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Investigation of NK cell function and their modulation in different malignancies

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Abstract NK cells have become a subject of investigation not only in the field of tumor immunology and infectious diseases, but also within all aspects of immunology, such as transplantation, autoimmunity, and hypersensitivity. Our early studies aside from investigating NK cell activity in experimental animals and humans included studies of perforin expression and modulation in this lymphocyte subset. As NK cell activity is modified by their environment, we showed clinical stage–dependent impairment of their activity and in vitro effect of different sera, Th1 cytokines, and their combination in breast cancer, Hodgkin's disease, and non-Hodgkin's lymphoma patients, especially with respect to metabolic and cell membrane changes of peripheral blood lymphocytes evaluated by spontaneous release of the enzyme lactate dehydrogenase (LDH) that led to the correction of the LDH enzyme release assay for natural cytotoxicity. By long-term immuno-monitoring of patients with malignancies, we also showed the kinetics of NK cell modulation during chemo-immunotherapy. In our more recent studies, we give data of NK function and novel families of NK cell receptor expression in healthy individuals that may be of help in NK cell profiling, by giving referent values of basic and cytokine-induced expression of some NK cell receptors either in evaluation of disease or in immuno-monitoring during cytokine therapy of patients with malignancies. Moreover, we give novel aspects of modulation of NK cell activity by cytokines approved for immunotherapy, IFN and IL-2, in melanoma and other malignancies with respect to alterations in new activating (NKG2D and CD161) and inhibitory (CD158a and CD158b) receptor characteristics and signaling molecules in CD16- and CD56-defined NK cells and their small immunoregulatory and large cytotoxic subsets in peripheral blood and lymph nodes, as NK cell-mediated killing of tumor cells depends on the balance between stimulatory and inhibitory signaling.

Keywords NK cells · Malignancies · Perforin · Cytokine modulation · Activating and inhibitory receptors

Natural killer cells

During 1980s, early investigations have started on a newly defined subpopulation of peripheral blood lymphocytes, natural killer cells (NK cells), that have been shown to be large granular lymphocytes distinct from T and B cells and initially regarded as an "experimental artifact" in T-cell cytotoxicity assays. NK cells were shown to be important effectors of the innate immune system that have a unique ability to directly lyse transformed or virus-infected cells without prior sensitization or MHC class restriction. NK cells were first discovered in mice by Kiessling et al. [1], who named them natural killer cells and in parallel by Herberman et al. [2]. Human NK cells were initially described as non-adherent, non-phagocytic, CD16 (low affinity FcγR+), large granular lymphocytes [3]. The identification of the NKR-Pl and NK1.1 [4] made it possible to define the murine NK cells roughly as NK1.1+ TCR-sIg-CD16+. Today, human NK cell are defined as

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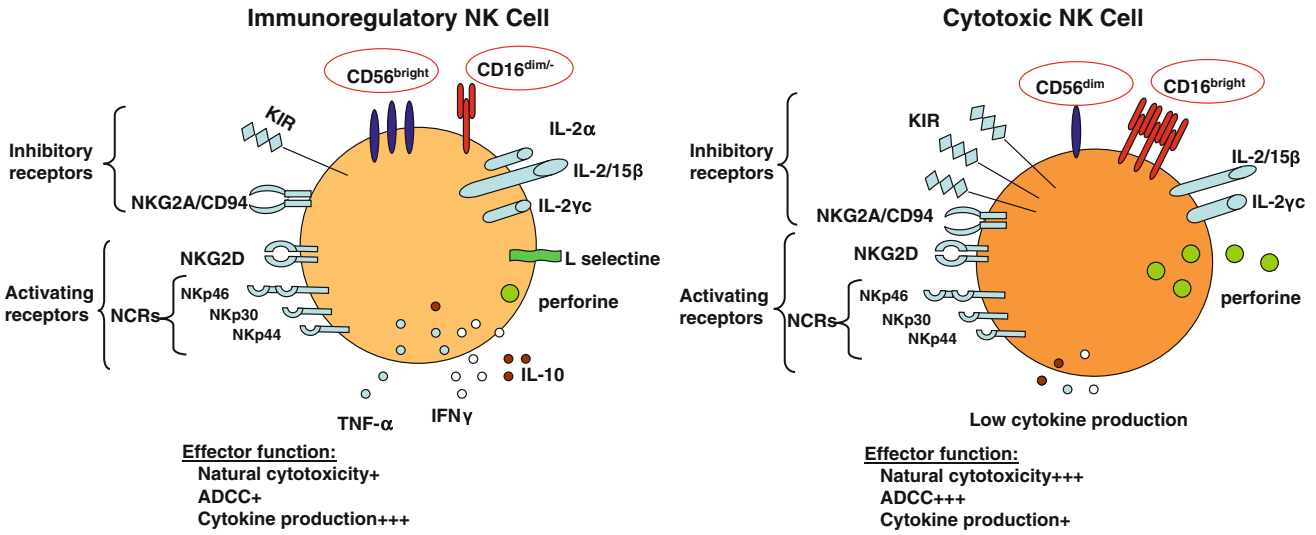

Fig. 1 Human NK cell subsets. $CD16^{dim}/CD56^{bright}$ NK cells are predominantly immunoregulatory cells that produce high levels of cytokines following monokine stimulation. This subset has low expression of CD16 and low natural cytotoxicity and ADCC. This subset expresses inhibitory NK cell receptors: low KIR, high NKG2A/CD94, and activating NK cell receptors: NKG2D and NCRs. The $CD16^{bright}/CD56^{dim}$ NK cells are essentially cytotoxic cells that produce low levels of cytokines in response to monokine stimulation. This subset has high expression of CD16 and high natural cytotoxicity and ADCC. This subset expresses inhibitory NK cell receptors: high KIR, NKG2A/CD94, and activating NK cell receptors: NKG2D, high NCRs, and perforin

$CD3^{-}16^{+}CD56^{+}$ lymphocytes [3]. They comprise 10-15% of all circulating lymphocytes and are also found in lymph nodes, spleen, and peripheral tissues. Resting NK cells circulate in the blood, but following activation by cytokines, they are capable of extravasation and infiltration into most tissues that contain malignant or pathogen-infected cells [5]. As NK cells are, therefore, engaged in the first line of defense against arising malignantly transformed cells, they came into focus of studies of both immunologists and oncologists (Fig. 1).

Now several reports show that NK cells also interfere with the shaping of the adaptive immune response [6]. Concerning primary responses, NK cells can meet dendritic cells (DC) in peripheral tissues, as well as in secondary lymphoid organs, and can act on them in two distinct ways [7]. Upon interaction, NK cells can kill immature DCs, thereby influencing DC homeostasis, but also potentially limiting DC-based vaccination efficacy. Conversely, the killing of target cells by NK cells can lead to the cross-presentation of antigens from apoptotic NK cell targets by subsets of DCs. This NK cell-mediated cytotoxicity of target cells induces robust antigen-specific adaptive immune responses involving $CD8^{+}$ T cells, $CD4^{+}$ T cells, and antibodies [8]. Recognition and killing of target cells by NK cells might thus provide a new and powerful strategy for vaccine development [9].

Therefore, after less than a hundred publications related to NK cells between 1975 and 1979, it was inconceivable that after 30 years, these cells will be a subject of investigation not only in the field of tumor immunology and infectious diseases, but also within all aspects of immunology, such as transplantation, autoimmunity, and hypersensitivity [10–12]. As the understanding of the molecular mechanisms governing NK cell activity increases, the expectations concerning NK cell-based immunotherapeutical approaches in cancer (in vivo modulation of NK cell activity, ex vivo purification/expansion, and adoptive transfer) grow [13].

We started investigating NK cells in the middle of 1980s and published first reports dealing with evaluation and modulation of NK and natural cytotoxic-NC cell function (Table 1) in various murine experimental strains and in humans [14, 15], and after that we proceeded with numerous basic and clinical aspects of NK cell investigation in oncology.

Our basic investigation regarding NK cells as important effectors of the innate immune system in the early 1990s was based on investigation of cytolytic molecule perforin [16]. Perforin plays a central role in lymphocyte-mediated cytolysis as it induces pore formation in the target cell membranes and their osmotic lysis. Only a few reports at that time demonstrated the direct presence of perforin in the murine system by an anti-mouse perforin monoclonal antibody (mAb) [17]. Concerning the expression of perforin protein in human cytotoxic cells, there existed very limited data, obtained with this same anti-mouse perforin mAb, and the detection of perforin was mostly performed in specific lymphocyte subsets, mostly in $CD8^{+}$ T cells and

Table 1 NK and NC cell activity of splenocytes in different strains of mice

	% Cytotoxicity	
	NK activity	NC activity
C57Bl/6	45.50	59.35
Balb c	49.90	41.47
C3H/HeJ	29.40	27.27
C2H/HeSn	34.37	26.33

% Cytotoxicity represents the mean activity of five tested animals of each strain [15]

much less in $CD56^{+}$ NK cells. Our study showed for the first time in human lymphocytes that, contrary to $CD8^{+}$ T cells that have low perforin level and up-regulate it after IL-2 treatment, NK cells constitutively contain perforin and express it in their cytotoxic $CD3^{-}CD56^{dim}$ and regulatory $CD3^{-}CD56^{bright}$ subsets (Fig. 2a, b) [18, 19].

