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NK cells and ILCs in tumor immunotherapy

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ABSTRACT

Cells of the innate immunity play an important role in tumor immunotherapy. Thus, NK cells can control tumor growth and metastatic spread. Thanks to their strong cytolytic activity against tumors, different approaches have been developed for exploiting/harnessing their function in patients with leukemia or solid tumors. Pioneering trials were based on the adoptive transfer of autologous NK cell-enriched cell populations that were expanded *in vitro* and co-infused with IL-2. Although relevant results were obtained in patients with advanced melanoma, the effect was mostly limited to certain metastatic localizations, particularly to the lung. In addition, the severe IL-2-related toxicity and the preferential IL-2-induced expansion of Treg limited this type of approach. This limitation may be overcome by the use of IL-15, particularly of modified IL-15 molecules to improve its half-life and optimize the biological effects. Other approaches to harness NK cell function include stimulation via TLR, the use of bi- and tri-specific NK cell engagers (BiKE and TriKE) linking activating NK receptors (e.g. CD16) to tumor-associated antigens and even incorporating an IL-15 moiety (TriKE). As recently shown, in tumor patients, NK cells may also express inhibitory checkpoints, primarily PD-1. Accordingly, the therapeutic use of checkpoint inhibitors may unleash NK cells against PD-L1$^+$ tumors. This effect may be predominant and crucial in tumors that have lost HLA cl-I expression, thus resulting "invisible" to T lymphocytes. Additional approaches in which NK cells may represent an important tool for cancer therapy, are to exploit the unique properties of the "adaptive" NK cells. These CD57$^+$ NKG2C$^+$ cells, despite their mature stage and a potent cytolytic activity, maintain a strong proliferating capacity. This property revealed to be crucial in hematopoietic stem cell transplantation (HSCT), particularly in the haplo-HSCT setting, to cure high-risk leukemias. T depleted haplo-HSCT (e.g. from one of the parents) allowed to save the life of thousands of patients lacking a HLA-compatible donor. In this setting, NK cells have been shown to play an essential role against leukemia cells and infections. Another major advance is represented by chimeric antigen receptor (CAR)-engineered NK cells. CAR-NK, different from CAR-T cells, may be obtained from allogeneic donors since they do not cause GvHD. Accordingly, they may represent "off-the-shelf" products to promptly treat tumor patients, with affordable costs. Different from NK cells, helper ILC (ILC1, ILC2 and ILC3), the innate counterpart of T helper cell subsets, remain rather ambiguous with respect to their anti-tumor activity. A possible exception is represented by a subset of ILC3: their frequency in peri-tumoral tissues in patients with NSCLC directly correlates with a better prognosis, possibly reflecting their ability to contribute to the organization of tertiary lymphoid structures, an important site of T cell-mediated anti-tumor responses. It is conceivable that innate immunity may significantly contribute to the major advances that immunotherapy has ensured and will continue to ensure to the cure of cancer.

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1. Introduction

The evolution of the immune system is mainly consequent to the pressure exerted by environmental pathogens. However, both innate and adaptive immunity may control and prevent tumor growth and spreading, exploiting different and complementary mechanisms. For example, while cytolytic T lymphocytes recognize tumor-derived peptides in the context of MHC class I molecules, thus requiring MHC expression on tumor cells, natural killer (NK) cells sense and are activated by the lack of MHC molecules. These different properties provided important clues for the development of therapeutic strategies in cancer treatment. Thus, the identification of tumor antigens and of immunogenic tumor peptides allowed the development of tumor vaccines to better target tumor cells. On the other hand, NK cell ability to kill MHC class I defective tumors has been exploited in different adoptive cell therapy approaches, including the infusion of lymphokine-activated NK cells (Rosenberg et al., 1987), hematopoietic stem cell transplantation (HSCT), particularly the HLA-haploidentical HSCT (haplo-HSCT) to treat high-risk leukemia. T and, more recently, NK cells engineered to express chimeric antigen receptors (CAR) also represent a particularly interesting novel strategy to treat both hematologic and solid tumors.

A major breakthrough in cancer immunotherapy has been the use of immune checkpoint inhibitors (ICI). Immune checkpoints (IC) are represented by inhibitory receptors which, physiologically, control the function of effector cells, such as T and NK cells (e.g. PD-1), or exert a broader control of immune response (e.g. CTLA-4), ensuring peripheral tolerance. The tumor-guided expression of such receptors on tumor-reactive effector cells and their ligands on tumor cells results in a potent, primary mechanism of evasion from anti-tumor immunity. By disrupting the receptor-ligand interaction, ICI unleash T and/or NK cells and restore the anti-tumor immune response. Thus, the armamentarium already available for cancer treatment, including surgery, chemotherapy and radiotherapy, has recently been enriched and integrated by potent immunotherapeutic tools. Indeed, immunotherapy has marked an important and decisive progress in cancer treatment. In this review, we will address the main biological characteristics of the innate lymphoid actors (including NK and innate lymphoid cells, ILC) playing a role in immunotherapy and discuss the settings in which immunotherapy involving these cells have played an important role in the cure of tumors. Particular emphasis will be given to the use of checkpoint inhibitors and other engagers aimed at harnessing NK/ILC effector function, the haplo-HSCT to treat high-risk leukemia, the novel approaches using adoptive transfer of allogeneic NK cells engineered with CAR and the use of highly-proliferating “adaptive” NK cells characterized by a strong anti-tumor activity.

2. NK cells

2.1. Biology of NK cells: development, education, function

NK cells are key elements of the innate immunity able to mediate powerful antitumor and antiviral responses, both directly killing cancer or infected cells and indirectly improving the responses mediated by antibodies and T cells. Indeed, NK cell-mediated cytotoxicity and cytokine release can affect the activity of additional innate immune cells (dendritic cells, macrophages and neutrophils) and confer to NK cells regulatory function, capable of influencing the subsequent antigen-specific, HLA-restricted T and B cell responses (Vivier et al., 2011).

Since their identification in the ‘70s, NK cells have been classified as lymphocytes based on their morphology, their expression of various lymphoid surface markers, and their differentiation from the common lymphoid progenitor cell (CLP) in the bone marrow (BM). However, at difference with T and B cells, NK cells do not express receptors encoded by rearranging genes and, for this reason, they are generally considered as components of the innate immune system (Abel et al., 2018).

From the beginning NK cells have been described as “naturally” cytotoxic cells, since, different from cytotoxic T cells, they do not require prior antigen exposure to mediate effector functions. However, more recently this concept has been partially revisited when it was demonstrated that NK cells require the priming by various factors (e.g. soluble factors released by other innate immune cells) to reach their full effector potential (Vivier et al., 2011).

Similar to T and B cells, NK cells might be self-reactive, even though NK receptor genes do not undergo somatic rearrangement. Thus, to allow their efficacy against tumor and infected cells but to ensure also self-tolerance, NK cells must undergo a process called NK cell “licensing” or “education” (Elliott and Yokoyama, 2011). By this process, only NK cells expressing inhibitory receptors for self HLA class I molecules acquire full functional potential, while the “autoreactive” NK cells acquire a state of hyporesponsiveness. The education process is necessary for ensuring self-tolerance by NK cells because the array of receptors that NK cells acquire during their development is largely random, and the HLA molecules recognized by these receptors are inherited independently from the receptor genes. Thus, as the result of the education process, the majority of mature and functional NK cells is equipped with at least one inhibitory receptor for self HLA class I antigens.

Initially, it was thought that human NK cells undergo development exclusively in BM. However, more recent evidences suggested that they can also develop and mature in secondary lymphoid organs (SLOs) such as tonsils and lymph nodes, thymus (Mingari et al., 1997) and even liver (Poggi et al., 1993; Yu et al., 2013). These data suggest that hematopoietic stem cells (HSC), CLP or both can migrate from BM to different peripheral tissues where they undergo differentiation towards NK cells (Abel et al., 2018; Caligiuri, 2008). The progenitor cells and the various differentiation stages that give rise to NK cells can be distinguished on the basis of the expression of different lineage-specific surface markers. In particular, $Lin^{-}CD34^{+}$ HSC differentiate first into $CD45RA^{+}$ lymphoid-primed multipotential progenitor (LMPP) and then into $CD38^{+}$, $CD7^{+}$, $CD10^{+}$, $CD127^{+}$ CLP that are potentially capable of generating Pro-B, Pre-T, NK cell progenitors, or other ILCs. CLP express the ID2 transcription factor, while the developmentally regulated expression of the T-box transcription factors (T-bet) and Eomesodermin (Eomes) control NK cell differentiation (Luetke-Eversloh et al., 2013; Spits et al., 2016).

Notably, during the process of NK cell differentiation some surface receptors may display functional properties opposite to those of mature NK cells. For example, CD161 functions as an inhibitory receptor in mature NK cells or as a triggering receptor during early stages of NK/ILC differentiation (Montaldo et al., 2012). On the contrary, 2B4 functions as an activating co-receptor in mature NK cells (Sivori et al., 2000), while it mediates strong inhibition at early stages of differentiation (Sivori et al., 2002). This opposite function of 2B4 is a consequence of the late appearance of the intracytoplasmic SLAM-associated protein (SAP) that plays an important role in the generation of the positive signals mediated by 2B4 engagement. Remarkably, deletion or loss of function mutations of SAP molecule are responsible of the X-linked lymphoproliferative disease (XLP) (Parolini et al., 2000).

Another molecule playing an important role in the process of NK cell development is NKp80 (Vitale et al., 2001). Indeed, it marks functionally mature NK cells which mature in SLOs. Thus, on the basis of NKp80 expression, in SLOs, it is possible to distinguish an $NKp80^{-}$ population showing both NK- and ILC3-associated features and an $NKp80^{+}$ population similar to PB $CD56^{bright}$ NK cells (Freud et al., 2016).

