Molecular Therapy
Oncolytics
Review

Natural killer cells and immune-checkpoint inhibitor therapy: Current knowledge and new challenges

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The discovery of immune checkpoints (ICs) and the development of specific blockers to relieve immune effector cells from this inhibiting mechanism has changed the view of anti-cancer therapy. In addition to cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed death 1 (PD1), classical ICs of T lymphocytes and recently described also on a fraction of natural killer (NK) cells, several NK cell receptors, including killer immunoglobulin-like inhibitory receptors (KIRs) and NGK2A, have been recognized as checkpoint members typical of the NK cell population. This offers the opportunity of a dual-checkpoint inhibition approach, targeting classical and non-classical ICs and leading to a synergistic therapeutic effect. In this review, we will overview and discuss this new perspective, focusing on the most relevant candidates for this role among the variety of potential NK ICs. Beside listing and defining classical ICs expressed also by NK cells, or non-classical ICs either on T or on NK cells, we will address their role in NK cell survival, chronic stimulation or functional exhaustion, and the potential relevance of this phenomenon on anti-tumor immune response. Furthermore, NK ICs will be proposed as possible new targets for the development of efficient combined immunotherapy, not forgetting the relevant concerns that may be raised on NK IC blockade. Finally, the impact of epigenetic drugs in such a complex therapeutic picture will be briefly addressed.

INTRODUCTION

The discovery of immune checkpoints (ICs) and their role in the regulation of anti-tumor immune responses has determined the development of pharmacological tools, namely monoclonal antibodies (mAbs), able to release immune effector cells from this blocking mechanism. The two most relevant ICs reported so far are the PD (programmed death)-1 receptor expressed on immunocompetent cells, which binds the programmed death-ligand (PDL) 1 on cancer cells (Figure 1), and cytotoxic T lymphocyte antigen 4 (CTLA4), which competes with the CD28 costimulatory receptor for the ligands B7-1 (CD80) or B7-2 (CD86) on tumor cells (Figure 1).

In recent years, the use of IC blockers led to considerable successes in the treatment of several neoplasias.[1–6] James P. Allison and Tasuku Honjo, the two scientists whose work mostly contributed to gaining crucial knowledge for the development of checkpoint inhibitor therapies, were awarded with the Nobel Prize in Physiology or Medicine in 2018.[7,8] The anti-CTLA4 ipilimumab was the first IC blocker approved as anti-cancer drug in 2011 in the United States for the treatment of metastatic melanoma.[9,10] In the following years, several PD1/PDL blockers, after the first anti-PD1 nivolumab, have been approved for the treatment of solid tumors and hematological malignancies, including non-small cell lung cancer (NSCLC), melanoma, renal cell carcinoma, and high-grade Hodgkin lymphoma (HL)[1,11] (Table 1). The theoretical basis of the numerous encouraging clinical results relies on the re-activation of $CD8^+$ T cell activity, consequent to IC blockade with specific mAbs, to allow the assembly and trigger of an anti-tumor immune response, avoiding or limiting tumor escape (Figure 1).[12,13]

However, a great variability of response among patients has been documented in several clinical trials, with a large fraction of non-responders, suggesting that the complexity of the IC network is not fully reealed.[14]

Recent evidence for the involvement of natural killer (NK) cells in the picture sheds new light on this topic, also widening the possible targets of pharmacological intervention.[14,15] That PD1 or CTLA4 are expressed at least by a fraction of NK cells is commonly accepted: of note, PD1/PDL1 blockade in NK cells turned out to be essential to guarantee the effectiveness of IC-based immunotherapy in animal models.[15,16]

In addition to the classical ICs, which remain typical of the T cell population, an increasing number of NK cell receptors are now considered as ICs, including killer immunoglobulin-like inhibitory receptors (KIRs); C-type lectin-like inhibitory receptors, such as natural killer group 2 (NKG2)A/CD94 complex; and leukocyte immunoglobulin-like receptors (LILRs). NGK2A is recognized as a checkpoint member

https://doi.org/10.1016/j.omto.2021.11.016.

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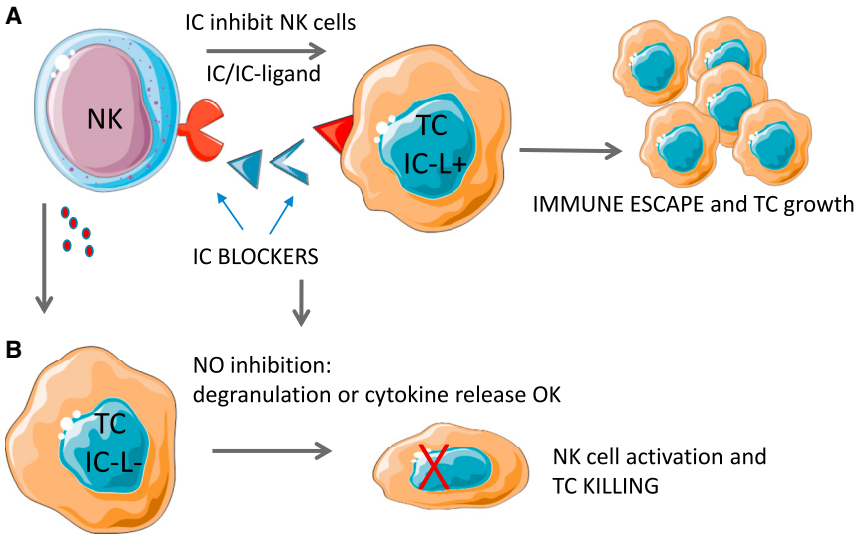

Figure 1. Schematic representation of IC network
(A) IC ligands (IC-L) expressed on tumor cells (TCs) bind to ICs on NK cells and deliver an inhibiting signal that impairs cytotoxicity or anti-tumor cytokine production, allowing tumor cell growth. (B) IC-negative TCs cannot inhibit NK cell function and TC growth and expansion is limited by NK cell activity. IC blockers can prevent IC/IC-L interaction and the consequent inhibiting signal delivery.

typical of the NK cell population. The coexistence of ICs typical of T lymphocytes and other ICs peculiar to NK cells has led to the proposal of a dual-checkpoint inhibition approach, to obtain a synergistic therapeutic effect, already proposed to overcome the problem of non-responder patients to PD1/PDL1 and/or CTLA4 blockade.[14,15,17] Extending the combination to NK-specific ICs, to restore a peculiar anti-tumor cytotoxic function, could strengthen the efficacy of IC-based immunotherapy.