Investigation of NK cells in different malignant diseases

In the following period, we extensively investigated NK cell cytotoxic activity in various malignancies including breast cancer, Hodgkin's disease (HD), non-Hodgkin's lymphoma (NHL), multiple myeloma, in solid tumors, that is, head and neck, ovarian cancer, thyroid cancer, and in melanoma (MM) in all clinical stages of disease using as targets for determination of NK cell cytotoxicity the standard NK cell-sensitive K562 erythromyeloid tumor cell line in the cytotoxicity assay, as well as many other cell lines [14, 20–24]. Considering that NK cells can detach from tumor cells and kill novel tumor cells [25], we found that determination of NK cell activity in malignancies is more reliable than their number. In this sense, we showed that breast cancer patients in all clinical stages of disease had significantly decreased NK cell activity and that it was most profound in advanced stage of this disease (Table 2; Fig. 3a) [14, 26, 27].

Modulation of NK cell activity of patients with malignancies

We showed that the treatment of both controls' and breast cancer patients' peripheral blood lymphocytes (PBL) with sera of patients with different clinical stages of this disease changed NK cell activity. The sera of the first three stages of breast cancer increased this activity, while sera of advanced disease caused a progressive decrease compared to control treatments with fetal calf serum (FCS). These results indicated the presence of inhibitory factors in sera

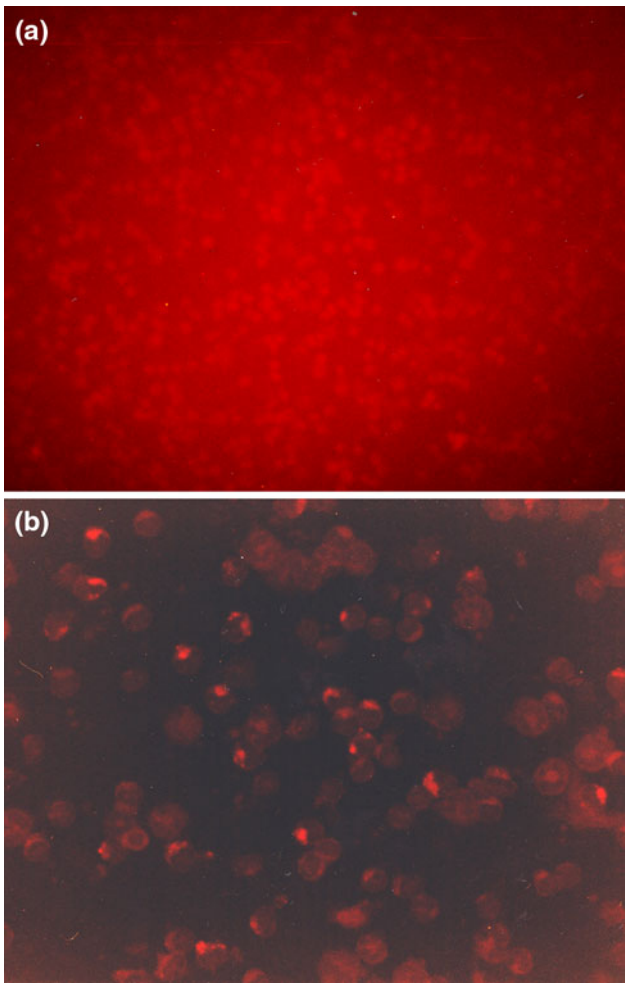

Fig. 2 Immunocytochemical staining of freshly isolated PBL from a healthy individual, using the anti human perforin monoclonal antibody, anti-P1. **a** Negative control, freshly isolated peripheral blood mononuclear cells treated with mouse IgG/biotin and SA/PE, X100. **b** Fresh PBL treated with anti P1-biotin and SA-PE are 23% perforin positive cells generally showing patchy staining and finer, granular staining, ×200

of patients with advanced disease. Moreover, as the sera of patients in advanced clinical stage gave a similar degree of inhibition of NK cell function of both patients and healthy controls, we determined, by dialysis, serum fraction that was responsible for this effect [20, 28–32]. We showed that as this serum fraction retained molecules from 8 to 12 kDa, that this could be the effect of TGFβ or IL-10, and not sIL-2 receptors, as they were rarely increased in sera [33], as well as the fact that sera interfered in a reversible manner with NK cell activation by rhIL-2 (Fig. 3b), [30, 33]. Further investigation showed that the inhibitory effect of the pooled metastatic breast cancer sera was not due to TNFα, as PBL treatment with TNFα alone or in combination with IL-2 did not significantly alter NK cell activation. Blocking of TNFα during IL-2 stimulation also did not

Table 2 NK cell activity in breast cancer patients in different clinical stages of disease

NK cell activity	Clinical stage of disease	*n*	NK cell* cytotoxicity (%)
Healthy controls		18	57.75 ± 2.21
Breast cancer	I–III	77	36.64 ± 1.92[a]
Patients	IV	11	18.31 ± 1.38[b]

* NK cell cytotoxicity estimated for effector to target ratio (E:T) 80:1

[a] Statistically significant ($p \leq 0.01$, Mann Whitney test) decrease compared to healthy controls

[b] Statistically significant ($p \leq 0.01$, Mann Whitney test) decrease compared to patients in I-III clinical stage

result in a significant change in NK cell potentiation in either cancer patients or healthy controls [33].

Our investigation showed that in vitro treatments of PBL with IL-2, IL-7, and IL-12 stimulated less NK cell activity of breast cancer patients than healthy controls, while the greatest enhancement in both groups was obtained with IL-2 (Fig. 3c) [30]. Our subsequent studies indicated that PBL of breast cancer patients, compared to controls, express lower level of pSTAT1, 3 and 5, and that pSTAT1 and IFNγ levels were low in T and NK cell subsets of these patients suggesting their association with decreased NK cell cytotoxicity that is associated with the progression of the disease (Fig. 3d) [34, 35].

Breast cancer and NK cell activity relevant to immunotherapy

We did not find any difference in the number and functional capacity of T and B lymphocytes, macrophages, or NK cell activity between breast cancer patients with primary solitary and asynchronous bilateral breast cancer, despite indications of risk factors of genetic, immunological, and endocrine nature [36]. As breast cancer belongs to the group of hormone-dependent tumors [37], we found that NK cell activity in patients with either solitary or bilateral tumors was influenced by the hormone status of the tumor, as the activity was decreased in breast cancer patients with functional estrogen receptors (ER) defined by the expression of progesterone receptors (PR) [38–40], probably owing to the unfavorable effect of estrogen on