During the process of NK cell differentiation the surface density of CD56 changes. Indeed, as shown in mice with a humanized immune system, $CD34^{+}$ cells give rise first to $CD56^{bright}$ and subsequently to $CD56^{dim}$ NK cells that also acquire CD16 expression. The $CD56^{bright}$ $CD16^{neg/dim}$ NK cells express only CD94/NKG2A as HLA-specific receptor, whereas the $CD56^{dim}$ $CD16^{bright}$ NK cells become KIR^{+} and/or $CD94/NKG2A^{+}$. At later stages of differentiation $CD56^{dim}$ KIR^{+} NK

cells also express high levels of CD57, lose the expression of CD94/NKG2A and decrease their proliferative capacity (Del Zotto et al., 2017; Moretta, 2010). Finally, the generation of “adaptive” or “memory-like” NK cells occurs as a result of the continuous stimulus during herpesvirus infections. Indeed, it has recently been shown that NK cells, in addition to kill transformed cells, can also sense pathogens and adapt their responses to the local environment, even mounting a sort of immunological memory (Sun et al., 2011) (see section 7).

2.2. HLA-specific inhibitory and activating NK receptors

NK cell tolerance towards autologous healthy cells is mediated by HLA-specific inhibitory NK receptors (iNKR), mainly represented by KIRs and CD94/NKG2A (Moretta et al., 1996). Inhibitory KIRs (iKIRs) are type I molecules of Ig superfamily, characterized by 2 or 3 extracellular domains and a long cytoplasmic tail (indicated as KIR2DL/KIR3DL). This tail contains immunoreceptor tyrosine-based inhibitory motifs (ITIM), capable of recruiting tyrosine phosphatases and transducing inhibitory signals (Long, 2008; Marsh et al., 2003; Parham, 2005). The main iKIRs are specific for allotypic determinants (i.e. epitopes) shared by distinct groups of HLA class I molecules, referred to as KIR-ligands (KIR-L). In detail, KIR2DL1 is specific for HLA-C^{Lys80} allotypes carrying HLA-C2 epitope, KIR2DL2/L3 recognize HLA-C^{Asn80} allotypes carrying HLA-C1, KIR3DL1 binds to HLA-B and HLA-A molecules sharing the Bw4 epitope, and KIR3DL2 recognizes HLA-A∗03 and -A∗11 (Moretta et al., 1996; Pende et al., 2019; Uhrberg et al., 1997; Vilches and Parham, 2002). CD94/NKG2A heterodimer is composed by type II proteins, which belong to the C-type lectin superfamily. It is specific for the non-classical HLA-E molecules, which are characterized by limited polymorphism. They bind peptides which are derived from the leader sequences of HLA-A, -B, or –C molecules (Braud et al., 1998; Lee et al., 1998) or from CMV (Hammer et al., 2018; Ulbrecht et al., 2000).

The activating counterpart of these receptors are represented by activating KIRs (aKIRs) and CD94/NKG2C. The aKIRs, named KIR2DS/KIR3DS, are characterized by short cytoplasmic tail without ITIM and by a transmembrane region containing a charged amino acidic residue which associates with KARAP/DAP12 adaptor molecule, bearing immunoreceptor tyrosine-based activating motifs (ITAM) (Lanier et al., 1998; Olcese et al., 1997). Among aKIR, KIR2DS1, like the inhibitory counterpart KIR2DL1, recognizes HLA-C2 (Moretta et al., 1995). Limited information is available on the ligands recognized by the other aKIRs (Blunt and Khakoo, 2020; Pende et al., 2019).

The *KIR* gene family is organized in haplotypes, termed A and B, divided by a recombination hot spot into centromeric (Cen) and telomeric (Tel) regions (Pyo et al., 2010). Group A haplotypes are characterized by a limited and fixed number of genes, mostly encoding iKIRs (*KIR2DL1*, *KIR2DL3*, *KIR3DL1*, and *KIR3DL2*), only 1 aKIR (*KIR2DS4*), in addition to the 2 pseudogenes (*KIR2DP1* and *KIR3DP1*). Differently, group B haplotypes contain a more variable and greater gene content, including more aKIRs (*KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5* and *KIR3DS1*). High degree of diversity is found in *KIR* repertoires, due to variability in presence/absence of *KIR* genes, *KIR* gene copy numbers and allelic polymorphism (Hsu et al., 2002; Jiang et al., 2012; Parham, 2005).

2.3. Non HLA-specific inhibitory NK receptors

NK cells can also express other inhibitory receptors which function as IC of NK cell activation, including PD-1, TIGIT, CD96, TIM-3 and CD161 (Sivori et al., 2019b). Some of these receptors are not constitutively expressed by resting NK cells, but they may be induced/upregulated under pathological conditions, primarily tumor encounter and CMV infection. PD-1, a member of Ig superfamily, binds PD-L1 and, with higher affinity, PD-L2, which can be up-regulated on certain tumor cells (Pesce et al., 2019). PD-1^{+} NK cells have been detected in patients with ovarian carcinoma, Kaposi sarcoma and Hodgkin lymphoma (Beldi-Ferchiou et al., 2016; Pesce et al., 2017; Vari et al., 2018). It is conceivable that the tumor microenvironment, through signals delivered by soluble factors and/or cells, can induce PD-1 expression. TIGIT and CD96 (Dougall et al., 2017; Zhou et al., 2018) can exert an inhibitory effect by competing with the activating receptor DNAM-1 (CD226) for binding to PVR (CD155) and Nectin-2 (CD112), often upregulated on tumor cells. Blockade of TIGIT has been shown to prevent NK cell exhaustion and to elicit potent T and NK cell anti-tumor immunity (Zhang et al., 2018). TIM-3 has been suggested to require cis and/or trans interaction with CEACAM-1 to exert an inhibitory function (Huang et al., 2015). Various ligands have been proposed, including galectin-9, phosphatidylserine, and HMGB1. CD161, belonging to C-type lectin superfamily, is an inhibitory receptor recognizing LLT1, which is expressed by various tumors including non-Hodgkin's lymphoma (NHL) (Bialoszewska and Malejczyk, 2018).

2.4. Activating NK receptors and their ligands

Human NK cells express a large set of receptors that, upon interaction with specific cellular ligands present on stressed, virus-infected or transformed cells, are responsible of NK cell triggering. The natural cytotoxicity receptors (NCRs) (Moretta et al., 2001), called NKp46/NCR1/CD335 (Pessino et al., 1998; Sivori et al., 1997, 1999), NKp44/NCR2/CD336 (Vitale et al., 1998) and NKp30/NCR3/CD337 (Pende et al., 1999), are among the main activating NK receptors. They are type I transmembrane molecules that belong to the Ig superfamily. NKp46 and NKp30 are expressed on virtually all resting NK cells, whereas NKp44 is constitutively expressed only on CD56^{bright} NK cells, but it is acquired by essentially all NK cells after cytokine-mediated activation. The presence of a positively-charged aminoacid in the NCR transmembrane domain allows NCR association with ITAM-bearing adaptor proteins, in particular CD3-ζ and/or FcεRI-γ (for NKp30 and NKp46) and KARAP/DAP12 (for NKp44), that are essential for the transduction of positive signals inside NK cells.

Although several molecules have been described to interact with NCRs, only few membrane-bound ligands have been identified. For example, B7-H6 (Brandt et al., 2009) represents a NKp30 ligand whereas a splice variant of mixed-lineage leukemia 5 (21spe-MLL5) (Baychelier et al., 2013) and a specific HLA-DP molecule (HLA-DP401) (Niehrs et al., 2019) have been identified as NKp44 ligands. Other NCR ligands, such as BAT3/BAG6 (Pogge von Strandmann et al., 2007; Reiners et al., 2013) (NKp30 ligand) and PCNA (Rosental et al., 2011) (NKp44 ligand), are intracellular proteins that may be expressed at the cell surface of tumor or stressed cells. In addition, some NCR ligands are molecules of viral origin that are capable of triggering NK cell function against infected cells (such as influenza virus-derived HA), recognized by NKp46 or NKp44, or are capable of inducing inhibitory signals (such as the CMV-encoded pp65 protein, recognized by NKp30 (Arnon et al., 2006). Finally, NKp46 and NKp44 can also recognize extracellular molecules. In particular, NKp46 can bind to the soluble plasma glycoprotein, the complement factor P/properdin (Narni-Mancinelli et al., 2017), whereas NKp44 can recognize Nidogen-1/Entactin (Gaggero et al., 2018) and PDGF-DD (Barrow et al., 2019).

Importantly, hypoxia and different soluble factors present in the tumor microenvironment (TME), including Indoleamine 2,3 dioxigenase (IDO) (Della Chiesa et al., 2006), Tumor growth factor-beta (TGF-β) (Castriconi et al., 2003), Prostaglandin E2 (PGE2) (Pietra et al., 2012), or the soluble form of NCR ligands (such as sBAT3 or sB7-H6) (Pesce et al., 2015; Reiners et al., 2013; Schlecker et al., 2014) can lead to impairment of NCR expression and function. Indeed, NK cells characterized by a NCR^{low} phenotype can be detected primarily at the tumor site (see section 2.6).

Another important NK receptor is NKG2D that transduces an activating signal thanks to the association with the transmembrane adaptor protein DAP10. NKG2D recognizes ULBPs and MICA/B that are HLA

class I homologues upregulated in infected, stressed and tumor cells (Bauer et al., 1999; Lanier, 2015; Raulet et al., 2013). Notably, soluble forms of NKG2D ligands shedding from tumor cells may be involved in a mechanism of tumor escape (Dhar and Wu, 2018).

NK cells express also several co-receptors, such as 2B4 (Sivori et al., 2000), NTBA (Bottino et al., 2001), DNAM-1 (Shibuya et al., 1996), CD59 (Marcenaro et al., 2003), and NKp80 (Vitale et al., 2001), that are capable of enhancing the NK cell triggering induced by NCRs or NKG2D.

Finally, $CD56^{dim}$ NK cells can mediate the antibody-dependent cell-mediated cytotoxicity (ADCC) (Ochoa et al., 2017) through the engagement of CD16, the low affinity receptor for the Fc fragment of IgG (FcγR).

