Unleashing Anti-Tumor Activity of Natural Killer Cells Via Modulation of Immune Checkpoints Receptors and Molecules

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Abstract. As vital innate lymphocytes, natural killer (NK) cells suppress cancer progression chiefly by inducing cell lysis and secreting pro-inflammatory cytokines. NK cell activation relies on the balance between inhibitory and stimulating signals mediated by a wide range of surface receptors. Specific receptors initiate intracellular signaling pathways, which are negatively regulated by specific checkpoint molecules. Synergistic activation is controlled by Cbl proteins and GSK-3β, while the downstream signaling pathways induced by ITIM-bearing receptors are regulated by SHP-1. These intracellular NK checkpoints are attractive targets for immune checkpoint blockade therapies, but not enough attention has been given. Hence, this paper discusses the major signaling pathways regulated by the intracellular checkpoints and their potential clinical application. The current progress in the investigation of NK checkpoint receptors is also summarized. This paper aims to promote the development of novel immunotherapies that optimize the tumor-suppressive activity of NK cells while suppressing tumor immunological evasion.

Keywords: Checkpoint Inhibition Therapy, Cancer Immunotherapy, Cancer Immunology, Natural Killer Cells.

1. Introduction

Constituting a predominant subset of the innate lymphoid cells (ILC) family, natural killer (NK) cells have demonstrated great anti-tumor potential and promising clinical activities, making them an emerging platform for cancer immunotherapy. They target cancerous cells with a wide range of effector functions, primarily the induction of cell lysis and the secretion of pro-inflammatory cytokines [1]. Furthermore, sufficient evidence has supported the functions of NK cells in sustaining cancer dormancy and altering tumor architecture, which aid in tumor suppression and metastasis control [2, 3].

Unlike T cells that possess major histocompatibility complex (MHC)-restricted activation, NK cells possess an array of stimulatory receptors with diverse ligand specificity [4]. Effective NK response against cancerous cells often requires signaling from specifically grouped activating receptors, whose signals must converge on certain molecules in the downstream pathways [4]. The common downstream signaling molecules that negatively regulate synergistic activation of NK cells may serve as intracellular immune checkpoints, which can be therapeutically inhibited to enhance cytotoxicity [4]. Apart from activating receptors, the activation of NK cells is also regulated by a pool of inhibitory receptors that share intracellular signaling cascades [1]. The key regulators in the common inhibitory signaling cascades serve as additional checkpoints, which may enhance tumor suppression when effectively blocked [4].

Though the rapidly growing field of NK cell-based therapeutic strategies now constitutes a significant area of cancer immunotherapy, the therapeutic potential of intracellular checkpoint molecules in NK cells has been underappreciated. Nevertheless, these understudied checkpoints may be crucial for overcoming the current limitations of NK cell-based immune checkpoint blockade therapy. Firstly, considering the heterogeneity of ligands expressed by tumor cells for NK activating receptors, targeting signaling molecules common to multiple activating receptors can greatly improve the efficiency of checkpoint immunotherapy [5]. Moreover, since NK cells express multiple inhibitory checkpoint receptors, even if one of them is effectively blocked, NK cell activation can still be suppressed via alternative pathways. However, targeting the intracellular checkpoints in the
shared inhibitory signaling cascades would avoid compromising the effectiveness of checkpoint immunotherapy. Herein, this review describes the current progress within the field of NK cell-based checkpoint inhibition, with an emphasis on the intracellular checkpoint molecules. The major signaling pathways mediated by the intracellular checkpoints and their potential therapeutic application are discussed, with an aim to promote the development of novel approaches that optimize the tumor-suppressive response mediated by NK cells.

