/TAM phenotype). Thus, macrophages isolated from malignant pleural effusions, as well as TAMs, exhibit weak cytotoxic activity against tumor cells, increase their proliferative activity and may protect tumor cells from apoptosis [257]. It has also been shown that the percentage of CD163+ TAMs

(CD163 is a marker for the M2 macrophage phenotype [258]) in malignant PE was higher than that found in non-malignant pleural effusion. CD163+ TAMs in malignant PE patients is a prognostic factor for progression-free survival and M2-related cytokines were more expressed in malignant PE-derived CD163+ TAMs than in malignant PE-derived CD163- macrophages [259]. In addition, it was demonstrated that CD163+ CD14+ macrophages could be used as an immune diagnostic marker for malignant PE [260].

It was also found that, regarding soluble factors present in malignant PE, the amount of TGFβ is higher than in PB. TGFβ is mainly produced by TAMs in malignant PE, plays an important role in impaired T cell cytotoxicity and *in vitro* treatment with anti-TGFβ antibody restored the impaired T cell cytotoxic activity in malignant PE [240].

In the solid tumor microenvironment, macrophage-derived TGFβ is able to exert an immunosuppressive action on NK cells, combined with expression of adenosine A2A receptor, that can suppress NK cell responses [261].

TGFβ is also involved in NK cell inhibition mediated by Treg cells [262] and dendritic cells [263]. Furthermore, NK cell activity was found to be inversely correlated with MDSC expansion. In addition, MDSC-mediated inhibition of NK cells was found to be cell contact dependent via membrane-bound transforming growth factor-β (TGFβ) on MDSC or inhibition of perforin and signal transducer and activator of transcription 5 (Stat5) activity in NK cells [264, 265].

In this work, our specific interest is focused on Natural Killer cells, characterized by CD3-CD56+ expression and further divided depending on the density of CD56 surface molecule expression and based on the presence or absence of activating receptor CD16 [142].

Lung tissues are moderately rich in Natural Killer cells [223, 226] and it was found that the predominant subset in normal parenchyma is the CD56dim CD16+ phenotype [216, 226, 266]. NK cells infiltrating non-small cell lung cancer (NSCLC), if compared to autologous Natural Killer cells isolated from peripheral blood and peri-tumor lung tissues, are predominantly CD56bright CD16- [216, 226]. A similar phenotype has been

observed at the level of Natural Killer cells infiltrating breast cancer [267] and in the case of colorectal cancer [268]. In the present work, it was demonstrated that a similar pattern, characterized by abundance of poorly cytotoxic Natural Killer cells, was finally observed at the level of different types of pleural effusions.

We have analyzed Natural Killer cells isolated from patients with pleural effusions caused by inflammatory diseases or primary or metastatic tumors of different origins. It has been shown that NK cells isolated from all types of pleural effusions analyzed are characterized by an expansion of CD56bright CD16- NK cell subset, compared to NK cells isolated from autologous peripheral blood or peripheral blood of healthy donors, in which CD56dim CD16+ cytotoxic NK cells prevail.

Our investigation analyzing NK cell functions would indicate that cytotoxic NK cells can be strongly conditioned by tumor microenvironment of patients with pleural effusion and become cells involved in the genesis of new tumor vasculature. Taking into consideration the pro-angiogenic properties of Natural Killer cells it is important to clarify that at the level of developing decidua, in the first trimester of pregnancy, Natural Killer cells are converted from "killers" to "builders" [269]. Specifically, the CD56superbright CD16- Natural Killer cell subset forms the human immune element that predominates in the first trimester of pregnancy [270]. Once the embryo is anchored at the level of the uterine mucosa, a crucial event for the correct formation of fetus-mother interface is the ability of the embryo to adopt an invasive phenotype to induce a vascular remodeling, two crucial steps for the growth of the placenta [271, 272]. dNK cells display an important regulatory role in the placenta development processes during pregnancy: purified dNK cells can produce angiogenic factors such as VEGF, PlGF and IL-8 [225].

The present work demonstrates that PE-NK cells display an increased expression of CD49a decidual NK cell surface marker, were poorly mature (low expression of CD57) and yet activated (high expression CD69). NK cells from PE display a higher amount of intracellular VEGF if compared to healthy and autologous PB-NK cells; this difference is accentuated in tmPE, in which NK cells are predominantly VEGF+, thus pro-angiogenic

NK cells. These data, derived from intracellular cytokine staining, are confirmed by an *in vitro* vessel formation assay (Figure 8) that reveals an important ability of tmPE-NK cells to induce capillary like structures on HUVECs.

The results obtained by performing cytotoxicity assays *ex vivo* and the phenotypic analysis of the marker CD69 lead us to hypothesize that the concept of Natural Killer cell activation that probably not only includes the establishment of a mechanism of cytotoxicity because of the interaction with the appropriate target, but can also be the response to different nature stimuli produced by other TUMIC resident cells. These phenomena could justify the partial activation and the poor cytotoxicity of analyzed NK cells isolated from pleural effusion samples. We can consequently assume that, for the full activation of NK cells, more activatory stimuli are needed.

Unexpected data obtained in this study is the concept that Natural Killer cells isolated from inflammatory pleural effusion exhibit features very like those obtained from the two type of malignant pleural effusions analyzed: it may be interesting to examine in depth, in future experiments, the role of Natural Killer cells in this kind of environment, to establish if they differ in some characteristics to those found when in a TUMIC.

NK cells Isolated from buffy coats from healthy donors, after 7-day culture with IL-15 + PE, appear to increase CD56bright CD16- subsets and VEGF production. Moreover, PE supernatants can induce a decreased production of IFNγ. It is known that the IFNγ produced by Natural Killer cells can enhance the microbicidal activity of macrophages and promote the differentiation of lymphocytes into Th1-subtype to ensure an effective adaptive response against tumors [273]. In a recent study the levels of cytokines, chemokines and angiogenic proteins from human malignant pleural effusions were analyzed demonstrating a presence of an up-regulation of pro-angiogenic proteins in malignant pleural effusions analyzed in comparison of those not malignant [239]. If this trend will be validated for our clinical cases, it would confirm the effectiveness of using an anti-angiogenic therapy in the treatment of malignant pleural effusions.

These data reinforce the idea to use the immune system as a weapon to fight tumors. If in the tumor microenvironment a change in normal immune protective functions occurs, it becomes indispensable to develop drugs that, coupled to the conventional chemotherapy/radiotherapy, could re-switch the anti-tumor cytotoxic function of NK cell. On the other hand, a good target for cancer immune therapy must be necessarily the inhibition of tumor angiogenic process to limit the formation of blood vessels that contribute to tumor progression.

As reported by Carrega P. and colleagues, the fact that different human cancers show a lymphocytic infiltrate enriched in non-cytotoxic CD56bright NK cells may represent immunoediting process orchestrated by tumor that leads to a defective immune surveillance [216]. Therefore, immuno-therapies based on Natural Killer cells could be discovered looking at factors that induce CD56bright NK cells to differentiate, *in vivo*, into CD56dim NK cells, or factors that increase the cytotoxic NK cell chemo attraction to the tumor.

Vacca et al. demonstrated that, upon culture in IL-2, PE-NK cells acquired a potent cytolytic activity against both allogeneic and autologous tumor cells and thus, they are not functionally impaired. They conclude that a short-term IL-2 activation may offer important clues for the development of novel approaches in tumor immunotherapy [274]. In the present work, we have confirmed that IL-2 can restore cytotoxicity of PE-NK cells, but if these cells are cultured in a medium containing IL-2 along with TGFβ or autologous PE, this benefic action of IL-2 diminished significantly. This phenomenon suggests that in PE there are soluble factors present able to contrast the action of IL-2 and to maintain NK cells in a non-cytotoxic condition and in a pro-tumor and anti-inflammatory state. Therefore, it will be of great interest to characterize the soluble factors present in tumor pleural fluids and to estimate the amount of TGFβ. To evaluate the role of TGFβ in the counteracting the action of IL-2 treatment or in the process of NK cells pro-tumor polarization it will be appropriate to neutralize it with anti-TGFβ antibodies. A similar strategy might help, in future, to develop a therapy able to make NK cells permanently cytotoxic despite the TUMIC stimuli.

Furthermore, recent reports have shown that, in some situations, activated NK cells can express programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte associated antigen 4 (CTLA-4), two immunosuppressive surface molecules, which are the target of currently investigated cancer immunotherapeutic drugs (Ipilimumab against CTLA-4 and Nivolumab and Pembrolizumab against PD-1) that restore T cell activation [275, 276]. These data may suggest that an immunotherapeutic strategy using this type of molecules could be designed also for modulation of anti-tumor functionality of Natural Killer cells in the context of tumor pleural effusions. In conclusion, we think that a better understanding of Natural Killer cell behavior could be an important checkpoint for innovative immunotherapeutic approaches for primary or metastatic tumors presenting pleural effusion-associated condition.

LEGENDS OF TABLES AND FIGURES

Table 1.

Characteristics of all healthy controls and patients with pleural effusion analyzed (n=77). Healthy Controls (h): n=14 (M: n=8; F: n=6); age: *mean±SEM*=58,1±3,4. Inflammatory Pleural Effusions (iPE): n=18 (M: n=16; F: n=2); age: *mean±SEM*=72,2±2,2. Malignant Pleural Effusions from Primary Tumors (ptPE): n=18 (M: n=9; F: n=9); age: *mean±SEM*=72,8±2,5. Malignant Pleural Effusions from Tumor Metastasis (tmPE): n=27 (M: n=11; F: n=16); age: *mean±SEM*=68,7±2,4.

Figure 1.

Representative dot plots that underlines CD56bright CD16- NK cells population, *ex vivo*, in peripheral blood samples of healthy individuals (hPB) (A), peripheral blood and pleural effusion from patients with inflammatory disease (iPB and iPE) (B), peripheral blood and pleural effusion from patients with primary tumor (ptPB and ptPE) (C) and peripheral blood and pleural effusion from patients with tumor metastasis (tmPB and tmPE) (D).

Figure 2.

Comparative analysis of the percentage of NK cells (within total lymphocytes) found, *ex vivo*, in peripheral blood samples of healthy individuals (hPB), peripheral blood and pleural effusion from patients with inflammatory disease (iPB and iPE), peripheral blood and pleural effusion from patients with primary tumor (ptPB and ptPE) and peripheral blood and pleural effusion from patients with tumor metastasis (tmPB and tmPE) (A). Percentage of CD56bright CD16- NK cells (B). Percentage of CD56bright and CD16- NK cells separately (C-D). The data are represented as *mean±SEM* of 64 samples

(hPB: n=13; iPB/PE: n=16; ptPB/PE: n=15; tmPB/PE: n=20). * p <.05, ** p <.01, ****p < .0001.

Figure 3.

Comparative analysis of the percentage, evaluated *ex vivo*, of NK cells CD9+ (A) and relative MFI (B) and CD49a+ (C) and relative MFI (D) found in peripheral blood samples of healthy individuals (hPB), peripheral blood and pleural effusion from patients with inflammatory disease (iPB and iPE), peripheral blood and pleural effusion from patients with primary tumor (ptPB and ptPE) and peripheral blood and pleural effusion from patients with tumor metastasis (tmPB and tmPE). Data are shown as *mean±SEM* of 39 samples (hPB: n=7; iPB/PE: n=9; ptPB/PE: n=11; tmPB/PE: n=12). * p <.05, ** p <.01.

Figure 4.

Ex vivo comparative analysis of the percentage of NK cells CD57+ (A) and relative MFI (B) and CD69a (C) and relative MFI (D) found in peripheral blood samples of healthy individuals (hPB), peripheral blood and pleural effusion from patients with inflammatory disease (iPB and iPE), peripheral blood and pleural effusion from patients with primary tumor (ptPB and ptPE) and peripheral blood and pleural effusion from patients with tumor metastasis (tmPB and tmPE). Data are shown as *mean±SEM* of 37 samples (hPB: n=7; iPB/PE: n=9; ptPB/PE: n=11; tmPB/PE: n=10). * p <.05, ** p <.01.

Figure 5.

Comparative *ex vivo* analysis of the percentage of NK cells, displaying NKp30 (A) and NKG2D (B) activating receptor and NKG2A inhibitory receptor (C) found in peripheral blood samples of healthy individuals (hPB), peripheral blood and pleural effusion from patients with inflammatory disease (iPB and iPE), peripheral blood and pleural effusion from patients with primary tumor (ptPB and ptPE) and peripheral blood and pleural effusion from patients with tumor metastasis (tmPB and tmPE). Data are shown as *mean±SEM* of 18 samples (hPB: n=4; iPB/PE: n=4; ptPB/PE: n=5; tmPB/PE: n=5).

Figure 6.

Spontaneous VEGF and CXCL12/SDF-1 *ex vivo* production was evaluated after 1h-incubation with monensin (2mM, BD). Comparative analysis of the percentage of NK cells producing VEGF (A) and CXCL12/SDF-1 (B) found in peripheral blood samples of healthy individuals (hPB), peripheral blood and pleural effusion from patients with inflammatory disease (iPB and iPE), peripheral blood and pleural effusion from patients with primary tumor (ptPB and ptPE) and peripheral blood and pleural effusion from patients with tumor metastasis (tmPB and tmPE). Data are shown as *mean±SEM* of 34 samples (hPB: n=6; iPB/PE: n=8; ptPB/PE: n=10; tmPB/PE: n=10). ** p <.01.

Figure 7.