In this review, we will deal with this new perspective, discussing the most relevant candidates for this role among the variety of potential NK ICs. In particular, we will describe the presence of NK cells at the site of lesion under IC-based therapy and the modifications in their function possibly due to the treatment; then, we will list and define classical and non-classical ICs expressed by NK cells, and, finally, we will address this last point as a possible source of new IC targets to be proposed for the development of efficient immunotherapy.

NK cell fate under IC therapy

The actual role of NK cells as anti-cancer effectors has been a controversial issue for many years due to the paucity of clinical studies reporting their presence in the immune infiltrate in the tumor microenvironment (TME). Nevertheless, the importance of this effector lymphocyte subset in anti-cancer immunosurveillance is now accepted, due to the huge number of reports supporting the contribution of NK cells in the spontaneous or antibody-induced killing of tumor cells.[18–21] Indeed, NK cells can respond to solid and hematopoietic cancers by releasing anti-tumor cytokines and chemokines; in addition, they can identify tumor cells lacking self-related molecules, including the major histocompatibility complex (MHC) class I molecular pattern, or recognize antigen expressed by stressed cells (induced stress-related recognition).[22–26] Finally, Fc-mediated effector functions of NK cells can be triggered by therapeutic mAbs, resulting in antibody-dependent cellular cytotoxicity (ADCC).[18–21] Thereby, NK cells are able to mediate strong antileukemia effects,[24,27,28] and now their presence in solid tumors is not only documented but also represents a good prognostic factor.[20,21]

Recent evidence, derived from clinical studies in solid tumors, revealed that the degree of effector lymphocytes, including NK cell infiltration, influences the outcome of immunotherapy, notably that of IC-based therapy (ICT), becoming a proposed marker for the eligibility of patients potentially susceptible to such treatment.[29] In particular, the differential immune cell infiltrate within the tumor or in the stromal area of the TME was shown to affect the response to ICT in melanomas and pancreatic cancer.[30] In turn, ICT itself can produce TME modifications, some affecting NK cell distribution and function, that may change the picture of the suitable ICT targets.[29,30]

NK cell localization during tumor progression and ICT

At variance with hematological malignancies, where NK cell contribution to mAb-based immunotherapy is documented, in solid tumors the efficiency of natural cytotoxicity or ADCC mediated by NK cells is variable and questionable. Despite the reported correlation between NK cell infiltration and tumor progression, inhibitory signals arising from TME and cancer cells seem to impair NK cell localization to the site of the lesion.[31] Strategies to increase infiltration of NK lymphocytes into tumors have been adopted to enhance the efficacy of anti-tumor mAbs that elicit NK cell-based ADCC, including trastuzumab in breast cancer or cetuximab in colorectal carcinoma; however, NK cell infiltration does not always lead to desirable results due to downregulation of cytotoxic T cell function.[29,32] Transfer of NK cells has been introduced to improve persistence in the tumor, as reported in a recent phase I trial showing stable disease in lymphoma and solid tumor patients following three infusions of allogenic NK cells.[33] However, this approach produced only partial and temporary results, so the trial failed to reach definitive clinical results. This could be dependent on the fact that, after infiltration into a tumor mass, NK cells undergo phenotypic and metabolic changes ultimately leading to defects of transient localization in the tumor and to decreased or limited effector function.[34,35] The discovery of IC expression on NK cells has revealed an important mechanism whereby such functional impairment takes place. While, on one hand, administration of mAbs targeting ICs can block these pathways and rescue the

Table 1. ICT: IC blockers approved or in active phase II/III clinical trials

Receptor	mAb	Type	Disease
CTLA4 (CD152)	Ipilimumab	human	melanoma, NSCLC
	tremelimumab (ticilizumab)	human	
PD1 (CD279)	Nivolumab	Human	metastatic melanoma, lung cancer, renal cell carcinoma, lymphomas
	Pembrolizumab	humanized	
PDL1 (B7-H1/CD274)	Avelumab	Human	Merkel cell carcinoma, metastatic urothelial cancer, NSCLC, TNBC, HCC
	atezolizumab	humanized	
B7-H3 (CD276)	enoblituzumab	humanized	neuroblastoma, rhabdomyosarcoma, Ewing sarcoma, Wilms tumor, melanoma
	orlotamab	humanized bispecific (CD3)	
TIM3 (CD366)	cobolimab[a] LY3321367[a] BGB-A425[a]	Human	liver cancer, metastatic melanoma, NSCLC, refractory solid tumors
LAG3 (CD223)	Sym022[a]	Human	advanced solid and hematological tumors, melanoma
	relatlimab[b]	Human	
TIGIT (CD226)	tiragolumab[a]	Human	advanced solid and hematological tumors, melanoma
	etigilimab[a]	Human	
KIR (CD158)	IPH2101[a] (KIR2DL1)	Human	MM, AML, relapsed/ refractory lymphomas
	lirilumab (IPH2102-KIR2DL1/2/3)[a]	Human	
	IPH4102 (KIR3DL2)[a]	Human	
NKG2A (CD159a)	monalizumab[a]	humanized	oral squamous cell carcinoma, gynecological malignancies, relapsed hematological malignancies

HCC, hepatocellular carcinoma; TNBC: triple negative breast cancer: MM:multiple myeloma; AML:acute myeloid leukemia.
[a]Phase I/II clinical trials.
[b]Phase III clinical trials.

anti-tumor activity of NK cells,[31,36] on the other hand, a distinction between ICT responders and non-responders has become evident.[17] As we discuss later, NK cells express not only classical ICs delivering negative signals but also a variety of non-classical molecules, mainly inhibitory receptors distinguished into conventional and non-conventional, that are able to negatively modulate their ability to reach cancer cells and destroy them. More and more scientists agree on the hypothesis that these non-classical ICs on NK cells can affect the fate of ICT and mAb-based immunotherapy.

Direct/indirect effects of ICT on NK cell function

Reciprocal effects of anti-cancer therapies and immune cell functions have been reported involving not only T lymphocytes or antigen-presenting cells but also NK cells.[37] In this context, the ultimate result of this interplay would be directed by the modulation of the IC network. In murine models, engagement of PD1 on activated NK cells by PDL1-expressing tumor cells reduces anti-tumor cytotoxicity, allowing tumor growth.[16] The blockade of PD1/PDL1 interaction leads to a rescue of NK cell activity (direct effect), indispensable for the control of neoplasia development, as treatment after depletion of NK cells was ineffective. Evidence for NK cell activity rescue upon IC blockade led to good clinical results in human cancers as well, including gastric cancers, lung tumors, and melanomas, acting both on the PD1/PDL1 axis and on CTLA4, another classical IC.[36] Restored NK cells recover not only their cytolytic potential but also the ability to produce interferon (IFN)γ or other cytokines or chemokines.[29,36] In any case, blocking of inhibiting signals may be insufficient to obtain an effective anti-cancer response, as NK cell activity is strictly related to the good and complete function of activating receptors.[18,20,37,38] In the next section, we will complete this complex picture by describing how the modulation of other important ICs, classical or non-classical, involving activatory or inhibitory receptors on NK cells, is needed to reach a reliable and stable rescue of complete anti-tumor effector cell function.

