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REVIEW

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Human NK cells and cancer

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ABSTRACT

The long story of NK cells started about 50 y ago with the first demonstration of a natural cytotoxic activity within an undefined subset of circulating leukocytes, has involved an ever-growing number of researchers, fascinated by the apparently easy-to-reach aim of getting a "universal anti-tumor immune tool". In fact, in spite of the impressive progress obtained in the first decades, these cells proved far more complex than expected and, paradoxically, the accumulating findings have continuously moved forward the attainment of a complete control of their function for immunotherapy. The refined studies of these latter years have indicated that NK cells can epigenetically calibrate their functional potential, in response to specific environmental contexts, giving rise to extraordinarily variegated subpopulations, comprehensive of memory-like cells, tissue-resident cells, or cells in various differentiation stages, or distinct functional states. In addition, NK cells can adapt their activity in response to a complex body of signals, spanning from the interaction with either suppressive or stimulating cells (myeloid-derived suppressor cells or dendritic cells, respectively) to the engagement of various receptors (specific for immune checkpoints, cytokines, tumor/viral ligands, or mediating antibody-dependent cell-mediated cytotoxicity). According to this picture, the idea of an easy and generalized exploitation of NK cells is changing, and the way is opening toward new carefully designed, combined and personalized therapeutic strategies, also based on the use of genetically modified NK cells and stimuli capable of strengthening and redirecting their effector functions against cancer.

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Introduction

Different effector cells are involved in anti-tumor activity, including αβ and γδ T lymphocytes, natural killer (NK) cells, and M1-polarized macrophages. In addition, dendritic cells (DC) instruct T lymphocytes by presenting tumor peptides, while phagocytes may participate in eliminating apoptotic and necrotic tumor cells. Despite the potential effectiveness of all these cellular mechanisms, tumors can counteract and escape the immune response, primarily by creating a hostile environment, both by directly impairing immune effector cells or by instructing different immune and nonimmune cells to become immunosuppressive.[1] These effects lead to a suppressive tumor microenvironment (TME), which may subvert the potential anti-tumor effect. Among defensive cells, NK lymphocytes play a major role in anti-tumor activity. They were discovered in the mid '70s, but only in the late '80s, the inspiring "missing self hypothesis"[2] led to the identification in the early '90s of the Human Leukocyte Antigen (HLA)-specific killer Ig-like receptors (KIRs), CD94/NKG2A, and of activating receptors.[3–7] These latter receptors allow the detection and killing of tumor cells, including the Natural Cytotoxicity Receptors (NCRs: NKp46, NKp44, NKp30), NKG2D, and DNAM-1.[8–10]

This contribution intends to briefly review the main interactions occurring between NK cells and tumor cells, the effect of suppressive activity of TME, and the immunotherapeutic approaches which may restore and/or potentiate the anti-tumor activity of NK cells. Some essential notions of NK cells and their receptors will be discussed to allow a deeper understanding of the NK/tumor cell interactions.

NK cell receptors and subsets

NK cells contribute to a first line of innate defenses: they are naturally equipped with a lytic machinery which allows the killing of some pathological target cells, including tumor and virus-infected cells. While innate immunity can frequently stop infections at a subclinical level, in more severe cases, it can contain infection allowing the intervention of the more efficient and specific, adaptive immunity, which involves T lymphocytes and, subsequently, B-cell-derived antibodies.[11]

The molecular mechanisms which allow NK cells to discriminate between healthy and abnormal cells remained a mystery for a long time. The main questions were as follows: 1) which receptors do allow NK cells to selectively hit

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tumor and virally infected cells? 2) what makes these target cells susceptible to NK cells? Capitalizing on the development of a sophisticated technique, allowing high cloning efficiency of T, and, subsequently, NK cells, Alessandro Moretta and coworkers discovered the prototypes and most important inhibitory receptors, i.e., KIRs.[3] These receptors, recognizing groups of HLA class I (HLA-I) allotypes, inhibit NK cell functions, avoiding killing healthy self cells. The major families of inhibitory receptors for HLA-I are the KIRs and the CD94/NKG2A heterodimer. In particular, KIR2DL1 and KIR2DL2/3 recognize HLA-C molecules carrying lysine and asparagine at position 80, respectively. KIR3DL1 binds to HLA-B and -A allotypes sharing the Bw4 epitope, and KIR3DL2 recognizes HLA-A *03 and -A *11. Differently, CD94/NKG2A is specific to the non-classical HLA-E molecule. The discovery of KIRs attracted the attention of immunogenetics researchers to study the *KIR* gene family and allowed the identification of two different haplotypes (termed A and B) differing in gene content (i.e., number and type of *KIR* genes).[12–14] The evidence of an "off" signal implies the existence of an "on" signal occurring when NK cells interact with abnormal target cells. This led to the discovery, by the same research group, of activating receptors involved in the recognition of abnormal cells and in the induction of the functional program of NK cells, i.e., cytotoxicity and cytokine production (primarily IFN-γ and TNF-α).[8–10,15] Importantly, the molecular structures recognized by activating NK receptors on target cells are either absent or expressed at low levels on healthy cells, while they become overexpressed in "stressed" cells. While also healthy cells can express such ligands for activating NK receptors upon "stress" induced by cytokines and cell proliferation/activation, they do express normal, or even increased levels of HLA-I molecules. The latters, upon interaction with KIR or CD94/NKG2A inhibitory receptors, inactivate NK cells, thus protecting them from NK-mediated attack. In contrast, tumor cells, as well as virally infected cells, may lose HLA expression or display an altered structure of HLA-I.

Some basic concepts regarding NK cell development and subsets are mentioned below.

Traditionally, two main NK cell subsets have been distinguished in peripheral blood (PB) based on the cell surface density of CD56 and CD16 molecules, termed $CD56^{bright}CD16^{dim/-}$ and $CD56^{dim}CD16^{+}$, with the former believed to be less mature but capable of producing high levels of cytokines (e.g., IFN-γ and TNF-α) and the latter more mature and cytotoxic.[16,17] Furthermore, $CD56^{dim}$ NK cells can mediate antibody-dependent cell-mediated cytotoxicity (ADCC) through the engagement of CD16, the low-affinity receptor for the Fc fragment of immunoglobulin G (FcγRIIIA, CD16A).[18] PB $CD56^{bright}CD16^{-}$ NK cells are uniformly positive for the HLA-E-specific inhibitory receptor CD94/NKG2A but lack KIRs. Differently, PB $CD56^{dim}CD16^{+}$ NK cells show variable expression of CD94/NKG2A and KIRs. Further distinctions were made within the $CD56^{dim}CD16^{+}$ NK cell subset which led to the description of a fully differentiated NK cell subset, characterized by the expression of CD57 and the absence of CD94/NKG2A, and a subset of mature NK cells, expressing the HLA-E-specific CD94/NKG2C activating receptor and displaying "adaptive" immune features in the context of CMV infection (called adaptive NK cells).[19,20] Indeed, under certain conditions NK cells undergo antigen-specific clonal expansion and become long-lived memory cells, features considered hallmarks of adaptive immunity lymphocytes.