Analysis of the capacity of pleural effusion fluid from patients with inflammatory disease (iPE), primary tumor (ptPE) and tumor metastasis (tmPE) to induce endothelial cell capillary-like morphogenesis on HUVECs, after 6 hours of incubation on matrigel. Negative and positive controls (CTRL- and CTRL+) were obtained using a serum-free medium and a medium containing 10% of FBS, respectively (A). Total master segments length (sum of the length of the detected master segments in the analyzed area) (B) and Nb meshes (number of meshes in the analyzed area) (C) are evaluated with ImageJ software and Angiogenesis Analyzer Tool.

Figure 8.

Analysis of the capacity of NK cell supernatant to induce endothelial cell capillary-like morphogenesis on HUVECs, after 6 hours of incubation on Matrigel. Supernatants are obtained from NK cells isolated from peripheral blood and pleural effusion from patients with inflammatory disease (iPB and iPE), peripheral blood and pleural effusion from patients with primary tumor (ptPB and ptPE) and peripheral blood and pleural effusion from patients with tumor metastasis (tmPB and tmPE), after 6 hours of incubation in a serum-free RPMI medium. Negative and positive controls (CTRL- and

CTRL+) were obtained using a serum-free medium and a medium containing 10% of FBS, respectively (A). Total master segments length (sum of the length of the detected master segments in the analyzed area) (B) and Nb meshes (number of meshes in the analyzed area) (C) are evaluated with ImageJ software and Angiogenesis Analyzer Tool.

Figure 9.

Spontaneous perforin *ex vivo* production was evaluated after 1h-incubation with monensin (2mM, BD). Comparative analysis of the percentage of CD56dim CD16+ NK cells producing Perforin (A) and relative MFI (B) found in peripheral blood samples of healthy individuals (hPB), peripheral blood and pleural effusion from patients with inflammatory disease (iPB and iPE), peripheral blood and pleural effusion from patients with primary tumor (ptPB and ptPE) and peripheral blood and pleural effusion from patients with tumor metastasis (tmPB and tmPE). Data are shown as *mean±SEM* of 34 samples (hPB: n=6; iPB/PE: n=8; ptPB/PE: n=10; tmPB/PE: n=10). * p < .05, ** p <.01.

Figure 10.

Ex vivo analysis of CD56dim CD16+ CD107a+ NK cells after 4h *in vitro* stimulation with the human erythroleukemia K562 tumor cell line target. Comparative analysis of the percentage of CD56dim CD16+ cytotoxic NK cells that express CD107a surface marker (A) and relative MFI (B) found in peripheral blood samples of healthy individuals (hPB), peripheral blood and pleural effusion from patients with inflammatory disease (iPB and iPE), peripheral blood and pleural effusion from patients with primary tumor (ptPB and ptPE) and peripheral blood and pleural effusion from patients with tumor metastasis (tmPB and tmPE). Data are shown as *mean±SEM* of 38 samples (hPB: n=6; iPB/PE: n=10; ptPB/PE: n=11; tmPB/PE: n=11). * p <.05, ** p <.01, ***p < .001.

Figure 11.

Analysis of CD56dim CD16+ CD107a+ NK cells after 4h *in vitro* stimulation with the human erythroleukemia K562 tumor cell line target, *ex vivo* and after 3-day *in vitro*

treatment with IL-2 (100 U/ml), IL-2+TGFβ (10 ng/ml) and IL-2+autologous PE (33% of culture media). CD56dim CD16+ CD107a+ NK cells from A: patients with inflammatory diseases; B: patients with primary tumors; C: patients with tumor metastasis. D: CD56dim CD16+ CD107a+ NK cells from healthy donors, *ex vivo* and after 3-day *in vitro* treatment with IL-2 (100 U/ml), IL-2+TGFβ (10 ng/ml) and IL-2+iPE, IL-2+ptPE and IL-2+tmPE (PE: 33% of culture media). Data are shown as *mean±SEM* of 38 samples (hPB: n=6; iPB/PE: n=10; ptPB/PE: n=11; tmPB/PE: n=11). * $p < .05$, ** $p < .01$, *** $p < .001$.

Figure 12.

Comparative analysis of the percentage of CD56bright CD16- NK cells (A) on total NK cells purified from 13 buffy coats of healthy donors and treated for 7 days with IL-15 (10 ng/ml), IL-15 with pleural effusion from patients with inflammatory disease (IL-15+iPE), IL-15 with pleural effusion from patients with primary tumor (IL-15+ptPE) and IL-15 with pleural effusion from patients with tumor metastasis (IL-15+tmPE). Each kind of PE used is a pool of 10 supernatants derived from 10 different patients and it is placed in the culture medium not diluted, constituting the 33% of culture media. Percentage of CD56bright and CD16- NK cells separately (C-D). The data are represented as *mean±SEM* of 13 buffy coat samples treated with different pool of PE supernatants (IL-15: n=13; IL-15+iPE: n=13; IL-15+ptPE: n=13; IL-15+tmPE: n=13).

Figure 13.

Comparative analysis of MFI of VEGF produced by NK cells (A) from buffy coats of healthy donors treated for 7 days with IL-15 (10 ng/ml), IL-15 with pleural effusion from patients with inflammatory disease (IL-15+iPE), IL-15 with pleural effusion from patients with primary tumor (IL-15+ptPE) and IL-15 with pleural effusion from patients with tumor metastasis (IL-15+tmPE) (A). IL-15 was used at the concentration of 10 ng/ml and the pool of PE supernatants constituted the 33% of the culture media. Percentage of CXCL12/SDF-1+ (B) and IFNγ+ (C) NK cells with same treatments. The data are represented as *mean±SEM* of 13 buffy coat samples (IL-15: n=13; IL-15+iPE: n=13; IL-15+ptPE: n=13; IL-15+tmPE: n=13). * $p < .05$, ** $p < .01$.

TABLES AND FIGURES

Table 1.A.

HEALTHY CONTROLS (h) n=14 (M: n=8; F: n=6)			
	GENDER	AGE	DIAGNOSIS
1	M	45	Healthy subject
2	M	61	Healthy subject
3	M	57	Healthy subject
4	M	57	Healthy subject
5	M	65	Healthy subject
6	M	46	Healthy subject
7	M	76	Healthy subject
8	M	46	Healthy subject
9	F	47	Healthy subject
10	F	59	Healthy subject
11	F	65	Healthy subject
12	F	70	Healthy subject
13	F	58	Healthy subject
14	F	62	Healthy subject
AGE: mean+SEM=58,1+3,4			

Table 1.B.

INFLAMMATORY PLEURAL EFFUSIONS (iPE) n=18 (M: n=16; F: n=2)			
	GENDER	AGE	DIAGNOSIS
1	M	64	Acute pleurisy
2	F	74	Chronic pleurisy
3	M	45	Chronic pleurisy
4	M	72	Chronic pleurisy
5	M	74	Chronic pleurisy
6	M	80	Chronic pleurisy
7	M	80	Chronic pleurisy
8	M	83	Chronic pleurisy
9	M	85	Chronic pleurisy
10	M	59	Inflammatory pleural effusion
11	M	72	Inflammatory pleural effusion
12	M	77	Inflammatory pleural effusion
13	F	75	Pachypleuritis
14	M	65	Pachypleuritis
15	M	69	Pachypleuritis
16	M	71	Pachypleuritis
17	M	75	Pachypleuritis
18	M	79	Pachypleuritis
AGE: mean+SEM=72,2+2,2			

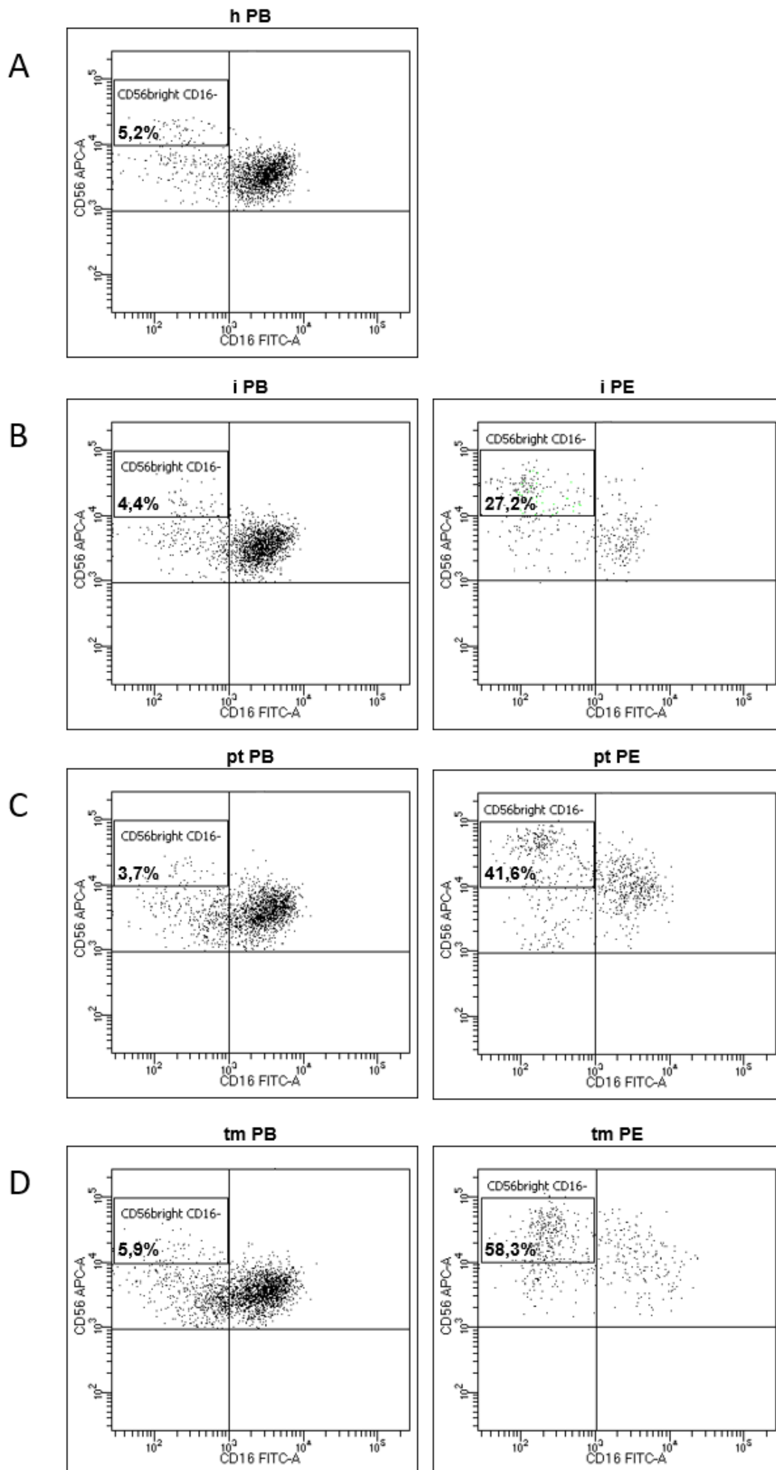
Table 1.C.

MALIGNANT PLEURAL EFFUSIONS FROM PRIMARY TUMORS (ptPE) n=18 (M: n=9; F: n=9)			
	GENDER	AGE	DIAGNOSIS
1	F	70	Epithelioid and sarcomatoid mesothelioma
2	M	66	Epithelioid and sarcomatoid mesothelioma
3	F	66	Epithelioid mesothelioma
4	F	77	Epithelioid mesothelioma
5	F	82	Epithelioid mesothelioma
6	M	49	Epithelioid mesothelioma
7	M	52	Epithelioid mesothelioma
8	M	73	Epithelioid mesothelioma
9	M	73	Epithelioid mesothelioma
10	M	80	Epithelioid mesothelioma
11	F	80	Epithelioid mesothelioma
12	F	90	Mesothelioma
13	M	67	Mesothelioma
14	F	72	Mesothelioma
15	F	79	Mesothelioma
16	F	85	Mesothelioma
17	M	70	Mesothelioma
18	M	79	Mesothelioma
AGE: mean+SEM=72,8+2,5			

Table 1.D.

MALIGNANT PLEURAL EFFUSIONS FROM TUMOR METASTASIS (tmPE) n=27 (M: n=11; F: n=16)			
	GENDER	AGE	DIAGNOSIS
1	F	53	Hepatocellular carcinoma
2	F	74	Ovarian carcinoma
3	M	82	Lung squamous cell carcinoma
4	F	52	Breast cancer
5	F	64	Breast cancer
6	F	59	Unknown cell carcinoma
7	F	73	Melanoma
8	M	88	Neuroendocrine carcinoma
9	F	68	Pancreatic cancer
10	F	49	Lung adenocarcinoma
11	F	50	Lung adenocarcinoma
12	F	67	Lung adenocarcinoma
13	F	68	Lung adenocarcinoma
14	F	69	Lung adenocarcinoma
15	F	75	Lung adenocarcinoma
16	F	79	Lung adenocarcinoma
17	F	88	Lung adenocarcinoma
18	F	91	Lung adenocarcinoma
19	M	46	Lung adenocarcinoma
20	M	58	Lung adenocarcinoma
21	M	64	Lung adenocarcinoma
22	M	70	Lung adenocarcinoma
23	M	75	Lung adenocarcinoma
24	M	77	Lung adenocarcinoma
25	M	78	Lung adenocarcinoma
26	M	79	Lung adenocarcinoma
27	M	58	Renal carcinoma
AGE: mean+SEM=68,7+2,4			

Figure 1.