Classical IC surface receptors of T lymphocytes expressed on NK cells

A necessary requirement for an inhibiting receptor to give a negative signal to effector cells is its expression at the cell surface. Herein, we will summarize some evidence on the expression and function of the two classical T cell inhibitory receptors PD1 and CTLA4 on NK cells.

NK cell expression and function of PD1 and CTLA4

Recently, a growing interest has been raised on the molecular mechanisms involved in NK cell exhaustion and/or anergic state[39–41]; this is related to the better-characterized mechanisms, which involve T lymphocytes response to the chronic exposure to a specific antigen, in particular during immune response against tumor cells.[42–44] It has been reported that PD1 antigen is minimally expressed on both human and mouse NK cells.[45] Nevertheless, applying different methodological approaches, such as cytofluorimetry, quantitative reverse transcriptase reaction, and RNA sequencing for PD1 antigen, it has been shown that PD1 is expressed on a minor fraction of peripheral and tumor-associated NK cells. Furthermore, PD1 is not upregulated on NK cells upon activation, unlike CD69 antigen and other inhibitory receptors, such as T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibition motif (ITIM) domains (TIGIT).[45] The finding that PD1 is only minimally expressed on tumor-infiltrating lymphocytes would suggest that the use of anti-PD1 humanized antibodies in clinical trials to relieve the inhibition of NK cell-mediated anti-tumor activity is premature and a greater knowledge of the biological relevance of NK cells with low expression of PD1 should be acquired.[45] However, some reports are in contrast with these findings and claim that PD1 can be expressed at high levels on a fraction of NK cells and PD1-mediated inhibition of NK cells is relevant, at least in some specific instances.[14,46–55] Indeed, it has been shown that immune evasion through the engagement of PD1 on NK cells by PDL1 on tumor cells is marked in HLs, more than in diffuse large B cell lymphomas (DLBCLs).[56] The PD1 expressed on NK cells of HL patients, interacting with PDL1 on

tumor-associated macrophages (TAMs), can deliver an inhibiting signal on NK cells evaluated by impaired CD137 antigen upregulation and tumor cell killing.[56] Restoration of NK cell activation and killing was obtained by using an anti-PD1 antibody.[56] It is of note that the increment in NK cells expressing PD1, compared with healthy donors, was reverted by the specific therapy of HL and DLBCL.[56] The finding that the role of PD1 on NK cells was more evident in HL compared with DLBCL could be dependent on the strong upregulation of PDL1 expression in the TME found in HL and related to the gene amplification at the chromosome 9p24.1 locus that involves the PDL1 and PDL2 genes and consequent upregulation at the cell surface of HL.[57]

Furthermore, it has been reported that PD1 is strongly upregulated on NK cells of patients with esophageal, liver, colorectal, gastric, and biliary cancer.[58] Importantly, a poor survival was observed in esophageal and liver cancers. Interestingly, the percentage of $PD1^{+}CD56^{+}$ NK cells was markedly increased in patients' peripheral blood within the $CD56^{bright}$ and $CD56^{dull}$ NK cell subsets.[58] The blockade of PD1 with a specific anti-PD1 antibody could increase the production of IFNγ and CD107a expression at the cell surface, indicating a role of PD1 in regulating cytokine release and NK cell degranulation.[58] Also, the engagement of PD1 with surface-bound anti-PD1 mAbs could induce the apoptosis of $PD1^{+}$ NK cells. In nude mice, blocking of PD1 led to increased phosphorylation of Akt and tumor growth inhibition enhancing NK cell activity.[58] Altogether these findings would indicate a key role of PD1 on NK cells in digestive tract cancers; this hypothesis was further confirmed by the increase of $PD1^{+}$ NK cells infiltrating these tumors.[58]

It has been reported that PD1 is specifically expressed at high levels on serologically human cytomegalovirus (HCMV)-positive donors and in about one-fourth of peripheral NK cells from healthy donors.[14] These $PD1^{+}$ NK cells are fully mature and terminally differentiated, bearing KIR and CD57 antigen but not natural kilelr group 2 member A (NKG2A) and with a low expression of CD56.[14] It is of note that $PD1^{+}$ NK cells expressed low levels of activating receptors, such as NKp30 and NKp46, while NKG2D and DNAM1 receptors did not show remarkable differences between $PD1^{+}$ and $PD1^{-}$ NK cell subsets.[14] Functional experiments have demonstrated that $PD1^{+}$ NK cells showed a lower ability than $PD1^{-}$ NK cells to mobilize CD107a at the cell surface; the mobilization of CD107a was partially restored using blocking anti-PD1 and anti-PDL1 mAbs.[57] Also, it appeared that anti-human leukocyte antigen (HLA) class I and anti-PDL1/2 mAbs used in combination further increased the mobilization of CD107a, suggesting that both PD1 and HLA-I NK cell receptors can deliver independent inhibiting signals to NK cells.[14] $PD1^{+}$ NK cells produced lower amounts of pro-inflammatory cytokines, such as IFNγ and tumor necrosis factor alpha (TNFα), and display a lower proliferation rate than $PD1^{-}$ NK cells in response to low doses of interleukin (IL) 2 or IL15.[14] Finally, $PD1^{+}$ NK cells with an impaired functional behavior were enriched in peritoneal effusion associated with seropapillary ovarian carcinoma, indicating that TME can trigger the expression of PD1 on NK cells or favor the selection of $PD1^{+}$ NK cells.[14]

An increment of peripheral blood and tumor-infiltrating $PD1^{+}$ NK cells have been reported not only in ovarian cancer but also in multiple myeloma, sarcoma, and head and neck cancers (HNCs).[14,52,54,55] Of note, a better overall survival was associated with high frequency of peripheral blood circulating $PD1^{+}$ NK cells over the mean value of expression of this receptor in HNC.[55] The expression of PD1 on NK cells was upregulated upon activation with the anti-epidermal growth factor receptor (EGFR) humanized antibody cetuximab and PD1 blockade increased cetuximab-mediated activation of ADCC of HNCs expressing high amounts of PDL1.[55] These findings suggest that $PD1^{+}$ NK cells are activated and the increase in NK tumor-infiltrating cells would indicate that PDL1-bearing tumor targets can impair NK cell-mediated cytolysis. Furthermore, the contemporary blocking of PD1 with the specific mAb and triggering ADCC with cetuximab can enhance anti-tumor activity against HNC.[55]