Recent studies have also characterized NK cells in different tissues, each with distinct phenotypic and functional profiles. Indeed, the presence of tissue-resident NK cells has been shown in certain tissues, where they are retained thanks to the expression of the CD103 (αE integrin) and CD49a (α1 integrin) tissue residency markers.[17,21] In addition, the development of advanced single-cell technologies, such as scRNA-seq and CITE (Cellular Indexing of Transcriptomes and Epitopes)-seq, has accelerated the dissection of NK cell subset heterogeneity, revealing an intricate and nuanced NK cell landscape as well as different trajectories in their differentiation from hemopoietic precursors. Nevertheless, data based on these technologies still require a careful comparison with established markers and, more in general, with the proteins that are actually expressed. Another important concept related to NK cell maturation is the process of NK cell licensing or education, during which only NK cells expressing KIRs and/or CD94/NKG2A recognizing self HLA-I alleles become fully functional.[22–24]

Tumor escape mechanisms in the tumor microenvironment

To understand the potential efficacy of NK cell-mediated antitumor activity, the accessibility of NK cells into the tumor and the role of TME have to be taken into consideration Figure 1.[25–28]

NK chemokine receptors and tissue chemoattractants

NK cell migration into inflamed tissues is controlled by adhesion molecules, chemokine receptors, and chemokine gradients. While $CD56^{dim}$ NK cells express CXCR1, CX3CR1, CXCR2, and low levels of CXCR3, $CD56^{bright}$ NK cells express L-selectin (CD62L), CCR7, CCR5, and CXCR3.[29] Therefore, these two NK cell subsets show different migration capabilities. Chemokine receptor expression differences account for the differential migration of $CD56^{dim}$ cells and $CD56^{bright}$ NK cells into inflamed tissues and secondary lymphoid organs, respectively. However, so far, it is not known whether $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets are differentially recruited to the tumor because of their diverse chemokine receptor repertoire.[30,31] At least in some tumors, it has been described that the presence of high NK cell infiltration is related to a more favorable outcome.[32] It is conceivable that NK cell recruitment into the tumor bed might be regulated by CXCR3 and CX3CR1 chemokine receptors. Experimental cancer models have shown that gene therapy with CX3CL1/fractalkine and CCL2 can stimulate tumor rejection by boosting NK cell infiltration and activation.[33,34] In addition, it has been suggested that NK cells exposed to IL-15 and glucocorticoids express a high level of CXCR3, thereby increasing their potential to infiltrate CXCL10-positive melanomas. Thus, in humans, $CD56^{bright}$ could be preferentially recruited to the tumor due to their surface expression of CXCR3.[35,36] Transforming Growth Factor beta (TGF-β) promotes the

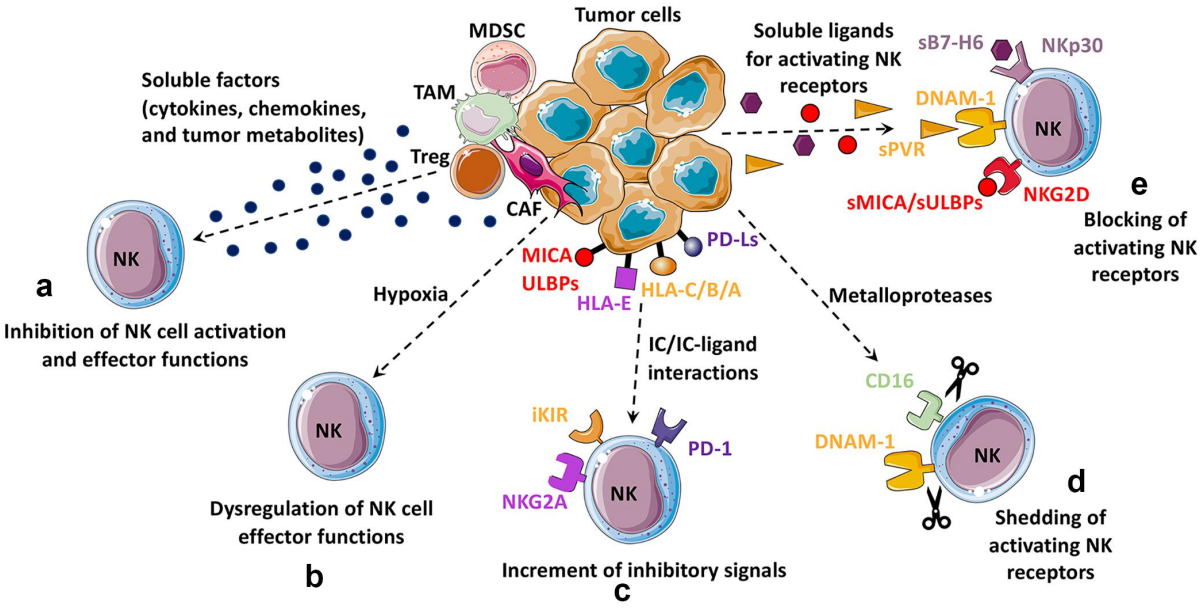

Figure 1. Inhibition of NK cell-mediated anti-tumor response by tumor microenvironment. The tumor microenvironment inhibits NK cell activation and functions through various mechanisms: a) Production of soluble factors (cytokines, chemokines, and tumor metabolites); b) Generation of hypoxic conditions; c) Increment of IC/IC-ligand interactions; d) Release of soluble activating receptors by metalloproteases; e) Release of soluble ligands for activating NK receptors. Abbreviations: CAF, cancer-associated fibroblasts; IC, immune checkpoint; iKIR, inhibitory killer Ig-like receptors; MDSC, myeloid-derived suppressor cells; NK, natural killer cells; TAM, tumor-associated macrophages; Tregs, regulatory T cells. Figure contains modified images from Servier Medical Art (https://smart.servier.com) licensed by the Creative Commons Attribution CC BY 4.0 International License (https://creativecommons.org/licenses/by/4.0/).