2. The role of NK cells in cancer immunology

NK cells restrict the progression of malignant cells via multiple mechanisms. Upon entry into the tumor microenvironment (TME), NK cells primarily use the ‘missing-self’ mechanism to identify and respond to cancerous cells, which frequently downregulate MHC-I expression to evade the attack by cytotoxic T cells [6]. The loss of MHC I-induced inhibitory signaling results in a relative increase in activating signaling, leading to NK cell activation [6]. Another strategy NK cells employ to detect tumor cells replies on the recognition of stress ligands upregulated in malignant cells by activating receptors expressed on NK cells (‘induced self’) [7]. Lastly, NK cells can identify tumor cells that are tagged by tumor antigen-specific antibodies and eliminate them via antibody-dependent cell-mediated cytotoxicity (ADCC) [7]. In addition to direct induction of cytolysis via perforin and granzymes, NK cells suppress cancer progression by secreting interferon-γ (IFN-γ) and tumor-necrosis factor-α (TNF-α) [7]. These pro-inflammatory cytokines suppress tumor proliferation and angiogenesis, while promoting apoptosis of malignant cells and cytotoxic CD8+ T cell activity [7]. The role of NK cells depends on the signaling from surface checkpoint receptors, such as CTLA-4 and PD-1, and intracellular checkpoint molecules, such as c-Casitas B-lineage lymphoma (c-Cbl) and glycogen synthase kinase 3 beta (GSK-3β).

3. Current progress in NK-based checkpoint blockade therapies targeting surface receptors

One of the most promising cancer immunotherapies is checkpoint inhibition, which is widely co-opted by cancer cells as a primary mechanism to evade immune attack. As the role of NK cells in anti-tumor immunity becomes increasingly appreciated, more and more inhibitors targeting NK checkpoint receptors entered clinical investigation. Such targets include NK cell-directed checkpoint receptors (Killer immunoglobulin (Ig)-like receptors (KIRs) and CD94/NKG2A) and the T cell-associated checkpoints (programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibition motif (ITIM) domains (TIGIT), CD96, and T cell immunoglobulin- and mucin-domain-containing molecule 3 (TIM-3)) [8]. The following section discusses the current progress in the development of NK-based checkpoint inhibitors, as well as the outcomes of relevant clinical trials.
3.1. NK cell-directed checkpoint receptors

Although KIRs antagonists successfully promoted NK cell cytotoxicity against tumors in mouse models, clinical trials of these inhibitors have yielded inconsistent results. One suspended phase II trial of IPH2101, a monoclonal antibody (mAb) blocking KIR2DL1, KIR2DL2, and KIR2DL3, in patients with smoldering multiple myeloma (SMM) revealed NK cell dysfunction, which was characterized by reduced degranulation and cytokine secretion [10]. In addition, the outcomes of a recent phase Ib trial that combined lirilumab and nivolumab to inhibit KIR and PD-1, respectively, suggested no significant enhancement in the efficacy against multiple myeloma, classic Hodgkin lymphoma, or non-Hodgkin lymphoma, in comparison to PD-1 inhibition alone [11]. Nonetheless, KIR inhibitors showed encouraging therapeutic activity in several other clinical trials. The most recent evidence is that a Phase I/II study of lirilumab and PD-1-inhibiting nivolumab obtained enhanced clinical benefits in patients with advanced-stage squamous cell carcinoma of the head and neck (SCCHN) [12]. Besides, lacutamab (IPH4102) targeting KIR3DL2 has demonstrated both safety and promising clinical activity in patients with relapsed/refractory cutaneous T-cell lymphomas (CTCL) in a Phase I trial (NCT02593045). This KIR3DL2-inhibiting mAb is being tested in further clinical research and may be a candidate treatment strategy for these CTCL patients in the future.

Monalizumab (IPH2201) is a therapeutic mAb specific to NKG2A, whose safety has been validated in many cancer patients [13]. Although monalizumab as a single agent showed little efficacy against gynecologic cancers and subcutaneous mouse tumors, it enhanced the tumor-suppressive effect of anti-PD-L1 mAbs [14, 15]. This further encouraged clinical trials that investigate monalizumab combined with inhibitors targeting other immune checkpoints and chemotherapy [13].

3.2. T cell-associated checkpoint receptors

PD-1 and PD-L1 pharmaceutical inhibitors have obtained significant successes in promoting T cell response against human cancers. Till now, anti-PD-1 mAbs that are approved for clinical use
include nivolumab, pembrolizumab, and tislelizumab. Approved mAbs targeting PD-L1 include atezolizumab, durvalumab, avelumab and dostarlimab. Additionally, spartalizumab, a novel mAb specific to PD-1, is being clinically evaluated across a range of cancer types. Importantly, preclinical research has demonstrated that anti-PD-L1 mAbs-induced suppression of several tumors is primarily mediated by NK cells [6]. Furthermore, PD-1 upregulation in NK cells was linked to a worse prognosis of multiple gastrointestinal cancers, gaining confidence for inhibiting PD-1-PD-L1 signaling on endogenous or adoptive NK cells [6].