The expression of CTLA4 on NK cells in HNC was very low both in peripheral blood and tumor-infiltrating NK cells but more homogeneous among the different donors[55]; furthermore, The Cancer Genome Atlas (TCGA) data for the expression of the *CTLA4* gene did not correlate with the expression of *NCR1* (NKp46) NK cell-specific markers by contrast to *PDCD1*. The very low expression of CTLA4 in HNC is consistent with the finding that peripheral NK cells do not express surface or intracytoplasmic CTLA4.[59,60] In addition, it has recently been reported that innate lymphoid cells (ILC)s can express CTLA4.[61] Indeed, CTLA4 appeared to be expressed by less than 10% of ILC1 but more than 10% of ILC2 and ILC3 cell subsets.[61] Of note, an increment of CTLA4 expression on ILC1 cells and a decrement of ILC3 cells was detected in melanoma patients upon treatment with the anti-CTLA4 antibody ipilimumab; this suggests that, during ipilimumab therapy, some ILC subsets can be selected. It has not been defined yet which role these selected cells may have in melanoma patients.[61]

Based on these findings, it is evident that there are conflicting results reported in the literature on the expression and function of PD1 on NK cells, and the reports on CTLA4 are scanty. It is clear that the reactivity of mAbs with PD1 at the NK cell surface is generally low and evident in a minor fraction of NK cells.[14,50,52,61] However, there is some evidence on the relevance of PD1 mainly on infiltrating NK cells. This would support the idea that the classical IC molecules of T lymphocytes can influence NK cell behavior, although this effect can target a small subset of NK cells and/or ILC cells.[14,50,52,61]

Other T cell inhibitory receptors expressed on NK cells

The T cell immunoglobulin and mucin-domain-containing-3 (TIM3), also known as hepatitis A virus cellular receptor 2 (HAVCR2), and the lymphocyte-activation gene 3 (LAG3) have been described as relevant ICs for T cells, but they are shared by NK cells during activation on specific subsets[62,63] (Table 1).

TIM3 is expressed by both $CD4^+$ and $CD8^+$ T cells and can interact with several ligands, such as galectin 9, carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 1, phosphatidyl serine (PtdSer), and the high-mobility group box (HMGB) 1, and influences intracellular signaling.[64,65] Of note, TIM3 does not bear intracytoplasmic ITIM domains but, on the contrary, several aminoacidic residues can be phosphorylated by Src-related kinases, such as Fyn and Lck.[66,67] This finding, together with the reversion of NK cell exhaustion by engagement of TIM3 in some tumor models, would suggest that TIM3 can be a costimulatory molecule instead of an IC receptor.[40,68] Overall, the functional significance of TIM3 upregulation on several tumor-infiltrating T and innate cells, such as NK cells and dendritic cells (DCs), within tumors is still to be completely defined.[69–80]

LAG3 was discovered in 1990 as a novel activation gene expressed by both $CD4^+$ and $CD3^-$ NK cells.[81] Also, LAG3 is similar to the CD4 molecule in its extracellular portion; for this reason, it can interact with stable peptide-major histocompatibility class II antigens.[82,83] Furthermore, LAG3 can bind other ligands such as Galectin-3, liver sinusoidal endothelial cell lectin, and fibrinogen-like protein 1.[84–91] Of note LAG3 can be induced on NK cells by the NK stimulating factor IL12, and this induction increases along the incubation time with this cytokine.[92] Furthermore, the molecular mechanism of inhibition is not related to ITIM, and it is still to be defined how LAG3 can deliver inhibiting signals. The effect of LAG3 engagement on NK cell surface with specific mAbs does not affect NK cell-mediated cytotoxicity, suggesting that this receptor is involved in the regulation of other NK cell functions. The finding that LAG3 can be coexpressed with PD1 on the $NKG2C^+$ NK cell subset would suggest its involvement in the generation of memory-like NK cells.[93]

An additional IC receptor is the T cell immunoreceptor with Ig and ITIM domains (TIGIT), also named Washington University Cell Adhesion Molecule (WUCAM)[94] or Vstm3.[95] TIGIT[96–98] is expressed on some T, NK, and dendritic cells[99,100] (Table 1).

Two main ligands for TIGIT have been reported: CD155 (PVR) and CD112 (PVRL2, nectin-2), which can be expressed on APCs, T cells, and non-hematopoietic cells such as tumor cells.[95,96,98] Of note, PVR and PVRL2 are the ligands of the DNAX adhesion molecule (DNAM) 1 (CD226) and of the T cell activation increase late expression molecule (TACTILE; also named CD96), which may deliver either a positive or a negative signal respectively.[101,102]

Of note, TIGIT contains an ITIM and an immunoglobulin tail tyrosine (ITT)-like motif, both relevant to mediate inhibition of TIGIT-expressing cells by the recruitment of the SH2-domain-containing inositol-5-phosphatase 1 (SHIP1). This recruitment leads to the reduction of granule polarization, cytotoxicity, and cytokine production in NK cells.[103,104] A role has not been reported so far for TIGIT in NK cells, with regard to the induction of Bcl-xl anti-apoptotic molecule and receptors for pro-survival factor such as IL15, IL7, and IL2, as described in T lymphocytes. Thus, its role is still to be defined in NK cell survival.

TIGIT is strongly expressed in tumor-infiltrating lymphocytes in several tumors; the blockade of TIGIT, LAG3, and TIM3 can synergize with the blockade of PD1 in relieving the $CD8^+$ T cell exhaustion in several different tumor models.[98,100,105–110] This indicates that, at least for T cells, the co-blockade of different IC receptors can promote anti-tumor immunity, leading to tumor regression.[99,100]

Old and new concepts on NK cell IC

Herein, we will analyze the biological role of other inhibitory receptors that have been described first on discrete subsets of innate cells, such as NK cells, and afterward on T lymphocytes.[101–118] These molecules (from now on conventional inhibitory receptors) are represented by KIRs, C-lectin type inhibitory receptors (CLIRs), and the leukocyte immunoglobulin-like receptor subfamily B member (LILRB) 1, which recognize specific MHC class I alleles (Table 2).[111–121] Then, we will focus on other (non-conventional) inhibitory receptors identified in NK cells but expressed on several types of cells of lymphoid and, in some instances, non-lymphoid origin, such as the leukocyte-associated immunoglobulin-like receptor (LAIR) 1, sialic acid-binding Ig-like lectin (Siglec) 7 and 9, inhibitory receptor protein (Irp) 60, immune receptor expressed on myeloid cells (IREM) 1, the killer cell lectin-like receptor subfamily B member (KLRB) 1, and the killer cell lectin-like receptor subfamily G member (KLRG) 1 (Table 2).[122–125]