deregulation of the CX3CL1–CX3CR1 axis thus inducing the recruitment of $CD56^{bright}$ NK cells at the expense of the $CD56^{dim}$ subset. On the same line, increased levels of CCL19, CXCL9, and CXCL10 and decreased levels of CXCL2 in lung tumors foster the recruitment of $CD16^{-}$ NK cells, to the detriment of the more cytotoxic $CD16^{+}$ NK cells.[37] Also, $CD56^{dim}$ NK cells have been found in human lung tumors, colorectal cancer, and lymph node melanoma metastases.[38–40] In addition to chemokines, other factors could boost the recruitment of NK cells in tumors. In a melanoma model, chemerin, an important chemoattractant, enhances the infiltration of immune cells expressing the chemerin receptor CMKLR1 (i.e., NK cells, T cells, and DCs) into tumors.[41] In addition, our group has demonstrated that NK cells, upon interaction with melanoma cells, can release a form of high mobility group box-1 protein able to chemoattract activated NK cells.[42] Moreover, NK cells can be recruited into the tumor DC capable of prime T cell-mediated immunity. In human cancers, intratumoral CCL5, XCL1, and XCL2 transcripts closely correlate with gene signatures of both NK cells and conventional type 1 DC and are associated with increased overall patient survival in several cancer types.[43] In addition, these NK cells, through secretion of FLT3L, control the abundance of intratumoral stimulatory DC and further the responsiveness of melanoma patients receiving anti-PD-1 therapy.[44]

On the other hand, it has been shown that tumor cells may also affect the ability of NK cells to enter a solid tumor site. In this context, it has been shown that neuroblastoma-derived TGF-β could skew the NK cell chemokine-receptor repertoire.[45]

Tumor-infiltrating cells

In the TME, different immune and stromal cells are present, as well as a heterogeneous array of soluble factors endowed with an immune suppressive activity.[46,47] Several tumor-infiltrating cell types can shape the TME and affect NK cell functions through different mechanisms. In the TME, the major cellular components are represented by myeloid cells, namely tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), and tumor-associated neutrophils (TAN) (Figure 1).[48–50] In particular, the subset of MDSC of polymorphonuclear lineage (PMN-MDSC) has recently been found not only in TME but also in PB in tumor patients. Importantly, their frequency in PB directly correlated with the severity and the prognosis of lung carcinoma patients. Notably, NK cells in these patients were significantly impaired in their functional activities.[51] Thus, it is conceivable that targeting or inactivating PMN-MDSC may represent a promising therapeutic tool. In mouse, it has been described that TAN can impair the cytotoxicity and infiltration capability of NK cells by CCR1 down-regulation. Moreover, neutrophils can decrease the responsiveness of the NKp46 and NKG2D activating receptors. Enhanced expression of PD-L1 on neutrophils and of PD-1 on NK cells, and subsequent PD-L1/PD-1 interactions were the main mechanisms determining the neutrophil-mediated suppression of NK cell immunity.[52] Other cells can contribute to the development and maintenance of an immune suppressive microenvironment, such as regulatory T lymphocytes (Tregs), frequently expanded in tumor patients and found both in PB and at the tumor site. Their presence in tumor infiltrates

correlates with impaired immune function and bad prognosis. In Gastrointestinal Stromal Tumor (GIST) and Hepatocellular Carcinoma (HCC) patients, low NK cell functional capabilities correlate with high numbers of Tregs. Along this line, Tregs isolated from GIST patients inhibit NK cells through membrane-bound TGF-β.[53] In addition, since Treg cells express the high-affinity IL-2 receptor alpha (CD25, IL-2 rα), they could also interfere with NK cell activation by competing for IL-2 availability.

Interestingly, the presence of tumor-associated adaptive NK cells with tissue-resident traits has recently been evaluated in some solid tumors, but the available data are still few and partially conflicting. In particular, tumor-associated NKG2C^{+} inhibitory self-KIR^{+} NK cells, expressing the tissue residency marker CD94a, have been described to be present in lung cancer patients but only in CD56^{bright} CD16^{-} NK cells and not in CD56^{dim} CD16^{+} NK cells as occurring in PB. In addition, these cells were hyperresponsive toward K562 cells in terms of both cytokine release and CD107a degranulation.[54] On the other hand, CD56^{dim} FCεRγ^{-} adaptive NK cells have been detected with increased frequencies in HCMV^{+} HBV-associated hepatocellular carcinoma patients. These cells exhibited low expression of tissue-resident markers (including CD49a, CXCR6, and CD69) and limited anti-tumoral activity toward liver cancer cells, suggesting that TME can influence their anti-tumor capabilities.[55]

Among tumor-associated stromal cells, activated fibroblasts, often termed cancer-associated fibroblasts (CAF), are considered to play a role in mediating suppressive activity toward NK cells in the TME.[25] CAF have been shown to display distinct phenotypes and features with respect to fibroblasts residing in healthy tissues.[56,57] For example, CAF derived from melanoma, HCC, and colorectal carcinomas were shown to inhibit NK cell activity through cell-to-cell contact and through secretion of Prostaglandin E2 (PGE2), which could abrogate IL-2-induced upregulation of NKp44, DNAM-1, and NKp30 activating receptors.[58–60] In addition, in acute myeloid leukemias (AML), aberrant bone marrow (BM) mesenchymal stromal cells (α-SMA^{+} MSC) and hypoxia may contribute to generate a pro-tumoral BM niche that can affect NK cell differentiation and lead to an impairment of NK cell cytolytic activity against neoplastic cells.[61]

Suppressive soluble factors at TME

Tumor-associated cells in the TME and tumor cells themselves can inhibit NK cell anti-tumor effector functions both by establishing cell-to-cell contacts and through the release of several cytokines, inflammatory mediators, reactive oxygen species, and other soluble molecules (Figure 1).