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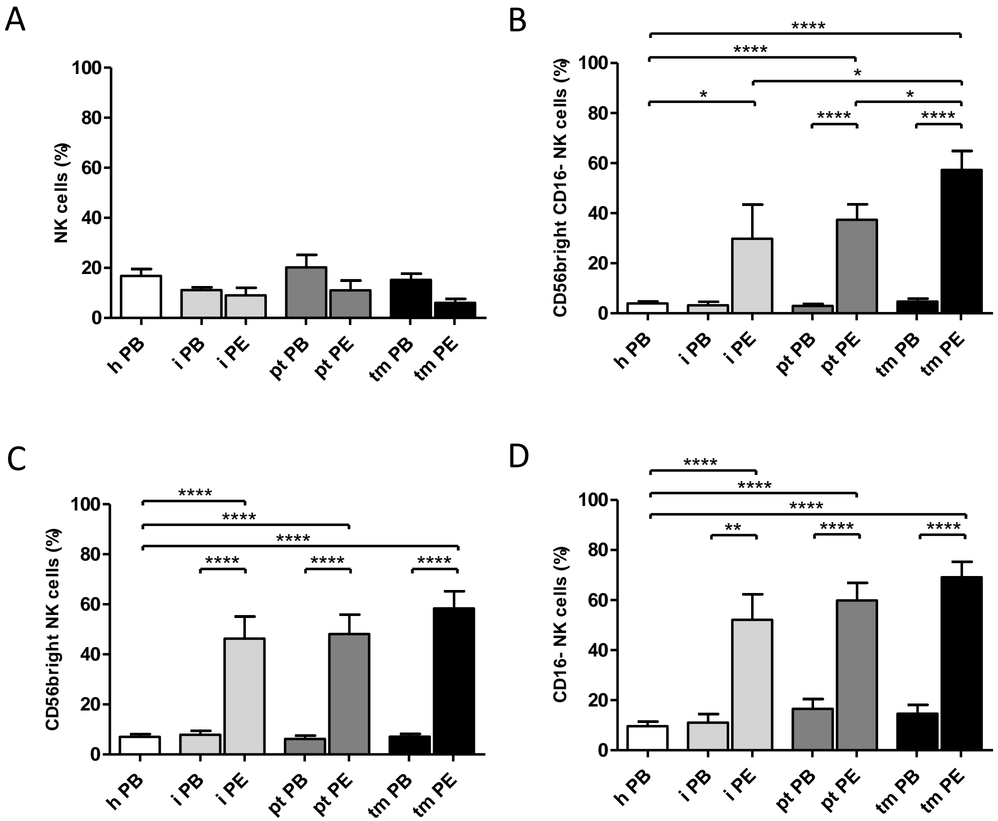

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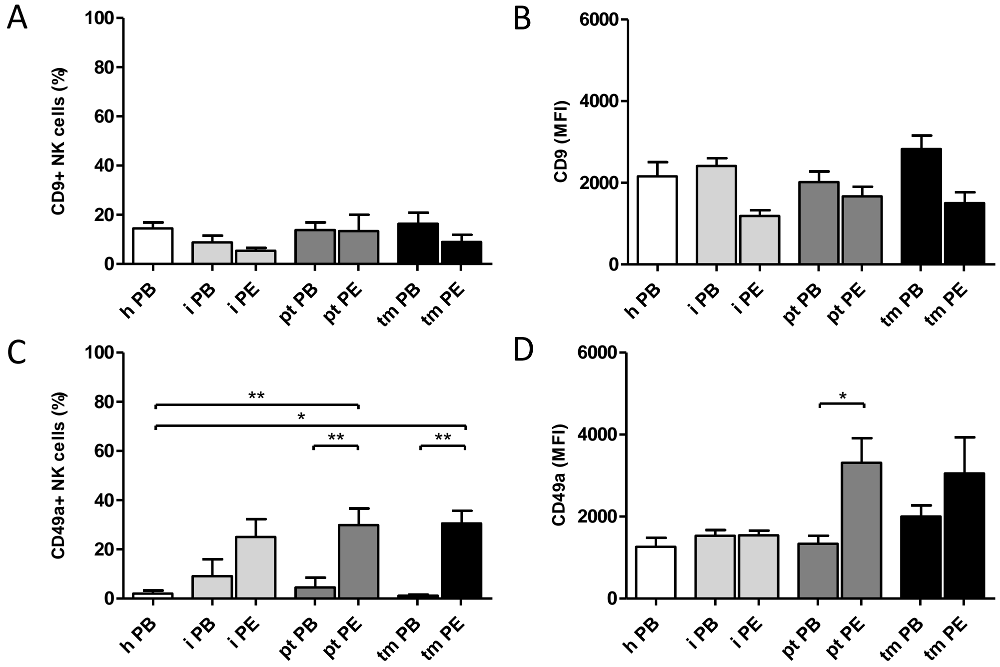

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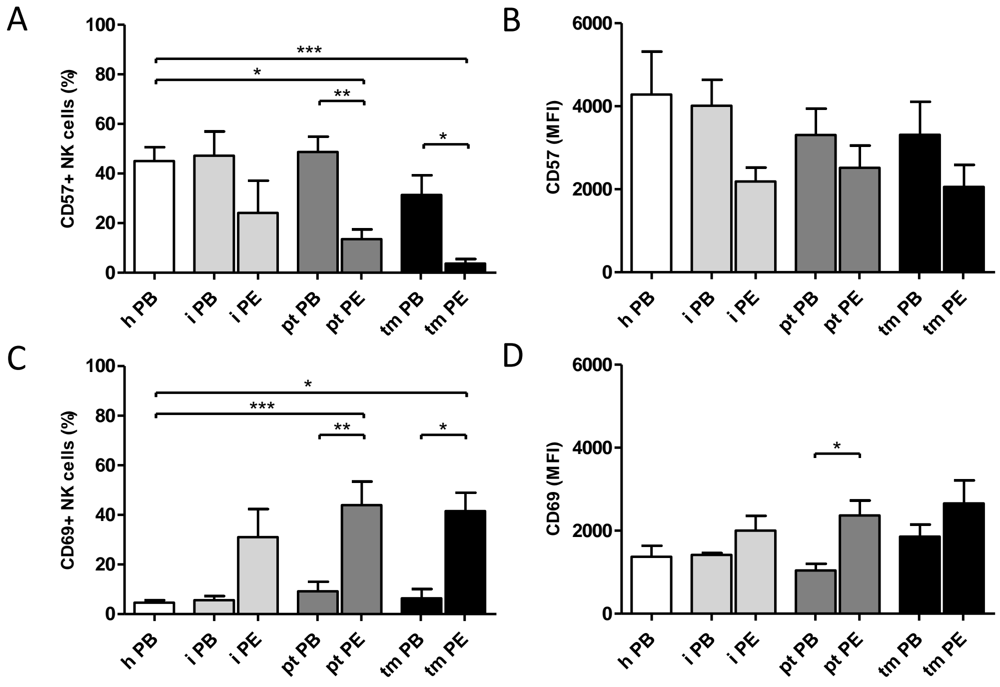

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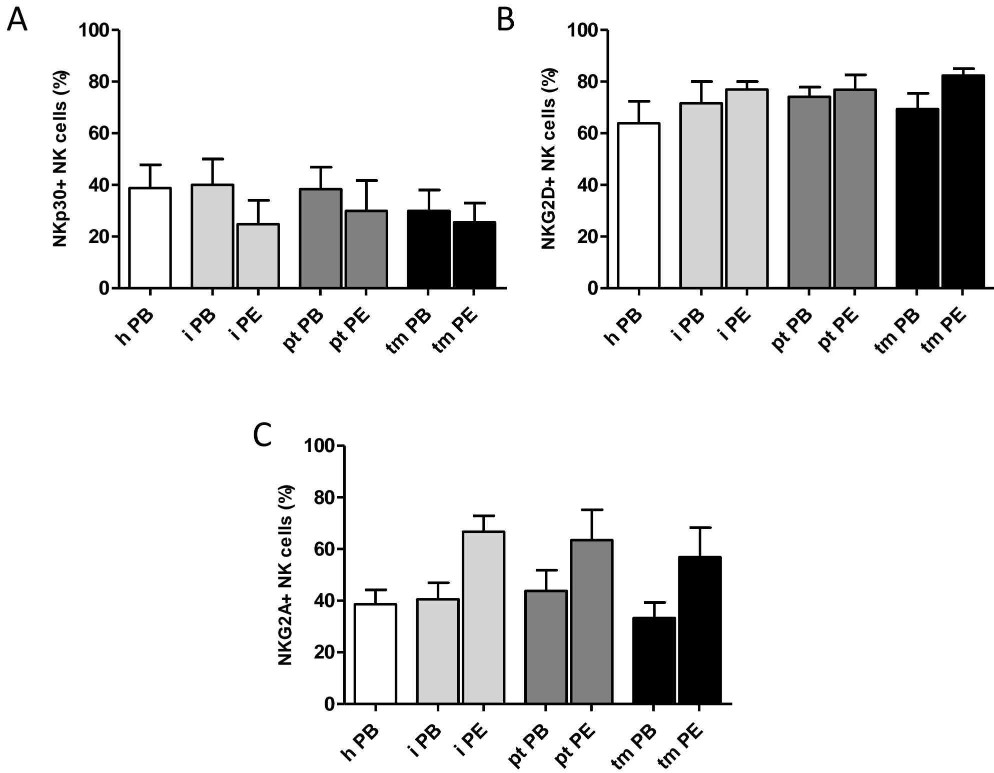

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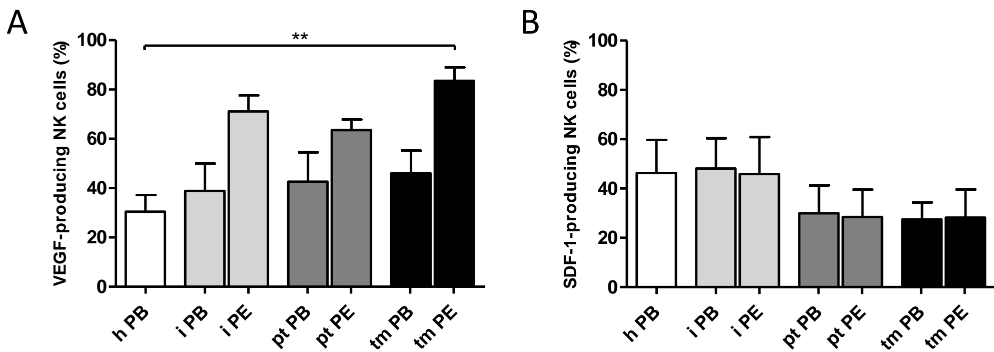

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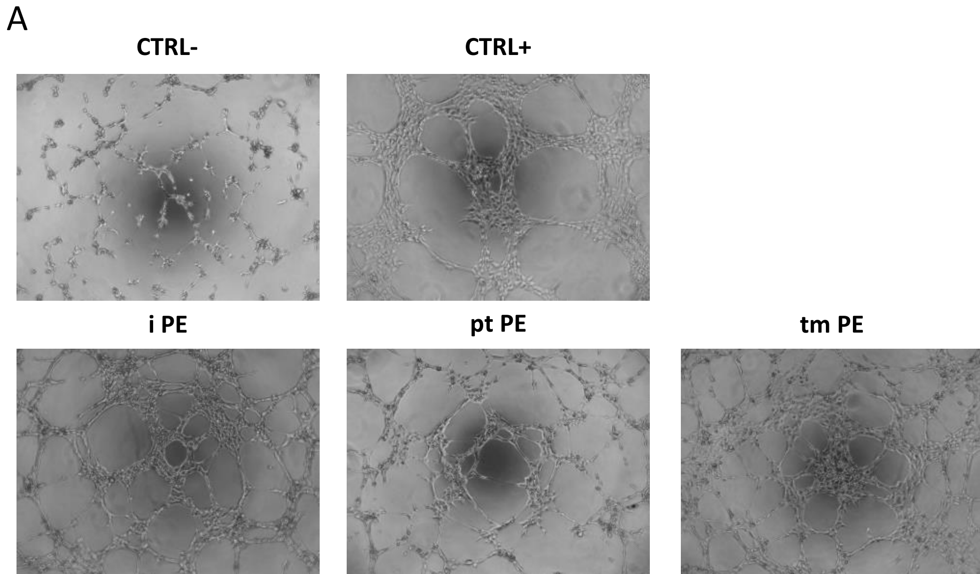