Conventional inhibitory receptors as potential ICs

It is well known that NK cells express at the cell surface clonally distributed receptors that recognize self-MHC class I alleles. KIR, CD94 associated with NKG2A, and LILRB1 are the main inhibitory receptors involved in the recognition of self-HLA class I alleles (Table 2).[111–114] The interaction between these receptors and discrete groups of self-HLA class I alleles delivers in NK cells an inhibitory signal through the recruitment of tyrosine phosphatases (Figure 2) and impedes the NK cell-mediated killing of autologous cells.[111–114]

It has been demonstrated that the use of anti-NKG2A antibodies can efficiently trigger anti-tumor cell elimination, leading to a strong anti-tumor effect in combination with anti-PD1 or anti-EGFR antibodies.[126] Indeed, the binding of NKG2A/CD94 to the non-classical MHC class I molecule HLA-E in humans, and $Qa\text{-}1^b$ in mice, leads to the engagement of Src homology region 2 domain-containing phosphatase (SHP)-1 tyrosine phosphatase to the ITIM phosphorylated tyrosines of NKG2A,[127–129] and this binding induces the inhibition of effector cell functions of NKG2A-expressing NK and T cells. The development of a humanized anti-NKG2A blocking mAb, termed monalizumab (Figure 2), has demonstrated, in different mouse models, that the combination of anti-NKG2A antibody and anti-PDL1 (or anti-PD1) antibodies can result in a therapeutic anti-tumor effect.[126] The finding that solid tumors of lung, head, and neck (SCCHN), gastrointestinal tract, and female genital tract expressing HLA-E are infiltrated by $NKG2A^+$ NK and $CD8^+$ T cells would suggest that the NKG2A blockade, alone or in combination with PD1/PDL1 blockade, can increase the anti-tumor effect of tumor-infiltrating lymphocytes.[126] Also, the humanized anti-NKG2A

Table 2. Some examples of inhibiting receptors and their activating counterpart on NK cells

Receptor	Ligand	Function
KIR2DL1[a]	HLA-Cw2, w4, w5, w6	inhibition
KIR2DL2	HLA-Cw1, w3, w7, w8	
KIR2DL3	HLA-Cw1, w3, w7, w8	
KIR3DL1	HLA-Bw4	
KIR3DL2	HLA-A3, A11	
KIR2DS1[a]	HLA-Cw2, w4, w5, w6	activation
KIR2DS2	HLA-Cw1, w3, w7, w8	
KIR2DL4	HLA-G	
CLIR/CD94/NKG2A/B[b]	HLA-E	inhibition
CLIR/CD94/NKG2C[b]	HLA-E	activation
LILRB1/ILT2/CD85J	HLA-G	inhibition
NKRP-1A/CD161/KLRB1	Clr-g (NKR-P1F)	activation
	Clr-b (NKR-P1D)	inhibition
LAIR-1/CD305	collagens, SP-D, C1q	inhibition
Siglec 7 and 9	α2-6-linked sialic acids and to α2,8-disialic acid	inhibition
KLRG1	E-, N-, R-cadherin	inhibition

[a]KIRs are composed of either two or three Ig-like domains (2D or 3D) with a long (L) or a short (S) cytoplasmic tail. This portion of the KIR molecule can be associated with SHIP-1 phosphatase or DAP12 transducing molecules leading to inhibition or activation of NK cell-mediated functional activities (e.g., cytolysis and cytokine production).
[b]CLIRs are composed of a molecular complex between CD94 and NKG2 (A or B for inhibiting forms, C for activating isoforms), and their cytoplasmic tail is associated with SHIP-1 phosphatase or DAP12 molecule to transduce inhibiting or activating signals respectively.

antibody monalizumab can enhance the expression of the CD137 activation marker on NK cells, when these cells are co-cultured with the Cal27 SCCHN cell line and incubated with the anti-EGFR antibody cetuximab.[126] More importantly, the combination of monalizumab and cetuximab was used to define its safety and efficacy in a phase II clinical trial (NCT02643550) for the treatment of SCCHN. This study confirmed RECIST (response evaluation criteria in solid tumours) partial response in 31% of patients (8 of 26) and a stable disease in 54% (14 of 26) when the two antibodies were used together, without additional side effects in patients treated with either monalizumab or cetuximab.[126]

The relevance of KIR for the recognition of tumor cells was pointed out by Ruggeri and coworkers almost 20 years ago.[130] Indeed, it has been shown that donor-versus-recipient NK cell alloreactivity could eliminate leukemia relapse and graft rejection and protect patients against the graft-versus-host disease (GVHD). A major determinant of NK cell alloreactivity is linked to the expression, on donor NK cells, of KIRs that do not recognize the HLA-C allele on recipient tissues.[130] Haploidentical hematopoietic stem cell transplantation (HSCT) is characterized by the mismatch of HLA-C between the stem cell donor and the recipient. Of note, donor NK cells that differentiate in the recipient can efficiently kill acute myeloid leukemia cells because the recipient HLA-C does not interact with the donor KIR, thus avoiding NK cell inhibition.[130] Also, donor NK cells can eliminate residual recipient T and dendritic cells, leading to a better engraftment and reduced GVHD.[130] In addition, it has been shown that KIR-ligand incompatibility can trigger anti-tumor cytotoxicity against melanoma and renal cell carcinoma: this killing is stronger than that exerted by autologous or allogeneic KIR-matched NK cells.[131] Similarly, a role for KIR mismatch has been proposed to promote the killing of glioblastoma cells.[132,133] Altogether these findings suggest that KIRs are a suitable target to relieve NK cells from MHC class I engagement and consequent inhibiting signals, using specific blocking antibodies (Figure 2). Of note, the humanized anti-KIR antibody IPH2101 (lirilumab; Table 1), recognizing a wide range of KIR members, has been proposed for the treatment of multiple myeloma (MM) or acute myeloid leukemia (AML).[134–138] Indeed, IPH2101 antibody was administered to 32 MM patients and was safe and well tolerated in patients suffering from advanced MM. Furthermore, the NK cells of these patients increased the expression of CD69 and CD25 at the cell surface, beside triggering NK cell-mediated cytotoxicity of MM cell lines *in vitro*.[134,136] Similar results have been obtained in AML patients with limited side effects dependent on the administration of IPH2101.[135] Importantly, in both studies, the dose administered can easily reach the almost complete occupancy of KIR on NK cells.[134–136] After these promising findings, which indicate a possible use of IPH2101 antibody as a therapeutic agent, it was reported that nine patients with smoldering myeloma treated with IPH2101 showed a clear contraction of $KIR2D^+$ circulating NK cells, accompanied by an evident reduction of their cytolytic activity against K562 target cells.[139] Indeed, KIR2D receptor was reduced on the surface of NK cells because $Fc\gamma RI^+$ monocytes, or IFNγ-stimulated granulocytes, could take up by trogocytosis the KIR2D receptors expressed on NK cells after the binding to IPH2101 antibody.[139] This suggests that, during administration of IPH2101, trogocytosis mediated by monocytes and/or macrophages either in peripheral blood or spleen and liver can reduce the number of KIR2D molecules on NK cells; in turn, this reduction detunes and anergizes the NK cell function of the $KIR2D^+$ NK cell subset instead of leading to an increase of the cytolysis of $HLA\text{-}C^+$ autologous tumor cells.[139,140] Based on these findings, anti-KIR antibody therapy might have a limited therapeutic effect, at least when used as a single agent.