Among soluble factors, TGF-β plays a pivotal role in tumor-mediated immune suppression. It is produced by different cell types in the TME, including tumor cells themselves, and is exposed on the surface of regulatory immune cells; it can also be delivered to extracellular vesicles or stored as an inactive component inside the extracellular matrix and released through the action of some metalloproteases.[62,63] Products of tumor cell metabolism can also be released. For example, both in solid tumors and in leukemias, enhanced tryptophan catabolism leads to an immunosuppressive environment. Thus, overexpression of the indoleamine 2,3-dioxygenase (IDO) enzyme catalyzes tryptophan degradation by producing l-kynurenine, which can directly affect NK cells. Our group showed that melanoma-derived IDO and/or PGE2 could inhibit NK cell surface expression of NKp30, NKp44, and NKG2D that are required for target cell recognition and killing.[64] Besides TGF-β, also IL-4, macrophage migration inhibitory factor (MIF), MUC-16, and adenosine can affect NK cell function.[65] In particular, it has been described that TGF-β is able to down-regulate NKG2D and NKp30 activating receptor expression, while IL-4 alters the capability of NK cells to produce cytokines.[66,67] Ovarian tumor cells can release/express both MIF and MUC-16 glycoprotein: MIF can down-regulate NKG2D expression, while MUC-16 can interfere with the formation of immunological synapses between NK and tumor cells.[68,69]

The chronic exposure to soluble factors and cell-to-cell contacts in the TME can deeply modify NK cells, inducing an "exhausted" phenotype. Exhausted tumor-associated (TA)-NK cells exhibit upregulation of inhibitory receptors such as PD-1, TIM-3, TIGIT (T cell immunoreceptor with Ig, and Immunoreceptor tyrosine-based inhibition Motif (ITIM) domains), LAG-3, and NKG2A, while they display a decreased expression of activating receptors (such as NKG2D)[70,71] and of Eomesodermin and T-bet transcription factors.[72,73] Exhausted TA-NK cells exhibit lower ability to proliferate, degranulate, and produce cytokines. In the TME, several metabolic stressed conditions such as nutrient depletion, low oxygen, and low pH can also impair NK cell functions, thus favoring exhaustion.[74] It has been shown that NK cell activity, proliferation, and survival rely on either oxidative phosphorylation (at steady-state) or glycolysis (upon activation).[75] Along this line, it has been described that during lung cancer progression, the presence of TGF-β in TME-induced inhibition of glycolysis results in a decreased cytotoxicity and viability of NK cells.[76] Altogether, these features explain the reduced anti-tumor effector functions frequently displayed by NK cells in the TME.[77–84] Although exhausted NK cells do not necessarily display alterations in the content of cytotoxic molecules, the impairment of NK cytotoxic function can also occur through the modulation of the effector molecules normally stored inside lytic granules. In different solid and hematologic tumors, patients' NK cells have been shown to display alterations in perforin and/or granzyme B expression, mainly mediated by TGF-β.[85] In addition, perforin and granzyme expression can be modulated by microRNA (miRNA) carried to NK cells via tumor-derived exosomes. Interestingly, tumor-derived exosomes can deliver several miRNAs with immune-modulating effects, such as miR-544, inhibiting NKp46 expression or miR-146a, down-regulating IFN-γ and TNF-α.[86,87]

Tumor-NK cell interactions

As described above, tumor cell recognition takes place through multiple NK receptor–ligand interactions dictating whether target cells will be spared or killed by NK cells. Tumor escape

mechanisms targeting these interactions represent an effective strategy to limit tumor cell recognition and subsequent cytotoxic activity by NK cells. On the NK cell side, the expression of the main activating NK receptors responsible for tumor cell killing can be reduced by different factors found in the TME. NCRs, NKG2D, and DNAM-1 surface expression can be variably affected by TGF-β, PGE2 or L-kynurenine produced by the IDO enzyme[64,66,88,89] In addition, hypoxia, which often characterizes the tumor and its microenvironment, can be responsible for the modulation of activating NK receptors (Figure 1).[90–92] Interestingly, tumor cells undergoing epithelial–mesenchymal transition (EMT), a process favored by hypoxia and other factors present in TME, display an increased ability to induce the down modulation of activating receptors on NK cells.[93] NK receptor modulation can also occur following persistent stimulation of NK cells with ligands expressed by cancer cells; in this case, receptor engagement by the corresponding ligand(s) induces receptor endocytosis, resulting in a decreased ability of NK cells to recognize and kill tumor cells.[94,95] Receptor modulation has been demonstrated for NCRs, NKG2D, and DNAM-1.[96–100] An additional mechanism by which activating NK receptors can be down-regulated involves the activity of metalloproteases, which mediate the shedding of the receptor from the cell surface through proteolytic cleavage. In particular, enzymes belonging to the matrix metalloproteinases (MMP) and ADAM (a disintegrin with metallopeptidase domain) families have been implicated in this process, for example, in the case of CD16 and DNAM-1 receptors (Figure 1).[101–106] In this context, the use of MMP and/or ADAM inhibitors could improve the ADCC function and increase the efficacy of therapeutic monoclonal antibodies (mAbs).[107,108] On the other hand, it has also been reported that the cleavage of CD16 mediated by ADAM17 could increase the efficiency of NK cell response, since CD16 shedding favors NK cell detachment from the target cell, which in turn improves serial killing.[109]

Regardless the mechanisms involved, it is of note that NK cells displaying a decreased surface expression of activating receptors have been detected in patients affected by different types of both solid and hematological cancers.[39,96,110–112]

Dysregulation of tumor-NK cell interactions can also be achieved through the upregulation of receptors delivering inhibitory signals, i.e., receptors for immune checkpoints (IC), including PD-1, TIM3, TIGIT, and CD96 (Figure 1).[113] Indeed, several studies demonstrated that in cancer patients these receptors can be upregulated in both PB-derived and tumor-infiltrating NK cells.[78–83] In addition, NK cells express a still unknown receptor able to recognize B7-H3, a member of the B7 family behaving as an immune checkpoint, given its broad expression in different tumor types and its ability to inhibit NK cell function following receptor ligation.[114] It has also to be considered that cancer cells can frequently display high levels of classical and non-classical HLA class I molecules, as a result of IFN-γ stimulation, further favoring the inhibitory signals conveyed to NK cells through inhibitory KIRs and CD94/NKG2A upon ligand engagement.[115,116] In addition, IFN-γ is also responsible for the upregulation of PD-L1/-L2 expression on tumor cells via the JAK/STAT pathway.[117]