2.5. NK alloreactivity: towards NK-based therapies

The high polymorphism of *KIR* and *HLA* class I genotypes, which segregate independently leading to diverse compound genotypes, primarily determines the great variability of the receptor phenotypic repertoire of the circulating NK cell pool among different donors (Parham, 2005). Another important feature of the iNKR repertoire is that KIR and CD94/NKG2A are clonally distributed and epigenetically regulated. For each individual, the KIR expression pattern is the result of a stochastic event but is highly influenced by self-HLA class I molecules during "education". Thanks to this process NK cells become competent only if they express at least one iNKR specific for self-HLA (Elliott and Yokoyama, 2011). In addition, the strength of iKIR/KIR-L binding influences the expression levels of the interacting iKIR, as well as its co-expression (or lack of expression), with other iNKR. While a strong iKIR/KIR-L interaction positively impacts on NK cell education, aKIR/KIR-L induces down-regulation of NK cell responsiveness, as shown for KIR2DS1 in HLA-C2/C2 individuals (Fauriat et al., 2010). The presence of NK cells expressing a single self-iKIR confers an advantage to the host, since it allows to sense the loss or peptide-induced alteration of even a single HLA class I allotype, and thus to kill the damaged cell, through the mechanism of "missing self recognition". By the same mechanism, educated NK cells can be alloreactive when expressing exclusively iKIR(s) that are not engaged by the HLA class I molecules (i.e. KIR-L) present on allogeneic cells. NK cell alloreactivity can occur in the context of haplo-HSCT, according to the presence of donor iKIR(s) specific for the KIR-L(s) present in the donor and absent in the recipient, identifying a KIR/KIR-L mismatch in graft-versus-host (GvH) direction (Locatelli et al., 2018). This phenomenon of NK alloreactivity occurs in around 50% of donor/recipient pairs. Genetically defined by donor *KIR* gene profile and donor/recipient KIR-L, the actual size of the alloreactive NK cell subset can greatly differ among different donors. Alloreactive NK cells can exert an efficient graft-versus-leukemia (GvL) effect by killing leukemia blasts escaping the preparative regimen to the allograft due to engagement of activating pathways without any "off" signal. In non-transplantation settings, adoptive NK cell therapy can be particularly efficient when donors have alloreactive NK cells for patients with either leukemia or solid tumors (Curti et al., 2016; Miller et al., 2005).

2.6. Effect of TME on NK cell function

Besides specific T lymphocytes, also NK cells are thought to play an important role in cancer immune-surveillance. NK cells are potentially capable of eliminating tumors with reduced or absent MHC class I expression that evade $CD8^+$ T cell–mediated control. Therefore, they are playing a complementary role in anti-tumor activity. Indeed, their role in tumor surveillance is well-established, since a lower degree of NK cytotoxic activity has been associated with cancer incidence in long survey subjects (Imai et al., 2000). Besides the role of NK cells in the control of hematological malignancies and tumor metastases (Messaoudene et al., 2014; Pasero et al., 2015), several studies have provided evidence that, in a variety of different solid tumors (including lung, gastric, colorectal, head and neck, and renal cell carcinoma), the presence of tumor-infiltrating NK (TI-NK) cells also affects tumorigenesis (Habif et al., 2019) and correlates with improved patient outcome (Coca et al., 1997; Eckl et al., 2012; Ishigami et al., 2000; Villegas et al., 2002). In addition to their cytotoxic function, upon interaction with melanoma cells, NK cells can also amplify their recruitment to tumors by releasing a chemotactic form of High Mobility Group Box-1 (HMGB1) protein capable of attracting additional activated NK cells (Parodi et al., 2015). Moreover, NK cells are found to recruit to tumor sites dendritic cells (DC) able to effectively 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 (cDC1) and are associated with increased overall patient survival in several cancer types (Bottcher et al., 2018). In addition, these innate cells, through secretion of FLT3L, control the abundance of intra-tumoral stimulatory DC and their frequency that directly correlates with survival in patients with melanoma receiving anti-PD-1 therapy (Barry et al., 2018).

The importance of the NK cell anti-tumor effector activity in tumor surveillance is corroborated by many studies showing that NK cells are frequently suppressed/altered in both solid tumors and hematological malignancies. NK cells present in solid tumors (including lung, gastric, colorectal, and head and neck cancers) are usually scarce. Few exceptions are represented by Renal Cell Carcinoma (RCC) and GastroIntestinal Stromal Tumors (GIST), which are infiltrated by a significant number of $NKp46^+$ cells (Rusakiewicz et al., 2013; Schleypen et al., 2003). Of note, TI- NK cells are usually not in direct contact with neoplastic cells, but they are rather located within the stroma. Besides their relative frequency and numbers, a key-limiting factor of the tumor-killing capacity of NK cells is their functional impairment in tumors. Solid-tumor infiltrating NK cells often display a $CD56^{bright}$ phenotype and/or a reduced expression of the activating receptors resulting in a de-potentiated anti-tumor activity. In breast cancer, despite TI-NK cells are enriched in $CD56^{bright}$ cells expressing NKp44 and CD25 and CD69 activation markers, they exhibit low levels of NKp30, NKG2D, DNAM-1 and CD16 and poor cytotoxic potential (Mamessier et al., 2011). In early-stage non–small cell lung cancers (NSCLC) TI-NK cells are mostly $CD56^{dim}$, but express limited amounts of activating receptors (namely NKp30, NKp80, CD16, and DNAM-1) and display a low degranulation and cytokine release potential (Platonova et al., 2011). In RCC, infiltrating NK cells express high levels of CD94/NKG2A inhibitory receptor, this contributing to decreased NK cell activity (Schleypen et al., 2003), while in GIST patients, NK cells are found to express predominantly the immunosuppressive NKp30c isoform and display a $CD56^{bright}CD16^-KIR^-$ phenotype (Delahaye et al., 2011; Rusakiewicz et al., 2013).

In order to gain further insight about the functional states of human NK cells in solid tumor, a recent study analyzed the transcriptome of TI-NK cells isolated from human melanoma metastases compared with circulating NK cells (de Andrade et al., 2019). Single-cell RNA-seq analysis of TI-NK cells has identified different NK cell subsets with specialized gene expression programs. Some NK cell subsets are found to express high levels of *XCL1* and *XCL2* chemokines genes that are critical for cross-presenting $XCR1^+$ DCs recruitment into tumors (Bottcher et al., 2018), whereas other subsets show a higher perforin and granzyme B expression. This analysis also reveals that TI-NK cells express higher levels of the *KRCL1* gene encoding NKG2A inhibitory receptor in comparison to circulating NK cells (de Andrade et al., 2019).

Indeed, the local tumor microenvironment (tumor residing cells or tumor cells themselves) can directly exert suppressive effects on the NK cell effector mechanisms. Immature myeloid cells and tumor associated macrophages (TAM) can polarize a Th2 response and/or produce suppressive factors such as IL-10, TGF-β and reactive oxygen species (ROS) (Gabrilovich et al., 2012). Treg cells represent another cell subset whose accumulation in tumors correlates with impaired immune

function and poor prognosis. A Treg increase and a low NK cell activity has been described in GIST and Hepatocellular carcinoma (HCC) bearing subjects (Ghiringhelli et al., 2005). Also Tumor-associated fibroblasts (TAF) derived from different solid tumors were shown to inhibit NK cell function through both cell-to-cell contact and release of PGE2, which abrogate the IL-2-induced up-regulation of NKp44, DNAM-1 and NKp30 (Balsamo et al., 2009; Li et al., 2012, 2013). Tumor cells can hamper NK immune response by different inhibitory mechanisms, including IDO expression and/or PGE2 production in metastatic melanoma, modulating expression of NKp30, NKp44 and NKG2D (Pietra et al., 2012). Other soluble tumor-derived factors such as TGF-β, IL-4, Macrophage migration inhibitory factor (MIF), MUC-16 and adenosine (Hoskin et al., 2008) can hamper NK cell functions. In neuroblastoma, TGF-β inhibits NK cell functions by modulating both the activating receptor expression and chemokine-receptor repertoire, possibly interfering with their ability to migrate and accumulate into tumor nest (Castriconi et al., 2013; Regis et al., 2017). MIF and MUC-16 glycoprotein, expressed in ovarian tumor, are able to down-regulate NKG2D and to interfere with the formation of the synapses between tumor cells and NK cells. In addition, shedding of MICA (NKG2D ligand) or of BAT3/BAG6 and B7H6 (ligands of NKp30) represents a commonly reported tumor escape mechanism (Groh et al., 2002; Kaiser et al., 2007; Salih et al., 2002). Finally, down-modulation of NK cell activity by tumor cells can also be mediated by inhibitory signals triggered by the engagement of NKp44 receptor with its ligand PCNA expressed in different tumor types.

Hypoxia, a condition which often characterizes tumor tissues, can both favor the selection of tumor cells with increased invasive and metastatic potential and alter the phenotypic and functional features of tumor-infiltrating immune cells. Along this line, hypoxia can significantly alter both the expression and function of major activating NK receptors with the remarkable exception of CD16, thus allowing NK cells to maintain their capability of mediating ADCC (Balsamo et al., 2013).

As far as hematological malignancies, different reports revealed that mature NK cells isolated from peripheral blood of patients with Acute Myeloid Leukemia (AML) display a low expression of major activating NK receptors, paralleled by an increased expression of CD94/NKG2A inhibitory receptors, low production of TNF-α and IFN-γ and an impaired cytolytic activity (Costello et al., 2002; Fauriat et al., 2005; Khaznadar et al., 2015; Stringaris et al., 2014). In serum of AML patients, the presence of soluble NKG2D-L (including MICA, MICB and ULBP2) is associated with a down-regulation of the surface NKG2D expression leading to an impairment in NKG2D-mediated NK-cell activity (Hilpert et al., 2012). Soluble ligands of NK cell activating receptors can be also detected bound to tumor-derived exosomes in patient's serum. Exosomes from leukemia/lymphoma cells can express NKG2D-L, leading to an inhibition of the NK cell activation (Clayton et al., 2008). In addition, chronic lymphocytic leukemia (CLL) may release soluble BAG6 (i.e. NKp30-ligand) capable of competing with the exosome-bound BAG6 for the induction of NK cell activation via NKp30 (Pogge von Strandmann et al., 2007; Reiners et al., 2013). Soluble inhibitory factors, such as IDO or TGF-β, may also play a significant inhibitory role (Curti et al., 2009; Szczepanski et al., 2011). In this context, sera derived from human AML patients have been shown to contain micro-vesicles bearing TGF-β at their surface, resulting in impairment of NK-cell function.