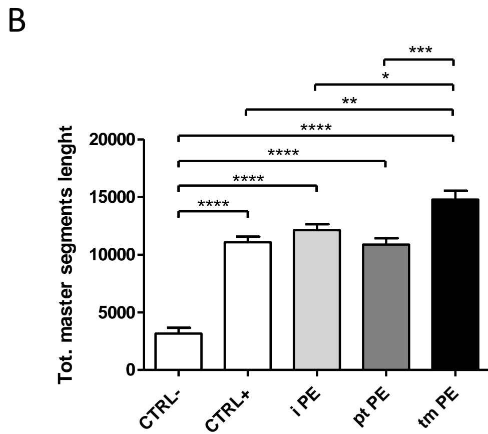

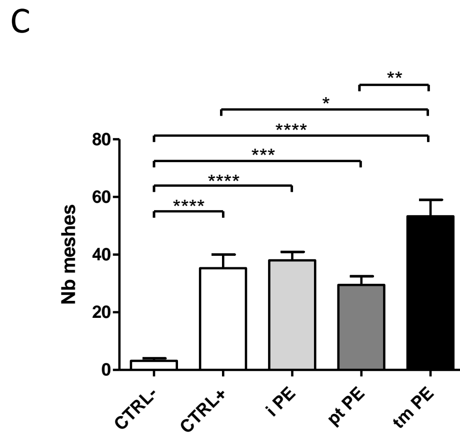

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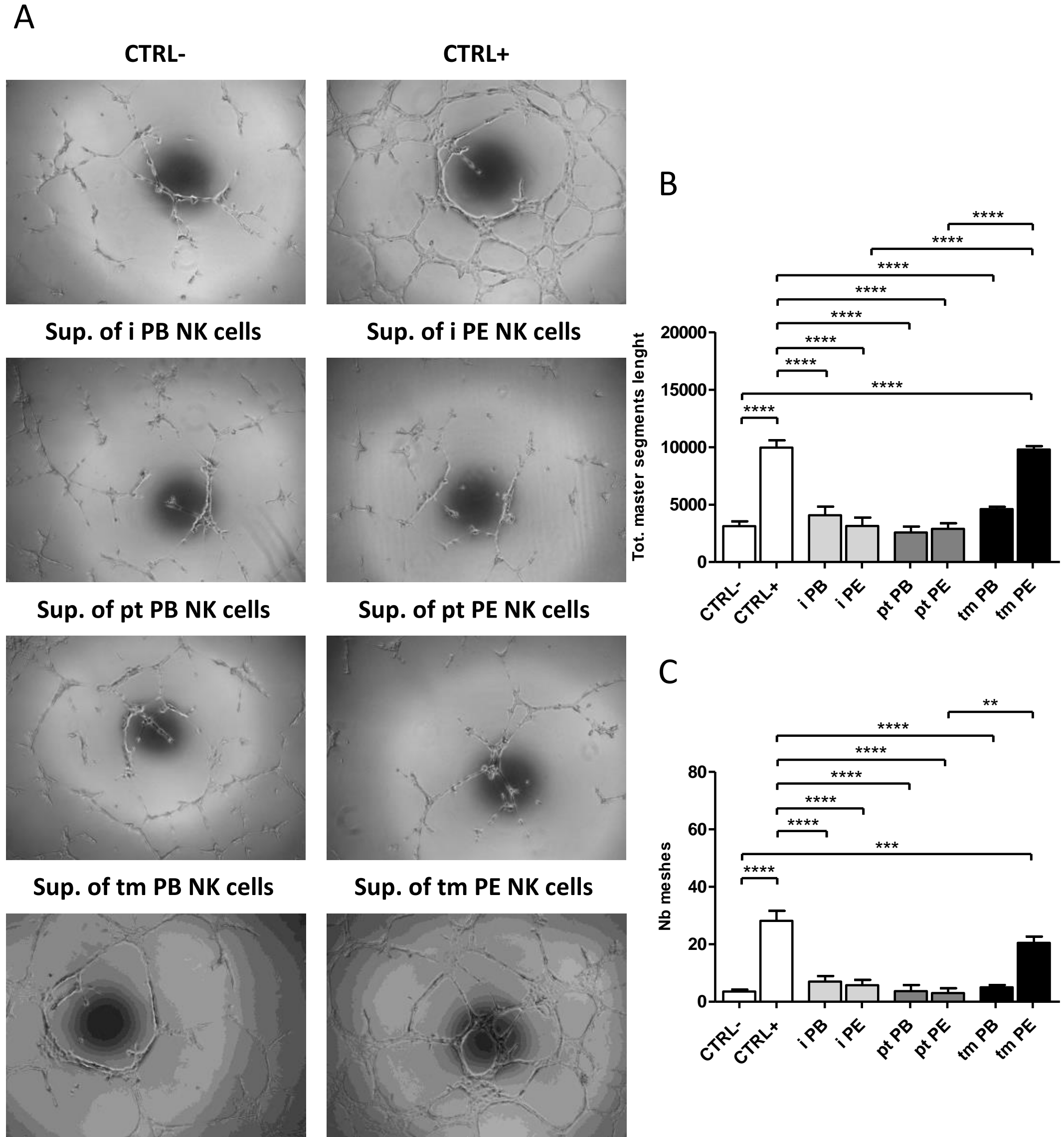

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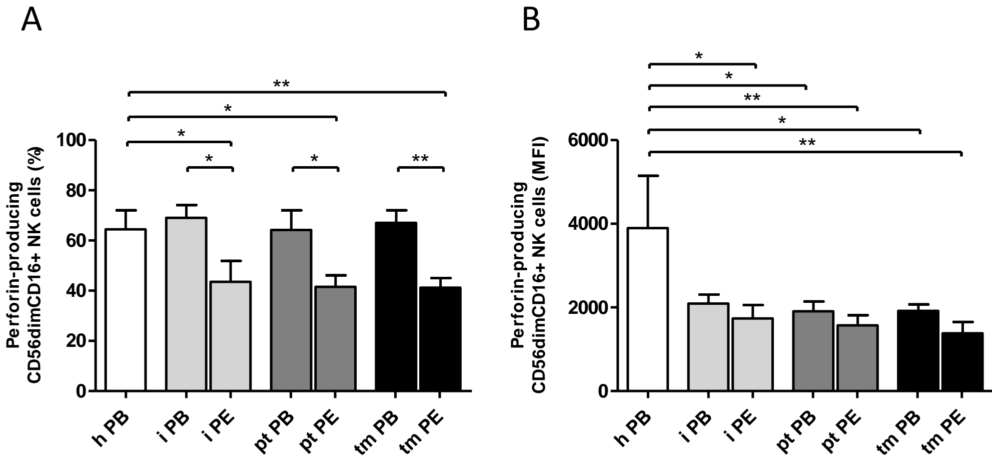

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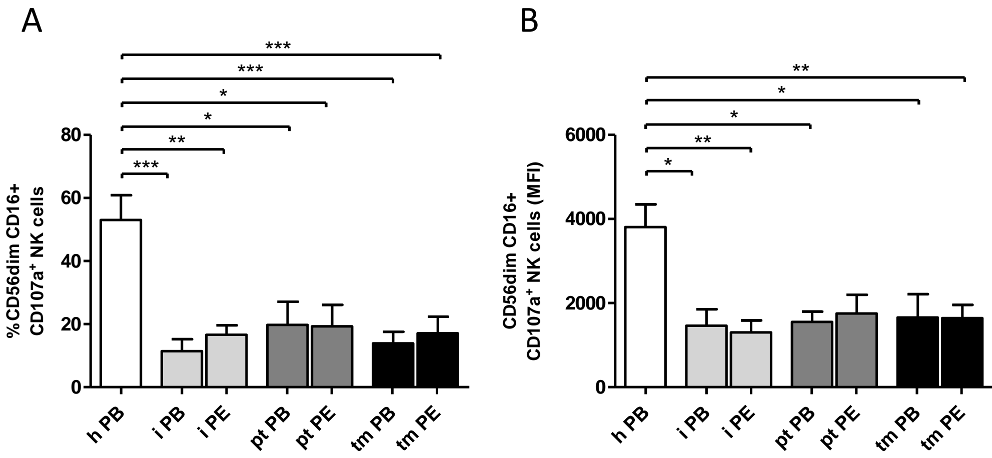

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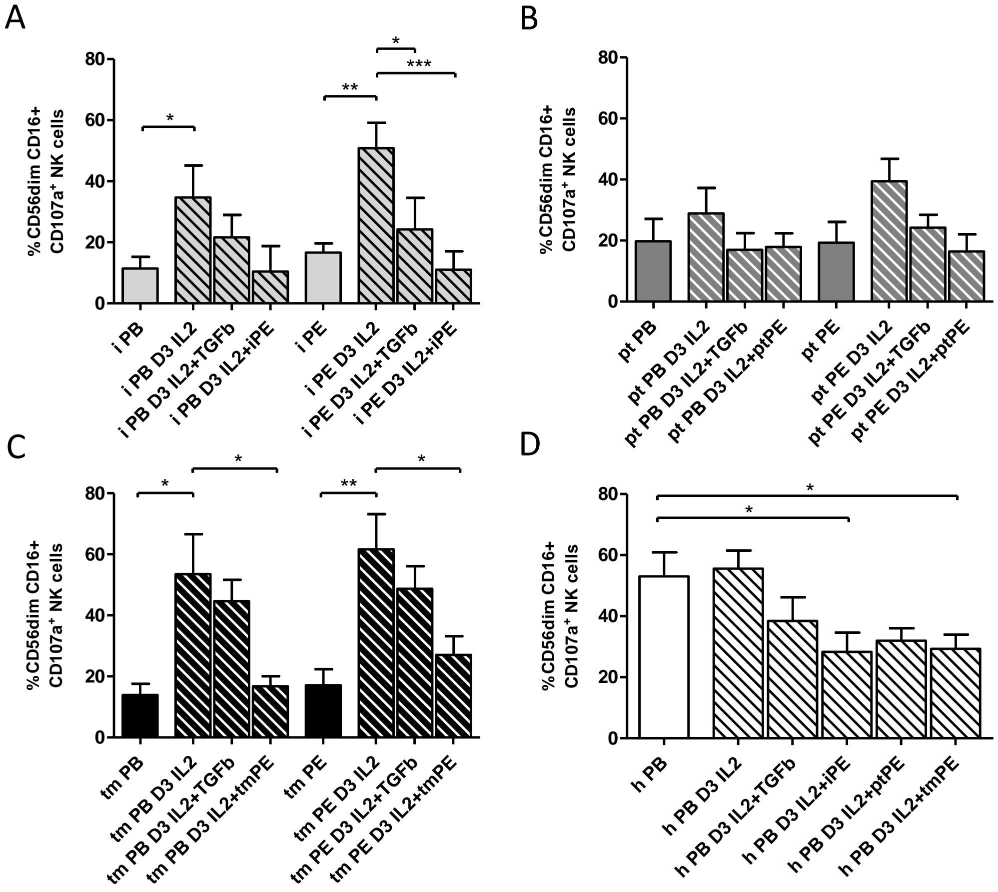

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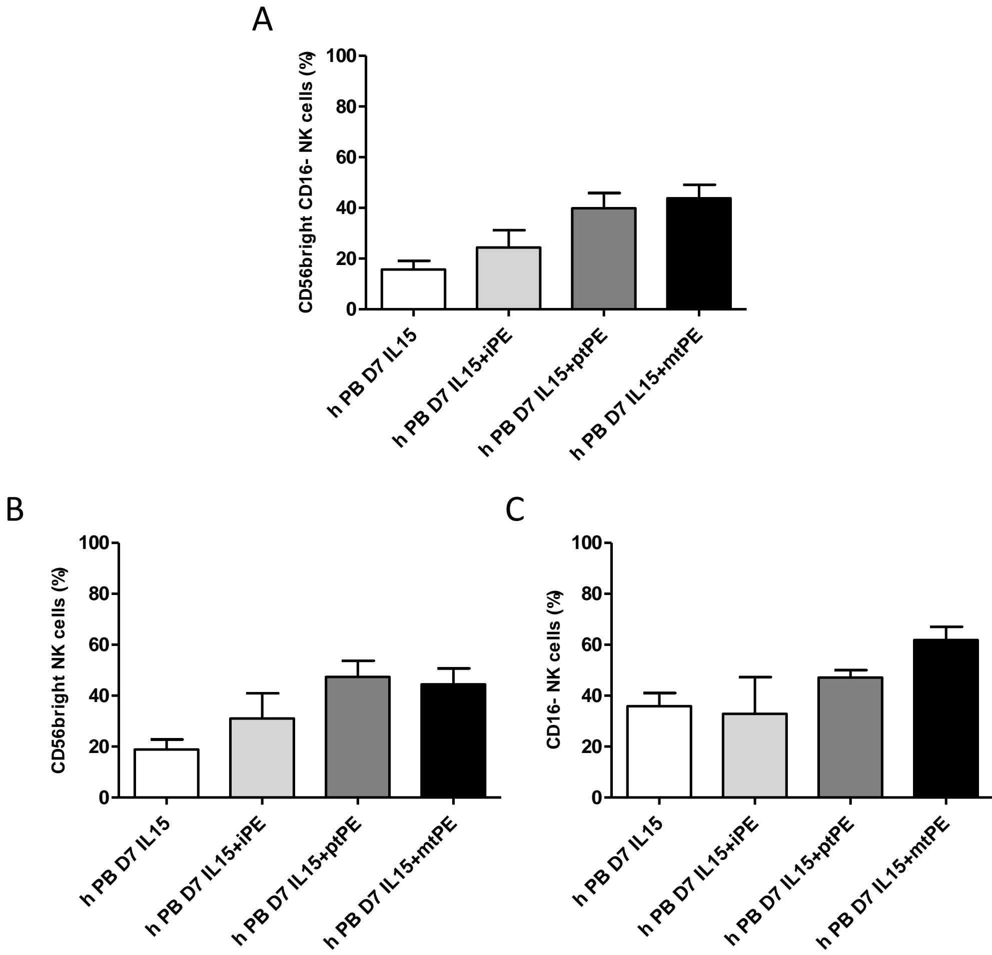