Indeed, focusing on target cells, the alteration of ligands expressed on tumor cells is an effective strategy to limit recognition and killing by NK cells. Regarding this issue, ligands for NKG2D receptor represent a good paradigm, as their modulation from tumor cell surface can occur via different mechanisms. NKG2D ligands, namely MICA/B and ULPBs, are generally overexpressed or de novo expressed upon cellular stress, neoplastic transformation or viral infection.[104,118,119] Their expression can be diminished following the release of soluble ligands in exosomes, as a result of proteolytic shedding or translation of alternatively spliced transcripts. Interestingly, the inhibition of MICA/B cleavage can be obtained with a mAb that binds MICA/B in the α3 domain including the site of proteolytic shedding. This mAb has been shown to inhibit tumor growth in multiple fully immunocompetent mouse models and reduce human melanoma metastases in a humanized mouse model.[120] In addition, a Phase 1 dose-escalation clinical trial is showing promising results with a humanized anti-MICA/B mAb (cln-619), both as monotherapy and in combination with pembrolizumab in patients with solid tumors (NCT05117476). Alternatively, the disappearance of NKG2D ligands from the cell surface can be due to intracellular retention or to internalization and subsequent proteasomal degradation (Figure 1).[121–125] Ligand expression can also be regulated at the transcriptional level; for example, tumor cells can overexpress several miRNAs targeting MICA.[126–130] Regarding DNAM-1 ligands, soluble poliovirus receptor (PVR or CD155) can be obtained by alternative splicing, while post-translational modifications affecting PVR or Nectin-2 (CD112) may induce intracellular retention and protein degradation.[106,131–133] Although the knowledge of NCR ligands is still incomplete, several tumor escape strategies targeting molecules recognized by NCRs have been described so far. In particular, NKp30 ligands, represented by B7-H6 transmembrane molecule and by BAT3/BAG6 nuclear protein,[134,135] can be released in soluble form or through exosomes;[136–138] in addition, B7-H6 can undergo proteolytic cleavage by ADAM-10 and -17 proteases.[139] On the other hand, NKp44-mediated NK cell activation can be impaired by tumor cells, thanks to the overexpression of proliferating cell nuclear antigen, delivering an inhibitory signal upon NKp44 recognition or through the release of Nidogen-1, an extracellular matrix protein most likely acting as a decoy ligand.[140,141] Remarkably, soluble forms of different NK receptor ligands are under investigation as predictive biomarkers, since they can be detected in sera of tumor patients, and their levels are frequently associated with disease progression and poor prognosis.[136,139,142–144]

A peculiar, and still poorly explored, mechanism by which the tumor can become more resistant to NK-mediated lysis involves cytoskeletal changes within cancer cells, deeply affecting the formation of the lytic immunological synapse. In this context, tumor escape from NK-mediated cytotoxicity driven by actin cytoskeleton remodeling has been clearly demonstrated in breast cancer.[145]

Other tumor-associated intrinsic and extrinsic factors can induce an increased resistance to NK cells. For instance, it has been shown that NK cells can promote EMT in tumor cells, while EMT can favor the escape from NK cell attack.[93] In particular, lung cancer cells undergoing an intermediate EMT

state have been recently shown to avoid NK cell attacks by both reducing chemokine production and inhibiting NK cell cytotoxic response.[146] In the case of pancreatic adenocarcinoma (PA), it has been demonstrated that PA cell lines deriving from primary or metastatic tumors display a different susceptibility to NK cells, with metastatic ones displaying EMT-related gene expression and phenotype and an increased resistance to NK cell-mediated lysis.[147]

Therapeutic approaches exploiting NK cells

In spite of the many tumor-related mechanisms of immune suppression and escape, the generation of NK-based immunotherapeutic tools remains a fascinating and promising option for the research of new, more effective, treatments of malignancies.[148] However, the grade of NK cell infiltration into solid tumors is crucial when considering the use of NK cells for cancer immunotherapy, as NK cells generally have a low ability to infiltrate. Poor NK cell infiltration into the TME may be attributed to the interference with NK chemotactic signaling and activation, and to properties of the tumor bed (i.e., vasculature density and ECM structure).[73] Strategies to increase NK homing and infiltration into tumors would be essential to improve NK anti-tumor efficacy and prevent resistance and relapse. In these latter years, several groups have been conducting preclinical and clinical studies trying to optimize the anti-tumor effectiveness of NK cells over the barriers of the KIR/HLA-I and NKG2A/HLA-E inhibitory interactions, the suppressive and poorly accessible TME, and the limited persistency of NK cells after transfer. To overcome these limitations, several strategies are under study, including the evaluation of enhancers of NK cell activity (e.g., cytokines, IC blockers, and specific NK cell engagers), the generation of NK cells engineered to express specific chimeric activating receptors (i.e. CAR-NK cells), and the study of the most effective NK cell subset in hemopoietic stem cell transplantation (HSCT).

Cytokine-activated NK cells and CIML NK cells

Several cytokines have been evaluated to expand *in vitro* NK cells with enhanced anti-tumor properties and to sustain persistency and activation of adoptively transferred NK cells.[149]

The first cytokine used was IL-2, which, however, has shown negative side effects at the bed side. Specifically, the induction of Tregs, the activation-induced cell death on NK cells, and the possible contribution of IL-2 to the vascular leak syndrome,[150–152] represented important issues. Moreover, the adoptive transfer of Lymphokine Activated Killer cells (LAK) (induced in vitro by IL-2 stimulation) showed limited efficacy.[153,154] Therefore, the use of IL-2 in therapy required careful revision of doses and protocols, and stimulated the search for new activating means. In this context, engineered IL-2 molecules (superkines) have been developed to selectively enhance the therapeutic effects of IL-2. IL-2 superkines induced prolonged and intense NK cell activation, expansion of cytotoxic T cells, rather than Tregs, and improved anti-tumor responses in mouse models.[155–158] Another option to stimulate NK cells in therapeutic settings is represented by IL-15, which shares two of its receptor subunits with the IL-2 receptor complex, showing similar NK cell activation properties. At variance with IL-2, however, IL-15 does not stimulate Tregs and, for this reason, it has been considered a promising alternative to IL-2.[159] As this cytokine shows optimal effect when it is associated with the IL-15 Rα and trans-presented by IL-15 Rα^{+} cells,[160,161] a super-agonistic molecular complex comprising the trans-presenting IL-15 Rα sushi domain and IgG1-Fc has been generated (*N*-803/ALT-803) and demonstrated to be effective in animal models and well tolerated in patients.[162–164] On the other hand, it has also been recently discussed how IL-15 or *N*-803 could be less effective than IL-2 in supporting the persistence of transferred allogeneic NK cells in AML patients, due to its propensity to activate host cytotoxic T lymphocytes (CTLs) and CTL-mediated rejection of transferred cells.[165,166] It is also important to consider that long-term exposure to IL-15 can lead to NK cell dysfunction through metabolic and epigenetic reprogramming.[167,168]

Another interesting cytokine for NK cells is represented by IL-18, but the anti-tumor activity of this cytokine is limited by IL-18BP, a high-affinity IL-18 decoy receptor frequently upregulated in diverse human and mouse tumors. To maintain the signaling potential of IL-18, an IL-18 variant ('decoy-resistant' IL-18, DR-18) resistant to the inhibition mediated by IL-18BP has been developed. DR-18 has been shown to exert potent anti-tumor effects in mouse tumor models by enhancing the activity and maturation of NK cells, which can be exploited to effectively treat tumors that are resistant to anti-PD-1 therapy and have lost surface expression of MHC class I molecules.[169]

