

Inhibitory checkpoints in human natural killer cells: IUPHAR Review 28

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Immune checkpoint inhibitors have revolutionized cancer therapy leading to exceptional success. However, there is still the need to improve their efficacy in non-responder patients. Natural killer (NK) cells represent the first line of defence against tumours, due to their ability to release immunomodulatory cytokines and kill target cells that have undergone malignant transformation. Harnessing NK cell response will open new possibilities to improve control of tumour growth. In this respect inhibitory checkpoints expressed on these innate lymphocytes represents a promising target for next-generation immunotherapy. In this review, we will summarize recent evidences on the expression of NK cells receptors in cancer, with a focus on the inhibitory checkpoint programmed cell death protein 1 (PD-1). We will also highlight the strength and limitations of the blockade of PD-1 inhibitory pathway and suggest new combination strategies that may help to unleash more efficiently NK cell anti-tumour response.

Abbreviations: AbE, abscopal effect; AML, acute myeloid leukaemia; BM, bone marrow; CAR, chimeric antigen receptor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DCs, dendritic cells; Gal-9, galectin-9; GvDH, graft versus host disease; HMGB-1, high-mobility group box 1; ICIs, immune checkpoint inhibitors; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; IDO, indoleamine 2,3-dioxygenase; iPSCs, induced pluripotent stem cells; NKG2A, killer cell lectin-like receptor C1; KIRs, killer immunoglobulin (Ig)-like receptors; LILRB, leukocyte Ig-like receptor subfamily B; LAG-3, lymphocyte activation gene-3; MHC-I, major histocompatibility complex class I; mAbs, monoclonal antibodies; NCRs, natural cytotoxicity receptors; NSCLC, non-small-cell lung cancer; PB, peripheral blood; PtdSer, phosphatidylserine; PD-L1, programmed cell death 1 ligand 1; PD-L2, programmed cell death 1 ligand 2; PD-1, programmed cell death protein 1; RT, radiotherapy; SHP-1 and SHP-2, SH2 domain-containing phosphatase; sPD-1, soluble form of PD-1; SCCHN, squamous cell carcinomas of the head and neck; TIGIT, T-cell immunoglobulin and ITIM domain; TIM3, T-cell immunoglobulin and mucin domain-containing protein 3; TAAs, tumour-associated antigens; TILs, tumour-infiltrating lymphocytes; TMB, tumour mutational burden; UCB, umbilical cord blood.

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1 | INTRODUCTION

Natural Killer (NK) cells are potent effector cells that play a pivotal role in the innate response against infections by viruses and, more importantly, against tumours growth, preventing tumour spreading and metastases. Upon activation, NK cells elicit a strong cytolytic activity and release chemokines and cytokines able to orchestrate early inflammatory responses. Thus, NK cells have an essential role in the first-line defence of the innate immune responses and modulate the subsequent activation of the adaptive immune system (Moretta, Bottino, Mingari, Biassoni, & Moretta, 2002; Moretta et al., 2004; Sivori, Vacca, et al., 2019). Originally, NK cells were thought to reside primarily in peripheral blood, bone marrow and spleen but recent evidences could demonstrate their presence in lymph nodes and other non-lymphoid organs such as the uterus, liver and lung (Shi, Ljunggren, La Cava, & Van Kaer, 2011).

The mechanisms of action of NK cells remained a mystery for many years until the 'missing self' hypothesis, proposed in the late 1980s, revealed that NK cells, by sensing the absence of major histocompatibility complex class I (MHC-I) on target cells, are able to discriminate between healthy and virus-infected or tumour cells (Ljunggren & Karre, 1990). This hypothesis was confirmed by the discovery, in mice and human NK cells, of MHC-specific receptors able to deliver inhibitory signals that block NK cell cytotoxicity (Moretta et al., 1990; Ciccone et al., 1992; Moretta et al., 1993; Moretta, Bottino, et al., 1996). Recognition of self-MHC-I molecules represents the most important mechanism to protect self-cells from NK cell killing. The discovery that 'off' signals are required to prevent NK-mediated autoreactivity suggested that 'on' signals should be present as well and be responsible for NK cell activation. Indeed, several surface receptors able to promote NK cell cytotoxicity were subsequently identified and characterized (Moretta et al., 2001; Moretta et al., 2004). Triggering of NK activating receptors occurs through binding with specific (non-MHC) ligands *de novo* expressed or overexpressed in stressed cells and, more importantly, in virus-infected or tumour-transformed cells. However, both tumour cells and tumour micro-environment can dampen NK cell-mediated anti-tumour activity by modulating the membrane expression of activating receptors (see below). The following paragraphs will analyse the NK cell receptors with particular regard to the inhibitory checkpoints and their important role as attractive therapeutic targets to enhance anti-tumour immune responses. In addition, we will discuss recent data indicating that different combined immunotherapies may represent new therapeutic approaches.

2 | NATURAL KILLER CELL RECEPTORS

2.1 | Inhibitory and activating receptors

NK cell function is regulated by an array of inhibitory and activating receptors. As mentioned before, the inhibitory receptors specific for

human leukocyte antigen class I (HLA-I) molecules provide the most important regulation of NK cells activity. Two main different types of HLA-I-specific inhibitory receptors have been identified in NK cells and are represented by the CD94/killer cell lectin-like receptor C1 (NKG2A) heterodimer and the members of the killer immunoglobulin (Ig)-like receptor (KIR) family (Moretta et al., 2014). Killer cell lectin like receptor C1 (NKG2A), as designated by International Union of Pharmacology (IUPHAR) guide to IMMUNOPHARMACOLOGY (Armstrong et al., 2020), is a type II transmembrane protein containing a lectin-type domain in the extracellular portion, which specifically recognizes HLA-E, a non-classical HLA-I molecule with a limited polymorphism (Lopez-Botet et al., 1997). KIRs are type I transmembrane receptors that specifically recognize shared polymorphic determinants of HLA-A, HLA-B and HLA-C molecules (Moretta et al., 1993). Importantly, KIR and NKG2A may be expressed also by T lymphocytes upon prolonged activation (Mingari et al., 1995; Mingari et al., 1996; Mingari, Moretta, & Moretta, 1998; Mingari, Ponte, et al., 1998) or exposure to TGF β (Bertone et al., 1999). Their expression may limit Tcell function, a critical event in tumour-infiltrating T lymphocytes.