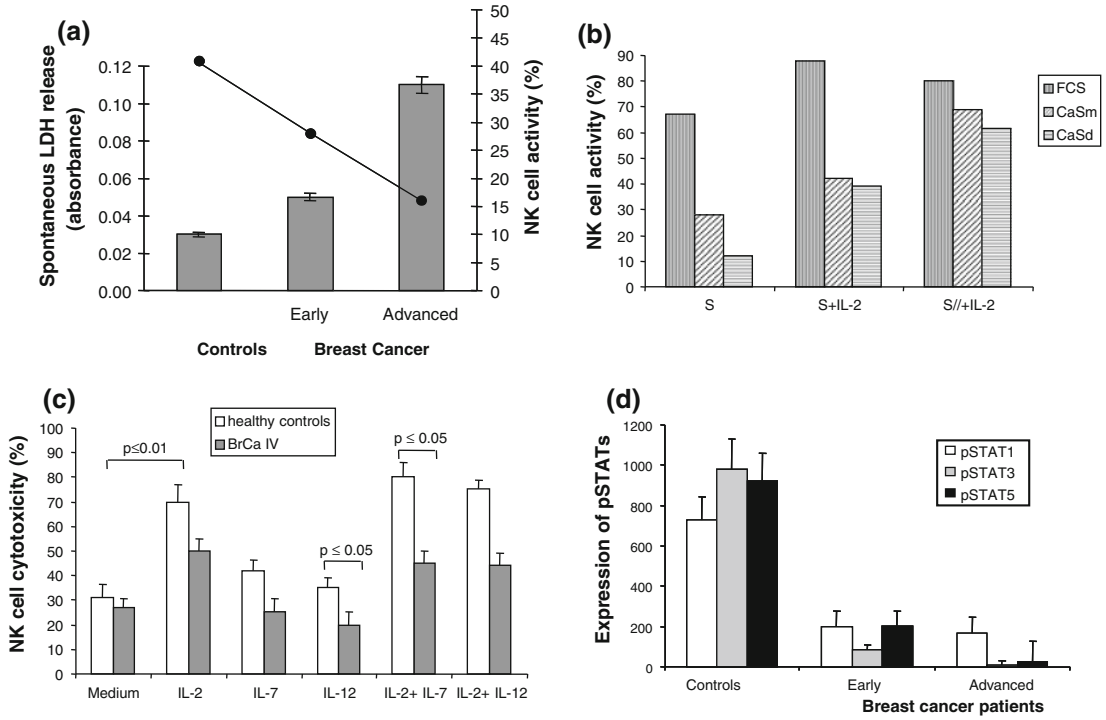

Fig. 3 Investigation of NK cell activity in breast cancer patients. **a** The relationship between NK cell activity (*filled circle*) and spontaneous PBL LDH release activity (mean values ± SE) for healthy controls and breast cancer patients given with respect to early and advanced stage of disease [22]. **b** Effect of sera of breast cancer patients in clinical stage IV, before (CaSm) and after (CaSd) dialysis, on NK cell activity of healthy individuals. Results represent NK cell activity after treatments with sera (S) alone, together with IL-2 (S + IL-2) and IL-2 treatments after removal of sera after 4 h treatments (S// + IL-2) [32]. **c** The effect of rhIL-2, IL-7, or IL-12, or their combination, on NK cell activity of healthy controls and breast cancer patients in clinical stage IV (BrCa IV) [33]. **d** PBL of breast cancer patients compared to controls express lower baseline level of pSTAT1, pSTAT3, and pSTAT5 [34]

immune reactivity, including suppression of NK cell activity by down-regulation of NK cells activating receptors–CD69, NKp46, NKG2D, and 2B4 (CD244), which directly inhibits NK cell activation, resulting from the reduced secretion of the soluble factors granzyme B, as well as FasL [41].

Her-2 characteristics of breast cancer

HER2 (also termed Her-2/neu), a 185-kDa transmembrane glycoprotein encoded by the ERBB-2 proto-oncogene, can be regarded as a "prototype" for this type of tumor antigen. HER2 belongs to the epidermal growth factor receptor (EGFR) family, with four closely related surface receptors (HER-1 to HER-4) [42]. Both in vitro and animal studies showed that HER2 plays a key role in oncogenic transformation and tumorigenesis, tumor progression, and metastatic potential through interaction with other members of the EGFR family, leading to more potent intracellular signaling [43]. Amplification and/or overexpression of HER2 have been shown at a relatively high frequency in both cell lines and biopsies derived from a wide range of human cancers of distinct histology. Clinical efficacy of the HER2-specific humanized monoclonal antibody (mAb) trastuzumab in combination with chemotherapy or as a single drug has recently been heralded as a "triumph" for targeted cancer therapy.

Passive immunotherapy with monoclonal antibodies against Her-2/neu receptor

Novel classification of breast cancer according to growth factor, Her-2/neu receptor expression [44], suggests that NK cells are of crucial importance for the effect of passive immunotherapy with monoclonal antibodies, trastuzumab, as by binding through their characteristic CD16 antigen, cytotoxic NK cell subsets expressing high level of this FcγRIII have been considered as the main ADCC cellular effector to the antibodies bound to Her-2/neu-positive tumor cells [45]. When bound to their antigen on the cell surface, antitumor mAbs can recruit various FcγR-expressing immune cells present in the tumor microenvironment through their Fc region, triggering FcγR-dependent mechanisms of action. Subsequent tumor cell lysis is likely to allow the formation of immune complexes made of mAb and tumor-associated antigen, eventually followed by endocytosis, also through FcγR-expressing cells. Therefore, FcγR-expressing cells play a dual role, since they include not only potent cytotoxic cells, such as NK cells, but also DCs that by becoming activated allow the recruitment of specific antitumor T cells, possibly leading also to the generation of an adaptive immune response [46].

Active immunotherapy with Her-2 tumor vaccines

Efforts have spurred to actively induce a protective immunity against HER2-expressing tumors with vaccines based on peptides, plasmid DNA (pDNA), or recombinant proteins [47]. HER2 tumor vaccines have now also reached the clinic with several phase I and II studies based on different approaches. These studies show that HER2 can be immunogenic and also suggest an improved clinical outcome as the result of the vaccination. Experimental data provide strong evidence that antibodies alone are not sufficient and specifically point at the role of NK cells in the vaccine-induced HER2-specific tumor rejection. Mice depleted of NK cells through repeated administration of anti-asialo-GM1 antibody lost their ability to reject HER2-expressing tumor cells [48]. Novel clinical trials that combine trastuzumab with emtansine, a human (HER2 receptor)-targeted antibody–drug conjugate, composed of trastuzumab and the potent cytotoxic agent DM1 derivative of maytansine in phase III development for HER2-positive cancer in disease settings such as early stage HER2-positive breast cancer, are also under investigation. [48]. Patients with metastatic HER2-positive breast cancer are treated with IL-2 and vaccinated with a plasmid DNA encoding HER-2/neu together with low doses of GM-CSF that can induce long-lasting cellular and humoral immune responses, including NK cell activation and lysis of breast cancer cells [47, 49].

Modification of methods for evaluation of NK cell activity

We investigated NK cell function by three different methods: by standard 51-chromium release assay, by the original colorimetric uncorrected lactate dehydrogenase (LDH) release assay [50], and by corrected LDH release assay [51] in breast cancer patients in different clinical stages of disease prior to therapy. As a discrepancy appeared between the assays, with the original LDH assay showing, not only, much higher values, but no stage-dependent depression in NK cell activity compared to the chromium release assay, our further analyses of separately cultured PBL revealed that this difference arose from an increasing, clinical stage-dependent, spontaneous LDH release from PBL of breast cancer patients (Fig. 3a). Furthermore, a stage-dependent increase in intracellular LDH activity of PBL was also found, although without difference in LDH-H and LDH-M isotype ratio, compared to controls.

Therefore, correction of the original LDH release assay for the spontaneous LDH release activity from PBL present in the assay gave values of NK cell activity comparable to those determined by the chromium assay and indicated that breast cancer patients have significant depression in NK cell activity, which correlates with the stage-dependent increase in spontaneous LDH release from PBL (Fig. 3a). Moreover, as both assays measure the secretory, perforin-mediated, NK cell cytotoxic pathway against tumor cells, it can be concluded that the appearance of spontaneous LDH release is an indicator of cell membrane damage that not only allows the loss of LDH, but also the components of the secretory killing pathway, resulting in NK cell dysfunction with the progression of disease [22].

The novel findings obtained in this work revealed alterations characteristic of cell membrane damage of PBL and its association with clinical stage of disease that can, aside from reflecting NK cell dysfunction, also underlie the defect in other PBL subsets and subsequently facilitate progression of the malignant process. This finding could be the consequence of the established cytokine imbalance in advanced malignancies [22]. Moreover, we have shown that TNFα induces early alterations in membrane integrity that lead to an increase in spontaneous LDH release, not only from treated PBL but also from tumor cell lines [52–54]. Our further studies of the role of TNFα in this process indicated its unfavorable effect on membrane integrity, not only in healthy controls [54, 55], but also in patients with malignancies, other than breast cancer [52].

NK cell dysfunction and its association with LDH release from PBL

In patients with malignant lymphomas among biochemical parameters, LDH represents a very valuable enzyme, and its serum level has been considered as very important in the evaluation of disease extension in Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL) [56]. Considering that elevation of serum LDH level correlates with bulky tumor mass, it represents an important independent prognostic factor for patients with lymphomas. Evaluation of LDH owing to its very well–defined structural and kinetic characteristics gives valuable data considering cellar metabolic status. In that sense, analyses of the intracellular ratio of LDH isotypes, H to M, indicate the direction of pyruvate–lactate conversion and give data whether glycolysis is of aerobic or anaerobic type [57, 58]. In this sense, aside from very useful measurements of serum LDH level, we showed that the intracellular PBL characteristics of this enzyme and its isotype profile are very sensitive indicators of the cellular metabolic state, aerobic or anaerobic direction of glycolysis, activation status, malignant transformation, as well as membrane damage. Based on this in our studies, we investigated all of these parameters relevant to LDH enzyme activity and evaluated these findings with respect to clinical and histological forms of these lymphoproliferative diseases.