The use of the IL-12/15/18 cytokine cocktail was initially studied, more than 10 y ago, for its ability to activate NK cells that could persist *in vivo* and recall the initial stimulus.[170,171] Such, so-called cytokine-induced memory-like (CIML) NK cells have been extensively analyzed for their epigenetic signature and functional characteristics, and their potential therapeutic properties are under investigation for both hematologic and solid tumors.[172–175] In particular, in two recent studies, PB NK cells from haploidentical donors have been exposed for 12–16 h to the IL-12/15/18 cytokine cocktail and then transferred to AML patients directly after lymphodepletion or as supportive immunotherapy after donor-hematopoietic cell transplantation. In both cases, transferred cells have been demonstrated to proliferate and differentiate to memory-like phenotype in the patients and support anti-tumor activity.[176,177]

CAR-NK cells

Chimeric antigen receptor-engineered T cell (CAR-T) therapy has revolutionized the treatment of several types of cancer.[178] Despite this success, there are still major limitations and obstacles to the broad application of this therapy, including manufacturing complexity, high cost, and treatment-associated toxicities.[179] Furthermore, CAR-T cell therapy has good success in the treatment of hematological malignancies, but response rates are much lower in patients with solid tumors.[180]

CAR-NK cell therapy is emerging as a promising alternative to modified T cells and can represent a simpler and cheaper "off-the-shelf" treatment compared to CAR-T cells. In addition, unlike CAR-T cells, CAR-NK cells do not cause cytokine release syndrome, immune effector cell-associated

neurotoxicity syndrome and graft-versus-host disease (GvHD). They do not require HLA compatibility, are associated with minimal side-effects, can be produced on a large scale from several sources, can be applied in an allogeneic setting, and, different from what should be required for CAR-T, do not need gene editing to avoid TCR engagement. CAR-NK cell administration has been shown to be safe and effective for patients with multiple myeloma, AML, and $CD19^+$ lymphoid tumors, thus supporting further investigation in a wide range of tumors.[181,182]

Many of the initial studies used NK cell lines, instead of NK cells, due to their superior proliferative capacity. Nevertheless, NK cell lines have significant limitations in their clinical applications.[183,184] Indeed, since NK cell lines derive from lymphomas, they must be irradiated or inactivated before the infusion into the patient, thus limiting their *in vivo* persistence and the effectiveness of the therapy.[185] Thus, recent interesting alternative sources are represented by NK cells isolated from PB or differentiated from cord/PB $CD34^+$ stem cells, CIML-NK cells, or induced pluripotent stem cell-derived NK cells.[186–191] Furthermore, several preclinical studies have been conducted to increase the clinical efficacy of CAR-NK cells through different approaches: i) prolonging the *in vivo* persistence by modifying CAR-NK cells to ectopically express cytokines such as IL-15; ii) promoting homing and trafficking to the tumor site (crucial point for the efficacy of cell therapy against solid tumors) by engineering cells to express peculiar chemokine receptors; iii) overcoming the immunosuppressive action of TGF-β in the TME (e.g., by deleting the TGF-β receptor) thanks to the use of inducible promoters, which become active upon recognition of a tumor-associated antigen, metabolite, or drug; and finally iv) metabolically optimizing NK cells. However, the long-term persistence of CAR-NK cells may not be necessary for a long-lasting anti-tumor response. Indeed, a standardized CAR-NK therapy that uses multiple infusions could be hypothesized to maintain enough circulating cells for the control of the ongoing disease.[182] Moreover, CAR-NK cells, in addition to CAR-mediated antigen recognition, take advantage of their innate killing capabilities and the possibility to trigger ADCC without further manipulation.[149,181]

NK cells in the context of hematopoietic stem cell transplantation

The capability of NK cells to recognize and eliminate transformed cells led to the investigation of the anti-tumor activity of these lymphocytes in the setting of HSCT. To take advantage of donor NK anti-leukemic effect (graft versus leukemia, GvL), the knowledge of the interactions between NK receptors and ligands expressed on tumor cells is crucial. The interaction between leukemic blasts and NK cells occurs in both directions. Indeed, malignant cells may impair their NK-mediated recognition by producing soluble ligands of NK activating receptors (e.g., MICA) or expressing ligands of inhibitory checkpoints, leading to NK cell exhaustion, as well as by modifying patient NK cell cytolytic capabilities by decreasing the expression levels of activating receptors (i.e., NCRs, NKG2D, and DNAM-1)[96,111,192–196] or increasing the expression of ICs (namely, TIGIT, PD-1, TIM3, and LAG3)[149,197,198] The relevance of NK/leukemic cell interactions is well documented in AML patients, in whom the anti-leukemic NKmediated activity was suppressed at diagnosis and relapse but restored when they achieved complete remission.[199] Moreover, many studies have demonstrated that early NK cell reconstitution following HSCT and higher NK cell numbers in the stem cell graft resulted in improved patient outcome by increasing the overall survival and reducing the incidence of relapse, GvHD, and viral post-transplant infections.[200]

Being donor-derived NK cells significant effectors of anti-tumor and anti-infective responses in the recipients, the relevance of both NK receptor and ligand polymorphisms was investigated to design donor selection algorithms and stratify patients with worse post-transplant complications. One of the mechanisms involved in the GvL NK-mediated effect is driven by inhibitory KIRs. The impact of KIR/KIR-ligand mismatch was first examined in a cohort of adult AML patients receiving haploidentical HSCT (haplo-HSCT) with grafts composed of megadoses of highly purified $CD34^{pos}$ hematopoietic precursors. In this pioneering study, the authors reported a better outcome in the patients receiving a transplant from a donor characterized by an "alloreactive" NK subset.[201] NK alloreactivity occurs when the donor NK cells express only an inhibitory KIR recognizing a self-KIR ligand (thus allowing NK cell education), which is lacking in the patient (thus providing NK-mediated allorecognition).[202–204] Moreover, the alloreactive NK subset decreases the incidence of GvHD by reducing patient antigen-presenting cells and prevents graft rejection by eliminating host T cells.[201] More recently, novel methods of graft manipulations, based on *ex vivo* or *in vivo* T cell depletion (i.e., T cell-depleted and T cell-repleted graft, respectively) were developed, and the effects of KIR/KIR-ligand mismatch have been examined in different HSCT settings. ,[205–208]