LIR-1 (LILRB1), another type I transmembrane molecule, belonging to the Ig-like receptor superfamily is another HLA-specific receptor interacting with both classical (HLA-A, HLA-B and HLA-C) and non-classical (HLA-G) HLA-I ligands (Cosman et al., 1997; Ponte et al., 1999; Vitale et al., 1999). Despite molecular differences, these inhibitory receptors, upon interaction with HLA-I ligands, activate common downstream pathways that transduce inhibitory signals determining a strong inhibition of the polarization and release of lytic granules (Das & Long, 2010). Indeed, they share the cytoplasmic domain called immunoreceptor tyrosine-based inhibitory motif (ITIM) that is phosphorylated upon ligand interaction and recruits the tyrosine phosphatase SH2 domain-containing phosphatase (SHP)-1 and (SHP)-2 (Lanier, Corliss, Wu, Leong, & Phillips, 1998; Moretta, Bottino, et al., 1996). In turn, these phosphatases activate a downstream cascade to turn off NK cell function. It must be noted that at least one receptor specific for classical HLA-I molecules is expressed by NK cells allowing the whole NK cell pool to detect the loss of even a single allele on self-cell, an event frequently occurring in transformed tumour cells (Garrido, 2019).

The activity of inhibitory receptors is required to counterbalance the function of activating receptors. The major activating NK receptors are represented by the Natural Cytotoxicity Receptors (NCRs) that consist of three molecules named NKp46 (Pessino et al., 1998; Sivori et al., 1997), NKp44 (Vitale et al., 1998) and NKp30 (Pende et al., 1999). NCRs are type I transmembrane receptors, belonging to the immunoglobulin-like family and their transmembrane domains contain a positively charged amino acid required for the interaction with the Immunoreceptor tyrosine-based activation motif (ITAM) sequence present in adaptor polypeptides (Barrow, Martin, & Colonna, 2019). NCRs, selectively expressed by NK cells (with the exception of NCR⁺ ILC3), represent the most reliable markers to identify these cells and play a pivotal role in NK-mediated tumour cells killing. In addition, it has

been demonstrated that the magnitude of the cytolytic activity towards NK-susceptible target cells correlates with NCRs surface density on NK cells (Sivori et al., 2000). Different ligands, such as virus-derived molecules and/or intracellular proteins (i.e. the nuclear protein PCNA) that are expressed in response to stress or during tumour transformation, have been shown to interact with NCRs extracellular regions. In addition, it has been recently demonstrated that also the extracellular ligand Nidogen-1 and the soluble plasma glycoprotein called complement factor P/**properdin** can interact with NKp44 and NKp46, respectively (Gaggero et al., 2018; Narni-Mancinelli et al., 2017; Sivori, Vacca, et al., 2019).

NKG2D (HER2), a type II transmembrane and C-type lectin-like molecule, represents another important NK cell-activating receptor. Also, its ligands, namely, ULBPs and MICA/B, are overexpressed in infected and tumour cells (Lanier, 2015). Other triggering surface molecules are represented by co-receptors that include 2B4 and NTB-A, as well as DNAM-1, NKp80 and CD59 whose function is to amplify NK cell triggering (Moretta et al., 2014). However, to exert their activity, they rely on the simultaneous engagement of one or another triggering receptor (such as NCRs and/or NKG2D).

Thus, it can be concluded that the balance of signals derived from activating and inhibitory receptors determines if NK cells will exert their cytotoxic activity or remain inactive. As mentioned before, the inhibitory receptors specific for HLA-I, KIRs and NKG2A, control NK cells autoreactivity. Under normal conditions, the interaction between inhibitory receptors and their ligands inactivates NK cells, preventing killing of healthy cells, with the remarkable exception of the NK-mediated editing of Dendritic cells (DCs), which failed to undergo proper maturation (Moretta et al., 2002). During virus infection or tumour progression, transformed cells down-regulate or even lose HLA-I expression, becoming susceptible to NK-mediated cell lysis. For example, this is particularly important during the initial phase of viral infection, when T lymphocytes have not expanded sufficiently to mediate protection and NK cells play a primary role in limiting virus spreading. It is well known that also tumour cells may express low levels of HLA-I molecules. However, loss of HLA-I expression is not sufficient to induce NK-mediated cell killing, which will occur only if triggering receptors have also been properly engaged by specific ligands present on the surface of target cells (Moretta et al., 2004). Indeed, such ligands for activating receptors are usually absent or expressed at low levels in resting healthy cells, while they are up-regulated or even expressed *de novo* at the surface of stressed normal cells or virus-infected or transformed tumour cells. In this context, while stressed healthy cells express normal or increased levels of HLA-I molecules, which efficiently engage the inhibitory receptors, in virus-infected or tumour cells, the activating signal will overcome the signalling mediated by the inhibitory receptors promoting target cell killing and cytokine release.

Notably, KIRs and the other HLA-I-specific inhibitory receptors should be considered as the first discovered inhibitory checkpoints controlling NK cells and, in some instances, T-cell function (relevant to anti-tumour defence).

2.2 | Inhibitory checkpoint receptors

In addition to the inhibitory receptors specific for HLA-I molecules, additional inhibitory checkpoints (including PD-1, TIM-3, LAG-3, etc.), primarily involved in the maintenance of immune cell homeostasis, have been described in NK cells. Most of these inhibitory checkpoints are not expressed by resting NK cells. However, under pathological conditions, their expression can be *de novo* induced upon interaction with specific ligands frequently expressed on the surface of tumour cells. In this context, checkpoint receptors can affect NK cell function facilitating tumour immune escape. A major inhibitory checkpoint is represented by **programmed cell death protein 1** (PD-1) that was originally discovered to be expressed on T cells and to induce programmed cell death during T-cell thymic selection (Ishida, Agata, Shibahara, & Honjo, 1992). Even though PD-1 was initially described on T, B and myeloid cells, it has been recently described also on NK cells (see below) (Pesce et al., 2017) (Huang, Francois, McGray, Miliotto, & Odunsi, 2017). PD-1 is a type I transmembrane glycoprotein composed of an IgV-type extracellular domain, which belongs to the CD28/**cytotoxic T-lymphocyte-associated protein 4** (CTLA-4) subfamily of the Ig superfamily (Chamoto, Al-Habsi, & Honjo, 2017). Structural and biochemical analyses demonstrated that, due to the lack of membrane-proximal cysteine residues required for homodimerization, PD-1 is monomeric in solution as well as on cell surface (Zhang et al., 2004). Its cytoplasmic domain contains two tyrosine residues, the membrane-proximal one constitutes the ITIM region, while the other domain is represented by the immunoreceptor tyrosine-based switch motif (ITSM) (Okazaki & Honjo, 2007) (Figure 1). Despite ITIM is often associated with inhibitory receptors (including KIRs and NKG2A) being required for the activation of the inhibitory cascade, the tyrosine residue in the ITSM has been shown to be essential for PD-1 inhibitory function (Okazaki, Maeda, Nishimura, Kurosaki, & Honjo, 2001). Indeed, upon antigen stimulation, this tyrosine residue is phosphorylated and recruits SHP-2, which through dephosphorylation of downstream effector targets, initiates the inhibitory cascade. The PD-1 ligands are represented by the type I transmembrane glycoprotein **programmed cell death 1 ligand 1** (PD-L1) and **programmed cell death 1 ligand 2** (PD-L2). Expression of PD-L1 is detectable on haematopoietic cells such as T and B cells, macrophages and bone marrow-derived mast cells. In addition, PD-L1 may be expressed also in non-haematopoietic cells like mesenchymal stem cells, lung, keratinocytes and vascular endothelium cells (Keir, Butte, Freeman, & Sharpe, 2008). On the contrary, PD-L2 shows a more restricted expression which is limited to macrophages, bone marrow-derived mast cells, dendritic cells and peritoneal B1 cells (Zhong, Tumor, Gao, Bai, & Rothstein, 2007). Importantly, the expression of PD-L1 and PD-L2 in both lymphoid and non-lymphoid tissues suggests that this pathway could regulate immune responses not only in target organs but also in secondary lymphoid organs. In normal condition, the engagement of the PD-1 pathway regulates immune cell activation and ensures peripheral tolerance. However, PD-1 ligands (PD-L1 and PD-L2) have been shown to be often expressed also by cells of different tumours. In this context, the interaction of PD-1 with its