LILRB1, also called ILT2/CD85J, belongs to the leukocyte immunoglobulin-like receptor subfamily B together with several other inhibitory receptors.[119–121] LILRB1 is expressed on both innate cells and adaptive immune cells and can bind HLA-G with strong affinity, inhibiting cell proliferation, cytotoxicity, cytokine production, and phagocytosis.[119–121,140,141] The humanized anti-LILRB1 antibody BND-22 can induce a macrophage and lymphocyte-mediated anti-neoplastic response in several *in vitro* and *in vivo* models.[142–144] Its safety profile, as well as the tolerability and immune effects, are under investigation, in a clinical trial, in cancer patients expressing the immunoregulatory ligand HLA-G (NCT04717375).[142–144] In the near future, results of this clinical trial will provide the basis to use this antibody, which blocks

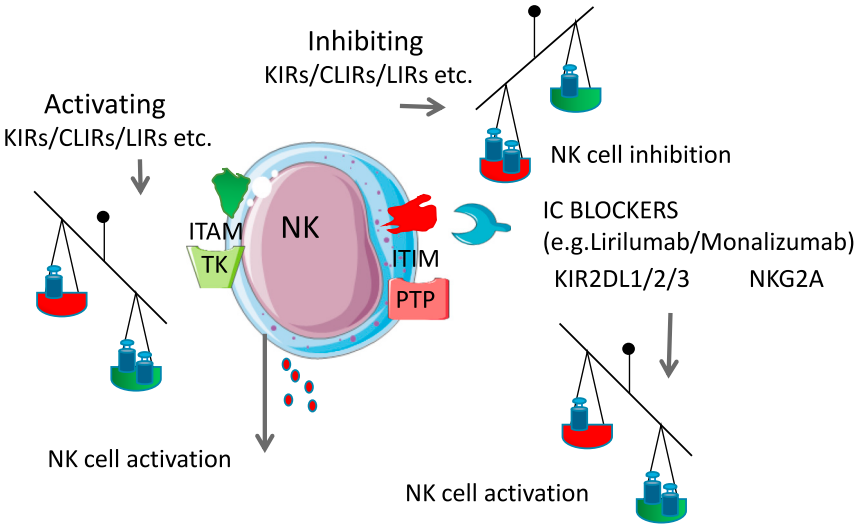

Figure 2. Schematic representation of KIRs as an IC network in NK cells

Inhibiting KIRs/CLIRs/LILRs and so forth delivers a signal that shifts the balance toward NK cell inactivation, so that they function as ICs. The use of blockers of these ICs can relieve the inhibition and push the balance toward NK cell activation. ITAM, immunoreceptor tyrosine-based activation motif; TK, tyrosine kinases; PTP, tyrosine phosphatases.

the activity of an inhibitory receptor mainly expressed on monocyte/macrophages involved in delivering a "don't eat me signal" to these cells. Of note, the presence of high numbers of tumor-infiltrating type 2 macrophages, expressing very high levels of LILRB1 receptors, in gastric cancer is associated with a poor prognosis and a pro-tumor microenvironment. Thus, it is conceivable that the interaction of LILRB1 with HLA-G on tumor cells leads to the impairment of macrophage-mediated anti-tumor activity; it is possible that impairing the immunosuppressive effect of this population of tumor macrophages could be a useful tool to relieve and reawake both innate and adaptive anti-tumor immune responses.[145–147]

Non-conventional inhibitory receptors as ICs

We can consider the receptors mentioned below as non-conventional NK cell inhibitory receptors because they do not recognize HLA class I alleles; nevertheless, their inhibiting signal is usually mediated, upon their engagement by the corresponding ligand, by the recruitment of tyrosine phosphatases to the intracytoplasmic ITIM, like KIR, CLIR, and LILRB members[122,148–154] (Table 2).

Siglec7, Siglec9, LAIR1, Irp60 (CD300a), and IREM1 (CD300f) are widely expressed not only on lymphoid but also myeloid cells, and the known ligands are widely distributed components of cell membranes, such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) for Irp60,[154-157] extracellular matrix proteins such as collagen for LAIR1,[155] or α2-6-linked sialic acids and α2,8-disialic acid, which are present in ganglioside GD3 for Siglec7 and Siglec9.[156] This would indicate that the inhibitory signal delivered by these receptors can be evoked in several different microenvironments where innate leukocytes may interact with apoptotic cells, collagens of epithelia, basal membranes and parenchymal tissues, or widely distributed sialic acid residues.

Conceivably, the blockade of one of these inhibitory receptors can have a strong effect within the TME; indeed, tumor apoptotic cells and mesenchymal stromal cells producing collagens may deliver negative signals to immune effector cells, and the impairment of these signals may wake the immune response up. However, the blockade of these receptors could also trigger reactions against several tissues different from the tumor itself, and it is not clear whether the undesired effects of this therapy can be limited. To date, no humanized antibody to these molecules have been used in phase I/II clinical trials, thus it is not easy to predict whether their blockade is not toxic and well tolerated.