Evidence supporting the preferential selection of donors characterized by *KIR* B/x genotypes was produced for patients with AML or myeloid malignancies when unrelated donors are the stem cell source, leading to the KIR B content scoring model.[209–212] This algorithm stratifies the different *KIR* genotypes by analyzing the number of centromeric and telomeric regions containing B haplotype-defining genes. A positive effect of donor *KIR* haplotype B was also observed in pediatric haplo-HSCT.[207] More recently, the evaluation of donor *KIR* genotype in a cohort of transplanted children with high-risk acute lymphoblastic leukemia suggested that a different centromeric presence and telomeric absence of *KIR* B motifs were associated with reduced relapse risk.[213]

Thus, several studies indicate that the donor *KIR* gene repertoire analysis may influence patient outcome, although with variable impacts among diverse HSCT platforms, suggesting that it should be introduced in donor selection searches when alternative donors are available. Many groups have attempted to apply an algorithm to quantify the complex interactions of activating and/or inhibitory KIR in the context of their ligands. Despite this, currently, there is no algorithm that can be applied to select the best donors in all haploidentical transplant platforms.[214] This lack is probably due to differences among transplantation settings, including graft manipulation approaches, conditioning regimens, and post-transplant immunosuppressive therapies. Nevertheless, *KIR*

repertoire analysis and assignment of the presence of A and/or B haplotypes based on *KIR* gene content has been recently included in the British Society for Histocompatibility and Immunogenetics guideline among the donor selection in HSCT, especially for C2/C2 group recipients having worse outcome in all HSCT settings.[215]

The post-transplant infusion of donor NK cells may result in an improved GvL effect. This advantage was demonstrated in the haplo-HSCT with post-transplant cyclophosphamide platform, in which the administration of high doses of *in vitro* expanded donor NK cells in early post-transplant time decreased the relapse rate, improving patient survival without any increase of GvHD.[216]

Biologicals triggering NK cell-mediated tumor recognition

Tumor recognition by NK cells can be triggered through the use of mAbs targeting IC–IC ligand interactions or by engagers bridging NK cell receptors to tumor-expressed surface proteins.

Immunotherapy with blocking monoclonal antibodies

As mentioned above, NK cells express IC which may compromise their anti-tumor activity upon interaction with their ligands on tumor cells. Although studies have been primarily focused on IC^+ T lymphocytes, also IC^+ NK cells are frequently present in the TME, and may be drastically impaired in their anti-tumor activity.

A promising therapeutic approach to treat HLA-I^+ solid tumors is represented by the use of monoclonal antibodies (mAbs) capable of disrupting the interactions between the HLA-specific inhibitory receptors (expressed on NK cells) and their ligands (expressed on tumor cells). In this context, the use of monalizumab, a first-in-class immune checkpoint inhibitor (ICI) targeting NKG2A and blocking the interaction between NKG2A and HLA-E, is achieving encouraging results in clinical studies in solid tumors, especially when combined with other checkpoint inhibitors, such as durvalumab (anti-PD-L1) in a variety of solid tumors (NCT02671435), or when used together with mAbs inducing ADCC, such as cetuximab (an EGFR-targeting antibody) in recurrent or metastatic head and neck squamous cell carcinoma (NCT04590963), and trastuzumab for HER2-positive breast cancer (NCT04307329). In non-small cell lung cancer (NSCLC), durvalumab plus monalizumab treatment has been associated with enhanced effector immune infiltration of tumors, interferon responses and markers of tertiary lymphoid structure formation, and systemic functional immune cell activation, as compared to durvalumab alone.[217]

However, the recovery of NK cell function appears crucial, particularly in tumors that have lost (e.g., neuroblastoma) or express low levels of HLA-I molecules (e.g., melanoma). These tumors are "invisible" to classical αβ T cells, while they may be susceptible to NK cells. For this reason, we felt important to briefly discuss therapies based on the use of ICI.

In recent years, molecules that block the PD-1/PD-L1 pathway have been developed to re-activate the immune system's response to cancer cells. This breakthrough led to the creation of several mAbs targeting PD-1 (like pembrolizumab and nivolumab) and PD-L1 (such as atezolizumab, avelumab, and durvalumab). These drugs have become foundational to contemporary cancer immunotherapy, serving as primary treatments for various tumor types and frequently outperforming traditional chemotherapy (Figure 2).[218] Identifying patients most likely to benefit from anti-PD-1/PD-L1 therapies is challenging, as many patients exhibit no response to these treatments. This has spurred efforts to develop predictive biomarkers, with PD-L1 expression emerging as the most commonly employed indicator.[219,220] PD-L1 expression, detected via immunohistochemistry on tumor or immune cells, is critical for therapy selection. The advent of various assays for measuring PD-L1 expression, each using distinct antibodies, has introduced complexity and inconsistencies in treatment eligibility determinations.[221]

The FDA has designated several assays as companion diagnostics for the safe and effective application of their corresponding drugs. These include assays for atezolizumab in urothelial carcinoma and NSCLC, as well as for nivolumab in NSCLC and pembrolizumab across a range of solid tumors. Other assays are categorized as complementary diagnostics, helpful but not mandatory for drug administration.[222] The commercial availability of diverse assays for clinical decision-making highlights a significant

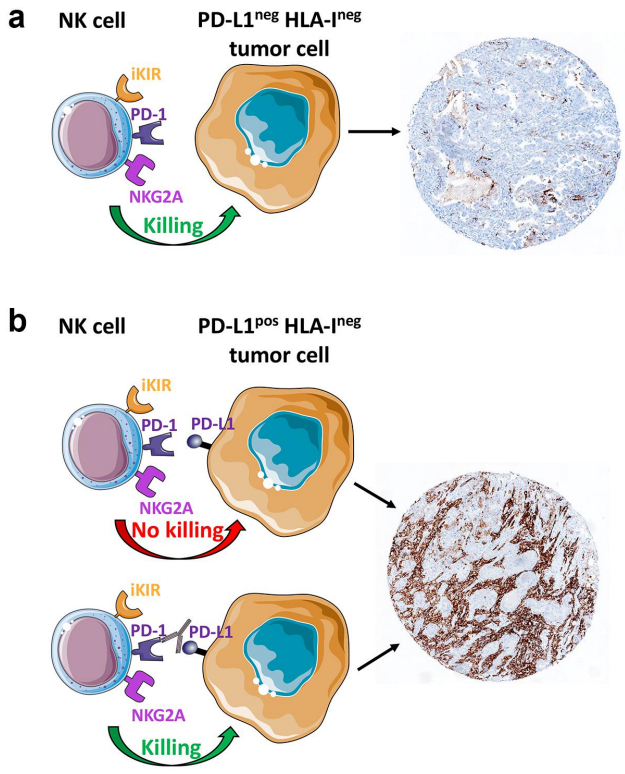

Figure 2. PD-L1 immunohistochemical expression in lung adenocarcinoma and contribution of NK cells in the anti-tumor response against HLA-I^{neg} tumor cells. a) In the presence of low PD-L1 expression (around 2%) on tumor cells, NK cells can kill HLA-I^{neg} tumor cells without any mAb-mediated blockade; b) In the presence of high PD-L1 expression (around 80%) on tumor cells, NK cells can kill HLA-I^{neg} tumor cells only upon mAb-mediated blockade of the PD-1/PD-L1 interaction. Figure contains modified images from Servier Medical Art (https://smart.servier.com) licensed by a Creative Commons Attribution CC BY 4.0 International License (https://creativecommons.org/licenses/by/4.0)