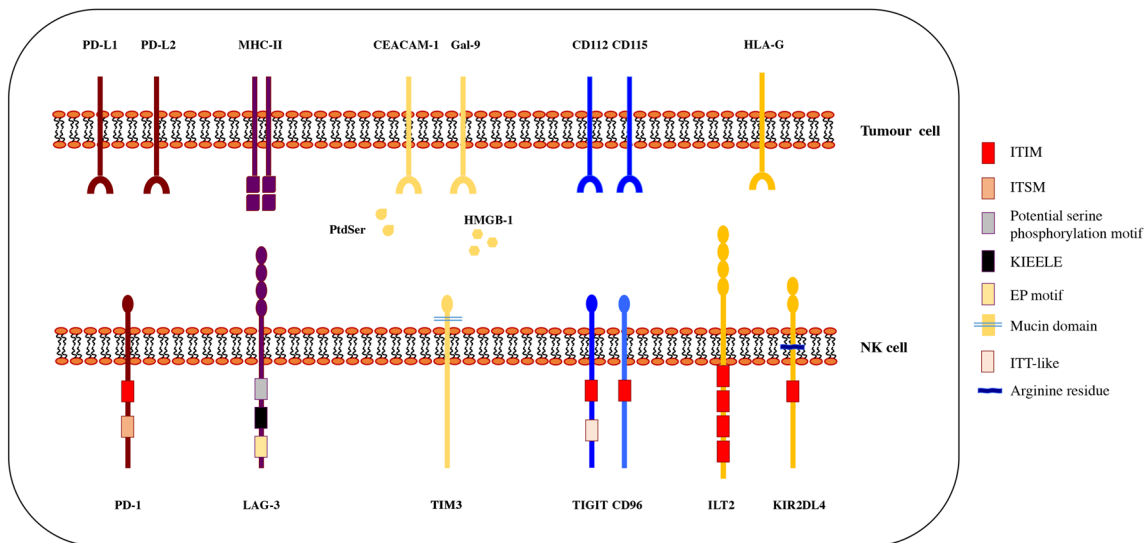


FIGURE 1 Schematic representation of inhibitory checkpoints and their ligands. NK cells express multiple inhibitory checkpoint receptors such as PD-1, TIM3, LAG-3, TIGIT, CD96, ILT2 and KIR2DL4 that interact with specific ligands expressed on virus-infected or tumour-transformed cells. The different extracellular and intracellular domains reported illustrate the molecular heterogeneity of these inhibitory checkpoints. The classical ITIM domain is shared between PD-1, TIGIT, CD96, ILT2 and KIR2DL4, while the ITSM- and ITT-like regions are present only in PD-1 and TIGIT, respectively. LAG-3 has an unusual cytoplasmic region containing a potential serine phosphorylation motif (S454), a glutamic acid–proline (EP) repeat and the conserved KIEELE motif that is indispensable for its inhibitory function. TIM3 presents a mucin domain in the extracellular region while lacks any conserved motifs in the intracytoplasmic tail. KIR2DL4 presents an arginine residue, in the transmembrane domain, able to interact with Fc ϵ RI

ligands inhibits NK cell-mediated killing, thus favouring tumour immune escape. Considering that the PD-1/PD-Ls axis is widely adopted by different tumour cells to escape the immune system, it is not surprising that this pathway has been extensively studied to develop new therapeutic approaches aimed to restore the anti-tumour immune response (see below). Of notice is also the discovery of a soluble form of PD-1 (sPD-1); recently different studies have been focused on sPD-1 possible role as an anti-tumour agent (Elhag et al., 2012; Nielsen, Ohm-Laursen, Barington, Husby, & Lillevang, 2005). In this context, clinical studies have investigated the presence of sPD-1 and its correlation with the overall survival of patients with different cancers (Kruger et al., 2017; Sorensen, Demuth, Weber, Sorensen, & Meldgaard, 2016).

In humans, in addition to PD-1, other inhibitory checkpoints may be expressed on the surface of NK cells. Lymphocyte activation gene-3 (LAG-3) is a type 1 transmembrane protein with significant homology to CD4. Compared with PD-1, LAG-3 has a larger extracellular domain that consists of four Ig-like domains and it is also characterized by an unusual cytoplasmic tail which contains domains not found in other inhibitory checkpoints (De Sousa, Leitner, Grabmeier-Pfistershammer, & Steinberger, 2018) (Figure 1). LAG-3 binds MHC class II molecules promoting an exhausted profile observed in tumour-infiltrating lymphocytes (TILs). While its role in the modulation of T-cell proliferation, activation and homeostasis has been extensively studied, LAG-3 effects on NK cell function are still to be clarified (Kim & Kim, 2018). As for PD-1, a soluble form of LAG-3, acting as an immune adjuvant has been identified and clinical-grade sLAG3 has been analysed in phase I

and II clinical trials to favour stimulation of the immune system (Chiossone & Vivier, 2017).

Tcell immunoglobulin and mucin domain-containing protein 3 (TIM3), a member of the TIM family, is a type I transmembrane protein containing an N-terminal IgV-like domain and a mucin domain (Figure 1). Similarly, to LAG-3, TIM3 does not contain classical inhibitory motifs, like ITIM, however phosphorylation of two tyrosine residues present in its cytoplasmic tail mediates the intracellular signalling pathway (De Sousa et al., 2018). Several ligands have been found to associate with TIM3 such as the high-mobility group box 1 (HMGB-1), galectin-9 (Gal-9), phosphatidylserine (PtdSer) and CEACAM-1 (Figure 1). TIM3 expression has been detected in different lymphoid cells and it has been suggested to be a marker for mature and/or activated NK cells. An increase of TIM3 expression in NK cells has been observed in patients with advanced melanoma and has been correlated with the NK cell exhaustion phenotype (Gallois, Silva, Osman, & Bhardwaj, 2014). Of note, TIM3 is often co-expressed with PD-1 during cancer progression and chronic infection.