Our investigations gave novel data indicating that PBL in numerous solid tumors and hematological malignancies, compared to healthy individuals, displayed a significant increase in spontaneous LDH release activity, which correlated with advanced clinical stage in all malignancies except in Hodgkin's disease, in which spontaneous LDH release was always increased as in patients in advanced clinical stages of breast, cervical, ovarian, thyroid cancer, and head and neck tumors [59]. Contrary to this, the total LDH activity was not increased in PBL in all investigated patients. However, we analyzed what part of total intracellular LDH activity constituted spontaneous LDH release in HD and NHL patients in all clinical stages and found that, except in HD, they had a clinical stage-dependent increase in spontaneous LDH release activity. The "percent of spontaneous LDH release" (percent of spontaneous LDH release = spontaneous LDH release from PBL/total intracellular PBL LDH activity × 100) was always increased in patients with these malignancies, regardless of the total intracellular LDH activity, indicating that spontaneous LDH release is independent of intracellular LDH and that it is actually the consequence of PBL membrane damage present in advanced stages of different solid tumors [52, 53, 60–62] that contributes to NK cell dysfunction.

Hodgkin's disease, non-Hodgkin's lymphoma and NK cell activity

Considering the lymphoid origin of malignant lymphomas and NK cells and that it has been established that the strongest known risk factors for lymphomas are immunosuppressed states, inherited or acquired, as well as scarce and contradictory data concerning NK cell function in these neoplasms in the early 1990s that did not give sufficient evidence with relation to histology or clinical stage of these diseases [63], we evaluated NK cell activity in NHL and HD patients, prior to therapy. We found by the lactate acid dehydrogenase (LDH) release cytotoxicity assay that decreased NK cell activity in NHL patients was essentially related to unfavorable histology, with the lowest activity in very aggressive forms, although within histological categories, clinical stage of the disease also contributed to the degree of NK cell dysfunction. In contrast, in HD, NK cell activity was persistently decreased compared to controls, irrespective of histological type and clinical stage (Fig. 4a). It is of interest also that the most

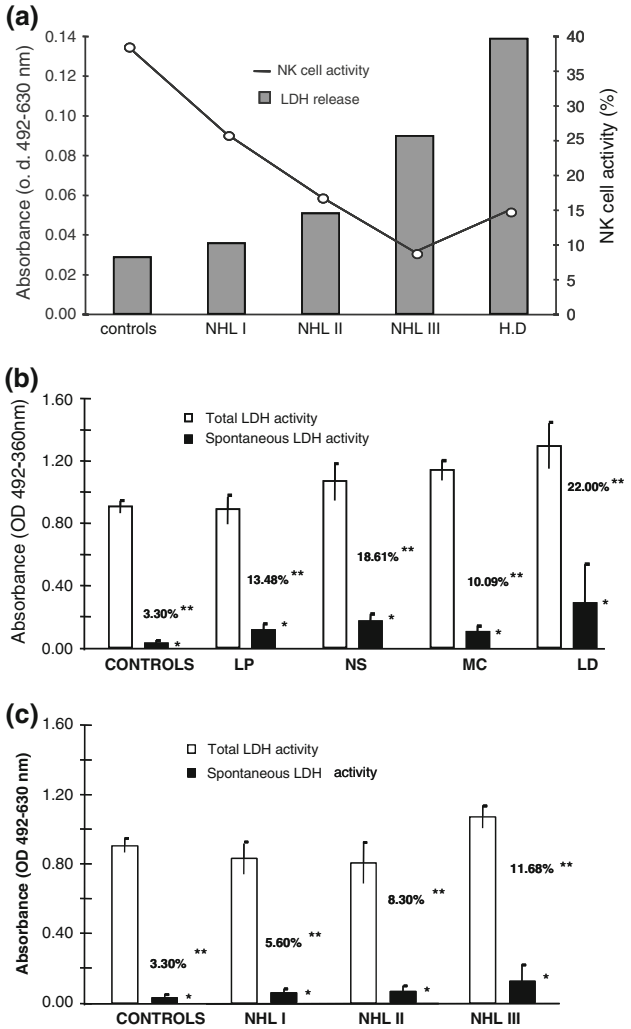

Fig. 4 Investigation of NK cell activity in NHL and HD patients. **a** The relationship between NK cell activity (mean values) and spontaneous PBL LDH release activity (mean values) for healthy controls, NHL patients given with respect to histological grade of malignancy (low grade: NHL I; intermediate grade: NHL II; high grade: NHL III) and HD patients [64]. Spontaneous LDH release activity of PBL is given as mean value ± SEM and, together with percentage of spontaneous release (% values), is significantly (* $p < 0.01$, ANOVA; ** $p \leq 0.05$, Mann Whitney test, respectively) increased in **b** HD (LP, NS, MC and LD are histological types of HD) and **c** NHL patients analyzed according to histological type and compared to controls [60]

profound NK cell dysfunction that is present and persistent from the onset of HD, and that is present in very aggressive NHL, was associated with the phenomenon of increased spontaneous LDH release activity from peripheral blood mononuclear cells (PBMC) of these patients [64].

Aside from the results of NK cell activity in hematological malignancies that showed the most profound NK cell dysfunction in HD and in very aggressive NHL, as well as in breast cancer patients with solitary or bilateral tumors and multiple myeloma patients [65, 66], we showed that impaired NK cell activity was associated with increased spontaneous release activity of LDH from patients' PBL. Spontaneous release activity of LDH indicates cell membrane damage that by allowing release of cytotoxic proteins can be responsible for impaired NK cell activity. Moreover, elevation of spontaneous LDH release precedes serum LDH increase in HD and NHL. The results obtained regarding enzyme LDH activity of PBL, LDH isotype H and M pattern [67], and PBL spontaneous LDH release activity in NHL, HD patients, and controls indicated that intracellular LDH-H and LDH-M isotype activity of PBL, and their spontaneous LDH release activity significantly increase in NHL with progressing histological grade of malignancy. Contrary to this, all classical HD patients had a significant elevation of each of these parameters. Furthermore, unlike HD, in NHL, increase in the level of spontaneous LDH release activity in each histological form was associated with increasing clinical stage of disease (Fig. 4b, c).We also showed that spontaneous LDH release activity of PBL for HD and NHL patients demonstrates positive correlation with serum LDH level, although elevation of spontaneous LDH release proceeds serum LDH increase in both diseases [60].

New families of NK cell receptors evaluated in healthy individuals

NK cell phenotype and function have been previously almost exclusively defined with respect to the expression of CD56, an adhesion molecule that is not directly involved in cytotoxicity, and not with respect to CD16 expression [68]. However, CD16 is one of the most important cytotoxic receptors [69] that is involved in both direct and in antibody-dependent, cellular cytotoxicity (ADCC) [70], cytokine production, proliferation, as well as post-activational NK cells apoptotic death [71]. Moreover, it has been established that CD16, as well as another prominent NK cytotoxic receptor, NKp46 mediate direct killing, that is, lysis of some virus-infected and tumor cells [72]. CD16, as well as NK p46, associates with two cytoplasmatic domains composed of FcεRIγ or TCRζ chains that comprise the immunoreceptor tyrosine-based activation motifs (ITAM), which upon ligand binding become phosphorylated and induce signal transduction by activation of non-receptor tyrosine kineses syk and ZAP-70 [73].

Based on CD16 cell surface expression, NK cells may be divided into two subsets that are functionally analogous to CD56 subsets [68]. In this sense, $CD56^{dim}$ NK cells have high expression of CD16 and are defined as $CD16^{bright}$, while $CD56^{bright}$ NK cells have low expression of CD16 antigen and are defined as $CD16^{dim}$ subset. In light of this, $CD16^{bright}$, like $CD56^{dim}$ subset, has a high expression of

the main cytotoxic receptor CD16, contains perforin and granzyme granules and is involved in cytotoxicity, while $CD16^{dim}$, like $CD56^{bright}$, subset has a regulatory function and produces abundant cytokines (IFN-γ, TNF-α, IL-10, IL-13, and GM-CSF) [24]. Based on our data obtained on PBL of healthy individuals, we have shown for the first time baseline level of CD16-based NK cell subset distribution with the average percentage of 78.97% for cytotoxic $CD3^{-}CD16^{bright}$ NK subset and 21.03% for the regulatory $CD3^{-}CD16^{dim}$ NK subset [74].

Despite the fact that formation of NK cell conjugates with tumor cells is a prerequisite for direct cytotoxicity, that is, necrosis, characterized by exocytosis of perforin and granzyme granules, NK-K562 target tumor cell conjugates have rarely been characterized and only with respect to $CD56^{+}$ NK cells [75], and not with respect to CD16-defined NK cells. We demonstrated for the first time not only that $CD16^{bright}$ NK cells have stronger target binding capacity, but our kinetic study also shows that this conjugate composition changes with time, so that after a longer (4h) follow-up, the conjugates lose $CD16^{bright}$ in favor of $CD16^{dim}$ NK cells [74]. These data confirm that following secretion of cytotoxic granules, NK cells become $CD16^{dim}$ or even CD16 negative, as suggested in one previous report [76].