Focusing on the LAIR1 receptor, it can interact with the surfactant protein D (SP-D) and the C1q complement component, besides different types of collagen.[157–160] The interaction with C1q is related to the presence of collagen-like motifs, as happens with SP-D.[157–160] It is well known that C1q, as well as other complement components, has been associated with inhibition of anti-tumor T cell responses by recruitment of myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), or TAM of type 2.[161] It has not been demonstrated, so far, that this immunosuppressive effect is dependent on the C1q interaction with LAIR1. Of note, LAIR1 can be associated with the leukocyte common antigen (CD45, previously called T200) at the NK cell surface.[162] Indeed, capping caused by an anti-CD45 antibody induced the co-capping of LAIR1 and immunoprecipitation of CD45 led to co-precipitation of LAIR1.[162] A similar association has not been shown for the other LAIR1$^+$ leukocyte populations. This finding would suggest that the LAIR1-mediated inhibition of some leukocyte functions can be related to the involvement of CD45 and vice versa.

Irp60 and IREM1 belong to the same CD300 family of receptors and display ITIM intracytoplasmic domain responsible for the downregulation of NK cell-mediated killing and cytokine production (for Irp60) or macrophage activation and induction of inflammatory responses (for IREM1).[163–165] Of note, IREM1 may regulate the myeloid differentiation factor 88 (MyD88) and toll-IL1 receptor-domain-containing adapter-inducing IFNβ in monocytes, while Irp60 exerts its activity just on MyD88.[165] This is dependent on the differential activation of SHP-1 and SHP-2 by these two receptors, indicating that inhibitory receptors with strong similarities may show different effects.[165] Irp60 and IREM1 ligands have not been

identified yet, and the use of their blockade is still to be assessed in clinical trials.

The relevance of Siglec7 and Siglec9 as myeloid IC[166–171] has recently been shown by using a humanized immunocompetent murine model where these receptors, together with the murine homolog Siglec-E, can impair the anti-tumor immune response.[171] Of note, the generation of a Siglec7/9/Siglec-E knockout mouse model has allowed evaluation of the therapeutic potential of anti-Siglec7 and anti-Siglec9 antibodies. This effect is mediated by the prevention of macrophage polarization to immunosuppressive TAM. Of note, the binding of Siglec9 to cancer-specific mucin can determine the generation of macrophages with immunosuppressive features that are lost by the blockade of Siglec-9.[171] These finding suggest that the blockade of Siglec7 and Siglec9 can reprogram TME, mainly influencing the myeloid arm of innate immune response. The role of NK cells in these experimental models has not been defined, but it is evident that Siglec7 and Siglec9 can be inhibited by either antibodies or sialoglycans such as lipid-conjugated glycopolypeptides[171–174]; it is conceivable that a role for NK cells in triggering anti-tumor immunity will be discovered in the near future, based on the previous demonstration of the key role of Siglecs in regulating NK cell activities.[166]

The KLRB1, also called NKRP1A/CD161,[175–180] can recognize the lectin-like transcript (LLT) 1 or C-type lectin domain family 2 member (CLEC2) D.[181,182] This receptor can apparently inhibit NK cell-mediated killing, and it regulates the transendothelial migration of at least $CD4^+$ T cells. More recently, it has been claimed that the interaction between KLRB1/CD161 and LLT1/CLEC2D in glioblastoma (GBM) may play a key role in the immune evasion of GBM cells from T lymphocytes.[183,184] Its role regarding infiltrating NK cells has not been defined yet. However, using specific anti-LLT1 antibodies, alone or in combination with anti-PD1/PDL1 antibodies, an evident reduction of androgen-independent growth of the cell line PC3 in a murine model has been detected.[185] This suggests that the impairment of KLRB1 interaction with LLT1 can be a suitable target to trigger an anti-tumor immune response in several kinds of cancers as LLT1 is widely expressed among tissues.[186]

KLRG1 is expressed on both NK and $CD8^+$ T lymphocytes interacting with E-, N-, and R-cadherin, present on epithelial and/or mesenchymal stromal cells.[187–197] This interaction leads to the inhibition of lymphocyte activities such as IFNγ production and proliferation, increasing NK cell apoptosis.[188] Of note, the engagement of cadherin by KLRG1 induces cadherin phosphorylation, influencing the adhesive properties of cadherin-positive cells.[187] Also, this inhibitory receptor can identify subsets of innate lymphoid cells[193,196] and it can be considered as a marker of some memory NK or T cells.[192–194] Also in this case, the combination of PD1 and KLRG1 blockade induced a sharp decrease of tumor size and increase of activation and frequency of tumor-infiltrating $CD8^+$ and NK cells.[125]

Relevant concerns on the blockade of IC receptors on NK cells

At least three additional main points should be taken into consideration when planning the blockade of IC on NK cells: (1) the presence on NK cells of activating isoforms of conventional IC, such as KIR with short-intracytoplasmic tail (Figure 2); (2) the role of soluble or exosome-associated IC or IC ligands within the host TME or biological fluids such as peripheral blood; (3) the functional significance of IC receptors on NK cells as potential anti-apoptotic regulators or their role in conserving memory NK cells.

It is well known that engagement of KIR isoforms on NK cells by the corresponding HLA-C allele ligand can deliver an activating signal (Table 2; Figure 2). These receptors can be clonally expressed, either together with their inhibiting counterparts or alone.[198–202] For some activating receptors, an inhibitory form has not been found; this is the case of the so-called p50.[198] Of note, some antibodies that recognize the inhibitory form of KIR can interact with the same antigenic epitopes present on the activating forms as well. Physiologically, it has been claimed that, although inhibiting and activating isoforms share the natural ligands, the inhibiting forms prevail in the binding because of a different affinity.[198–202] The anti-KIR antibody IPH2101 does not distinguish between inhibiting and activating isoforms, thus the conflicting effects observed (see above) may be dependent on the relative presence of different NK cell subsets displaying functionally different isoforms.[134–136]

A second point is the presence of IC receptors not only at the NK cell surface but also as soluble forms or exosome-associated molecules.[203–209] Indeed, IC receptors, including CTLA4, PD1, and their ligands, have been detected in the serum of cancer patients and supernatants of tumor cell cultures.[203–209] Of note, the magnitude of the increase in exosomal PDL1 in peripheral blood can be an indicator of the adaptive response due to T cell anti-tumor activity, and this parameter can stratify responder and non-responder patients.[209] Furthermore, soluble PDL1 can influence T cell response. No data regarding the effects of soluble or exosomal-associated IC receptors on NK cells have been shown to date in the literature, but it is conceivable that the efficiency of the blockade by specific antibodies can be altered by these IC soluble forms present in peripheral blood.

The functional significance of some NK inhibitory receptors is not fully understood. Indeed, it has been reported that the inhibitory signal mediated by KIR can downregulate the apoptosis that follows the engagement of soluble HLA class I antigens by CD8 on NK cells; also, the engagement of activating KIR with discrete soluble HLA class I alleles can induce NK cell apoptosis.[210,211] This would imply that KIR can play a role in regulating NK cell survival, and this function has not been tested for the other IC receptors herein reviewed.