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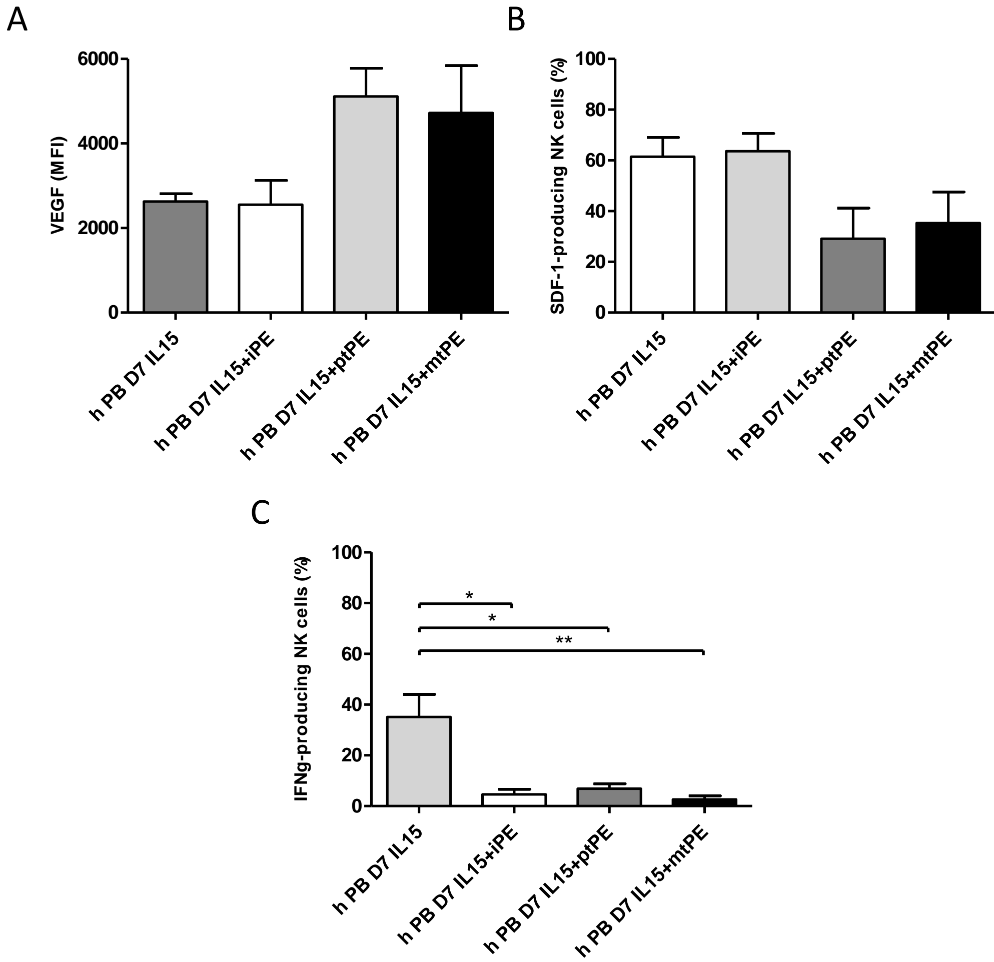

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APPENDIX

CD56^{bright}CD16^{-} NK Cells Produce Adenosine through a CD38-Mediated Pathway and Act as Regulatory Cells Inhibiting Autologous CD4^{+} T Cell Proliferation

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Recent studies suggested that human CD56^{bright}CD16^{-} NK cells may play a role in the regulation of the immune response. Since the mechanism(s) involved have not yet been elucidated, in the present study we have investigated the role of nucleotide-metabolizing enzymes that regulate the extracellular balance of nucleotides/nucleosides and produce the immunosuppressive molecule adenosine (ADO). Peripheral blood CD56^{dim}CD16^{+} and CD56^{bright}CD16^{-} NK cells expressed similar levels of CD38. CD39, CD73, and CD157 expression was higher in CD56^{bright}CD16^{-} than in CD56^{dim}CD16^{+} NK cells. CD57 was mostly expressed by CD56^{dim}CD16^{+} NK cells. CD203a/PC-1 expression was restricted to CD56^{bright}CD16^{-} NK cells. CD56^{bright} CD16^{-} NK cells produce ADO and inhibit autologous CD4^{+} T cell proliferation. Such inhibition was 1) reverted pretreating CD56^{bright}CD16^{-} NK cells with a CD38 inhibitor and 2) increased pretreating CD56^{bright}CD16^{-} NK cells with a nucleoside transporter inhibitor, which increase extracellular ADO concentration. CD56^{bright}CD16^{-} NK cells isolated from the synovial fluid of juvenile idiopathic arthritis patients failed to inhibit autologous CD4^{+} T cell proliferation. Such functional impairment could be related to 1) the observed reduced CD38/CD73 expression, 2) a peculiar ADO production kinetics, and 3) a different expression of ADO receptors. In contrast, CD56^{bright}CD16^{-} NK cells isolated from inflammatory pleural effusions display a potent regulatory activity. In conclusion, CD56^{bright}CD16^{-} NK cells act as "regulatory cells" through ADO produced by an ectoenzymes network, with a pivotal role of CD38. This function may be relevant for the modulation of the immune response in physiological and pathological conditions, and it could be impaired during autoimmune/inflammatory diseases. ***The Journal of Immunology*, 2015, 195: 965–972.**

Natural killer cells, which were originally characterized as cytotoxic cells, are a lymphocyte population belonging to the innate immune system, able to spontaneously lyse cancer cells and virus-infected cells (1). In the last two decades, the role of these cells in the control of the adaptive immune response, in particular through the production of proinflammatory and anti-inflammatory cytokines, has been characterized (2, 3).

In humans, two major subsets of NK cells can be defined according to their expression of CD16 (FcγRIIIA, low-affinity receptor for the Fc portion of Ig G) and of CD56 (an adhesion molecule that mediates homotypic interactions) (4). The main subset (90% of total NK cells) in the peripheral blood (PB) is represented by CD16^{+}CD56^{dim} NK cells, which display a high natural and Ab-dependent cytotoxicity and express killer Ig-like receptors and Ig-like transcript 2. In contrast, CD16^{-}CD56^{bright} NK cells are poorly represented in the PB (10%), but they are the main NK cell subset in secondary lymphoid organs, where they make up 75–95% of total NK cells (5). These cells display low cytotoxicity and secrete a variety of cytokines upon stimulation. Recent studies have demonstrated that CD16^{-}CD56^{bright} NK cells are a more immature subset of NK cells, developing toward the CD16^{+}CD56^{dim} phenotype. Yu et al. (6) have demonstrated that the surface expression of CD94 defines an intermediate stage between the two major subsets, represented by CD94^{high}CD16^{+} CD56^{dim} NK cells, that are on their way to developing into terminally differentiated CD94^{low}CD16^{+}CD56^{dim} NK cells.

Recent studies suggest a possible regulatory role for CD16^{-} CD56^{bright} NK cells (7–9). These cells release anti-inflammatory cytokines in response to different stimuli. Moreover, in patients with multiple sclerosis, treatment with IFN-β reduces inflammation in relapsing patients through an increase in the release of anti-inflammatory cytokines, which is associated with an increase of PB CD16^{-}CD56^{bright} NK cells (10). Similarly, treatment of multiple sclerosis patients with daclizumab, a humanized anti–IL-

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The online version of this article contains supplemental material.

Abbreviations used in this article: ADO, adenosine; ADOR, ADO receptor; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; HD, healthy donor; JIA, juvenile idiopathic arthritis; MRFI, mean relative fluorescence intensity; PB, peripheral blood; PEF, pleural effusion; SF, synovial fluid.

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2Rα Ab, led to the expansion of PB CD16^{-}CD56^{bright} NK cells, which was correlated with decreased T cell survival and better prognosis (11).

Recently, Laroni et al. (12) have demonstrated that CD16^{-}CD56^{bright} NK cells suppressed autologous CD4^{+} T cell proliferation in a contact-dependent manner, and that the inhibition increased upon IL-27 treatment. However, the mechanisms whereby CD16^{-}CD56^{bright} NK cells act as regulatory cells have not yet been identified.

Regulation of the immune responses may be achieved through the expression and/or release of different molecules by immunoregulatory cells. Among these, extracellular adenosine (ADO) is responsible for the control of the immune response in physiological and pathological conditions through the interaction with four different G protein–coupled receptors (ADO receptors [ADOR] A1, A2a, A2b and A3) that are expressed by T (13–15) and B (16–18) lymphocytes and NK cells (18–21). ADO is a purine nucleoside produced as the final product of a complex ectoenzyme network. This network is composed of surface molecules with an extracellular catalytic domain, including 1) ADP ribosylcyclases (CD38, CD157) (22), 2) ectonucleotide pyrophosphatase/phosphodiesterase-1 (CD203a/PC-1) (23), 3) ectonucleoside triphosphate diphosphohydrolase 1 (CD39) (24), and 4) ecto-5′-nucleotidase (CD73) (24).

The working hypothesis of this initial work is that the expression of selected ectoenzymes by CD16^{-}CD56^{bright} NK cells makes this subset able to produce ADO and support the regulatory functions of this cell subset. Thus, in this study we have analyzed the expression and function of a panel of ectoenzymes in CD16^{-}CD56^{bright} NK cells from healthy donors (HD) and from patients affected by juvenile idiopathic arthritis (JIA), demonstrating, to our knowledge for the first time, that the regulatory function of CD16^{-}CD56^{bright} NK cells is at least in part related to ADO production mediated by an ectoenzymatic network and might be altered during autoimmune/inflammatory diseases.

Materials and Methods

Normal donors and patients

This study was approved by the Ethics Committee of the G. Gaslini Institute, Genoa, Italy. Buffy coat preparations were obtained from five HD. Surgically removed tonsils from three patients and paired PB/synovial fluid (SF) samples from five JIA patients were obtained following informed consent of patients' parents or legal guardians. All JIA individuals were classified as limited oligoarticular JIA according to International League of Associations for Rheumatology Durban criteria (25). All samples were collected from untreated patients at diagnosis. PB and pleural effusion (PEF) samples were obtained from patients with pachypleuritis at the Ospedale di Circolo of Varese, following informed consent, with local Ethics Committee approval.

Cell isolation

Mononuclear cells (MNC) were isolated by Ficoll-Hypaque density gradients (Sigma-Aldrich, St. Louis, MO). Whole PEF cells were obtained by a single step of centrifugation. In some experiments, CD16^{-}CD56^{bright} NK cells and CD4^{+} T cells were isolated using a CD16^{-}CD56^{+} NK cell isolation kit and a CD4^{+} cell isolation kit, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's protocol. CD16^{-}CD56^{bright} NK cells were cultured overnight in the presence of 5 ng/ml IL-15 before use.

Flow cytometric analysis

The expression of ectoenzymes was evaluated on total PB or SF MNC from HD and JIA patients, gating on CD3^{-}CD16^{-}CD56^{bright} or CD3^{-}CD16^{+}CD56^{dim} NK cells, using the following Abs: anti-CD3 allophycocyanin (Becton Dickinson, Franklin Lakes, NJ), anti-CD16 PE (Beckman Coulter, Brea, CA), and anti-CD56 PC7 (Beckman Coulter). Ectoenzyme expression was analyzed using the following mAbs generated in our laboratory and FITC-conjugated by AcZon (Bologna, Italy): anti-CD38 (clone IB4), anti-CD73 (clone CD75), anti-CD57 (clone TB01), anti-CD157 (clone SY/11B5), anti-CD203a/PC-1 (clone 3E8, provided by J. Goding). CD39 expression was analyzed using anti-CD39 FITC mAb (Beckman Coulter). FITC-conjugated irrelevant isotype-matched mAbs were purchased from Beckman Coulter. In some experiments, the expression of ectoenzymes was evaluated on CD16^{-}CD56^{bright} NK cells that were purified as described above.

The expression of ADOR was evaluated on total MNC from JIA patients' PB or SF, gating on CD3^{-}CD16^{-}CD56^{bright} NK cells, using the following purified Abs: rabbit polyclonal anti-ADORA1 (LifeSpan BioSciences), rabbit polyclonal anti-ADORA2a (Thermo Scientific), and goat polyclonal anti-ADORA2b (Santa Cruz Biotechnology). FITC-conjugated goat-anti rabbit Ig (Abcam) and swine anti-goat Ig (Life Technologies) were used as secondary reagents.

Cells were run on a Gallios cytometer and analyzed using Kaluza software (Beckman Coulter). Data were expressed as mean relative fluorescence intensity (MRFI), obtained as follows: mean fluorescence intensity obtained with specific mAb/mean fluorescence intensity obtained with irrelevant isotype-matched mAb.

ADO production and nucleotide consumption

CD16^{-}CD56^{bright} NK cells (10^5 or 2×10^5 cells/well) were cultured in RPMI 1640/10% FBS at 37°C and 5% CO_2 in round-bottom 96-well plates (Costar Corning) in the presence or absence of AMP, ADPR, ATP, or NAD^{+} (20 or 50 μM). In some experiments, CD16^{-}CD56^{bright} NK cells were treated for 30 min with erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; inhibitor of ADO deaminase, 100 μM), NaN_3, or POM-1 (inhibitors of CD39, Sigma-Aldrich, 100 μM) before being cultured with the substrates. Supernatants were collected after 5, 30, or 45 min and acetonitrile was immediately added at a 1:2 ratio at 4°C to stabilize ADO. Samples were then centrifuged at 12,000 rpm and supernatants were collected and stored at −80°C until use. The presence of ADO, AMP, ATP, and NAD^{+} was investigated by HPLC.

HPLC analysis

Chromatographic analysis was performed with an HPLC System (Beckman Gold 126/166NM, Beckman Coulter) equipped with a reverse-phase column (Hamilton C18, 5 μm; 250 × 4.5 mm). Separation of nucleotides and nucleosides was performed using a mobile-phase buffer (0.125 M citric acid and 0.025 M KH_2PO_4 [pH 5.1]) with 8% acetonitrile (Sigma-Aldrich) during 10 min at a flow rate of 0.8 ml/min. UV absorption spectra were measured at 254 nm. HPLC-grade standards used to calibrate the signals were dissolved in AIM V serum-free medium (Invitrogen, Paisley, U.K.), pH 7.4, 0.2 μm sterile-filtered, and injected in a buffer volume of 20 μl. The retention times (in minutes) of standards were: AMP, 2.15; NAD, 2.8; ADPR, 3.2; and ADO; 5.56. Peak integration was performed using Karat software (Beckman Coulter).