challenge: integrating comprehensive testing into routine clinical practice. Efforts to harmonize results from different antibodies have shown some analytical performance similarity among certain assays, suggesting potential interchangeability.[223] However, discrepancies persist, particularly in identifying positive cases at clinically relevant cutoffs, emphasizing the complexity of PD-L1 testing and accurate biomarker assessment.[224,225]

To overcome resistance to immune checkpoint inhibitors (ICI), numerous trials are exploring combination treatment approaches, following the initial success of nivolumab and ipilimumab in melanoma and renal cell carcinoma.[226] Novel combination strategies are rapidly developing, with promising results from dual-checkpoint inhibition of PD-L1 and LAG-3 or TIGIT.[227] The potential for digital pathology and artificial intelligence to enhance patient selection for immunotherapy is promising.[228] These technologies could offer a more detailed evaluation of the immune context, potentially leading to robust predictive models. However, integrating such advanced methodologies into clinical practice requires substantial infrastructure updates and rigorous validation efforts.

NK cell engagers

The NK cell engagers are represented by engineered multivalent soluble molecules. Bi- or tri-specific killer engagers (BiKE or TriKE) consist of two associated single-chain fragment variables, targeting, respectively, a given tumor-specific antigen, and an activating NK receptor (usually CD16), and (in the case of TriKEs) an additional domain generally engaging cytokine receptors on NK cells. This field of research has received great impulse in these last years, and different platforms have been set up to generate ever more sophisticated molecules. These molecules, identified with different acronyms, depending on the originating lab and platform, are being evaluated in preclinical and clinical studies.[229,230] Thus, for example, TriKEs combining IL-15 and anti-CD16 fragment and targeting HER2 or mesothelin have been recently tested against ovarian and lung cancer cells, respectively, and an anti-B7-H3 TriKE has been evaluated against different tumor cell types.[231] In this latter case, as B7-H3 is an inhibitory ligand for NK cells, theoretically, this engager has the double effect of targeting tumor cells and unleashing NK cell activity by the impairment of an inhibitory interaction. Other engagers, presently under study in phase I/II clinical trials, have been set up to simultaneously trigger two different activating receptors such as CD16 and NKp46, or CD16 and NKG2D, and targeting given tumor-associated antigens.[230,232–234]

Conclusions

There is no doubt that tumor immunotherapy has made major progress during the past 10 y. Two game-changing approaches have been the use of ICI and CAR-engineered immune effector cells (CAR-T and CAR-NK cells). However, there are still many unanswered questions for which we have major expectations of substantial improvements. For example, why can disruptive PD-1/PD-L1 interactions lead to a significant restoration of anti-tumor responses despite the presence of other mechanisms of immune evasion? An explanation might be the existence of still poorly explored connections among different inhibitory mechanisms operating at the tumor site. A better understanding of the prevalent mechanisms in a given tumor and their connections may lead to the design of combined strategies to improve responses to ICI. In spite of more recent development, CAR-NK cells may offer advantages over CAR-T, especially considering the additional difficulties in supporting effective anti-tumor activity in solid tumors. As discussed above, the tumor microenvironment may result hostile to any effector cells. First, tumor-specific surface antigens are extremely rare. Second, the homing of CAR-T cells to tumor tissues requires the release of appropriate chemotactic factors which attract effector cells at the tumor site. In addition, engineering and expanding autologous T cells (which may also be compromised) from individual patients is time-consuming, particularly expensive, and uncertain in terms of cell numbers obtained in each procedure. Therefore, it is not possible to establish reliable and consistent protocols. Importantly, NK cells do not cause GvHD and, thereby, can be obtained from allogeneic healthy donors and stored in large numbers. Thus, they represent ideal off-the-shelf products, immediately available for treating tumor patients. Indeed, major expectations are also based on CAR-NK cells because of their strong cytolytic activity and homing ability. Thus, obtaining good expansions of cytotoxic NK cells under good manufacturing practice conditions is an important issue to improve the use of NK cells in clinical practice, both as unmodified and genetically modified cells.

On the whole, the recent advances in the knowledge of NK cell biology and their manipulation are unveiling the considerable previously unknown therapeutic potential of these cells, which, we do believe, will be crucial for a future breakthrough in the fight against cancer.

Abbreviations

ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
AML	Acute Myeloid Leukemias
BiKE	Bi-specific Killer Engagers
BM	Bone Marrow
CAF	Cancer-Associated Fibroblasts
CAR	Chimeric Activating Receptor
CIML	Cytokine-Induced Memory-Like
DC	Dendritic Cells
EMT	Epithelial-Mesenchymal Transition
FDA	Food and Drug Administration
GIST	Gastrointestinal Stromal Tumor
GvHD	Graft versus Host Disease
GvL	Graft versus Leukemia
haplo-HSCT	Haploidentical HSCT
HCC	Hepatocellular Carcinoma
HLA	Human Leukocyte Antigen
HLA-I	HLA class I
HSCT	Hemopoietic Stem Cell Transplantation
IC	Immune Checkpoints
ICI	IC Inhibitors
IDO	Indoleamine 2,3-Dioxygenase
KIR	Killer Ig-like Receptor
MMP	Matrix Metalloproteinases
MIF	Migration Inhibitory Factor

miRNA	microRNA
MDSC	Myeloid-Derived Suppressor Cells
NCRs	Natural Cytotoxicity Receptors
NID1	Nidogen-1
NSCLC	Non-Small Cell Lung Cancer
NK	Natural Killer
PA	Pancreatic Adenocarcinoma
PB	Peripheral Blood
PGE2	Prostaglandin E2
PVR	Poliovirus Receptor
TAM	Tumor-Associated Macrophages
TAN	Tumor-Associated Neutrophils
TGF-β	Transforming Growth Factor beta
TME	Tumor Microenvironment
Tregs	Regulatory T lymphocytes
TriKE	Tri-specific Killer Engagers

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