Based on all these considerations, the net effect of IC receptor blockade is the triggering of NK cell activity as a consequence of relieving the brake that impedes the self-killing; in turn, the same

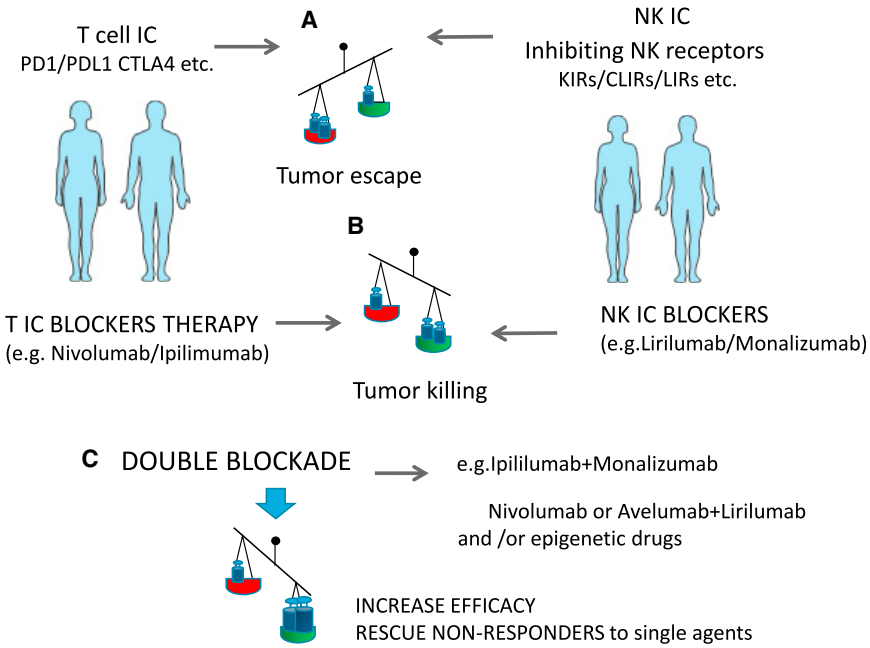

Figure 3. Scheme of some therapeutic approaches based on the blockade of T or NK ICs: Possible double blockade

(A) Effect of IC activation; (B) blocking of either T or NK cell ICs can push the balance from tumor escape to tumor killing; (C) double blockade (T + NK IC) and/or association with epigenetic drugs could potentiate the final anti-tumor effect and rescue non-responder patients to single agents.

mechanism can lead to a reduced persistence of anti-tumor effects due to a shortage of NK cell survival.

Mouse cytomegalovirus (MCMV) memory NK cells have been identified with a unique pattern of transcriptional signatures and functional properties similar to those ascribed to memory T cells.[212–224] In humans, anti-HCMV NK cells have been found, expressing NKG2C, CD57, and CD56^{low} antigens.[219,220] Besides viral infection-induced memory NK cells, it appears that cytokines are relevant to generating NK cell memory.[219,220] Of note, cytokine-induced memory NK cells expressing KIR can produce higher amounts of IFNγ interacting with primary self-MHC class I, compared with control or naive NK cells.[219–221] This would suggest that the KIR-mediated inhibitory signal is essential to NK cell memory; on the other hand, the blockade of KIR inhibitory signal may be detrimental for NK cell-mediated anti-tumor immune response.

Novel approaches for IC blockade: The role of epigenetics

It has been reported that *in vitro* chronic stimulation of CD3$^-$ CD56^{dim}CD57$^+$NKG2C$^+$ NK cells with anti-NKG2C antibodies and IL15 can drive strong activation and proliferation, accompanied by the high expression of LAG3 and PD1.[224] These chronically activated adaptive NK cells were altered in their response to tumor target cells and showed genome-wide alterations in DNA methylation, indicating a strong epigenetic effect of chronic stimulation.[224] If the goal of immunotherapy and IC blockade is to reverse the immune exhaustion and stimulate cytotoxic activity of effector cells against tumors, these findings can have relevant implications for this therapy.[224] Indeed, it appears that NK cell exhaustion is not a temporary state but is epigenetically imprinted, involving several genomic regions with a differential patterns of methylation.[224] The analysis of epigenetic pattern in tumor-infiltrating NK cells would help to understand whether these cells are dysfunctional because of exhaustion, as in chronic viral infections.[225,226]

It is conceivable that epigenetic drugs can influence the functional activity of NK cell subsets, such as cell migration and anti-tumor cytotoxicity.[227–232] As a consequence, NK cell anti-tumor effect could be maximized using both epigenetic drugs and IC blockade, thus affecting the insurgence of drug resistance or triggering immune response in the worst neoplasias, such as glioblastomas, pancreatic adenocarcinomas, and sarcomas.[233–239]

CONCLUSIONS

Targeting ICs has considerably improved cancer therapies. However, many patients have become non-responders to this therapy. To overcome this inconvenience, the recent focus on innate immunity, in particular NK cells, has brought some advancements in tumor treatment, providing the persistence of activated NK cells within a TME that is immunosuppressive. In this perspective, IC inhibitors are mostly effective in releasing NK cell activity, tuning the balance between activation and rescue from inhibition, and allowing the maintenance of anti-cancer natural immunity. This concept can also open the intriguing field of combinatory therapy, based on the dual blockade of classical T ICs and conventional NK ICs, with or without the addition of epigenetic drugs (Figure 3).

The most intriguing recent approach, however, is based on non-classical/non-conventional ICs preferentially expressed by NK cells; indeed, the huge number of these molecules enriches the panel of potential targets for antibodies designed to sustain such therapy, although they are still under investigation.

Furthermore, a selection among the plethora of inhibitory receptors and the different kinds of drugs available to date would be performed using three-dimensional culture systems, such as patient-derived tumor spheroids or organoids and self-NK cells, to evaluate the actual role of NK cells displaying inhibiting and activating isoforms of IC.[240,241] The association of these patient-derived models can help to select the appropriate combinations among blockade of

conventional and unconventional ICs together with the use of epigenetic drugs and/or targeted therapy.[242–244]

ACKNOWLEDGMENTS

This research was funded by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC) IG 21648, Compagnia di San Paolo (ROL 32567), 5xmille 2015 and 2016, and Ricerca Corrente 2018 from the Italian Ministry of Health to A.P.

AUTHOR CONTRIBUTIONS

Both authors A.P. and M.R.Z. contributed to the conceptualization, supervision, visualization, writing the original draft, and review and editing of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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