In chronic myeloid leukemia (CML) patients, PB NK cells are decreased in numbers and display defects in their proliferation potential and cytolytic functions in comparison with healthy donor blood NK cells (Pierson and Miller, 1996). Similarly, in patients with Myelodysplastic Syndrome (MDS), the cytolytic activity of NK cells is severely altered, even in the presence of IL-2 stimulation *in vitro* (Kiladjian et al., 2006).

Finally, AML blasts may activate the aryl hydrocarbon receptor (AHR) pathway that induces miR-29b expression in NK cell precursors impairing their maturation and functions (Scoville et al., 2018).

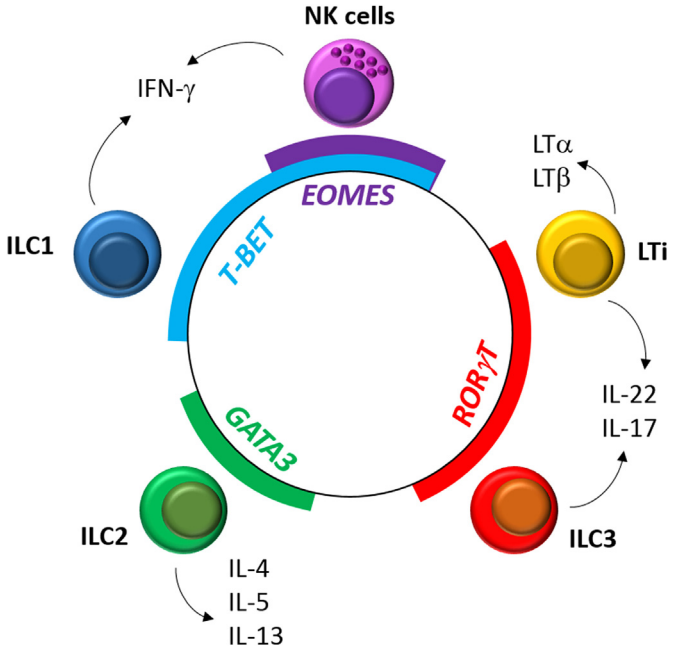

Fig. 1. NK cells and helper ILCs
NK cells and ILC1 are dependent on T-bet for their development and produce IFN-γ as signature cytokine. NK cells also require the transcription factor Eomes, and are characterized by cytotoxic activity dependent on the exocytosis of preformed lytic granules. ILC2 express GATA3 and produce IL-4, IL-5, and IL-13. Both LTi and ILC3 are dependent on RORγt for their development and function and produce IL-22 and IL-17, with LTi also capable of secreting lymphotoxin (LT) α and β.

3. Helper ILCs

ILCs represent the innate counterpart of T lymphocytes (Artis and Spits, 2015; Eberl et al., 2015; Spits et al., 2013). Thus, NK cells mirror the functions of $CD8^+$ cytotoxic T cells, whereas ILC1, ILC2, and ILC3 are referred to as "helper ILCs" (hILCs) because they mirror $CD4^+$ T helper (Th)1, Th2, and Th17 cells, respectively. Recently, ILCs have been classified in five subsets in terms of their function and transcription factor requirements (Fig. 1) (Vivier et al., 2018). Both NK cells and ILC1 produce IFN-γ as signature cytokine and are dependent on T-bet. While NK cells require also Eomes, ILC1 can develop in the absence of this transcription factor. ILC2 are defined by their capacity to produce the type-2 cytokines IL-4, IL-5, and IL-13 (Moro et al., 2010; Neill et al., 2010; Price et al., 2010) and contain larger amounts of the transcription factor GATA3 than the other ILC subsets (Furusawa et al., 2013; Hoyler et al., 2012; Klein Wolterink et al., 2013; Mjosberg et al., 2012). ILC3 are dependent on RORγt for their development and function, and the predominant cytokine they produce is IL-22 (Buonocore et al., 2010; Sanos et al., 2009; Satoh-Takayama et al., 2008). The fifth subset of ILCs is represented by lymphoid tissue inducer (LTi) cells, crucial for the formation of lymph nodes and Peyer's patches during embryonic development, mainly through the action of lymphotoxin (Mebius et al., 1997).

While the role of NK cells in controlling tumor growth and metastasis is now well established, the involvement of hILCs in tumors remains poorly understood. hILCs are mostly located in mucosal barriers and play an important role in the maintenance of tissue integrity and in innate defenses against pathogens. Nevertheless, their ability to contribute to tissue remodeling and induce neo-angiogenesis upon tissue damage through the production of large amounts of cytokines suggests that they may also contribute to tumor-associated inflammation

(Tumino et al., 2019c). In the following paragraphs, we summarize recent evidences on the role played by hILCs in tumor control. Their role is sometimes controversial because of the ILCs heterogeneity and plasticity possibly induced by the TME. Further investigations are thus needed to clearly understand how to exploit hILCs in cancer immunotherapy.

3.1. ILC1

ILCs plasticity, namely a shift from one to another ILC subset, has been reported especially for ILC1s, implying that their functional capability may change substantially in a given microenvironment, particularly at tumor sites under the influence of soluble factors (Bald et al., 2019). For instance, analogously to what has been shown in human decidua (Montaldo et al., 2015), molecules present in the tumor microenvironment, such as TGF-β can induce NK cell conversion to ILC1s with pro-angiogenic and immune tolerant features. This was suggested by the identification, in both murine (Gao et al., 2017) and human (Levi et al., 2015) tumors, of "ILC1-like" cells displaying intermediate features and capable of promoting metastasis by producing VEGF (Levi et al., 2015; Montaldo et al., 2015). On the other hand, ILC1-derived IFN-γ may have antitumor effects, including the recruitment and the activation of effector immune cells through the upregulation of costimulatory molecules, cytotoxicity and cytokine production (Castro et al., 2018).

3.2. ILC2

ILC2 respond to IL-25, thymic stromal lymphopoietin (TSLP), and IL-33 cytokines and are mainly involved in the innate immune response to parasites and in the damaged tissue repair after infection resolution (Monticelli et al., 2011, 2015). Type 2 responses have classically been associated to the establishment of a tumor-promoting microenvironment. Indeed, ILC2 have been shown to play a predominant detrimental role in various tumor settings (Bie et al., 2014; Trabanelli et al., 2019). For instance, ILC2 activity may correlate with the immunosuppressive function of myeloid-derived suppressor cells (MDSCs) in acute promyelocytic leukemia (Trabanelli et al., 2017) and in bladder cancer (Chevalier et al., 2017). However, a protective role in cancer has been reported for IL-33, the major cytokine promoting differentiation and function of ILC2. In a mouse model of lymphoma, IL-33 overexpression was shown to block tumor growth (Kim et al., 2016), and in primary and metastatic murine lung tumors IL-33 could enhance both MHC class I expression on dendritic cells and T cell-mediated killing of tumor cells (Saranchova et al., 2016). Therefore, these studies suggest that, in particular conditions, ILC2 may contribute to promote an anti-tumor immune response.

3.3. ILC3

ILC3 development depends on RORγt transcription factor. In humans and mice, two different subsets of ILC3 can be distinguished on the basis of their surface expression of the NCR, NKp44 and NKp46, namely NCR^- and NCR^+ ILC3. They mainly produce IL-17, IL-22, IL-8 and GM-CSF in response to appropriate stimuli including IL-23, IL-1β. In particular, in humans, IL-22 production is restricted to the NCR^+ ILC3 subset, while NCR^- ILC3 predominantly produce IL-17 (Cella et al., 2009; Hoorweg et al., 2012). NCR^+ ILC3 appear to contribute to the formation and/or maintenance of tertiary lymphoid organs (TLO) at the tumor sites. Importantly, the presence and frequency of TLO are associated with a better prognosis. In NSCLC, NCR^+ ILC3 were found to correlate with the density of TLO, produced the pro-inflammatory cytokines IL-22 and TNF-α, and the IL-8 chemokine. In addition, they induced the expression of the adhesion molecules ICAM-1 and VCAM-1 on mesenchymal stromal cells that, in turn, favor leukocyte infiltration (Carrega et al., 2015; Eisenring et al., 2010). In NSCLC, low numbers of NCR^+ ILC3 have been shown to be associated with more advanced tumor stages and, consequently, with a poorer prognosis (Carrega et al., 2015). On the contrary, ILC3 may also display a tumorigenic capability. Indeed, high levels of IL-23, and IL-17 have been associated with progression to fatal metastatic colorectal cancer (Wang and Karin, 2015). Moreover, in colorectal cancer, IL-22 correlated with tumor growth and metastasis (Jiang et al., 2013) and chemoresistance (Wu et al., 2013).

The ambiguity of helper ILC, including ILC3, in the control of human tumors, may pose serious doubts on their possible therapeutic use in cancer patients. On the other hand, their ability to promote tissue repair, maintenance of the tissue integrity and defense against pathogens, may result useful in patients receiving HSCT. Indeed, both the conditioning regimen and GvHD may cause serious tissue damages. In this context, LTi-like cells may contribute to the restoration not only of the mucosal integrity, but also to the re-organization of mucosa-associated TLO (see above). In this context, it has been shown that $CD34^+$ cells, used as a source of hematopoietic precursors in HSCT, derived from different sources including BM, PB of G-CSF-mobilized donors or umbilical cord blood (UCB), generate NK and ILC3 in remarkably different proportions. In particular, ILC3 are more represented in the lymphoid progenies of $CD34^+$ precursors derived from UCB or BM. In addition, a negative effect on the ILC3 generation is exerted by G-CSF, as shown by *in vitro* studies (Moretta et al., 2016). These data may be exploited for the development of culture conditions favoring the generation of ILC3 to be used in protocols of adoptive cell therapy in case of delayed mucosal tissue repair after HSCT or severe GvHD. In this context, it has been reported that a transient increase of circulating NCR^+ ILC3 correlates with a reduced incidence of GvHD (Munneke et al., 2014), thus further supporting the notion of a favorable effect of ILC3 in the prevention/cure of GvHD. Interestingly, IL-1β has been shown to affect the differentiation of $CD34^+$ precursors towards ILC3, favoring NK cell development, suggesting that inflammatory responses may interfere with ILC3 generation (Ambrosini et al., 2015; Vitale et al., 2015). In any case, a more precise knowledge of the conditions which influence the differentiation of ILC in HSCT recipients is important in view of their defensive role against different pathogens, which represent a major threat in transplanted patients.