LAG-3 is a checkpoint receptor expressed on a variety of immune cells. Though blocking LAG-3 has been demonstrated to improve T cell response against tumors, the potential role of LAG-3 modulation in NK cell-mediated tumor immunity remains unclear. Early results of studies revealed that inhibiting LAG-3 facilitated mouse NK cell killing of selected tumors but had no significant impact on human NK cells [4]. Hence, future research investigating how LAG-3 inhibitors (such as the newly approved relatlimab) affect the tumor-suppressive NK cell responses is necessary.

TIGIT and CD96 are key immune checkpoints found in NK cells and some T cells. Upregulation of both receptors has been discovered in tumor-infiltrating NK cells compared to NK cells in peritumoral tissues [4]. In addition, early results suggested that high expression of TIGIT/CD96 correlated with an unfavorable prognosis in patients with cancers. In multiple mouse models, TIGIT blockade promoted NK cell-mediated immunity against cancers and CD96 inhibitors limited tumor metastases [16, 17]. These promising findings have encouraged several different clinical trials of TIGIT inhibitors either as a monotherapy or combined with pharmaceutical inhibitors targeting other checkpoints, with an aim to treat multiple myeloma (NCT04150965), advanced solid tumors (NCT04047862), and lung cancers (NCT03563716, NCT04256421, NCT04294810). In addition, an anti-CD96 mAb recently entered a Phase I clinical trial both as a monotherapy and combined with dostarlimab (an antibody specific to PD-1) to treat advanced solid neoplasms (NCT04446351).

TIM-3 modulation has shown controversial effects on the effector functions of NK cells. Blocking TIM-3 in NK cells improved their cytotoxicity and IFN production in patients with lung adenocarcinoma or advanced-stage melanoma, indicating that this technique has therapeutic promise [18, 19]. Conversely, TIM-3 inhibition led to suppressed NK cell-induced elimination of pancreatic tumors, calling for further investigation to evaluate the impact of TIM-3 modulation on NK cells [20]. Pharmaceutical molecules blocking TIM-3 are now being tested for efficacy and safety in numerous clinical studies, but none of them include combinations with other NK cell-based therapies [6].

### 4. Intracellular checkpoint molecules in NK cells

Many signaling pathways in NK cells involve common downstream negative regulators, which serve as intracellular checkpoint molecules. Figure 2 shows a simplified illustration of three major pathways regulated the key checkpoint molecules including SH2-containing inositol phosphatase (SHIP-1), Src homology 2 domain (SH2)-containing protein tyrosine phosphatases (SHP-1/2), c-Casitas B-lineage lymphoma (c-Cbl), glycogen synthase kinase 3 beta (GSK-3β), and Cytokine-inducible SH2-containing protein (CIS).
Figure 2. The major signaling pathways in NK cells that involve common negative regulators (shown in red boxes) [4, 5, 9] (Created with BioRender.com). These negative regulators serve as intracellular checkpoints and can be modulated to enhance NK anti-tumor functions.

NK cells are equipped with diverse inhibitory receptors that have ITIMs on their cytoplasmic ends [1]. Upon the binding of specific ligands to these surface receptors, the ITIM motifs become phosphorylated, recruiting SHP-1/2 and SHIP-1 [21]. The interaction between SH2 domains of SHP-1 and phosphorylated tyrosine residues on ITIMs then activates SHP-1, which subsequently dephosphorylates the key molecules downstream of NK cell activating signaling pathways, such as Vav1, thereby downregulating NK cell activation [22].

Apart from inhibitory receptors, NK cells possess a pool of stimulatory receptors, which, when specifically combined, can lead to synergistic activation [4]. For instance, 2B4 combined with NKG2D or DNAM-1 lead to SLP-76 phosphorylation, thereby activating Erk in a Vav1-dependent manner [4]. As a vital signaling protein, Vav1 is regulated by both SHP-1 and c-cbl [4, 22]. Besides, NKG2D or DNAM-1 can independently induce another activating signal via PI3K and Akt [9]. The signal from Akt, as well as activated Erk, can induce phosphorylation that downregulates the regulatory activity of GSK-3β, which is a checkpoint molecule capable of limiting NK effector functions [9].