Acetonitrile-treated CD16^{-}CD56^{bright} NK cell supernatants (see above) were evaporated by SpeedVac, reconstituted in mobile-phase buffer, and assayed by HPLC.

The qualitative identity of HPLC peaks was confirmed by comigration of known reference standards. The presence of ADO was also confirmed by spiking standard (50 μM ADO), followed by chromatography. Quantitative measurements were inferred by comparing the peak area of samples with calibration curves for peak areas of each standard compound. Product concentrations were expressed as pmol/30 min/no. of cells (1×10^5 or 2×10^5 cells). Interpolation of data have been obtained using a Microsoft Excel algorithm.

CD4^{+} T cell proliferation

Autologous CD4^{+} T cell proliferation was assessed by CFSE dilution assay. Briefly, cells were stained with CFSE (Invitrogen, 1 μg/ml, 15 min at 37°C), washed, and then cultured in RPMI 1640/10% FBS at 37°C and 5% CO_2, alone or in the presence of anti-CD3/anti-CD28 mAb-coated beads (T cell activation/expansion kit, Miltenyi Biotec). Stimulated CD4^{+} T cells were cultured in the presence or absence of autologous CD16^{-}CD56^{bright} NK cells (NK/CD4 ratio ranged from 1:1 to 1:16). In some experiments, CD16^{-}CD56^{bright} NK cells were treated for 30 min with the following specific inhibitors before being cultured with autologous CD4^{+} T cells (at 1:1 NK/CD4 ratio): kuromanin (inhibitor of CD38, 10 μM, Sigma-Aldrich), POM-1, dipyridamole (inhibitor of nucleoside transporter, 50 μM, Sigma-Aldrich), EHNA, α-β-meADP (inhibitor of CD73, 300 μM, Sigma-Aldrich), and β-γ-meATP (inhibitor of CD203a/PC-1, 300 μM, Sigma-Aldrich).

After 5 d, cells were harvested and washed and then stained with anti-CD4 PE mAb (Beckman Coulter). After additional washes, cells were run

on Gallios cytometer, and CFSE dilution was analyzed by gating on CD4$^+$ cells, using Kaluza software (Beckman Coulter).

Statistical analysis

Statistical analysis was performed using Prism 5.03 software (GraphPad Software). Gaussian distributions of data were analyzed using a Kolmogorov–Smirnov test. The Student t test or Mann–Whitney U test was used to compare data, depending on data distribution. Data from paired SF/PB samples were analyzed using a paired t test. A p value <0.05 was considered to be statistically significant.

Results

The expression of ectoenzymes is different between PB CD16$^-$ CD56^{bright} and CD16$^+$CD56^{dim} NK cells

The expression of ectoenzymes was first analyzed by flow cytometry on PB MNC from four HD, gating on CD16$^-$CD56^{bright} or CD16$^+$CD56^{dim} NK cells (as shown in Fig. 1A). Additionally, we have evaluated the expression of CD57, a marker of NK cells with an unrelated ectoenzyme activity (β-1,3-glucuronyltransferase). As shown in Fig. 1B, the expression of CD38 was similar in the two subsets (mean ± SD: 39.45 ± 10.21 versus 40.44 ± 16.44), whereas the expression of CD73 (86.74 ± 5.42 versus 41.24 ± 13.74, $p < 0.05$), CD39 (2.27 ± 0.36 versus 1.45 ± 0.12, $p < 0.05$), CD157 (4.93 ± 0.34 versus 3.16 ± 0.67, $p < 0.05$), and CD203a/PC-1 (3.29 ± 0.75 versus 1.31 ± 0.18, $p < 0.05$) was higher in CD16$^-$CD56^{bright} than CD16$^+$CD56^{dim} NK cells. Conversely, the expression of CD57 (131.2 ± 54.88 versus 1.62 ± 0.87, $p < 0.05$) was higher in CD16$^+$CD56^{dim} than in CD16$^-$CD56^{bright} NK cells. A representative experiment is shown in Fig. 1A. The expression of ectoenzymes was then evaluated on purified PB CD16$^-$CD56^{bright} NK cells from three normal donors, obtaining similar results (Fig. 2A, mean ± SD: CD57, 1.6 ± 0.06; CD157, 5.27 ± 0.51; CD203a/PC-1, 4.04 ± 1.43; CD38, 552 ± 23.56; CD73, 69.33 ± 21.01; CD39, 17.18 ± 0.37). A representative staining is shown in Fig. 2B.

Finally, ectoenzyme expression was evaluated on CD16$^-$CD56^{bright} NK cells purfied from human tonsil. The pattern of expression was similar to PB CD16$^-$CD56^{bright} NK cells (Fig. 2C, mean ± SD: CD57, 1.61 ± 0.05; CD157, 12.09 ± 0.45; CD203a/PC-1, 4.04 ± 0.5; CD38, 53.7 ± 0.45; CD73, 236.2 ± 3.28; CD39, 7.71 ± 0.5).

PB CD16$^-$CD56^{bright} NK cells produce ADO from different substrates

We asked whether CD16$^-$CD56^{bright} NK cells could produce ADO from different substrates. As shown in Fig. 3A, CD16$^-$CD56^{bright} NK cells produced high amounts of ADO when cultured in the presence of AMP (234 ± 10 pmol ADO/30 min/10^5 cells) and NAD$^+$ (153 ± 160.3 pmol ADO/30 min/10^5cells), whereas low amounts of ADO were detected using ATP as substrate (4.09 ± 0.12 pmol ADO/30 min/10^5 cells). Pretreatment of NK cells with EHNA, a specific inhibitor of ADO deaminase, increased the production of ADO from AMP (262.9 ± 33.57 pmol ADO/30 min/10^5cells), NAD$^+$ (188.5 ± 157.4 pmol ADO/30 min/10^5 cells), and significantly from ATP (43.75 ± 50.54 pmol ADO/30 min/10^5 cells, $p = 0.02$). Taken together, these results demonstrate that PB CD16$^-$CD56^{bright} NK cells efficiently produce ADO from AMP and NAD$^+$, indicating that CD38, CD203a/PC-1, and CD73 are functional.

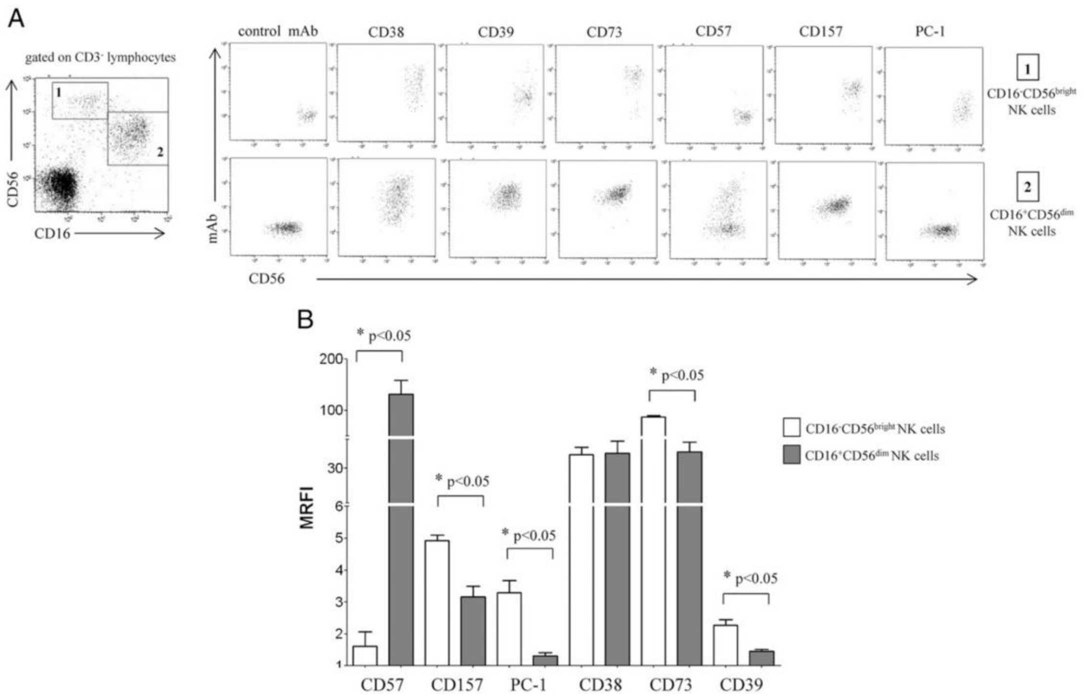

FIGURE 1. Expression of ectoenzymes on NK cells. Expression of CD57, CD157, CD203a/PC-1, CD38, CD73, and CD39 was evaluated by flow cytometry on PB MNC from HD, gating on CD3$^-$CD16$^-$CD56^{bright} (gate 1) or CD3$^-$CD16$^+$CD56^{dim} (gate 2) NK cells. Dot plots in (**A**) show a representative experiment. Histograms in (**B**) show pooled results from five independent experiments. Results obtained from CD3$^-$CD16$^-$CD56^{bright} (open bars) or CD3$^-$CD16$^+$CD56^{dim} (filled bars) NK cells are expressed as MRFI. Mean ± SD is shown. The p values are indicated when differences are significant.

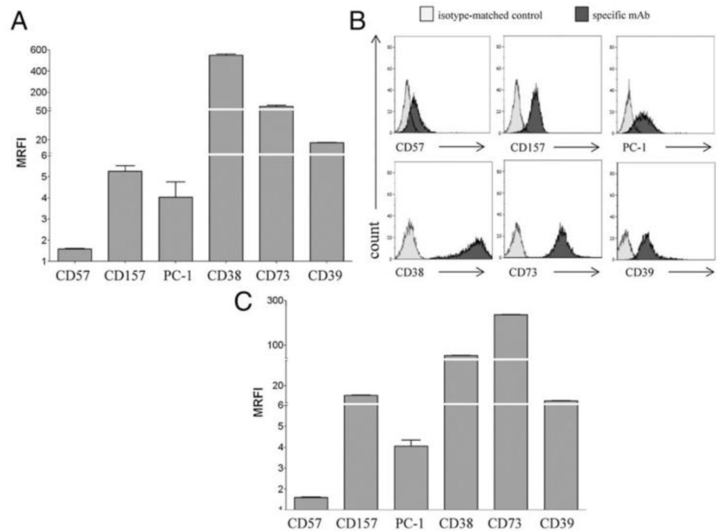

FIGURE 2. Expression of ectoenzymes on purified CD16^{-}CD56^{bright} NK cells. Expression of CD57, CD157, CD203a/PC-1, CD38, CD73, and CD39 was evaluated by flow cytometry on purified CD16^{-}CD56^{bright} NK cells from PB (**A**) or tonsil (**C**). Histograms in (**B**) show a representative staining on purified PB CD16^{-}CD56^{bright} NK cells. Black profiles indicate staining with specific mAb, and gray profiles indicate staining with irrelevant isotype-matched mAbs.

PB CD16^{-}CD56^{bright} NK cells inhibit autologous CD4^{+} T cell proliferation through ADO produced by CD38-mediated pathway

The immunosuppressive potential of CD16^{-}CD56^{bright} NK cells was next assessed by cocolture with autologous CD4^{+} T cells at different NK/CD4 ratios in the presence of anti-CD3/anti-CD28–coated beads. As shown in Fig. 3B, the percentage of proliferating CD4^{+} T cells (percentage of proliferating cells ± SD: medium alone, 80.55 ± 10.74) was significantly decreased in the presence of CD16^{-}CD56^{bright} NK cells at 1:1 (51.49 ± 19.53, $p < 0.0001$), 1:2 (61.51 ± 19.98, $p = 0.004$) and 1:4 (66.02 ± 18.87, $p = 0.015$) but not 1:8 (74.24 ± 14.27) or 1:16 (75.87 ± 11.72) NK/CD4 ratios. A representative experiment is shown in Fig. 3C.

To test the impact of each ectoenzyme on the regulatory function of CD16^{-}CD56^{bright} NK cells, the latter cells were pretreated with specific inhibitors of CD38 (kuromanin), CD39 (POM-1), CD73 (α-β-meADP), CD203a/PC-1 (β-γ-meATP). Additionally, specific inhibitors of ADO deaminase (EHNA) and ADO transporter NT (dipyridamole) were tested. As shown in Fig. 3D, inhibition of CD4^{+} T cell proliferation by CD16^{-}CD56^{bright} NK cells at 1:1 CD4/NK ratio (percentage of proliferating cells ± SD: medium alone, 79.57 ± 11.72; CD4 plus NK, 45.91 ± 18.53; $p = 0.001$) was significantly reverted in the presence of kuromanin (61 ± 25.39, $p = 0.05$), but not in the presence of POM-1 (34.25 ± 12.88), α-β-meADP (37.61 ± 17.94), β-γ-meATP (40.81 ± 22.56), or EHNA (31.07 ± 29.05). Conversely, NK cell–mediated inhibition was increased in the presence of dipyridamole (18.89 ± 8.24, $p = 0.001$).

These observations suggest that the function of CD38 is crucial for the regulatory activity of CD16^{-}CD56^{bright} NK cells. Moreover, the inhibition of the nucleoside transporter, which transports ADO across the plasma membrane (thus depleting it from the microenvironment), increases NK cell–mediated inhibition of CD4^{+} T cell proliferation, thus indicating that ADO is responsible for such inhibition.

Synovial CD16^{-}CD56^{bright} NK cells from JIA patients express reduced levels of CD38 and CD73 and lack detectable regulatory activity

To test whether the expression and function of ectoenzymes on CD16^{-}CD56^{bright} NK cells might be altered during autoimmune/inflammatory diseases, we analyzed the expression of ectoenzymes on NK cells from PB and SF obtained from JIA patients.