3.4. Immune checkpoints on hILCs

It has been shown in many inflammatory conditions that the expression of IC is induced not only on NK cells (see section 2.3), but also on hILCs. In vitro experiments and data on mouse models of infection demonstrate that the expression of IC on hILCs inhibits their cytokine production (Mariotti et al., 2019). In the context of cancer, there are many recent studies showing that ICs are expressed on both NK cells and hILCs. For instance, in mice, tumor-associated ILC1s express higher levels of inhibitory receptors (NKG2A, KLRG1, CTLA4, CD96, LAG3) compared to NK cells (Gao et al., 2017). In a study investigating IC expression on ILCs in breast tumors, it was shown that ILC1s isolated from malignant tissues express higher levels of CTLA-4 compared to circulating ILC1s, and that infiltrating ILC2s express higher levels of both CTLA4 and PD-1 (Salimi et al., 2018). In gastrointestinal tumors, a higher expression of PD-1 in malignant compared to para-lesional tissues was shown for ILC2s and ILC3s (Salimi et al., 2018). PD-1 expression has also been detected on both NK cells and ILC3s in malignant pleural effusions of patients with primary and metastatic tumors (Tumino et al., 2019b). ILC2s normally express the IC KLRG1, and its expression can be further upregulated in inflammatory conditions. High KLRG1 expression was found indeed on ILC2s in gastrointestinal tumors (Salimi et al., 2018), and on ILC2s isolated from colorectal cancers (Simoni et al., 2017).

The role of IC expression by specific ILC subsets and in particular types of cancer to date is unknown. We can speculate, for instance, that IC expression on tumor-associated ILC2s may favor tumor elimination by inhibiting a type 2 response, analogously to what was shown in

helminth infections (Taylor et al., 2017). Moreover, high IC expression by ILC1s suggests that TME-induced NK cell conversion to ILC1s could represent a mechanism by which tumor escapes immune surveillance. However, to date the actual impact of IC expression by hILCs on the efficacy of anti-tumor immune response is not clear. In addition to descriptive data on IC expression in hILCs in cancer, functional studies in this context are thus clearly needed. This information would help not only to understand the role of hILCs and their cytokines in the response to tumors, but also to predict the favourable or unfavourable side effects of targeting these cell subsets when ICI are used in the therapy to unleash NK cell activity (see section 4.4).

4. Harnessing NK cells

4.1. Cytokines

Therapeutic approaches based on the use of cytokines able to directly stimulate and promote NK cell activation, persistence, and expansion have been tested in several preclinical and clinical studies (Davis et al., 2015b; Hu et al., 2019).

The first cytokine that was used to improve NK cell antitumor responses was IL-2 (Rosenberg et al., 1985), but some problems due to the use of this cytokine have been reported in clinical studies. Indeed, the high doses of IL-2 that are necessary to allow an efficient antitumor activity can induce severe adverse reactions, including vascular leakage and organ injury caused by activation of the vascular endothelium (Dutcher et al., 1991; Kruit et al., 1994; Rosenberg et al., 1987). On the contrary, low doses of IL-2 can induce an efficient *in vivo* expansion of NK cells, but also a limited antitumor efficacy (Miller et al., 1997). Moreover, IL-2 acting on Treg cells can induce production of TGF-β and, consequently, immunosuppression (Ghiringhelli et al., 2005). For these reasons, mutant forms of IL-2 characterized by high affinity for the IL-2Rβγ present on NK cells, but reduced affinity for the IL-2Rα expressed on Treg cells, became necessary for inducing immunoactivation without immunosuppression (Carmenate et al., 2013; Levin et al., 2012; Sim et al., 2016).

Another cytokine that plays an important role in NK cell activation without inducing Treg-mediated immune suppression is represented by IL-15. Also in this case, modifications of the cytokine have been performed to improve IL-15 half-life, optimize its biological effects, and even reducing toxicity. One of these compounds is represented by the IL-15 superagonist ALT-803 that has been recently developed by binding an IL-15 mutant (called IL-15N72D) to a soluble, dimeric IL-15Rα-Fc fusion protein (IL-15Rα-Fc) (Han et al., 2011; Wong et al., 2013) (Fig. 2A). ALT-803 has been described to increase both NK and T cell-mediated anti-tumor responses both in preclinical and clinical studies. Notably, it is generally well-tolerated and it does not induce severe toxicities as well as GvHD (Romee et al., 2018). Interestingly, ALT-803 can function as scaffold for producing more active immunotherapeutic agents. For example, it has been fused with four single chains of rituximab (anti-CD20) to generate the 2B8T2M compound that is characterized by a trispecific activity: CD20 binding on tumor cells, cytokine-stimulation of NK cells, and induction of ADCC by binding FcγR on NK cells (Charych et al., 2016). Moreover, considering the IL-15 efficacy in NK cell expansion and the capability of IL-21 in boosting NK cell antitumor cytotoxicity, a protocol based on the sequential use of these two cytokines has been developed (Wagner et al., 2017).

A different approach capable of inducing expansion of NK cells *ex-vivo* is based on the use of cells genetically modified to express membrane-bound cytokines. For example, K562 feeder cells carrying membrane-bound IL-21 have been recently used in a phase I clinical trial for *ex vivo* expansion of donor NK cells in haplo-HSCT (Ciurea et al., 2017; Denman et al., 2012).

Another promising cytokine-based approach for antitumor clinical applications is represented by the “cytokine induced memory-like NK cells” (CIML-NK) (Romee et al., 2016). These cells are obtained by *ex vivo* pre-activation of allogeneic NK cells with a mixture of cytokines, in particular IL-15, IL-12, and IL-18, before the NK cell adoptive transfer, and represent a promising tool in cancer immunotherapy. Indeed, CIML-NK cells exhibit enhanced effector functions: higher IFN-γ production, increased expression of granzyme B and perforin, superior cytotoxicity in response to tumor targets (Fig. 2B). However, it is important to consider that cytokine-prestimulation induces phenotypic changes (including homing receptors) that may influence trafficking and proliferation of NK cells (Terren et al., 2018). Several clinical trials based on the use of CIML-NK cells are ongoing (NCT01898793, NCT03068819, and NCT02782546).

4.2. Toll-like receptors (TLRs)

Initially described in other innate immune cells, such as DCs and monocytes/macrophages, some TLRs, including TLR2 (Becker et al., 2003; Ghiringhelli et al., 2005; Marcenaro et al., 2008), TLR3 (Pisegna et al., 2004; Schmidt et al., 2004; Sivori et al., 2007), TLR5 (Chalifour et al., 2004), TLR7/8 (Alter et al., 2007; Hart et al., 2005) and TLR9 (Sivori et al., 2006), have been also described on human NK cells. TLRs allow NK cells to sense and respond to viral and bacterial patterns. In particular, the TLR-mediated recognition of PAMPs has been shown to induce NK cell activation both in terms of increment of cytotoxicity and cytokine release. However, PAMP-mediated activation of NK cells can only occur thanks to the activity of other immune cells (such as DCs) that offer the cytokine microenvironment necessary for inducing the NK cell response. In addition, in the context of the NK/DC crosstalk, TLRs can allow the NK cell-mediated editing of DC maturation, a sort of “quality control” process by which NK cells select those DCs that are undergoing appropriate maturation and, as a consequence, will be able to mediate the optimal T cell priming after migration to SLOs (Marcenaro et al., 2011; Sivori et al., 2004). Moreover, a peculiar cooperation between TLR and KIR has been demonstrated in human NK cells (Sivori et al., 2010a, 2010b). Indeed, KIR3DL2 can bind CpG-ODNs (TLR9 ligands) at the NK cell surface by its extracellular D0 domain and shuttle CpG-ODNs to endosomes where TLR9 is localized, thus allowing TLR-mediated activation of NK cell function. Notably, similarly to KIR3DL2, also other KIRs carrying the extracellular D0 domain have been shown to bind CpG-DNA. Considering that the D0 domain of KIRs was hypothesized to be expressed by the putative ancestral mammalian KIR, these data suggested that, originally, certain KIRs could exert a function different from recognition of HLA class I molecules and act as sensors for microbial products and as chaperones for TLR9 ligands. This fact may suggest that conservation of KIR3DL2-encoding gene in all haplotypes could depend on the need of prompt NK-mediated responses to microbial products.

On the basis of their capability of harnessing antitumor immune responses, CpG-DNA and other TLR agonists have been considered as possible adjuvants in immunotherapy (Circelli et al., 2017; Kim et al., 2010; Matsumoto et al., 2015). Indeed, the use of CpG-ODNs to promote Th1 immune responses in combination with chemotherapy may induce potent anti-tumor immune responses with relevant clinical benefits.

Recently, it has been shown that several miRNA can bind to TLRs (such as TLR3 and TLR7/8) and mediate immune responses by inducing immune and inflammatory gene expression. In addition, some miRNA can regulate TLR expression and signaling, and TLR signaling may modulate miRNA expression (Bayraktar et al., 2019). Thus, it could be interesting to evaluate whether similar effects may occur also in NK cells, where TLRs known to bind miRNA, such as TLR3 and TLR7/8, have been described to be present.