Other widely studied inhibitory checkpoints are represented by the T-cell immunoglobulin and ITIM domain (TIGIT) and CD96 (Figure 1). TIGIT and CD96 belong to the Ig superfamily, that includes also the activating co-receptor DNAM-1. Both receptors contain an ITIM sequence in their cytoplasmic domains that initiates the inhibitory cascade leading to inhibition of NK cell function, including granule polarization, cytotoxicity and cytokine secretion (Stanietsky et al., 2009). TIGIT and CD96 corresponding ligands are represented by the cell surface nectin and nectin-like CD115 (PVR) and CD112 (PVRL2, nectin-2) molecules that are up-regulated in different

tumours. Considering that CD115 and CD112 are also DNAM-1-ligands, it is evident that both these inhibitory checkpoints and the activating co-receptor may compete for the same ligands. Therefore, TIGIT engagement with CD115 inhibits NK cell function by counterbalancing the DNAM-1-mediated activating signals. TIGIT can be detected in resting NK cells and its expression is up-regulated upon NK cell activation and in tumour-associated NK cells of patients with colon cancer (Zhang et al., 2018). Indeed, high expression of TIGIT on tumour-infiltrating NK cells, has been shown to correlate with reduced cytotoxicity and cytokine release.

The immunoglobulin-like transcript 2, ILT2 (**LILRB1**) and KIR2DL4 represent two other important NK cell inhibitory receptors (Lin & Yan, 2018). While ILT2 (LILRB1) is expressed on NK cells, T cells, monocytes, B cells, dendritic cells and myeloid-derived suppressor cells (MDSCs), KIR2DL4 is predominantly found on decidual NK cells and cannot be detected on the surface of peripheral blood resting NK cells (Koopman et al., 2003; Rajagopalan & Long, 2012). Regulation of NK cell function by KIR2DL4 is quite complicated because it is characterized by one ITIM domain in its intracellular region and, by an arginine residue, in its transmembrane domain able to interact with **Fc ϵ RI**. Thus it presents features of both inhibitory and stimulatory receptors (Rajagopalan & Long, 2012). ILT2 (LILRB1) belongs to the leukocyte Ig-like receptor subfamily B (LILRB), a group of type I transmembrane glycoprotein, and its extracellular region consists of four immunoglobulin domains, while the cytoplasmic tail is characterized by four ITIM sequences (Kang et al., 2016). Both ILT2 and KIR2DL4 interact with the HLA-G molecule. HLA-G in normal condition is expressed at the fetal-maternal interface and few healthy adult tissues like cornea, pancreatic isles and thymic medulla (Carosella, Rouas-Freiss, Tronik-Le Roux, Moreau, & LeMaoult, 2015; Lin & Yan, 2018). However, its expression can be switched on in pathological conditions, such as cancer, viral infection autoimmune and inflammatory diseases (Lin & Yan, 2018). Indeed, aberrant HLA-G expression has been detected in several tumours like ovarian carcinoma, gastric cancer, breast and lung cancer, and colorectal cancer (Carosella et al., 2015), and it has been correlated with metastatic status, advanced tumour stage and poor disease outcome. Indeed, overexpression of HLA-G is a well-known mechanism used by tumour cells to avoid and inhibit both the innate and adaptive immune responses (Li et al., 2017). Thus, HLA-G/ILT2 interaction impairs NK cells chemotaxis, cytotoxicity, IFN- γ production and MICA/NKG2D-induced activation (Menier, Riteau, Carosella, & Rouas-Freiss, 2002; Morandi et al., 2011). For this reason, the HLA-G/ILT signalling pathway is considered as a new immune checkpoint and a promising immunotherapy target.

3 | INHIBITORY CHECKPOINTS AS TARGETS OF IMMUNOTHERAPY

Immunotherapy through immune checkpoint inhibitors (ICIs) results in increased activation of the immune response and has recently led

to the approval of several new drugs. The first developed approach was aimed at blocking two key inhibitory receptors that critically affect peripheral T-cell tolerance and T-cell function: CTLA-4 and PD-1. CTLA-4 is a CD28 homologue but with much higher affinity for B7. Thus, the inhibitory signal is mostly due to the 'subtraction' of B7 to the costimulatory signal physiologically provided by CD28: B7 binding and required for T-cell receptor (TCR)-mediated T-cell activation (Walunas et al., 1994). Anti-CTLA4 blockade with **ipilimumab** was the first treatment to prolong the overall survival in patients with advanced melanoma in randomized trials (Hodi et al., 2010; Robert et al., 2011). More recently, strategies aimed at blocking PD-1 have been developed. PD-1 blockade has been shown to improve the survival and the progression-free survival in patients with metastatic melanoma and in patients with metastatic squamous and non-squamous non-small-cell lung cancer (NSCLC) who have been previously treated with standard therapies. Recently, immunotherapy with monoclonal antibodies (mAbs) that target the PD-1/PD-L1 axis has successfully improved cancer treatment and has been approved in a variety of cancers, including several solid tumours (Baitsch et al., 2011; Hamid et al., 2013; Rizvi et al., 2015; Topalian et al., 2012). Both CTLA-4 and PD-1 have similar negative effects on T-cell activation, however, while CTLA-4 is rapidly expressed on the surface of T cells following activation, thus compromising early T-cell response, PD-1 is expressed on T cells upon exhaustion and acts later on long-term tolerance maintenance. Although both CTLA-4- and PD-1-deficient mice develop autoimmune phenotypes (Nishimura, Nose, Hiai, Minato, & Honjo, 1999; Walunas, Bakker, & Bluestone, 1996), the severity and timing is different, suggesting that the two receptors differentially shape the T-cell repertoire and the immune response. While PD-1-deficient mice take several months to develop autoimmunity, which it is targeted to specific organs, CTLA-4-deficient mice develop multi-organ disease and die within 3 weeks, consistent with the critical physiological role of this receptor for T-cell homeostatic expansion. Another important difference between the two checkpoints is that CTLA-4 is expressed by T cells only, while PD-1 is expressed by other immune cells, also including NK cells (Fife & Bluestone, 2008). Thus, PD-1 checkpoint blockade allows broadening the efficacy of immunotherapy by acting on multiple effector lymphocytes and, in particular, on the NK cell compartment, which has recently become the focus of interest for therapeutic interventions (Andre et al., 2018; Cerwenka & Lanier, 2018; Mariotti, Quatrini, Munari, Vacca, & Moretta, 2019; Mingari, Pietra, & Moretta, 2019; Pesce et al., 2019). In this context, a common strategy to elude T-cell-mediated immune responses is the down-regulation or loss of expression of HLA-I molecules, which as has been documented for a large range of epithelial cell cancers and melanomas (Garrido, 2019; So et al., 2005; Zitvogel, Tesniere, & Kroemer, 2006). Therefore, while initially immunotherapy was mainly focused on enhancing Tcell responses, targeting immune checkpoints expressed on NK cells is now extremely important for advanced tumours that become 'invisible' to T cells. Among the NK cell checkpoints, PD-1 expression has been documented in many tumour settings and will be the focus of the next paragraphs.