Our findings indicate also that the expression of a degranulation marker, CD107a, when evaluated after 30 min. and 4 h of PBL and K562 in vitro contact shows time-dependent increase on $CD3^{-}CD16+$ NK cell population, and on both $CD3^{-}CD16^{bright}$ and $CD3^{-}CD16^{dim}$ NK cell subsets. Moreover, besides greater cytotoxic potential of the $CD3^{-}CD16^{bright}$ NK subset, we have shown that expression of CD107a, a protein associated with the release of lytic lysosomal vesicles containing perforin, is strongly up-regulated on this subset after cytotoxic degranulation, as well as stronger conjugate-binding capacity of $CD3^{-}CD16^{bright}$ NK subset with K562 cell line [74].

Several new families of activating and inhibitory receptors have been recently identified on NK cells, including natural cytotoxicity receptors (NCR), c-lectin-like receptors, and killer immunoglobulin-like receptors (KIR), and it has been shown that NK cell activity is determined by the balance of these activating and inhibitory receptors [77]. One of the most prominent activating receptors, NKG2D [78], is a c-type lectin that upon binding stress-induced ligands on malignantly transformed cells, such as MHC class I-related molecules, MICA/MICB, and UL16-binding protein (ULBP) [79], induces cytotoxicity by recruiting phosphoinositide (PI)-3-kinases after association of its intracellular domain with DAP10 adaptor protein. We have shown that there is a significant positive correlation of NKG2D expression with NK cytotoxicity in healthy individuals, as well as in melanoma patients, in spite of changes in the expression of this receptor [27, 74]. In this sense, we have given new data showing that conjugate formation of NK cells of healthy individuals, and, moreover, advanced melanoma patients with decreased NK cell NKG2D activating receptor expression, and K562 or FemX tumor cells, down-regulates NKG2D expression [27, 74], indicating that NK cell contact with tumor cells, that is, melanoma, causes un unfavorable decrease in the expression of this important activating receptor. However, although NKG2D-mediated responses are beneficial in immune responses against tumors and pathogens, evidence is also emerging that this system may be deleterious by contributing to autoimmunity [80].

Also, one of the earliest markers of NK cells, CD161 (mouse NKR-P1A analogue) [81], encoded by a single non-polymorphic gene without precisely defined signaling pathway in humans is primarily designated as an activating receptor [82], even though recently, upon identification of its lectin-like transcript 1 (LLT1) ligand, its inhibitory potential has been introduced, although it remains controversial [83]. Our thorough investigation in healthy individuals indicates similar expression of the two activating receptors NKG2D and CD161 on both $CD3^{-}CD16^{dim}$ and $CD3^{-}CD16^{bright}$ NK subsets [74].

It has been shown that the novel NK cell KIR repertoire depends on both KIR and HLA gene polymorphisms [84]. Clinical studies have correlated KIR gene content with infection, cancer, autoimmunity, pregnancy syndromes, and transplant outcome [85]. Consequently, differential expression of these inhibitory receptors by subsets of human NK cells allows them to carefully monitor self (and foreign)-MHC molecules and uniquely regulate cytotoxicity when pathological processes perturb MHC expression [80]. KIR is divided into haplotype A and B, with a more frequent A haplotype that includes inhibitory CD158a (KIR2DL1) and CD158b (KIR2DL2,3) receptors [86] that recognize HLA-Cw 4,5,6 and HLA-Cw1,3,7 class I molecules, respectively [87]. These two KIRs inhibit NK cell activity through an immune tyrosine-based inhibitory motif (ITIM) by recruiting protein tyrosine phosphatases (SHP-1 and SHP-2) responsible for dephosphorylation of surrounding tyrosine kinases and adaptor proteins, including DAP-10. Furthermore, by blocking actin cytoskeleton-dependent raft recruitment of different receptors may be a general mechanism by which inhibitory receptors control NK cell activation [88]. Moreover, it has recently been shown that higher expression of CD158b on NK cells is implicated in greater susceptibility to melanoma and its metastasis [89].

We have shown for the first time in healthy individuals the distribution and density of a representative set of NK cell–activating NKG2D, CD161, and inhibitory CD158a

and CD158b receptors on $CD3^{-}CD16^{+}$ NK cells in PBL, on gated NK cells, as well as on $CD3^{-}CD16^{bright}$ and $CD3^{-}CD16^{dim}$ subsets. Our detailed analysis showed, contrary to CD158a and CD158b KIRs, a significant positive correlation of NKG2D and CD161 expression with NK cytotoxicity [74]. Considering that changes in receptor expression modify NK cell activity, these novel data may be useful in detecting predisposition for or existence of various diseases, as there is an urgent need for referent values for NK cell receptors since their changes could confer altered risk in a number of diseases including human immunodeficiency virus (HIV) susceptibility and progression, hepatitis C virus clearance, idiopathic bronchiectasis, autoimmunity, and cancer. Moreover, receptors as NKG2D, that are beneficial in surveillance against cancer and infections, while deleterious as in the development of autoimmune disorders, could also, together with their ligands, become targets for therapeutic intervention.

Melanoma and NK cell activity in association with NK receptor expression

We have been intensively investigating melanoma patients before and during the course of different chemo-immunotherapy, as this is the most lethal form of skin cancer, despite its immunogenicity. Although melanoma antigens do elicit desirable T-cell response, a significant role in the pathogenesis and clinical course of the disease, due to the down-regulation of HLA class I on melanoma cells, is mediated by NK cells, as NK cells preferentially target tumor cells with low HLA class I expression. This alternative NK cell recognition system, which benefits from HLA down-regulation on tumor cells, combined with their innate antitumor cytotoxic potential, offers a valid option for monitoring, as well as their use in adoptive cell therapy in this malignancy, in spite of the new findings that immunoselection can generate malignant cells with changes in NK cell–activating ligands that can escape these cells with a negative impact on the clinical course of the disease [90].

In this sense, we have shown tumor-induced suppression of NK cell cytotoxic activity (Fig. 5) and clinical stage-dependent decrease in the percentage of NK cells in melanoma patients, defined as $CD3^{-}CD16^{+}$ [91], with lowest values in metastatic disease. In agreement with suppressed NK cell activity in MM patients estimated against K562, FemX, HeLa, and Daudi target tumor cells and, accordingly, lower expression of CD107a marker of cytotoxic degranulation, we have shown an unfavorable distribution of NK cell subsets consisting of a reduction in the main effector cytotoxic $CD16^{bight}$ subset [92]. Despite this found redistribution of NK cell subsets with an increase in $CD16^{dim}$ immunoregulatory subset in MM patients, IFNγ

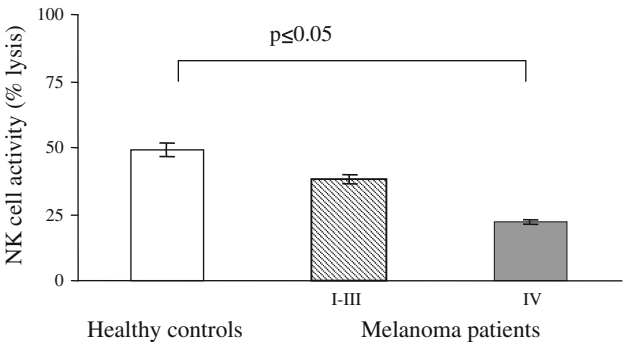

Fig. 5 Investigation of NK cell activity in melanoma patients. NK cell-specific lysis of healthy controls, MM patients (clinical stage I–III, $n = 9$), and MM patients (clinical stage IV, $n = 79$) evaluated against K562 tumor target cell line (E:T, 80:1), shows significant (* $p \leq 0.05$, Mann–Whitney test, respectively) impairment in NK cell activity in patients in clinical stage IV [91]

production was decreased. In this sense, the observed decrease in $CD3^{-}CD16^{bight}$ NK cell subset in MM patients, based on some novel findings of the rapid IFNγ production upon NK cell activation in $CD3^{-}CD56^{dim}$, that is, $CD3^{-}CD16^{bight}$, NK cell subset preferentially involved in NK cell cytotoxicity [93], along with the established tumor-derived production of immuno-suppressive cytokines (TGF_{β}, IL-10, etc.) might contribute to the found decreased IFNγ and TNF-α production in metastatic disease, cytokines that are also engaged in the immuno-regulatory role of NK cells [27].