As shown in Fig. 4A, CD16^{-}CD56^{bright} NK cells are significantly enriched in SF, as compared with paired PB samples (percentage of MNC ± SD: PB, 0.23 ± 0.07; SF, 2.75 ± 1.2; $p = 0.001$; percentage of CD3^{-}CD56^{+} NK cells ± SD: PB, 6.5 ± 0.72; SF, 73.55 ± 2; $p = 0.0003$). Conversely, CD16^{+}CD56^{dim} NK cells are more enriched in PB than in SF (percentage of cells ± SD: PB, 6.89 ± 2.3; SF, 0.72 ± 0.28; $p = 0.0004$; percentage of CD3^{-}CD56^{+} NK cells ± SD: PB, 52.9 ± 11.3; SF, 11.58 ± 1; $p = 0.01$).

Next, the expression of ectoenzymes was evaluated on MNC from PB or SF, gating on CD16^{-}CD56^{bright} NK cells (as shown in Supplemental Fig. 1). As shown in Fig. 4B, the expression of CD57 (mean ± SD: PB, 1.42 ± 0.19; SF, 1.22 ± 0.08), CD157 (mean ± SD: PB, 9.42 ± 4.7; SF, 4.88 ± 1.8), CD203a/PC-1 (mean ± SD: PB, 5.21 ± 1.51; SF, 4.47 ± 2), and CD39 (mean ± SD: PB, 2.88 ± 2.86; SF, 1.28 ± 0.2) was similar between PB and SF CD16^{-}CD56^{bright} NK cells. Conversely, the expression of CD38 (mean ± SD: PB, 77.5 ± 34.49; SF, 28.09 ± 8.1; $p = 0.02$) and CD73 (mean ± SD: PB, 173.8 ± 73.57; SF, 83.12 ± 29; $p = 0.04$) was significantly lower in SF than in PB CD16^{-}CD56^{bright} NK cells. A representative experiment is shown in Supplemental Fig. 1.

To test whether the lower expression of CD38 and CD73 may affect the regulatory activity of CD16^{-}CD56^{bright} NK cells, the latter cells were cocultured with autologous stimulated CD4^{+} T cells. As shown in Fig. 4C, SF CD16^{-}CD56^{bright} NK cells failed to inhibit autologous CD4^{+} T cell proliferation at 1:1 CD4/NK ratio (percentage of proliferating cells ± SD: CD4^{+} T cells alone, 94.12 ± 6.21; CD4/NK 1:1, 97.1 ± 2.48). In contrast, CD16^{-}CD56^{bright} NK cells from inflammatory PEF strongly inhibited autologous CD4^{+} T cell proliferation at 1:1 CD4/NK ratio (percentage of proliferating cells ± SD: CD4^{+} T cells alone, 79.82 ± 1.91; CD4/NK 1:1, 26.12 ± 5.83, $p = 0.05$).

Fig. 4D shows a representative experiment performed with CD16^{-}CD56^{bright} NK cells from HD PB, JIA patient SF, and inflammatory PEF cultured with autologous CD4^{+} T cells at different CD4/NK ratios. These data suggested that regulatory function of CD16^{-}CD56^{bright} NK cells was increased during in-

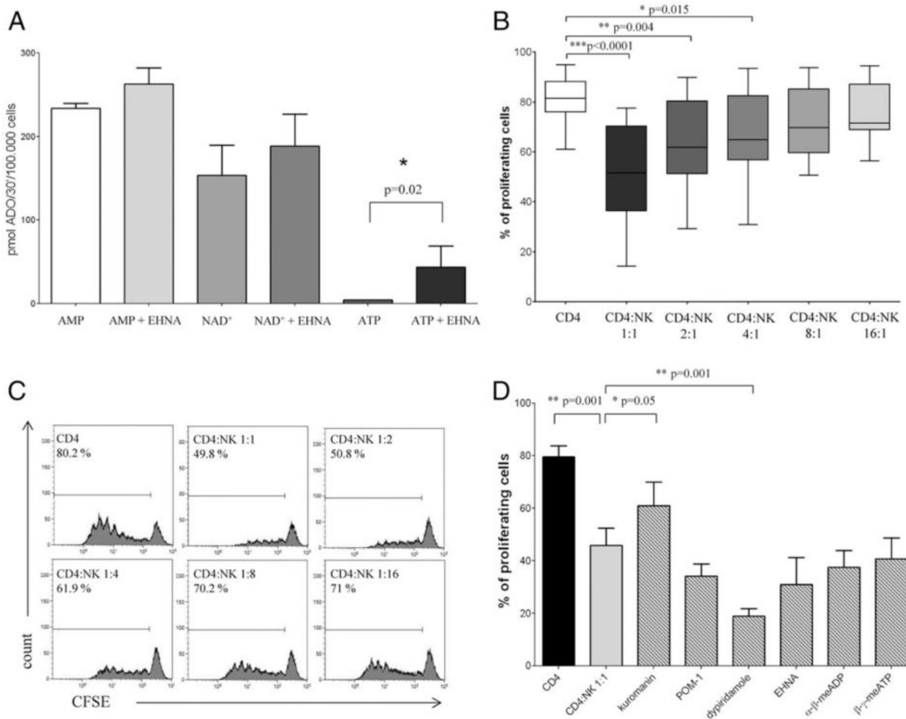

FIGURE 3. CD16$^-$CD56^{bright} NK cells produce ADO and inhibit CD4$^+$ T cell proliferation. (**A**) Production of ADO was assessed on CD16$^-$CD56^{bright} NK cells pretreated or not for 30 min with EHNA (ADO deaminase inhibitor) and then cultured in the presence of AMP, NAD$^+$, or ATP. Results are expressed as pmol ADO/30 min/10^5 cells (mean ± SD). Mean of five different experiments ± SD is shown. The *p* values are indicated when differences are significant. (**B**) Proliferation of CD4$^+$ T cells was analyzed by CFSE dilution after stimulation with anti-CD3/anti-CD28 mAb–coated beads, in the presence or absence of autologous CD16$^-$CD56^{bright} NK cells at different NK/CD4 ratios (ranging from 1:1 to 1:16). Results are pooled from 10 different experiments and are expressed as percentage of proliferating cells. Box indicated 25th and 75th percentile and median. Whiskers indicate minimum and maximum values. The *p* values are indicated when differences are significant. A representative experiment is shown in (**C**), where horizontal bars indicate proliferating cells, assessed by CFSE dilution. CD4/NK ratios and percentage of proliferating CD4$^+$ T cells are indicated. (**D**) Proliferation of CD4$^+$ T cells was analyzed by CFSE dilution after stimulation with anti-CD3/anti-CD28–coated beads in the presence or absence of autologous CD16$^-$CD56^{bright} NK cells (at 1:1 NK/CD4 ratios) in the presence or absence of kuromanin (inhibitor of CD38), POM-1 (inhibitor of CD39), dipyridamole (inhibitor of NT ADO transporter), EHNA (inhibitor of ADO deaminase), α-β-meADP (inhibitor of CD73), and β-γ-meATP (inhibitor of CD203a/PC-1). Results are expressed as percentage of proliferating cells. Mean of five different experiments ± SD is shown. The *p* values are indicated when differences are significant.

flammation, whereas such function was impaired during autoimmune diseases.

ADO production and the expression of ADO receptors are different between synovial and peripheral CD16$^-$CD56^{bright} NK cells from JIA patients

We next evaluated whether the loss of regulatory activity in SF CD16$^-$CD56^{bright} NK cells might be related to a different ADO production in these cells. ADO production from different substrates was evaluated at different time points using CD16$^-$CD56^{bright} NK cells from PB or SF of JIA patients. As shown in Fig. 5A, ADO production profile was similar in PB and SF NK cells using as substrate AMP (*upper left panel*) or ADPR (*upper right panel*). ADO production from AMP showed a peak at 5 min in both cell populations and then decreased, whereas a linear increase of ADO production from ADPR was detected.

A different behavior between PB and SF NK cells was observed using ATP as substrate, because SF NK cells displayed a peak of ADO production at 5 min, whereas PB NK cells showed a very low ADO production at 5 min, and then a linear increase after 30 min. Conversely, ADO production from NAD$^+$ in PB NK cells displayed a peak after 5 min and then increased again after 30 min, whereas in SF NK cells displayed a peak after 30 min and then rapidly decreased.

Taken together, these results suggest that CD73 and CD203a/PC-1 activities is similar in PB and SF NK cells. On the contrary, the activities run by CD38 and CD39 appears different. However, the ADO levels produced by both NK cell populations are high.

To analyze the impact of the classical (CD39 and CD73) and alternative (CD203a/PC-1 and CD73) ectoenzymatic pathway on ADO production by CD56^{bright} CD16$^-$ NK cells, we tested the consumption of ATP and the production of AMP in the presence of NaN_3 and POM-1, two CD39 inhibitors. As shown in Fig. 5B, ATP consumption and AMP production were only partially inhibited in the presence of NaN_3 (percentage of inhibition, mean ± SD: 35.6 ± 3.11 and 23.83 ± 3.58, respectively) or POM-1 (percentage of inhibition, mean ± SD: 37.25 ± 12.06 and 41.47 ± 2.07, respectively). These results indicate that CD203a/PC-1 is more efficient than CD39 in the conversion of ATP in AMP, the substrate used by CD73 for ADO generation by CD56^{bright}CD16$^-$ NK cells.

Finally, to test whether the lack of inhibitory activity of SF CD56^{bright}CD16$^-$ NK cells might be related to a different ex-

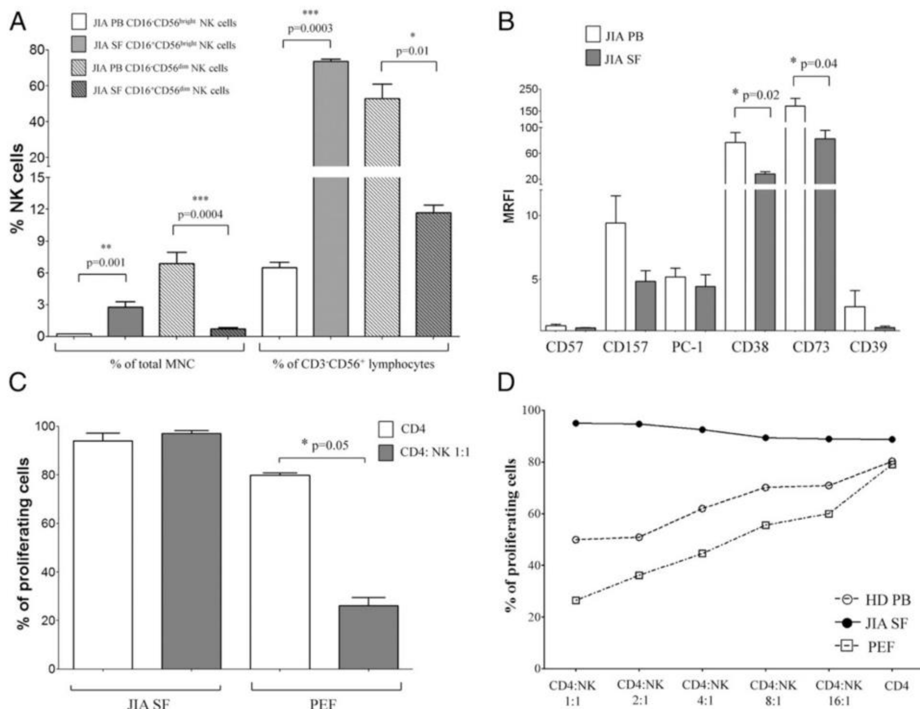

FIGURE 4. CD16^{-}CD56^{bright} NK cells from JIA patients' SF express lower levels of CD38/CD73 and fail to inhibit autologous CD4^{+} T cell proliferation. (**A**) The percentage of CD3^{-}CD16^{-}CD56^{bright} (empty bars) and CD3^{-}CD16^{+}CD56^{dim} (stripped bars) NK cells was assessed by flow cytometry on PB (white bars) and SF (gray bars) from JIA patients. Results are expressed as percentage of positive cells on total MNC or CD3^{-}CD56^{+} total NK cells. Mean of five different experiments ± SD is shown. The p values are indicated when differences are significant. (**B**) Expression of CD57, CD157, CD203a/PC-1, CD38, CD73, and CD39 was evaluated by flow cytometry on PB (open bars) and SF (gray bars) MNC from JIA patients, gating on CD3^{-}CD16^{-}CD56^{bright} NK cells. Results are expressed as MRFI. Mean of five different experiments ± SD is shown. The p values are indicated when differences are significant. (**C**) Proliferation of CD4^{+} T cells was analyzed by CFSE dilution after stimulation with anti-CD3/anti-CD28–coated beads in the presence (gray bar) or absence (open bar) of autologous CD16^{-}CD56^{bright} NK cells from SF or PEF at 1:1 NK/CD4 ratios. Results are expressed as percentage of proliferating cells. Mean of three different experiments ± SD is shown. (**D**) Proliferation of CD4^{+} T cells from JIA patients (●, continuous line), HD (○, dotted line), or patients with inflammation (□, dotted line) was analyzed by CFSE dilution after stimulation with anti-CD3/anti-CD28–coated beads in the presence or absence of autologous CD16^{-}CD56^{bright} NK cells (from SF, PB, or PEF, respectively) at different NK/CD4 ratios (ranging from 1:1 to 1:16). Results are expressed as percentage of proliferating cells. A representative experiment is shown.

pression of ADOR on such cells, we analyzed the expression of ADORA1, ADORA2a, and ADORA2b in PB and SF NK cells from JIA patients. As shown in Fig. 5C, the expression of such receptors was very low in these cell populations. However, PB NK cells display a significantly higher expression of ADORA1 (percentage of positive cells ± SD: 13.4 ± 7.8 versus 1.3 ± 1.3; p = 0.01) and ADORA2a (percentage of positive cells ± SD: 7 ± 4.3 versus 1.4 ± 0.7; p = 0.05) than SF NK cells. Conversely, the expression of ADORA2b was higher in SF NK cells than in PB NK cells (percentage of positive cells ± SD: 6 ± 3.9 versus 1.9 ± 2.3; p = 0.05).