4 | PD-1/PD-L AXIS BLOCKADE: AN EXAMPLE OF IMMUNOTHERAPY TARGETING AN NK CELL INHIBITORY CHECKPOINT

4.1 | PD-1/PD-L axis in tumours

The PD-1/PD-L1 axis plays a fundamental role in the inhibition of anti-tumour effector function of CD8⁺ T cells. Different from T cells, which express high levels of PD-1 at exhaustion, PD-1 is absent or expressed at very low levels on peripheral blood NK cells of healthy donors, with the exception of a minor fraction of cytomegalovirus (CMV)-seropositive individuals (Pesce et al., 2017). In mice, it was shown that endogenous glucocorticoids released upon infection with murine CMV (MCMV) induce PD-1 expression on spleen NK cells (Quatrini et al., 2018). Regarding human NK cells, recent data would suggest that persistent stimulation by tumour cells expressing ligands for activating NK receptors or soluble factors present in the TME may induce PD-1 surface expression (Pesce et al., 2019) with consequent impairment of anti-tumour NK effector function. Notably, human NK cells have been shown to display an intracytoplasmic pool of PD-1 mRNA and PD-1 protein localized in the Golgi (Mariotti et al., 2019); however, the stimuli required for its expression on the membrane are still undefined.

There is clear evidence that tumour cells express the natural ligands of PD-1 (PD-Ls), as a mean to evade immune attack (Munari, Zamboni, Lunardi, Marconi, et al., 2018). Recently, different independent studies provided evidence for the expression of PD-1 on human tumour-infiltrating NK cells including multiple myeloma (Benson et al., 2010), Kaposi sarcoma (Beldi-Ferchiou et al., 2016), ovarian carcinoma (Pesce et al., 2017) and digestive and lung cancers (Liu et al., 2017; Tumino et al., 2019). Of note, in malignant pleural effusions derived from patients with primary or metastatic lung tumours, PD-1 expression is not only confined to NK cells but is also expressed by tumour-infiltrating ILC3s (Tumino et al., 2019). It has been shown that NK cells isolated from pleural effusions could release cytokines and efficiently kill tumour cells (Croxatto et al., 2017; Vacca, Martini, Mingari, & Moretta, 2013). However, when these PD-1⁺ NK cells interacted with tumour cells expressing PD-Ls, their cytokine production was sharply inhibited (Tumino et al., 2019).

4.2 | Strengths and limitations of monoclonal antibodies targeting the PD-1/PD-L axis in cancer

Monoclonal antibodies acting through PD-1 blockade represent a major breakthrough in oncology, showing significant clinical success in the treatment of several types of cancer. There are currently five approved therapeutic agents on the market targeting the PD-1/PD-L1 pathway: **Nivolumab** and **pembrolizumab** are humanized IgG monoclonal antibodies directed at the PD-1 receptor, whereas **atezolizumab**, **durvalumab** and **avelumab** are humanized IgG monoclonal antibodies directed at the PD-L1 ligand. Several large-scale clinical trials showed significant response rates and improvements

in overall survival in a variety of patients with solid tumours including NSCLC, gastrointestinal carcinomas, squamous cell carcinomas of the head and neck (SCCHN), renal cell carcinomas, urothelial carcinomas, cervical carcinomas and breast carcinomas, as well as in patients with lymphoma or melanoma. For this reason, all of these five antibodies have been approved for use as the first- or second-line treatment (Garon et al., 2015; Reck et al., 2016) (Borghaei et al., 2015; Rizvi et al., 2015). Each of these drugs binds to a different epitope on their respective target and their mechanisms of action consist in blocking the PD-1/PD-L1 receptor–ligand interaction. A novel class of anti-PD-1 antibodies was recently identified with a different mechanism of action but with an antagonistic activity comparable with that of pembrolizumab and nivolumab. These antibodies bind to the opposite face of the PD-1 protein relative to the PD-1–PD-L1 interaction site and act predominantly through the restoration of the positive signalling transduced by the simultaneous engagement of CD28 co-receptor (Fenwick et al., 2019). Although the synergistic effect of these non-blocking anti-PD-1 antibodies with blocking PD-1 antibodies has only been demonstrated in vitro and in a mouse tumour model in vivo, their distinct mechanism of action suggests that they may complement classical immunotherapy by enhancing the recovery of anti-tumour activity of cytotoxic lymphocytes.

Despite the successes of the above-mentioned Food and Drug Administration (FDA)-approved antibodies, only ~30–40% of patients show a response to anti-PD-1 immunotherapy. Response rates vary widely between cancer types and there is a particular need for predictive biomarkers that can be used to identify patients more likely to respond to immune checkpoint modulators. To this end, different tumour features are evaluated prior to the design of the therapy. These include assays to analyse the level of PD-L1 expression in tumour cells and DNA sequencing to estimate tumour mutational burden (TMB) (Havel, Chowell, & Chan, 2019). One important critical limitation that may influence the response to the PD-1/PD-L1 axis blockade is the screening of tumours based on the expression of PD-L1, which is routinely evaluated by immunohistochemical staining using different methods (tumour proportion score, combined positive score and tumour-infiltrating immune cells). This diagnostic test may guide the decision of the appropriate therapeutic strategy to be adopted and helps to predict benefits in subgroups of patients, depending on the degree of biomarker expression at different cut-off (Sholl et al., 2016) (Figure 2). Higher PD-L1 expression on tumour cells generally correlates with higher rates of clinical response to treatment with PD-1/PD-L1 inhibitors. However, this is not always the case, as clinical responses are also observed in patients with tumours negative for PD-L1. It is reasonable to think that such discrepancy may be at least in part explained by the fact that a number of tumours could be misclassified, in terms of PD-L1 expression, due to different factors including expression heterogeneity in the tumours, interclone differences and interobserver/intraobserver variability, as demonstrated in several reports (Munari et al., 2017; Munari, Rossi, Zamboni, Lunardi, et al., 2018; Munari, Zamboni, Lunardi, Marchionni, et al., 2018).