4.3. BiKE, TriKE, engagers

Bi- and tri-specific killer cell engagers (BiKEs and TriKEs) have been

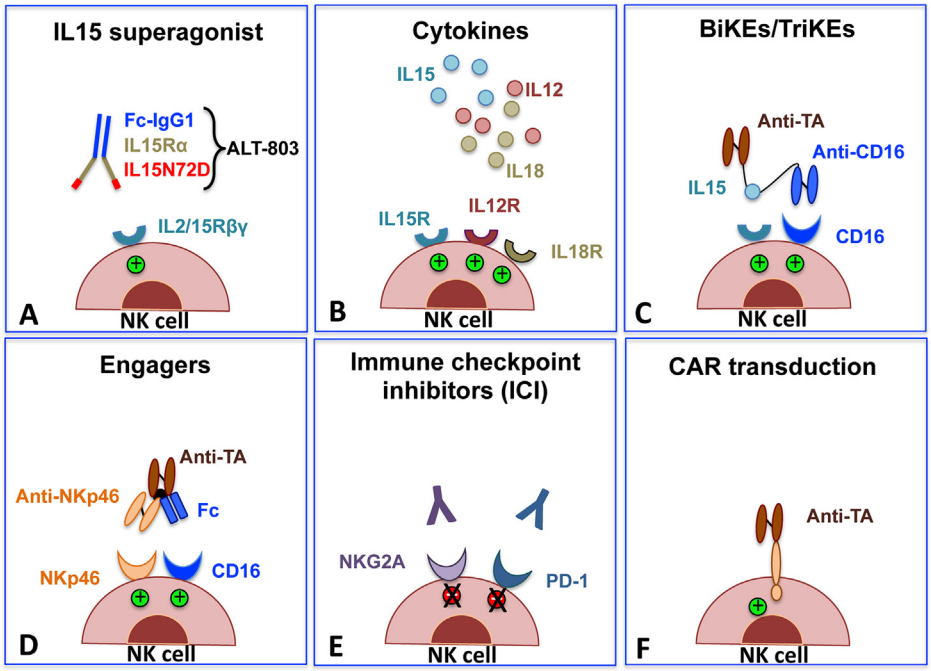

Fig. 2. Strategies to harness the antitumor activity of NK cells

A) The IL-15 superagonist ALT-803 is an engineered cytokine, an IL-15 mutant (IL-15N72D) bound to a soluble dimeric IL-15Rα-Fc fusion protein. **B)** IL-15, IL-12 and IL-18 cytokines can potentiate NK cells, named "cytokine induced memory-like" (CIML-NK). **C)** BiKEs and TriKEs are constructs composed of two or three single chain variable fragments (scFv), respectively, specific for the activating NK receptor CD16 and for tumor antigens (TA), to enhance NK/tumor cell interaction and NK cell killing. Depicted is a TriKE, incorporating an IL-15 moiety. **D)** Trifunctional NK cell engagers (NKCEs) act on two activating receptors, NKp46 (via scFv) and CD16 (via Fc), and a tumor antigen (via scFv) on cancer cells. **E)** Immune checkpoint inhibitors (ICI) such as anti-NKG2A and anti-PD-1 can unleash NK cells, allowing an antitumor response. **F)** NK cells can be genetically modified through the use of CAR constructs to redirect their specificity against antigens expressed on tumor cells.

designed to create an efficient immunological synapse between NK cells and tumor cells, by coupling NK cell engagement to tumor targeting (Davis et al., 2015b). These molecules are small constructs (50–75 kDa), composed by two (BiKEs) or three (TriKEs) single chain variable fragments (scFv) made up of a variable heavy and light chain (V_H and V_L) of antibodies, connected through short peptide linker(s). This methodology has been translated to NK cells from the experience on bi-specific T cell engaging antibodies (BiTE), such as blinatumomab, engaging CD3 on T cells and CD19 on B cell malignancies (Ribera, 2017; von Stackelberg et al., 2016). To efficiently trigger NK cell cytotoxicity, the engagement of CD16 has been chosen in the first generation of constructs targeting different tumor-cell antigens. BiKEs and TriKEs designed with anti-CD16 scFv can trigger NK cell function much more efficiently than the natural CD16-Fc interaction mediating ADCC of opsonized target cells (Moore et al., 2011), in view also of the CD16 polymorphism and the presence of low-affinity CD16 allotypes (Cartron et al., 2002) (Fig. 2C). In comparison to the use of mAbs, BiKEs and TriKEs display several advantages, including increased biodistribution for their smaller size, lower immunogenicity and greater flexibility. The engagement of CD16 has been combined with the recognition of CD19/CD22 on B cell Non-Hodgkin's lymphomas (Gleason et al., 2012), CD33 on AML and MDS (Gleason et al., 2014; Wiernik et al., 2013), EpCAM on various carcinomas (Vallera et al., 2016), and CD133 on cancer stem cells or EpCAM/CD133 for a broad spectrum molecule (Schmohl et al., 2016, 2017). TriKEs with multiple specificities have the advantage of targeting two different molecules, allowing to circumvent eventual down-regulation of one selected molecule on tumor cells. New generation of TriKEs and TetraKEs (e.g. molecules with four function domains) have been designed to incorporate an IL-15 moiety, with the aim of promoting NK cell activation, *in vivo* persistence and proliferation. This evidence has been provided with the anti-CD16 x IL-15 x anti-CD33 (abbreviated as 161533) TriKE targeting AML and MDS in both *in vitro* and *in vivo* preclinical models (Vallera et al., 2016). Moreover, a novel CD19 targeting 161519 TriKE has been developed, having a potential immunotherapeutic value in CLL (Felices et al., 2019). An interesting possibility is represented by the incorporation of an scFv blocking ADAM-17, a matrix metalloproteinase involved in CD16 shedding, to maximize CD16-mediated killing. In view of the high flexibility of the BiKE/TriKE platform, it can be also envisaged the use of blocking scFv targeting inhibitory checkpoint receptors or TGF-β to reduce negative signaling in the tumor microenvironment.

In addition to CD16, the engagement of other triggering NK cell receptors such as NCRs and NKG2D can be extremely valuable. In a recent study, trifunctional NK cell engagers (NKCEs) that co-engage NKp46 and CD16 on NK cells and recognize a surface antigen (e.g. CD19, CD20 or EGFR) on cancer cells have shown to elicit more potent anti-tumor activity than intact therapeutic mAb (e.g. rituximab, obinitumab and cetuximab) in preclinical models (Gauthier et al., 2019) (Fig. 2D). Importantly, while some activating receptors as CD16 appear down-regulated, NKp46 expression remains high in patients with many solid tumors, suggesting that its engagement can promote efficient NK cell function. Thus, NKCEs can be promising tools for cancer immunotherapy, complementing existing immune-oncology approaches.

4.4. Immune checkpoint inhibitors

In the tumor microenvironment, the fully anti-tumor potential of NK cells could be hindered by multiple IC, including HLA class I specific receptors (KIRs and NKG2A) and those recognizing ligands other than HLA class I molecules (PD-1, TIGIT, CD96, TIM-3, and LAG-3) (see section 2.3). Of note, a high expression of ligands for IC correlates with poor prognosis in patients with hematological malignancies (Guan et al., 2019). In addition, signals in the TME can influence or induce the *de novo* expression of some of these IC in tumor-associated NK cells, thus inhibiting NK cell functions. Indeed, all these IC are potential targets for drugs, the blocking of which may contribute to trigger/restore NK-mediated anti-tumor immunity.

Due to the impressive inhibitory effect exerted by KIRs on NK cell function, human mAbs blocking these molecules on NK cells were the first to enter clinical phase in AML, lymphoma and some solid tumors (Fig. 2E). In clinical trials, lirilumab (1–7F9, IPH2101) targeting KIR2DL1, KIR2DL2 and KIR2DL3 in patients with AML, myeloma or solid tumors showed limited side effects (Vey et al., 2018) but unfortunately also limited anti-tumor efficacy (Carlsten et al., 2016). By contrast, IPH4102, a first-in-class monoclonal antibody targeting KIR3DL2 is showing encouraging clinical activity in patients with cutaneous T-cell lymphoma, predominantly those with Sézary syndrome (Bagot et al., 2019).

A major progress towards the clinical use of antibodies against NK cell ICs has been achieved by Andrè et al. who explored whether

blocking of NKG2A (Andre et al., 2018), alone or in combination with other therapeutic mAbs, could unleash NK cell-mediated responses against tumor cells expressing HLA-E. Of note, several human solid cancers (including lung, colon, pancreas, stomach, head and neck, and liver tumors) express HLA-E, and NKG2A was found to be expressed by NK cells in the tumor nest. Monalizumab (an anti-NKG2A blocking mAb) not only promotes NKG2A$^+$ NK cell effector functions against HLA-E$^+$ target cells, but also improved the efficacy of durvalumab (an anti-PD-L1 blocking mAb) by increasing the reactivity of NKG2A$^+$PD-1$^+$ NK cells against HLA-E$^+$PD-L1$^+$ cells. Monalizumab is currently being tested in clinical trials as monotherapy or in combination with other antibodies (anti-EGFR or anti-PD-L1). A phase II clinical trial with monalizumab combined with cetuximab in patients with squamous cell carcinoma of head and neck (SCCHN, NCT26435509) has provided encouraging efficacy results.

Several studies in humans have shown that NK cells from cancer patients express PD-1, which correlates with a lower anti-tumor activity (Beldi-Ferchiou et al., 2016; Benson et al., 2010; Liu et al., 2017b; Pesce et al., 2019). Recently, it has been described that the therapeutic effect of PD-1 and PD-L1 blockade may rely also on the antitumor activity of NK cells (Hsu et al., 2018). Using several cancer mouse models, Hsu and coworkers found that activated NK cells express PD-1 and that PD-1 engagement by PD-L1$^+$ tumor cells potently suppresses NK cell–mediated immunity to tumors. Thus, releasing PD-1–imposed inhibition, through blockade of PD-1 or PD-L1, is able to activate an NK response that could be indispensable for the full effect of PD-1/PD-L1 blockade.

TIGIT is a checkpoint inhibitory receptor constitutively expressed by NK cells and usually up-regulated on tumor resident NK cells. This receptor was found to be associated with NK cell exhaustion in patients with colon cancer. TIGIT blockade is able to reverse the exhausted status of tumor infiltrating NK cells (Zhang et al., 2018), thus representing a potential new strategy to explore in immunotherapy. To date, clinical trials investigating the therapeutic potential of anti-TIGIT mAbs (used either alone or in combination with anti-PD-1/PD-L1) for the treatment of patient with advanced solid tumors are active.

CD96 is another important checkpoint for NK cell effector functions. In particular CD96$^+$ NK cells are significantly increased in the intra-tumor tissues of HCC (Sun et al., 2019). These CD96$^+$ NK cells are functionally exhausted with impaired IFN-γ and TNF-α production, and low gene expression levels of *Tbx21*, *Prf1*, and *GzmB*. Importantly, patients with higher intra-tumor CD96 expression exhibit poorer clinical outcomes, suggesting a possible therapeutic role of CD96 blockade in fighting liver cancer.