Our investigation demonstrated that MM patients not only had significantly decreased NK activity and NK cell IFN-γ production, but also a redistribution of NK cell subsets characterized by an increase in $CD16^{dim}$ and a reduction in $CD16^{bright}$ cells. Based on our novel findings for healthy individuals of the baseline level of distribution and density (MFIR) of a representative set of newly defined activating (NKG2D, CD161) and inhibitory (CD158a, CD158b) KIR receptors on $CD3^{-}CD16^{+}$ NK cells and their two subsets in peripheral blood (Table 3) [74], our extensive studies in MM patients showed that they have a decrease in NKG2D and CD161 and an increase in the expression of CD158a and CD158b KIR receptors on $CD3^{-}CD16^{+}$ (Fig. 6a) [89] or $CD3^{-}CD56^{+}$-defined NK cells [27]. A positive correlation was found between NKG2D expression and NK cytotoxicity, while we found a negative correlation between CD158b expression and NK cell cytotoxicity. Similarly, we also reported new data that patients with multiple myeloma had decreased NK cell activity that was associated with an increase in inhibitory KIR CD158a expression on NK cells of these patients [65]. Therefore, NKG2D, CD158a, and CD158b expression on NK cells in these patients may represent several clinically useful "biomarkers" of suppressed NK function in malignancies [92].

Modulation of NK cell activity in melanoma and other malignancies

The activity of NK cells can be modulated by a range of soluble factors, including type I interferons, IL-2, IL-12, IL-15, IL-18, and IL-21 [33, 61]. IFN-α enhances innate immune antitumor activity by inducing NK cell proliferation [94] and cytotoxicity [95, 96], as well as the regulatory Th1-cytokine (IFNγ, IL-2) production that enhances favorable, cell-mediated, antitumor reactions [97, 98]. In this sense, it has been shown that immunoregulatory activities of IFN-α, used in adjuvant immunotherapy of high-risk melanoma, are mediated by a set of cellular genes [99] responsible for its biological effects, including STAT1 (signal transducers and activators of transcription 1) and STAT2 [100, 101]. When phosphorylated, these STATs induce transcription of various IFN target genes, including interferon-regulating transcription factor-1 (IRF-1) that affects the cell cycle, induces cytokine secretion, and subsequently, through perforin and FasL synthesis, up-regulate NK cell cytotxicity [102, 103].

Another important cytokine, IL-2, also approved in metastatic melanoma therapy, facilitates NK cell–mediated antitumor cytotoxicity, as well as the production of Th1 cytokines. Signal transduction mediated by IL-2 involves STAT3 and more importantly STAT5 [104] that lead to many of IL-2 functional effects. In this sense, as IFN-α and IL-2 are crucial cytokines that are involved in NK cell activation against tumor cells, our studies have shown that NK cell cytotoxicity of MM patients, prior to therapy, is augmented in in vitro PBL treatments by these cytokines. Up-regulation of NK cell activity with IFN-α and IL-2 has been previously shown for healthy controls [22, 105, 106] and for patients with malignancies, including solid tumors and hematological malignancies [105–107]. However, studies showing up-regulation of NK cell activity in melanoma by IFN-α [95, 96] and IL-2 [26, 108], in spite of the fact that numerous NK cell receptors have been recently described, have investigated this phenomenon only with respect to NK cell cytotoxic mechanism [105].

Considering tumor-induced suppression of NK cell activity in advanced malignancies, we show in breast cancer and MM patients that cytokines applied in immunotherapy, IL-2 and IFN-α, not only enhance in vitro NK cell activity, but, also, according to our new findings of NK cell activation by these cytokines, show induction of activating NKG2D receptor by IL-2 and IFN-α, especially on cytotoxic $CD16^{bright}$ NK cell subset. This is in accordance with our novel findings for healthy controls, that both, IL-2 and IFN-α, up-regulate NKG2D on NK cells, while only IFNα up-regulates CD161 (Fig. 6a–c), without altering KIR expression [109]. There are no reports so far dealing with the effect of IL-2 on CD161 expression in MM patients, and its observed effect on NKG2D expression has

Table 3 Distribution of NK cell receptors in PBL, $CD3^-CD16^{dim}$ and $CD3^-CD16^{bright}$ NK cell subsets of healthy individuals and MM patients

Subsets	Healthy controls		MM patients	
	Mean	Range	Mean	Range
$CD3^-CD16^+$	13.68	2–39	12.79[a]	4–38
$CD3^-CD16^{bright+}$	9.51	1–31	8.30	2–19
$CD3^-CD16^{dim+}$	4.27	1–31	4.50	1–9
$CD3^-CD16^+NKG2D^+$	10.36	2–24	8.08[a]	2–18
$CD3^-CD16^{bright+}NKG2D^+$	7.97	1–24	6.63	3–17
$CD3^-CD16^{dim+}NKG2D^+$	2.49	0–8	2.90	1–10
$CD3^-CD16^+CD161^+$	4.06	0.1–11	3.11[a]	2–5
$CD3^-CD16^{bright+}CD161^+$	3.21	0–10	1.94	0–9
$CD3^-CD16^{dim+}CD161^+$	0.92	0–4	0.71	0–2
$CD3^-CD16^+CD158a^+$	2.34	0–6	14.66[b]	0–8
$CD3^-CD16^{bright+}CD158a^+$	1.90	0–6	1.46	0–8
$CD3^-CD16^{dim+}CD158a^+$	0.36	0–2	0.82	0–5
$CD3^-CD16^+CD158b^+$	5.69	1–14	4.02	0–14
$CD3^-CD16^{bright+}CD158b^+$	4.24	4–14	2.52	1–6
$CD3^-CD16^{dim+}CD158b^+$	1.46	0–10	0.75	0–2

Percentages of NK cell subsets in peripheral blood from healthy individuals and MM patients are expressed as mean and range values

[a] Percentage of $CD3^-CD16^+$, $CD3^-CD16^+NKG2D^+$ and $CD3^-CD16^+CD161^+$ NK cells is significantly ($p \leq 0.01$, Mann–Whitney test) lower in MM patients compared to healthy controls

[b] Percentage of $CD3^-CD16^+CD158a^+$ is significantly ($p \leq 0.01$, Mann–Whitney test) higher in MM patients compared to healthy controls (adapted from Konjević et al. [74])