STRATEGIES TO IMPROVE IMMUNE CHECKPOINT BLOCKADE RESPONSE

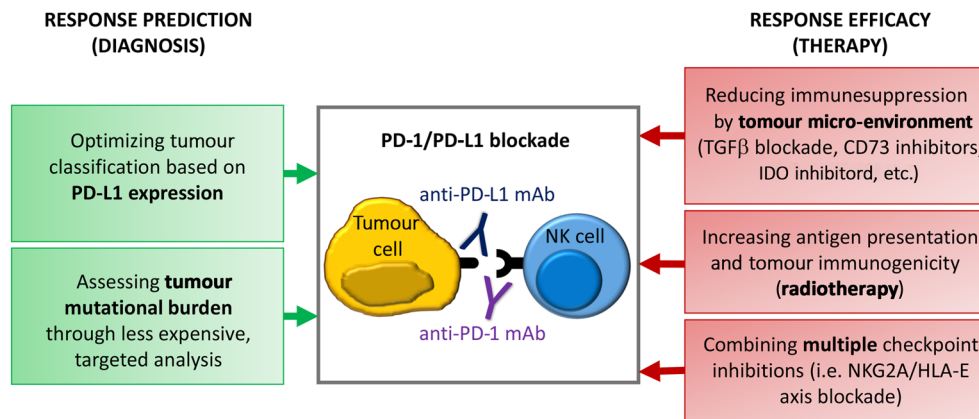


FIGURE 2 Different approaches to enhance immune response. Despite the development of new therapeutic approaches targeting the inhibitory checkpoints, the percentage of patients responding to anti-PD-1 immunotherapy remained low. Different strategies can be adopted to improve both the immune response prediction and efficacy. A more in-depth analysis of tumour mutational burden (TMB) and a better characterization of PD-L1 expression at tumour site will allow a more accurate diagnosis. In addition, improvement of immune checkpoint blockade response can be obtained combining different therapeutic approaches

High tumour mutational load has been correlated to better response to immune checkpoint blockade, because it may generate novel antigens that are not subject to immune tolerance, thus improving T-cell-mediated adaptive immune responses (Yarchoan, Hopkins, & Jaffee, 2017). Therefore, TMB has emerged as a promising novel biomarker to further aid in patient selection (Figure 2). High mutation burden is typically observed in carcinogen-driven tumours such as lung cancer and melanoma and in tumours caused by germline mutations in genes encoding for proteins involved in DNA repair and replication. The main limitation of TMB analysis is represented by the high costs of the whole genome sequencing; therefore, it is limited to malignancies that are more likely to be highly mutated, and it is often analysed indirectly through less expensive techniques. Indirect TMB assessment includes analysis of microsatellite instability status, chromosomal structural analysis and mutational analysis of selected genes (Galuppini et al., 2019).

The limitations of the efficacy of immune checkpoint blockade therapies highlight the need for improving predictive strategies and finding novel synergistic therapeutic targets to increase the response rate and/or the number of patients that can be treated.

5 | COMBINED THERAPIES TO IMPROVE IMMUNE CHECKPOINT BLOCKADE EFFICACY

A great effort has been done recently to explore the possible advantages of combining the blockade of multiple inhibitory signals at the same time, targeting NK cells together with T cells, neutralizing inhibitory molecules present at the tumour site or increasing tumour immunogenicity through radiotherapy (RT).

5.1 | Immune checkpoint inhibitor (ICI) and tumour immunosuppressive micro-environment

A major role in the failure of immune checkpoint therapies is played by the TME. For instance, hypoxia is responsible for the generation of adenosine, which decreases proliferation and effector function of cytotoxic lymphocytes (Cekic, Day, Sag, & Linden, 2014). Adenosine is generated by the sequential degradation of ATP to AMP, and AMP dephosphorylation, by CD39 and CD73, respectively. A combination of anti-CD73 and anti-PD-1 treatments has been tested in patients with advanced solid tumours and anti-tumour activity has been preliminary reported (Siu et al., 2018), and several other molecules that target the adenosine releasing pathway are currently in clinical development (Perrot et al., 2019). Another enzyme present in the TME is the **indoleamine 2,3-dioxygenase (IDO)**, which catalyses tryptophan degradation leading to deprivation of this amino acid and kynurenine production. Both these events contribute to tumour tolerance acting on the differentiation of regulatory T cells (Tregs) and directly suppressing T and NK cell function and proliferation (Della Chiesa et al., 2006; Munn & Mellor, 2007). Indeed, it was shown that IDO mediates resistance to anti-CTLA4 and PD-1 therapies (Holmgaard, Zamarin, Munn, Wolchok, & Allison, 2013), but to date, no significant benefit has been revealed by combined IDO and PD-1/PD-L1 axis inhibition in melanoma (Long et al., 2019). Another component of the TME that contributes to the evasion of immune surveillance and to the failure of immune checkpoint blockade is TGFβ (Mariathasan et al., 2018). TGFβ excludes immune cells from the tumour bed (Tauriello et al., 2018) and inhibits both T cells and NK cell cytotoxicity (Castriconi et al., 2013; Gao et al., 2017; Viel et al., 2016). A first-in-class bifunctional checkpoint inhibitor (the fusion protein M7824) comprising the extracellular domain of human TGFβRII (TGFβ Trap) linked to the C-terminus of human anti-PD-L1 heavy chain (αPD-L1)

has been developed and has proved to be efficient in promoting T-cell and NK cell activation in mouse tumour models (Knudson et al., 2018). These findings prove that simultaneous targeting of TGF β and PD-L1/PD-1 immunosuppressive pathways may represent a promising tool to promote anti-tumour responses (Figure 2).

5.2 | Combination of ICI targeting different pathways

Another strategy to improve the efficacy of checkpoint blockade is to target multiple checkpoints at the same time. Indeed, several studies demonstrated that the combined blockade of multiple inhibitory checkpoints resulted to be more effective in controlling tumour growth compared with blockade of a single receptor (Nirschl & Drake, 2013). These data confirmed the hypothesis that combined immune checkpoint blockade could be an innovative and potential treatment strategy to be applied to a wide variety of cancers (Figure 2). In addition, this notion revealed that inhibitory checkpoints might synergize to down-modulate the overall anti-tumour immune response. Among the inhibitory receptors expressed by NK cells, a promising target for combined therapy is NKG2A, which has the advantage of being expressed also by activated T cells (Bertone et al., 1999; Mingari, Ponte, et al., 1998) and whose ligand (HLA-E) is frequently overexpressed in tumours. NKG2A blockade with the mAbs **monalizumab** in combination with the blockade of the PD-1/PD-L1 axis was proved to enhance both T-cell and NK cell responses (Andre et al., 2018). Moreover, blocking NKG2A increased NK cell antibody-dependent cell-mediated cytotoxicity, thus enhancing the efficacy of cetuximab (an anti-estimated GFR [anti-EGFR] antibody) treatment in patients with squamous cell carcinoma of the head and neck SCCHN (Andre et al., 2018). These findings suggest that targeting NKG2A may represent a complementary tool to unleash NK cell response by combining checkpoint inhibitors with monoclonal antibodies targeting tumour antigens. In addition, combined immunotherapies targeting PD-1 and other inhibitory checkpoints are under investigation. Thus, IMP321, a clinical-grade sLAG-3 protein, is currently used in combination with anti-PD-1 therapy in phase II clinical trials. Moreover, the efficacy of an anti-LAG3 monoclonal antibodies (**relatlimab**) is currently being investigated in several types of cancer (NCT02061761; NCT02658981). As mentioned before, TIM3 and PD-1 are often found to be co-expressed on NK cells from cancer patients. Thus, anti-TIM3-blocking monoclonal antibodies have been used in combination with anti-PD-1 in phase I clinical trials to block tumour growth and restore the anti-tumour immune response (Chiossone & Vivier, 2017). Moreover, the inhibitory receptor T-cell immunoglobulin and ITIM domain has been shown to act in synergy with both PD-1 and TIM3 (Kurtulus et al., 2015). Indeed, combined blockade of TIGIT and TIM3 or PD-1 resulted in tumour regression and increased anti-tumour immunity. In this context, anti-PD-1 therapy combined with a fully human anti-TIGIT monoclonal antibody (MTIG7192A, RG6058) is tested in a phase I clinical trial towards different solid tumours.