Tim-3 is expressed on both adaptive and innate immune cells. It has been described that up-regulated expression of TIM-3 on circulating NK cells correlates with decreased overall survival in lung carcinoma patients (Xu et al., 2015) and advanced tumor stage in gastric cancer patients (Wang et al., 2015). In esophageal cancer patients, Tim-3$^+$ NK cells exhibit a phenotype with enhanced dysfunction. Accordingly, a high Tim-3 level on tumor-infiltrating NK cells is correlated with tumor invasion, nodal status and poor stage in these patients (Zheng et al., 2019). Furthermore, NK cells from melanoma patients are found to be functionally impaired, and Tim-3 blockade reversed this exhausted phenotype (da Silva et al., 2014). Moreover, Tim-3 expression levels correlate with the stage of the disease and poor prognostic factors. Taken together, these data indicate that Tim-3 can function as an NK-cell exhaustion marker in advanced tumors and support the development of Tim-3-targeted therapies to restore antitumor immunity. Nevertheless, other studies have also reported stimulatory function of Tim-3 in promoting T cell differentiation and activation (De Sousa Linhares et al., 2018; Sabins et al., 2017). Thus, the use in the clinic of anti-Tim-3 mAbs should be implemented carefully because Tim-3 might also play and activating role under certain circumstances.

Finally, another IC that may be expressed by activated NK cells is LAG-3 (Triebel et al., 1990). The main ligands of LAG-3 are MHC class II molecules and the recently described fibrinogen family protein (FGL1) (Wang et al., 2019). FGL1 is up-regulated in a variety of human solid tumors and high FGL1 plasma levels in patients with lung cancer or melanoma is associated with a poor prognosis and resistance to anti-PD-1 therapy. These data indicate that the FGL1/LAG-3 interaction may facilitate tumor immune escape. While in mice it has been shown that LAG-3 reduced NK cell-mediated anti-metastatic activity (Ohs et al., 2017), studies on human LAG-3$^+$ NK cells are limited. Relatlimab (and anti-LAG3 mAb) is being investigated, either alone or in combination with other ICI, in several types of cancers (NCT02061761; NCT02658981).

5. Exploiting NK cells in HSCT

Allogeneic HSCT represents a life-saving therapy for patients with high-risk acute leukemia (Copelan, 2006). However, timely availability of an HLA-identical sibling or HLA-matched unrelated donor to provide access to HSCT can be extremely challenging. The likelihood of finding an HLA-compatible donor can significantly vary among racial and ethnic groups, ranging from 75% for whites of European descent to 16% for blacks of South or Central American descent (Gragert et al., 2014). In contrast, most patients have a readily available family member with one identical HLA haplotype and the other mismatched (i.e. haploidentical). For pediatric patients both parents can be HLA-haploidentical donors, highly motivated to promptly donate hematopoietic stem cells. However, strategies must be applied to overcome the prohibitive immunological barriers due to the high degree of HLA incompatibility in the donor/recipient pair.

5.1. Haplo-HSCT with pure CD34$^+$ cells

Haplo-HSCT became successful in 1990s through the combined use of intensified myeloablative conditioning to prevent graft rejection, extensive T-cell depletion of the graft to avoid GvHD, and infusion of “mega-doses” of highly purified CD34 positively selected cells (Aversa et al., 1998). Seminal studies by the Perugia group reported that, in adult patients with high-risk AML given haplo-HSCT in complete remission (CR), the use of an NK-alloreactive donors (according to KIR/KIR-L mismatch in GvH direction model) was associated with better event-free survival (EFS) (67% vs. 18%) (Ruggeri et al., 2002, 2007). In pediatric patients receiving CD34$^+$ haplo-HSCT, the overall survival rate was very successful in the presence of NK alloreactivity, especially for the subset of patients with high-risk ALL (~70% survival). On the other hand, only ~40% of children with AML became long survivors in the presence of donor alloreactive NK cells. In the absence of NK alloreactivity, the survival rate was ~35% for ALL and ~20% for AML (Locatelli et al., 2018). The immune reconstitution in the first period of time after CD34$^+$ haplo-HSCT mainly relies on NK cells, representing the first lymphocyte population which reconstitute after the allograft, while the recovery of T cells requires months to be completed. The first wave of NK cells is composed of poorly cytolytic cells displaying an immature phenotype (mainly KIR$^-$, CD94/NKG2A$^+$), and, upon differentiation, the appearance of KIR$^+$ NK cells, including alloreactive cells, requires 2–3 months after transplant, these cells then persisting for years in the recipient (Pende et al., 2009). Regarding aKIR, a positive contribution of KIR2DS1$^+$ NK cells derived from HLA-C1/C2 donors was demonstrated in the recognition and killing of HLA-C2/C2 leukemia cells (Pende et al., 2009). The relevant role played by aKIRs has been also documented by the evidence that patients transplanted from donors carrying the B/x genotype and a high B content value, implying the presence of many aKIRs, have a significantly better EFS (Mancusi et al., 2015; Oevermann et al., 2014), consistent with previous findings on unrelated HSCT (Cooley et al., 2010).

While the survival rates in CD34$^+$ haplo-HSCT should be considered as a positive result in patients with no alternative therapeutic options, a further improvement was desirable. Notably, virtually all deaths consequent to leukemia relapses or transplant-related mortality (TRM)

occurred early, within few months after transplant, when the patients lack both adaptive immunity and mature NK cells, including alloreactive NK cells. This immune-deficient status may greatly compromise the control of leukemia relapses and infections (Reisner et al., 2011; Rocha and Locatelli, 2007).

5.2. The αβT- and B-cell-depleted haplo-HSCT

Taking into account, in CD34$^+$ haplo-HSCT, the delayed generation of efficient, highly cytolytic NK cells and the parallel early occurrence of life-threatening events, a novel method of graft manipulation has been developed, based on the negative selection of αβT lymphocytes, to avoid GvHD, and CD19$^+$ B cells, to prevent post-transplant EBV-related lymphoproliferative disorders. In αβT/B-depleted haplo-HSCT, the graft infused into the patients is composed of different cell types, besides HSC (Li Pira et al., 2016). Of major interest, a high number of donor mature NK cells (median dose infused 34.6 × 10^6/kg) could help prevent leukemia recurrence and also exert a protective role against virus reactivation/infection (Locatelli et al., 2017). Also the high number γδT cells infused with the graft (median dose 8.1 × 10^6/kg), may greatly contribute to the killing of residual leukemia blasts escaping the conditioning regimen and to protect patients against infections (Airoldi et al., 2015; Braza and Klein, 2013; Vantourout and Hayday, 2013). The clinical outcome of pediatric patients was particularly good with a great improvement in comparison to the CD34$^+$ haplo-HSCT (Locatelli et al., 2017). The most dramatic improvement in the overall probability at 5 years was detected in patients with AML, with a LFS of 67.5%. Slightly better results were obtained in ALL patients (LFS of 71.4%). In the αβT- and B-cell-depleted haplo-HSCT, donor NK alloreactivity did not appear to offer a significant advantage in terms of final outcome. In this cohort, a donor selection between mother and father of the patient has been applied, giving priority to NK alloreactivity. In addition, other criteria addressed the focus on activating pathways, associated with a better NK-cell based anti-leukemia activity. These novel criteria included the presence of KIR B/x genotype and high B content value (more aKIRs), *KIR2DS1* in HLA-C1$^+$ donor for HLA-C2$^+$ recipient, higher numbers of NK and γδT lymphocytes in donor peripheral blood, high expression of NKp46, and presence of NKG2C (Locatelli et al., 2017, 2018). The presence of mature NK and γδT from the beginning of the transplantation and their persistence in the recipient as efficient immune cells, not impaired by any post-transplant pharmacological GvHD prophylaxis, can explain the successful clinical results. To further boost γδT cell activity, several patients given αβT/B-depleted haplo-HSCT were treated with zolendronic acid, a drug that promotes Vδ2 cell differentiation and cytotoxicity against leukemia blasts (Bertaina et al., 2017). The detrimental role played by MDSC, particularly abundant in the graft, on NK cells has been recently investigated (see section 5.3) (Tumino et al., 2019a).

5.3. Myeloid derived suppressor cells in αβT- and B-cell-depleted HSCT

MDSC are a heterogeneous population composed of both immature and mature, activated myeloid cells capable of inhibiting both innate and adaptive immune responses. Previous studies have shown that human and murine MDSC are capable of interfering with T- and NK-cell proliferation and/or function (Bronte et al., 2016; Gabrilovich and Nagaraj, 2009). Human MDSC can be classified on the basis of surface marker expression in two major subsets, namely, monocytic MDSC (Mo-MDSC) and PMN-MDSC.

Our group has recently demonstrated that CD66b$^+$ PMN-MDSC are present in large amounts in the peripheral blood of G-CSF mobilized donors and, more importantly, in the graft infused in the patient after depletion of αβT and CD19$^+$ B cells (Tumino et al., 2019a). We investigated the possible effect of PMN-MDSC on the *in vitro* HSC proliferation and differentiation towards NK and other ILC subsets showing no substantial changes in the frequency and in the functional activity of NK/ILC cells generated *in vitro* from HSC. By contrast, PMN-MDSC were shown to exert a potent inhibitory effect on mature NK cells, inducing a downregulation of the expression of major activating NK receptors and their related transduction molecules. This effect appears to be mediated by the release of IDO-induced tryptophan catabolites, PGE2 and MDSC-derived exosomes.

In view of these results, a novel graft manipulation including depletion of CD66b$^+$ PMN-MDSC from the graft could restore/potentiate the GvL activity and allow an optimal protection against infections by infused-donor NK cells.

6. CAR-NK

Genetic modification of effector cells of the immune system is a promising approach for treatment of advanced cancers that are refractory to conventional therapies (Melaiu et al., 2019; Sivori et al., 2019a). In particular, the use of chimeric antigen receptors (CAR) targeting cell surface antigens provides a valuable approach to increase the efficacy of effector cells (Fig. 2F). Indeed, CARs confer to an immune cell a high affinity binding to the cells expressing the target antigen, thus lowering the threshold of cell activation and induction of effector functions. CAR is a genetically engineered protein, composed by an extracellular domain specific for the target antigen that is derived from a single-chain variable fragment (scFv), a transmembrane domain and, finally, an intracellular signaling domain, responsible for the transduction of the activating signal.