Discussion

Considerable evidence suggests that CD16^{-}CD56^{bright} NK cells may be involved in the control of the immune responses, mainly through the production of anti-inflammatory molecules (7–11). Moreover, a correlation between the clinical success of anti-inflammatory treatments and the expansion of CD16^{-}CD56^{bright} NK cells has been described in patients with different autoimmune and inflammatory diseases (7, 9–11). Recently, novel immunoregulatory properties have been ascribed to this NK cell subset, including the ability to suppress autologous CD4^{+} T cell proliferation (12). However, the body of information available concerning the mechanisms involved in the "regulatory" properties of CD16^{-}CD56^{bright} NK cells is quite limited.

The original findings of the present study are that CD16^{+}CD56^{dim} and CD16^{-}CD56^{bright} NK cells display different patterns of ectoenzyme expression, which in turn may reflect their different roles in controlling the immune response. In detail, CD16^{+}CD56^{dim} NK cells virtually lack CD203a/PC-1 and CD39, and they express lower levels of CD73. CD203a/PC-1 is a key molecule in the alternative ADO generation pathway, because it may convert ADPR (generated by CD38 from NAD^{+}) or ATP to AMP (26, 27). CD39 is involved in a classical pathway independent of CD203a/PC-1, because the former molecule generates AMP from ATP and ADP (28). Both pathways converge to CD73, which converts AMP to ADO.

Consistent with data obtained by Laroni et al. (12) we found that CD16^{-}CD56^{bright} NK cells suppress autologous CD4^{+} T cell proliferation at 1:1 NK/CD4 ratio. Notably, our data demonstrated that CD16^{-}CD56^{bright} NK cells significantly inhibited CD4^{+} T cell proliferation also at lower NK/CD4 ratios (1:2 and

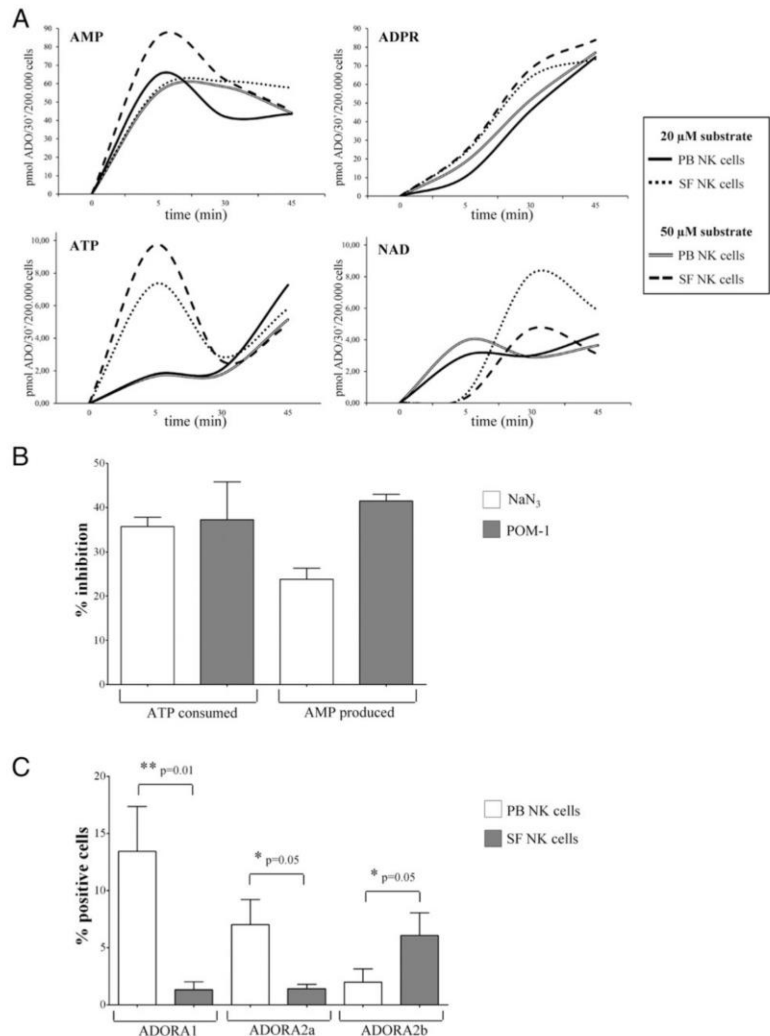

FIGURE 5. Production of ADO, consumption of ATP, and expression of ADOR in SF CD16^{-}CD56^{bright} NK cells. (**A**) Production of ADO was assessed on SF and PB CD16^{-}CD56^{bright} NK cells cultured in the presence of AMP, ADPR, ATP, or NAD^{+} (20 or 50 μM). ADO was measured in supernatants collected after 5, 30, or 45 min. Results are expressed as pmol ADO/30 min/2 × 10^5cells. (**B**) Consumption of ATP and production of AMP was evaluated on SF CD16^{-}CD56^{bright} NK cells, untreated or pretreated for 30 min with NaN_3 (black bars) or POM-1 (gray bars) and then cultured in the presence of ATP (50 μM). ATP and AMP were measured in supernatants collected after 30 min. Results are expressed percentage of inhibition. Mean of three different experiments ± SD is shown. (**C**) Expression of ADORA1, ADORA2a, and ADORA2b was evaluated on SF and PB MNC cells, gating on CD3^{-}CD16^{-}CD56^{bright} NK cells. Results are expressed as percentage of positive cells. Mean of five different experiments ± SD is shown. The *p* values are indicated when differences are significant.

1:4), which are closer to the physiological figures that can be achieved in secondary lymphoid organs or inflamed tissues.

We have demonstrated that CD16^{-}CD56^{bright} NK cells are able to secrete high amounts of ADO from different substrates. Among these, NAD^{+} is converted to AMP more efficiently than ATP, suggesting that CD38, rather than CD203a/PC-1, is the key molecule for ADO production in this context. This conclusion was reinforced by the finding that NK cell–mediated inhibition of autologous CD4^{+} T cell proliferation was dampened by kuromanin, a specific inhibitor of CD38. Such effect was not achieved using POM-1 or β-γ-meATP (specific inhibitors of CD39 and CD203a/PC-1, respectively). These results were not surprising, because we have demonstrated that ADO can be produced by CD16^{-}CD56^{bright} NK cells using either the canonical or the alternative pathway. Moreover, β-γ-meATP may also activate NK cells, either by interacting with P_2X receptor instead of inhibiting CD203a/PC-1 enzymatic activity (29), or inducing cAMP formation. Surprisingly α-β-meADP, a specific inhibitor of CD73, did not restore T cell proliferation. It is conceivable that, in our experimental conditions, the inhibition of CD73 was not total. Another possible explanation is the presence of alkaline phosphatase, which may act as a surrogate for the activity of CD73, leading to ADO production in the presence of CD73 inhibition (4).

CD16^{-}CD56^{bright} NK cells may function as regulatory cells in physiological and/or pathological conditions (such as cancer or immune deficiencies), and this feature may be dampened in autoimmune/inflammatory settings. This hypothesis was supported by the finding that CD16^{-}CD56^{bright} NK cells in PEF exert a potent regulatory activity. In contrast, CD16^{-}CD56^{bright} NK cells infiltrating the synovial tissue of patients with JIA displayed a lower expression of CD38 and CD73 (the key molecules for ADO production) than did their PB counterparts, and they failed to inhibit autologous CD4^{+} T cell proliferation. The lower expression of CD38 in synovial NK cells is apparently unrelated to a cleavage operated by metalloproteases, as confirmed by the absence of soluble CD38 in SF (data not shown). Despite lower CD38/CD73 expression, SF NK cells produced high amounts of ADO from different substrates. However, the kinetics of ADO

production from ATP or NAD$^+$ in PB was different from SF NK cells. NAD$^+$ was converted more rapidly in ADO by PB NK cells, thus reflecting that CD38 activity in PB is higher than that of SF NK cells. This feature may explain the defective immunosuppressive activity featured by SF NK cells. Another possible explanation might be related to the different expression of ADORA1, ADORA2a, and ADORA2b between PB and SF NK cells. Beavis et al. (30) have demonstrated that ADORA2a and ADORA2b display different signaling pathways and differential activity on NK cells. It is tempting to speculate that the higher expression of ADORA2b detected on SF NK cells may lead to an autocrine consumption of ADO produced by the latter cells, and it may cause a functional impairment, rendering them unable to inhibit autologous CD4$^+$ T cell proliferation.

In conclusion, this study has delineated a novel immunoregulatory function for CD16$^-$CD56^{bright} NK cells. Such function is related to the production of ADO by a complex network of ectoenzymes, a process in which CD38 plays a pivotal role, and may be altered during autoimmune/inflammatory diseases. These results may pave the way to characterization of the function of these cells in different pathological conditions, where the regulation of the normal immune response is altered.

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Disclosures

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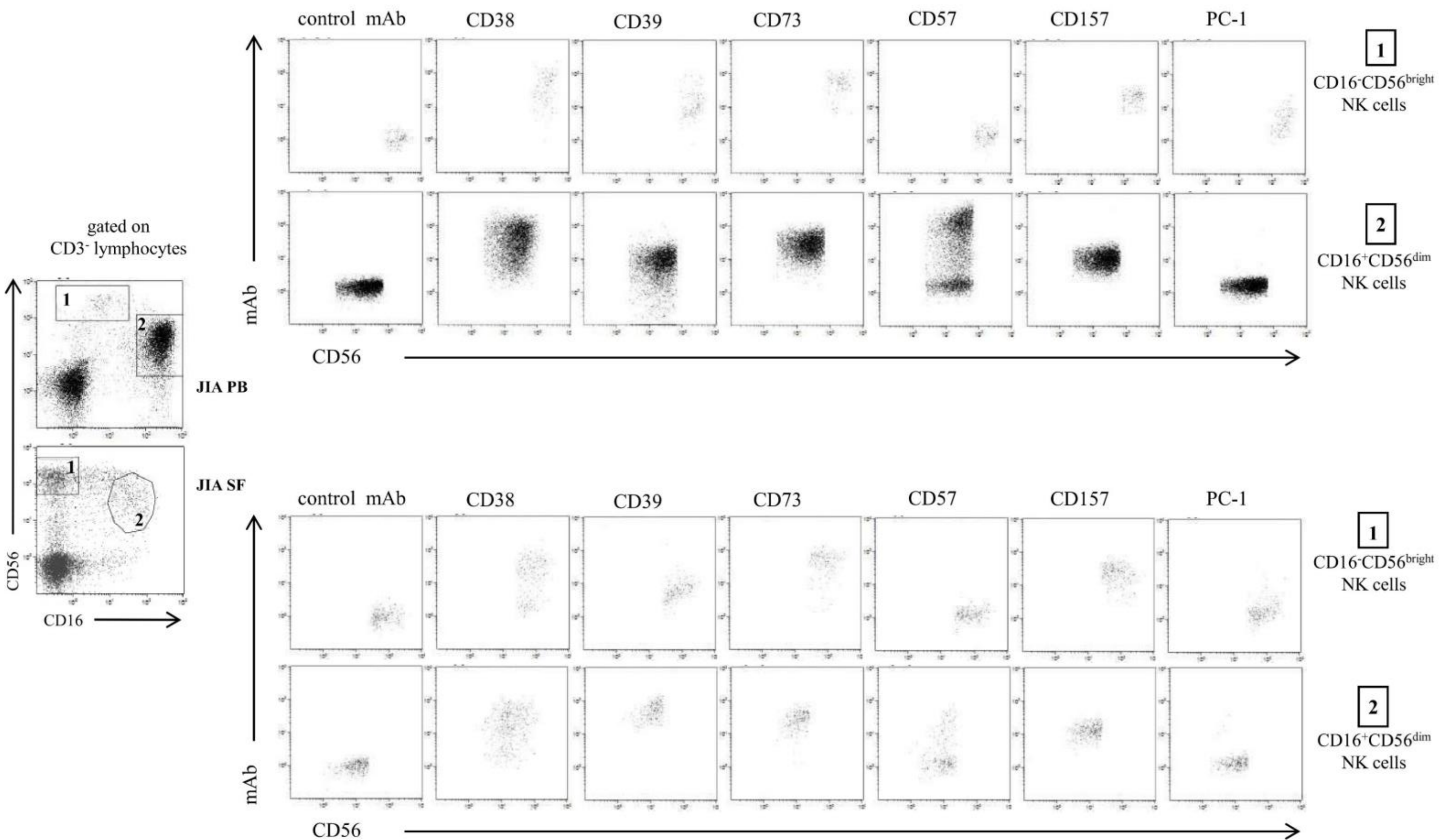

Supplemental Figure 1. Expression of ectoenzymes on NK cells from PB and SF of JIA patients

Expression of CD57, CD157, CD203a/PC-1, CD38, CD73 and CD39 was evaluated by flow cytometry on PB and SF MNC from JIA patients, gating on $CD3^{-}CD16^{-}CD56^{bright}$ (gate 1) or $CD3^{-}CD16^{+}CD56^{dim}$ (gate 2) NK cells. Dot plots show a representative experiment.