Moreover, due to the potent inhibitory effect exerted by KIRs on NK cell function, a human monoclonal antibodies blocking KIR2DL1, KIR2DL2 and KIR2DL3 on NK cells, namely, **lirilumab**, was developed. In clinical trials for acute myeloid leukaemia (AML), myeloma or solid tumours, lirilumab showed, however, limited anti-tumour efficacy (Carlsten et al., 2016) (Vey et al., 2018). By contrast, IPH4102, a first-in-class monoclonal antibodies targeting **KIR3DL2** is showing encouraging clinical activity in patients with cutaneous T-cell lymphoma (Bagot et al., 2019). In addition, several approaches have been developed to block the HLA-G/ILT signalling pathway, which take into account also the different mechanism regulating HLA-G surface expression. These approaches include HLA-G antagonists, blocking antibodies against HLA-G or its receptors, siRNA, inhibition of its intercellular transfer and HLA-G as target for drug delivery (Li et al., 2017; Lin & Yan, 2019; Zhang et al., 2014). Therefore, KIRs and HLA-G may also represent promising targets for combined therapy.

5.3 | ICI and radiotherapy

Radiotherapy (RT) is an essential treatment for cancer patients (Ngwa et al., 2018). It has been widely demonstrated that RT is able to modulate the anti-tumour response acting, through different mechanisms, on the TME. Indeed, it reduces the overall tumour burden increasing systemic responses to immunotherapy, and in addition, preclinical models showed several immune-stimulatory effects of ionizing radiations. These include activation of pro-death signalling in tumour cells and release of damage-associated molecular patterns and 'eat me' signals. Of note, RT can also serve as an in situ vaccine, promoting the release of tumour-associated antigens (TAAs) and enhancing the number of APC cells in both the tumour micro-environment and lymph nodes with consequent enhanced dendritic cell activation, T-cell priming and recruitment of CD8⁺ T lymphocytes (Hwang, Pike, Royce, Mahal, & Loeffler, 2018). In addition, RT can modulate the innate immune response, increasing NK cell-mediated cytotoxicity through the up-regulation of NK cell-activating receptor, NKG2D and NKp30, expression (Kim et al., 2006) (Matta et al., 2013). Moreover, it has been demonstrated that the interaction of RT with patient immune system is essential to promote the abscopal effect (AbE), which represents a promising tool to use abscopal effect for the treatment of not only localized tumours but also metastases (Ngwa et al., 2018). AbE was first observed in 1953 and it describes the systemic anti-tumour reactions upon RT treatment that can lead to regression of lesions in metastases outside the radiation field (Mole, 1953). However, on the other side, RT may also impair the anti-tumour immune responses increasing the influx of MDSC and Tregs and promoting the release of inflammatory cytokines, which can also induce PD-L1 expression (Gandhi et al., 2015) (Deng et al., 2014). Thus, considering the important achievements obtained with ICI in targeting the immune response and, at the same time, the fact that not all patients benefit of ICI or RT

treatment alone, increase interest raised for the integration of immune checkpoint blockade with RT for the treatment of patients with numerically and spatially restricted areas of metastasis (Pitroda, Chmura, & Weichselbaum, 2019). More importantly, combination of RT and ICI represents a great opportunity to boost AbE rates. Indeed, preclinical studies demonstrated that regression of non-irradiated lesions can only be observed in immunocompetent mice, indicating that despite the increase of tumour immunogenicity, RT alone is not sufficient to induce AbE (Demaria et al., 2004). Several reports indicated that AbE is mainly observed in highly immunogenic tumours, such as malignant melanoma, renal cell carcinoma, hepatocellular carcinoma and NSCLC. Importantly, several immunotherapeutic agents, targeting different mechanisms of immune-mediated responses, are able to boost AbE (Ngwa et al., 2018). In particular, preclinical studies indicate that PD-1/PD-L1 blockade is optimal in combination with RT. Indeed, an enhancement of anti-tumour effects and significant reduction of secondary non-irradiated tumours was observed in mice model, bearing solid or haematological tumours, treated with in-field RT in combination with PD-1 or PD-L1 blockers (Gong, Le, Massarelli, Hendifar, & Tuli, 2018). Clinical studies confirmed that combination of anti-PD-1 or anti-PD-L1 immunotherapies with RT was associated with clinical significant and durable tumour responses in different cancer patients (Haymaker et al., 2017) (Yuan et al., 2017) (Takamori et al., 2018) (Xie, Gu, Zhang, Chen, & Wu, 2017).

However, some limitations have also emerged from experimental and clinical data (Ngwa et al., 2018). Despite the boost of the immune response, the presence of immune suppressive environment (i.e. MDSC and Treg cells and/or immunosuppressive cytokines) or tolerance at the tumour sites can repress AbE. Immune-related adverse events can be also due to the overlapping toxicities derived from RT and ICI combination (Vanneman & Dranoff, 2012). More importantly, despite the numerous preclinical and clinical studies, the mechanisms underlying AbE have not yet been identified. Therefore, it is essential to develop new strategies to overcome these limitations and improve our understanding of the biological pathway regulating AbE. In this context, numerous preclinical studies have explored a range of radiation regimens combined with immune checkpoint blockade to optimize synergy with immunotherapy (Patel & Minn, 2018) (Figure 2). However, since the radiation dose, fractionation, timing and target organs are still not well defined, additional clinical studies are needed to fully realize the potential of integrated radio-immunotherapies. Under investigation studies, aimed to minimize the effect of immunosuppression, use different approaches to improve T-cell stimulation primed by RT and/or immunotherapy. Moreover, considering that AbE is more often associated to highly immunogenic tumours, it is essential to develop new strategies to increase immunogenicity. In this context, preclinical models demonstrated that RT, ICI and anticancer vaccine combination could increase abscopal effect response rates (Zheng et al., 2016). Overall, despite the multiple challenges in overcoming cancer immunosuppression, there is an increasing interest in developing new strategies to boost AbE in order to improve patient outcomes.