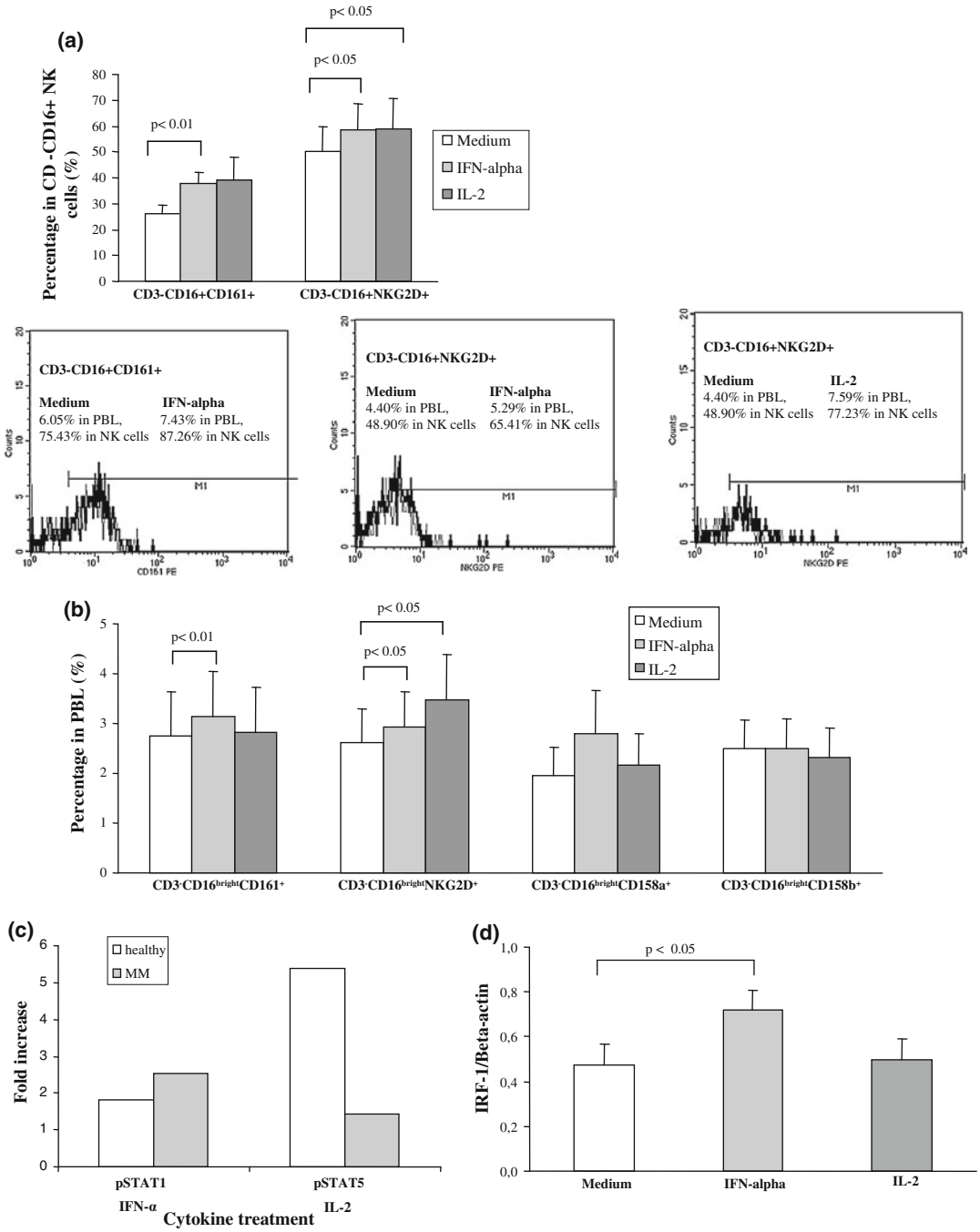

Fig. 6 Modulation of the expression of novel NK cell receptors on peripheral blood NK cells of healthy individuals and melanoma patients. **a** Expression of CD161 and NKG2D-activating NK cell receptors after IFN-α and IL-2 18 h in vitro treatment on gated $CD3^{-}CD16^{+}$ NK cells. IFN-α significantly ($p \leq 0.05$, Wilcoxon signed rank test) increases the percentage of CD161 and NKG2D activating receptors, while IL-2 significantly increases ($p \leq 0.05$, Wilcoxon signed rank test) only the percentage of NKG2D. Results are shown as mean for MM patients ± SE ($n = 26$). Representative flow cytometric histograms showing statistically significant ($p \leq 0.05$, Wilcoxon signed rank test) increase of NKG2D and CD161 expression on $CD3^{-}CD16^{+}$ NK cells after in vitro treatments. Marker (*M1*) position was set according to the isotype control. The *gray line* in each histogram represents treatment with culture medium alone while the *darker line* represents cytokine treatment. **b** The percentage of investigated NK cell receptor expression on $CD16^{bright}$ and $CD16^{dim}$ NK cell subsets after IFN-α and IL-2 18 h in vitro treatments. IFN-α significantly ($p \leq 0.05$, Wilcoxon signed rank test) increases the expression of CD161 and NKG2D, IL-2 increases only the expression of NKG2D ($p \leq 0.05$, Wilcoxon signed rank test), while there is no change in KIR CD158a and CD158b expression on $CD16^{bright}$ NK cells. Results are shown as mean ± SE. **c** IFN-α pSTAT-1 protein induction shows similar fold increase for healthy controls and MM patients, while for pSTAT-5 protein, IL-2-induction is greater for healthy controls compared to MM patients. **d** IFN-α significantly ($p \leq 0.05$, Wilcoxon signed rank test) induces IRF-1 transcription with respect to β-actin level, after 4 h in vitro treated PBL of MM patients (results are shown as mean ± SE for 15 MM patients) [109]

been shown so far mainly in experimental settings, that is, on NK cell lines [106], in healthy individuals [110] and in one report in myelodysplastic syndrome [107]. Although there are no such previous data, our analyses also show that, despite lower expression of NKG2D and CD161 in MM patients compared to healthy controls, the induction of NK cell receptors after in vitro treatments with IFN-α and IL-2 on NK cells of investigated MM patients was similar to that in healthy controls. Moreover, as there was no significant change in NK bright and dim subset distribution following these treatments, the novel observed induction of activating NKG2D and CD161 receptor expression by IFN-α or IL-2, and by IFN-α only, respectively, is most likely the consequence of direct regulation, on a single cell level, by these cytokines, rather than a reflection of subset shift within the NK cell compartment.

Our additional new data show that 18 h in vitro PBL treatments of MM patients, prior to chemotherapy or surgery that is known to induce immunosuppression [111], with IFN-α and IL-2 did not affect the expression of the two investigated KIR receptors, CD158a and CD158b, that is in agreement with most recent findings that describe their up-regulation only after prolonged 48, 72 h, and 7 days in vitro PBL treatment [112–114]. In this sense, IFN-α has been shown before in one report to up-regulate KIR expression after longer 48-h treatment on NK cell lines only [106], while up-regulation of these receptors with IL-2, aside from data obtained for healthy individuals, was reported in rheumatoid arthritis and breast cancer [115, 116]. As we have recently reported that MM patients have a decrease in the cytotoxic $CD16^{bright}$ subset and an increase in the regulatory $CD16^{dim}$ NK cell subset [92], we showed that the unfavorable distribution of these subsets is not affected by IFN-α or IL-2 short-term PBL in vitro treatments.

Immuno-monitoring during chemo-immunotherapy of melanoma patients

Our long-term clinical studies on the effect of chemo-immunotherapy (DTIC and/or IFNα) in MM patients, by showing the kinetics of NK cell activity, IL-2 production, CD4/CD8 ratio, $CD4^+$ T and $CD8^+$ T-cell number, expression of activation antigens CD69, CD38, and HLA-DR on $CD56^+$ NK cells and $CD8^+$ and $CD3^+$ T cells, show that the induction of antitumor immune response in treated metastatic melanoma patients appears early but is short-lived (Fig. 7a), possibly due to persistently high level of immuno-suppressive cytokines [24, 117] and that obtained stimulation should be augmented and prolonged by the timely introduction of additional immuno-modulating agents [118]. Our further clinical studies show that introduction of another agent, 13-*cis* retinoic acid that has previously been mostly investigated with respect to its antiproliferative activity [119, 120], into DTIC and IFNα protocols for MM patients, based on the induced expression of CD69 early activation antigen on NK cells (Fig. 7b), shows beneficial association with clinical response [95]. These results of immuno-monitoring are in accord with new concepts that chemotherapy can boost NK cell activity of either autologous or adoptively transferred NK cells by up-regulating activating ligands, such as MICA/MICB and ULBP, on tumor cells [90].

NK cells in lymph nodes of melanoma patients

Aside from circulating in peripheral blood where they comprise around 20% of total mononuclear cells [68], NK

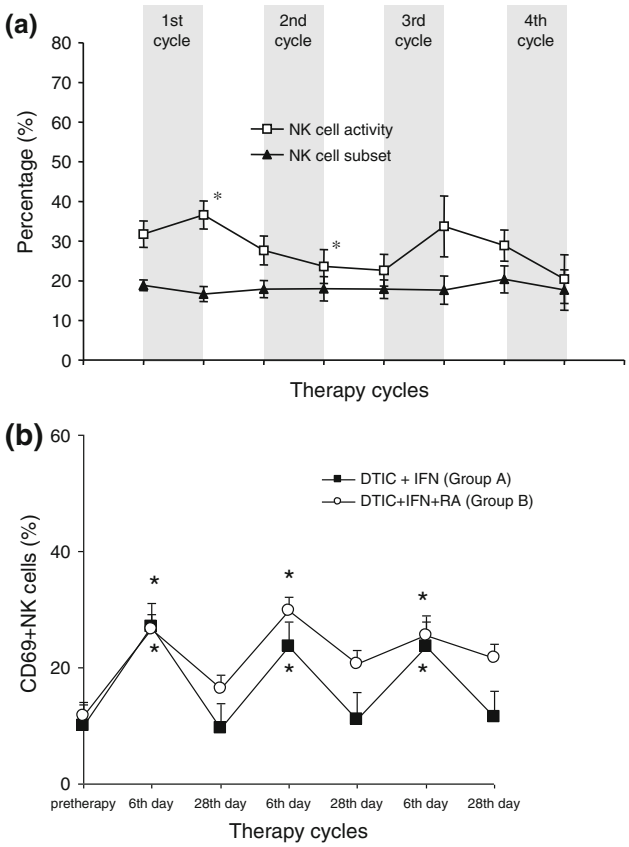

Fig. 7 Clinical immuno-monitoring of NK cell characteristics in melanoma patients undergoing therapy. **a** Changes in the number and activity of peripheral blood NK cells in melanoma patients receiving DTIC chemotherapy during treatment (the values are expressed as mean ± SE, * $p \leq 0.05$, Wilcoxon test) [117]. **b** Monitoring by flow cytometry of the expression of early activation antigen CD69 on $CD3^-CD56^+$ NK cells during the course of two chemo-immunotherapy protocols shows a significant (*$p \leq 0.05$, Student's t test) serial and transitory up-regulation of CD69 expression on the 6th day of every cycle of therapy, corresponding to the end of the 6-day period of administration of IFN-α in both investigated groups [95]

cells are also present in secondary lymphoid organs. Novel data indicate that NK cells are most abundant in spleen where they represent approximately 17% of mononuclear cells, while in lymph nodes (LNs), they comprise approximately 5% and in inflamed tonsills below 1% of overall mononuclear cells. In spite of the significantly lower percentage of NK cells in LNs compared to peripheral blood, LNs contain 40% of all lymphocytes in humans, while peripheral blood harbors only 2%, which makes NK cells from LN the dominant NK cell compartment in humans [121].