CAR technology has been originally applied to T cells, in view of the readiness of their *in vitro* expansion and manipulation. Thus, most of the clinical trials have been, or are being, performed using CAR-T cells (Maude et al., 2018; Neelapu et al., 2017; Park et al., 2018). Notably, however, only autologous (i.e. patient-derived) T cells can be employed due to the risk of inducing severe GvHD if allogeneic T cells are used. Thus, major limitations in the use of CAR-T are related to the high manufacturing cost, to the time needed for their generation and expansion and to the uncertainty of the cell numbers obtainable in a time compatible with the risk of tumor progression and relapses. In contrast, the use of CAR-NK would eliminate the challenges related to the preparation of patient-specific cell products. In fact, NK cells do not cause GvHD (Chiossone et al., 2017; Daher and Rezvani, 2018; Moretta et al., 2011; Ruggeri et al., 2002). Accordingly, they can be obtained from healthy third-party donors and may represent suitable “off-the-shelf” products to be promptly used to treat patients. Importantly, adverse reactions, such as the cytokine release syndrome (CRS) or neurotoxicity, have been observed in patients given CAR-T cells (Maude et al., 2018; Neelapu et al., 2017; Park et al., 2018), but not when NK-cell based adoptive therapies, including those with CAR-NK cells (Liu et al., 2020), were used in clinical trials. The other side of the coin is the difficulty, often experienced, in expanding and transducing NK cells isolated from donor PB, in order to obtain clinical scale products. This has greatly limited the number of clinical trials using CAR-NK cells. In addition, with few exceptions, cells were primarily obtained from NK cell lines (Chen et al., 2016; Chu et al., 2014; Esser et al., 2012; Han et al., 2015; Imai et al., 2004; Kloess et al., 2019; Mehta and Rezvani, 2018; Muller et al., 2008; Romanski et al., 2016; Tang et al., 2018; Tassev et al., 2012; Uherek et al., 2002; You et al., 2019; Zhang et al., 2016), a procedure which may be, at least in part, unsafe or of limited efficacy because of the need to irradiate immortalized cell lines prior infusion to prevent propagation in patients (Tonn et al., 2013). NK cells derived from UCB have been explored as platform for CAR approaches; indeed, they are characterized by low risk of viral transmission from donor to recipient, rapid availability of UCB units, less stringent requirements for HLA matching, and lower risk of GvHD (Liu et al., 2018; Sarvaria et al., 2017). In particular, UCB-derived NK cells have been considered to generate CAR.CD19 NK cells that, thanks to the inclusion in the CAR construct of the sequence coding for IL-15, are also capable of producing this cytokine to boost in an autocrine/paracrine manner

their *in vivo* expansion and persistence. Early data on the clinical efficacy of this type of CAR-NK therapy show that the administration of CAR.CD19-modified UCB-derived NK cells is not associated with the development of CRS, neurotoxicity, or GvHD. In addition, anti-tumor responses were rapid and occurred within 30 days after infusion at all dose levels. The infused CAR-NK cells expanded and persisted at low levels for at least 12 months (Liu et al., 2020).

Several attempts have been conducted to obtain high numbers of NK cells from PB (Ayello et al., 2017; Kweon et al., 2019; Leung, 2014; Miller et al., 2005; Sutlu et al., 2010) but few of them have been applied to produce CAR-modified NK cells (Fujisaki et al., 2009). Recently, an innovative and efficient, feeder-free, bovine serum-free protocol to genetically modify and expand PB-derived NK cells with high proliferative capacity has been developed (Quintarelli et al., 2019). Thus, this approach is beneficial both in terms of GMP production and safety requirements. In the same study, we have also demonstrated that genetic modification of NK cells with a CAR targeting CD19 molecule on leukemia cells did not modify the expression and function of the "native" activating NK receptors. In this context, it is important to consider that, different from CAR-T cells, CAR-NK cells can recognize and kill tumor cells, even by the use of their native activating NK receptors, such as NCRs. This aspect is relevant especially when tumor cells lose the expression of the antigen targeted by the CAR. Moreover, the expression of CD16 by these CAR-modified PB-derived NK cells can mediate the killing of tumor cells even by ADCC (Sivori et al., 2019a; Vitale et al., 2019). Finally, using an *in vivo* mouse model of human lymphoma, we have shown that CAR-NK cells are able to mediate strong antitumor responses against B-cell malignancies, comparable to that of CAR T cells, but with the advantage of being associated with a lower production of cytokines such as IFN-γ and TNF-α (Quintarelli et al., 2019).

7. Adaptive NK cells

As mentioned above, human NK cells are capable of adapting their receptor repertoire and functional program in response to certain environmental stimuli.

Cytomegalovirus (CMV) infection represents so far the most powerful stimulus capable of promoting the differentiation of functionally and phenotypically skewed NK cells, endowed with unexpected features typical of adaptive immunity (Rolle and Brodin, 2016; Sun et al., 2011). By interacting with CMV-infected cells, NK cells can undergo an oligoclonal, virus-induced expansion, hallmarked by the expression of the HLA-E-specific activating receptor CD94/NKG2C, a highly differentiated surface signature $CD57^+$selfKIR$^+$NKG2A$^-$ and broad epigenetic modifications (Guma et al., 2004; Lee et al., 2015; Schlums et al., 2015). This CMV-driven NK cell subset has been first observed in CMV-seropositive healthy individuals (Guma et al., 2004) and later on in CMV-infected immunocompromised subjects (Beziat et al., 2015; Brunetta et al., 2010; Della Chiesa et al., 2012; Foley et al., 2012; Kuijpers et al., 2008; Muccio et al., 2016). Interestingly, a protective anti-viral role of CMV-induced NK cell expansions has been observed *in vivo* in different clinical settings including HSCT (Davis et al., 2015a), severe T cell immunodeficiency (Kuijpers et al., 2008) and kidney transplant recipients (Ataya et al., 2020; Redondo-Pachon et al., 2017), while a direct killing of CMV-infected targets by NKG2C$^+$ NK cells is not easily reproducible *in vitro* (Pupuleku et al., 2017).

In the HSCT setting, the influence exerted by CMV on NK cells developing after transplant has also highlighted the long-term persistence of this CMV-induced population, that can be detected at high frequencies for up to 2 years after transplant (Della Chiesa et al., 2012; Foley et al., 2012; Muccio et al., 2018). Along this line, recent studies in paroxysmal nocturnal hemoglobinuria and GATA2-deficient patients supported the preferential survival and/or self-renewal of NKG2C$^+$ NK cells expansions as compared to canonical NK cells (Corat et al., 2017; Schlums et al., 2017). This adaptive trait, besides directly challenging the classical view of NK cells as short-lived innate lymphocytes, renders this peculiar subset an appealing population to be exploited in immunotherapeutic approaches where the longevity of the NK-cell product is crucial (e.g. adoptive transfer, CAR-engineering). Interestingly, recent clinical data have shown that CMV-driven expansion of NKG2C$^+$CD57$^+$ NK cells in HSCT patients correlated with reduced leukemia relapse rates suggesting an immunotherapeutic role of adaptive NKG2C$^+$ NK cells per se against leukemia (Cichocki et al., 2016, 2019; Russo et al., 2018). These clinical observations have prompted the development of protocols, based on the mechanisms underlying the generation of adaptive NK cells that involve a direct role of NKG2C triggering (Guma et al., 2006; Rolle et al., 2018), to efficiently expand NKG2C$^+$ NK cells that can be used to treat pediatric ALL (Liu et al., 2017a). As mentioned before, adaptive NKG2C$^+$ NK cells usually express a specific KIR and lack NKG2A; thus, it would be possible to expand alloreactive NK cell populations by the optimization of these expansion protocols.

Longevity and expansion capabilities are not the only features shown by adaptive NK cells that can be usefully harnessed in immunotherapy. Indeed, adaptive NK cells are also endowed with heightened ADCC abilities that can augment the efficacy of therapeutic mAbs, BiKE, TriKE or other immune engagers on NK-mediated killing of tumor targets by this population (Capuano et al., 2018; Schlums et al., 2015; Zhang et al., 2013). Interestingly, adaptive NK cells appear to be resistant to MDSC and Treg suppression, thus possibly retaining further advantage in the tumor microenvironment (Sarhan et al., 2016, 2018).

In view of their specialized features, adaptive NK cells hold important translational promise in novel immunotherapies (Capuano et al., 2019).

8. Future perspectives

Recently, major progresses in the cure of highly aggressive cancers have been achieved thanks to immunotherapeutic approaches that either target mechanisms controlling the immune response or harness potent effector cells belonging to the adaptive or to the innate immunity. Thus, the use of ICI has changed the clinical history of highly lethal cancers, while the use of CAR-engineered T cells allows targeting tumor cell surface antigens with high specificity. Despite these major advances, a relevant percentage of patients are poorly or non-responders to these innovative therapies. Therefore, there is clearly need of substantial therapeutic improvements. For example, failure to respond to the currently available ICI may require blocking of other inhibitory pathways, either receptor-mediated or involving soluble factors present in the TME. A therapeutic failure of CAR-T cells may reflect the loss of the tumor antigen targeted by CAR or the inhibitory effect of the TME. Therefore, it is clear that either the identification of more stably expressed tumor antigens or combination therapies allowing to prevent CAR-T (or CAR-NK) inhibition at the tumor sites may help improve/rescue the effectiveness of these effector cells. In addition, we believe that many of the limitations of the CAR-T cell therapies (including the need of generating the drug product from the patient, the fact that CAR-T cells are not immediately available, the frequent toxicity and the very high costs) may be overcome with the use of donor-derived CAR-NK cells which can now be expanded very efficiently and represent an immediately available, "off-the-shelf" product to be used for several patients. Although helper ILC need further characterization and a better understanding of their role in cancer control/promotion, it seems that a subset of ILC3 correlates with favorable prognosis, at least in NSCLC. Thus, one may consider the possible adoptive transfer of these cells in cancer patients.

In conclusion, we have now a wide armamentarium to treat cancers and leukemia. Immunotherapy has added (and will add) crucial tools that may further improve the prognosis of aggressive and still lethal tumors.

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Declaration of competing interest

All authors declare no conflicting financial interests.

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