6 | CHIMERIC ANTIGEN RECEPTOR-ENGINEERED NK CELLS

In the field of tumour immunotherapy, a complementary approach to unleash cytotoxic lymphocytes through ICI is represented by the development of chimeric antigen receptor (CAR)-cells. This approach is based on engineering cells with synthetic receptors in order to combine their high anti-tumour activity with the capability of tumour antigen recognition of antibodies. This promising technique was first used to engineer T cells generating CAR-T cells (Singh & McGuirk, 2020). The expression of synthetic receptors instead of the physiological TCR allows CAR-T cells to recognize any cell surface molecule independently of MHC presentation, thus broadening the array of antigens recognized by T cells. Several approaches to generate CAR-T cells have been developed in order to maximize their activity, persistence and expansion (Depil, Duchateau, Grupp, Mufti, & Poirot, 2020). Since their development, adoptive cell transfer of chimeric antigen receptor T cells revealed successful in the treatment of high-risk B-cell lymphoma and lymphoblastic leukaemia (Neelapu et al., 2017) (Park et al., 2018). Despite the important success in the treatment of haematological malignancies, similar results have not been obtained so far in solid tumours. However, in this context, it is worth to mention the recent development of a CAR-T cells against the HLA-G molecule in order to elicit immune response against HLA-G⁺ solid tumours (presentation at the ESMO Immunology Congress 2019, Geneva, Switzerland Volume 30, Supplement 11, Loustau, M. *Annals of Oncology*, Volume 30, xi12). Nevertheless, some obstacles limit chimeric antigen receptor T clinical application, such as: high cost, need of personalized production that requires a substantial delay between patient enrolment and treatment, cytokine release syndrome and neurotoxicity (Sivori et al., 2019). On the contrary, NK cells engineering could represent an appealing solution. Indeed, chimeric antigen receptor NK cells do not require HLA matching and can be administered in allogeneic settings without causing graft versus host disease (GvDH) providing a cost-effective 'off-the-shelf' product (Burger et al., 2019) (Zhang, Hu, & Shi, 2020). In addition, due to the expression of activating receptors, such as NCR, NKG2D, DNAM-1 and CD16, NK cells are able to induce cytotoxicity in both CAR-dependent and -independent manners reducing the eventuality of loss of CAR-targeting antigen-related relapses and increasing their killing activity (Hu, Tian, & Zhang, 2018). Different sources for the isolation and generation of genetically engineered NK cells exist, including peripheral blood and umbilical cord blood (UCB) NK cells, various human NK cell lines (NK92, YT, KHYG, etc.) and induced pluripotent stem cell (iPSC)-derived NK cells. Several clinical trials are evaluating the efficiency of chimeric antigen receptor NK92 cells in haematological malignancies and multiple myeloma as well as in glioblastoma (Chu et al., 2014) (Han et al., 2015) (Zhang et al., 2020). However, irradiated NK92 cannot expand *in vivo* after infusion, and lacking the expression of CD16 and NKp44, the ADCC and the natural cytotoxicity, proper of activated primary NK cells, are compromised, making the NK92 cell line not the most

reliable source for NK cell engineering (Sivori, Meazza, et al., 2019). For this reason, UCB-, PB- and iPSC-derived NK cells have also been used as possible source for the development of CAR-NK cells. Several attempts have been made to identify the best protocol for PB-NK expansion, and an innovative strategy, which does not require a feeder layer, has been developed and successfully tested for CAR-CD19 NK cells (Quintarelli et al., 2020). CAR-NK cells derived from iPSC also demonstrated potent ability to kill, both *in vitro* and *in vivo*, mesothelin-expressing tumours (Li, Hermanson, Moriarity, & Kaufman, 2018). iPSC-NK cells represent an innovative and particularly promising tool because they efficiently kill both haematological malignancies and solid tumours, and most importantly, they are reproducible and can be easily genetically modified and expanded on the clinical scale (Zhang et al., 2020).

However, low *in vivo* persistence, immunosuppression exerted by TME and difficulty to infiltrate into solid tumours represent other challenges for CAR-NK clinical applications (Zhang et al., 2020). Thus, CAR constructs have been optimized according to NK cell features. NKG2D-expressing CAR-NK cells gave encouraging results in a mouse model of osteosarcoma, as they could recognize different tumour cells as well as immunosuppressive cells (MDSC and Tregs) present in the TME (Parihar et al., 2019). Moreover, a chimeric PD-1–NKG2D construct was also used to increase NK92 killing against various tumour cells (Guo et al., 2019). CAR-NK cells can also be engineered with IL-15 and/or chemokine receptors in order to increase specificity, efficacy and safety properties.

Even though there are still relevant challenges that impair a widespread use of CAR-NK cell-based therapies including improvement of *ex vivo* expansion, CAR construct optimization and development of new strategies to dampen the TME immunosuppression, NK cell therapy represent a promising and powerful tool for a more affordable 'off-the-shelf' cancer immunotherapy.

7 | CONCLUDING REMARKS

Recent years have witnessed an extraordinary progress in cancer therapy. The major advances are related to the use of checkpoint inhibitors, which dramatically changed the clinical outcome of cancer patients. Notably, the most efficient responses have been obtained in highly aggressive, metastatic tumours, primarily melanoma and NSCLC. These results have been explained with the high rate of genetic mutations of these tumours. Indeed, a direct correlation has been established between responses to checkpoint inhibitory treatment and mutation rates of different tumour types. A likely explanation is that genetic mutations, upon altering proteins, generate new tumour antigens recognized by T cells in an HLA-restricted fashion. However, HLA-I partial or total losses are a major mechanism of tumour escape and selection of tumour cells clones that became 'invisible' to T lymphocytes. In this respect, NK cells, different from T cells, efficiently kill HLA-I-deficient tumour cells, representing useful

effectors to attack these tumours. In this context, harnessing NK cell function should represent a major focus of present and future immunotherapy. Indeed, the function of inhibitory checkpoints expressed by NK cells, their biology and role in tumour immunotherapy, particularly in combination therapies, should be further investigated. Other strategies should also be examined in depth. A more promising approach is represented by NK cells that have been genetically modified to express CAR directed to tumour surface antigens. Different from chimeric antigen receptor T cells that must be used in an autologous setting (allogenic T cells would cause a severe GVHD), allogenic NK cells do not cause GVHD. Thus, donor-derived CAR-NK cells may be safely used for cancer treatment. This current technology allows obtaining large number of CAR-NK cells *in vitro* with a 4-log expansion, while their survival and expansion *in vivo* may be overcome by the additional transfection with IL-15-inducing gene. Notably, CAR-NK cells may represent optimal 'off-the-shelf' donor-derived products, since they can be prepared in large scale, with markedly reduced costs. In addition, they are immediately available to cancer patients in need of treatment and may be administered according to well-defined protocols, as to the timing and the number of cell infused. The possible benefit of CAR-NK approach in combination with checkpoint inhibitors should also be examined.

In conclusion, both major discoveries in the past three decades and the continuous technological advances have provided a large armamentarium that allowed and will allow improving treatment of high-risk leukaemia and solid tumours. In this context, NK-based therapies may play a fundamental role.

7.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <https://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Armstrong et al., 2020) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/2020 (Alexander et al., 2019).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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