It has been established that NK cells reside in T-cell areas of inflamed lymph nodes [122] and, as LN became recognized as the sites of NK cell differentiation from hematopoietic progenitors, these secondary lymphatic organs have been recently introduced as a new field of NK cell research [123]. Previous investigations have been focused mainly on NK cells from peripheral blood of patients with malignant tumors [99, 101], while rare attempts for quantification have been performed on NK cells from regional LN of these patients. The scarce data on NK cells in LN were obtained on animal models, but the effect of tumor burden on human NK cell phenotype and distribution of two functional NK cell subsets in LNs remained unclear. A major problem in this type of studies is the determination of baseline values since ethical and practical considerations limit the availability of LN from healthy donors.

Based on our obtained data of the increased percentage of NK cells in involved regional LNs, compared to non-involved LNs of melanoma patients, we show that tumor invasion into LN tissue recruits mostly NK cells of $CD56^{dim}$ subset (Fig. 8) [124] into draining LNs where they can also interact with dendritic cells [125] and probably induce dendritic cell maturation and improve the efficiency of tumor antigen presentation. These NK cells invading LNs show counterbalance between the increased expression of CD16 receptor important for NK cell cytotoxic activity and increased inhibitory KIR CD158b expression [124] with an opposing effect that may impair NK cell cytotoxic activity. The other investigated and less abundant inhibitory KIR-CD158a receptor expression was found to be similar in both tumor infiltrated and uninfiltrated LNs. Furthermore, as it was

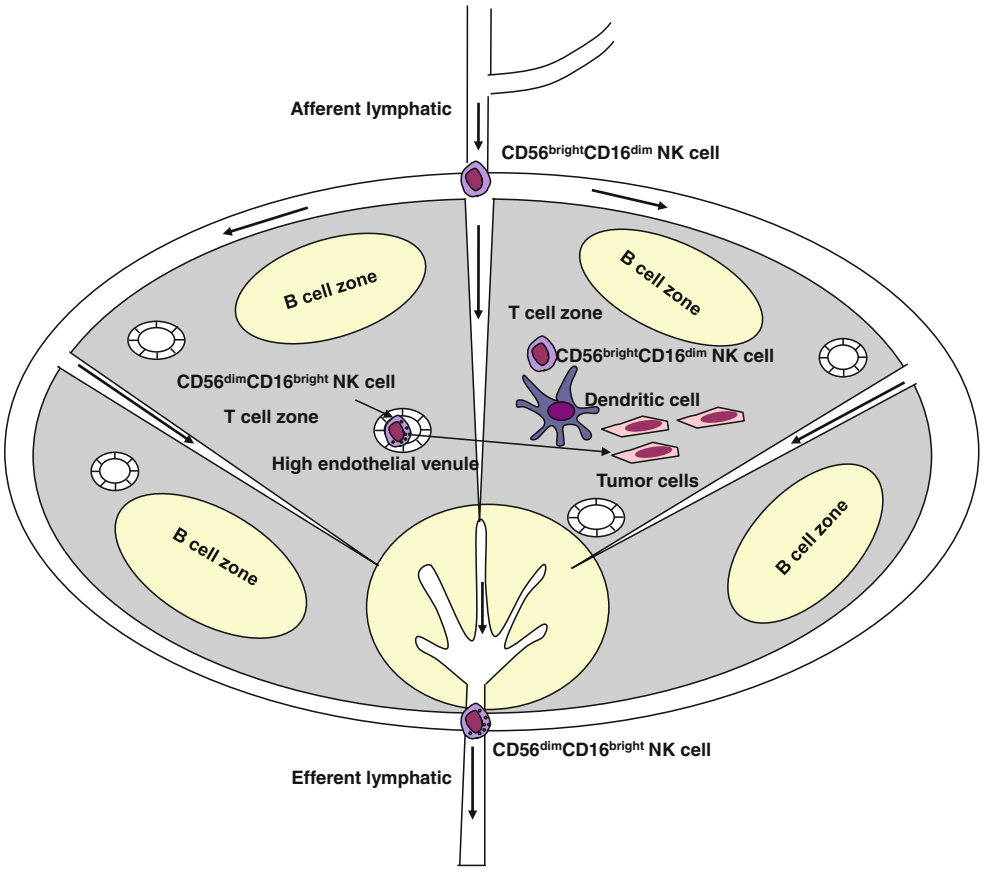

Fig. 8 NK cells in lymph nodes. Physiologically, $CD56^{bright}$ $CD16^{dim}$ NK cells enter a lymph node (*LN*) via afferent lymphatics, go to the T-cell zone, and eventually in cooperation with dendritic cells (*DC*) mature into $CD56^{dim}$ $CD16^{bright}$ NK cells that leave LN via efferent lymphatics. During malignant process, $CD56^{dim}$ $CD16^{bright}$ NK cells enter a metastatically invaded LN through high endothelial venules and interact with present tumor cells (*TC*)

previously reported for tumor tissue that NK cells in tumor-infiltrating lymphocytes (TIL) show evident CD69 activation antigen expression [126], and we also show that the presence of tumor cells in LNs is associated with higher percentage of NK cells expressing CD69 early activation marker [124].

NK cells in adoptive immunotherapy of cancer

Regarding immunotherapy in malignant diseases, even though it has been shown that adoptively transferred tumor-reactive T lymphocytes can mediate regression of metastatic cancers, many patients are ineligible for this type of treatment. Instead, patients are treated with adoptively transferred NK cells. This therapy of adoptively transferred in vitro activated autologous NK cells after the patients received a lymphodepleting but non-myeloablative chemotherapy regimens has been most commonly administered to patients with metastatic melanoma or renal cell carcinoma, as well as in other malignancies including breast cancer [127]. Moreover, new treatments of breast cancer patients have been initiated with allogeneic $CD3^+/CD19^+$-depleted haploidentical NK cells grafts with IL-2 that have shown to facilitate strong NK cell cytolytic responses and the rapid emergence of an NK cell receptor phenotype that is more prone to activation [90, 128]. Also, administration of chemotherapy before giving donor NK cells may stop tumor growth and decrease rejection of NK cells. In this sense, recognition of NK cell ligands on tumor cells could also be evaluated as possible prognostic markers [9].

Adoptive NK cell and monoclonal antibody therapy with cytostatic agents

NK cells persist for months after transfer and mediate ADCC suggesting that coupling adoptive NK cell transfer with monoclonal antibody administration deserves evaluation [129, 130]. In a more recent investigation, the observation was made that $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) inhibited NKG2D-mediated NK cell cytotoxicity and that depletion of Tregs in vivo significantly enhanced NK cell-mediated tumor rejection. All of these studies suggest that autologous NK cells may be efficacious for the treatment of patients with cancer, particularly if the patients are lymphodepleted prior to adoptive cell transfer [131].

Monoclonal anti-KIR receptor antibody therapy

A safer therapeutic strategy is to block NK cell-inhibitory KIR receptors in an autologous setting, and this is currently tested in phase II clinical trials with a fully human anti-KIR monoclonal antibody [132, 133]. This monoclonal antibody recognizes KIR2D inhibitory receptors and blocks their interaction with the human MHC class I molecule, HLA-C, leading to NK cell-mediated lysis of leukemic cells. However, one of the main concerns for using this therapeutic approach in humans is the risk of generating a strong reactivity against normal self-tissues and/or interfering with NK cell education [9].

Conclusion

As the role of NK cells has been shown in the loss of tolerance, in infection, and cancer, the data that we obtained on NK function and NK cell receptor expression, first of all in healthy individuals, may be of help in NK cell profiling, by giving basic referent values and those for cytokine-induced novel NK cell receptor expression, either in evaluation of disease or in immuno-monitoring during cytokine immunotherapy of patients with malignancies**.** Our extensive basic and for clinical practice novel investigations of NK cell cytotoxicity in numerous malignancies have shown that the found, clinical stage-dependent impairment of NK cell activity is associated with the change in the expression of certain newly defined NK cell activating and inhibitory receptors. We have also shown, in in vitro studies, that this impairment can be overcome with different cytokines. In this sense, we give insight into novel aspects of NK cell activation by most commonly used cytokines in immunotherapy with respect to characteristics of these NK cell receptors on CD16 and CD56-defined NK cells and their immunoregulatory $CD16^{dim}/CD56^{bright}$ and larger cytotoxic $CD16^{bright}/CD56^{dim}$ subsets, as it is now recognized that NK cell-mediated killing of tumor cells depends on the balance between stimulatory and inhibitory signaling initiated after ligation of these receptors.

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