Another major NK pathway is IL-15 pathway, whose signaling is critical to NK cell survival, proliferation, and natural cytotoxicity against tumors [23]. Interestingly, IL-15 signaling promotes the production of the checkpoint molecule CIS, which serves as a negative feedback regulation [23].

4.1. Cbl-b and c-Cbl

The Cbl family of ubiquitin E3 ligases, including Cbl-b, c-Cbl, are predominant suppressors of activating signaling pathways in NK cells. Cbl proteins attenuate signaling of NK receptors by facilitating the ubiquitylation of active tyrosine kinases and other proteins in the signaling pathways, hence inducing their lysosomal degradation or proteasomal degradation [24]. When phosphorylated by TAM receptors (Tyro3, Axl, and Mer), Cbl-b targets key signaling molecules such as LAT1/SLC7A5, which is essential for NK activation and cytokine secretion [25]. As shown in mice models, Cbl-b knockdown by both genetic mutation and a TAM kinase inhibitor (LDC1267) significantly promoted the anti-metastatic response of NK cells [26]. The same group of researchers further demonstrated that warfarin reduced tumor metastasis through Cbl-b/TAM receptors in NK cells, indicating that modulating the Cbl-b/TAM pathway may serve as a potential cancer treatment strategy [26].
The ubiquitin E3 ligase c-cbl inhibits Vav1, an important signaling molecule downstream of NK activating receptors [4]. The knockdown of c-Cbl in human NK cells amplifies the signal from a single stimulatory receptor, thereby lowering the activation threshold of NK cells [27]. Furthermore, by promoting the activating signal from the Vav1-dependent pathway, c-cbl knockdown in human NK cells markedly improved their cytotoxicity and pro-inflammatory cytokines secretion [27]. However, none of these promising outcomes were observed in human NK cells with Cbl-b knockdown. Thus, the clinical value of Cbl-b modulation in humans is still in need of further research [27].

Recently, a non-viral lipid nanoparticle (NP)-based delivery system containing small interfering RNAs (siRNAs) has successfully silenced the main intracellular NK checkpoints, c-Cbl, Cbl-b, and SHP-1, in vivo [28]. Such checkpoint blockade unleashes NK cell activity and enhances NK cytotoxicity in the tumor microenvironment [28]. Besides, since these nanoparticles directly target NK cells in vivo, there is no need for ex vivo expansion of NK cells, which has been a major drawback of current NK cell-based immunotherapies [28]. As a result, this innovative NP-based delivery system could be a useful tool for future NK cell-based cancer therapies.

4.2. GSK-3β

GSK-3 is a widely expressed serine/threonine protein kinase that regulates several physiological functions, including immunological responses [29]. Two isoforms of GSK-3 exist in mammals, named GSK-3α and GSK-3β, which are encoded by different genes [29]. GSK-3β negatively regulates synergistic activation of NK cells by acting as a convergence point downstream of unique combinations of stimulatory receptors (like NKG2D and 2B4) [9]. Such negative regulation is found in a variety of activation pathways initiated by either ITAM-coupled receptors or non-ITAM-coupled receptors [5]. Ultimately, GSK-3β suppresses the primary anti-tumor activities of NK cells like cytotoxicity and cytokine secretion, suggesting that GSK-3β could be therapeutically targeted to promote NK cell activation and activity [9]. Besides, various preclinical studies have illustrated the importance of GSK-3 in T cell regulation and the therapeutic potential of GSK-3 inhibitors. For instance, GSK-3β inhibition in CD8+ memory stem T cells in vitro increased their cytotoxicity against gastric tumor cells through the upregulation of FasL, IFN-γ, and granzyme B [30]. Additionally, GSK-3β blockade facilitated the survival and proliferation of activated glioblastoma (GBM)-specific IL13 chimeric antigen receptor-expressing T (IL13-CAR-T) cells [31]. Furthermore, IL13-CAR-T cells with GSK-3β inactivation had improved cytotoxicity against tumor cells upon activation by the target antigen [31]. These promising outcomes of GSK-3β blockade in T cell anti-tumor activity motivate the relevant research of NK-based GSK-3β inhibition therapy.

GSK-3β gene knockdown or inhibition with various pharmacological agents enhances NK cell activation via a variety of stimulatory receptors. Mechanistically, GSK-3β inhibition has been linked to the activation of Akt and Erk, which are both essential for effective NK cell activation [7]. In support, Parameswaran et al. discovered that GSK-3β overexpression in NK cells from AML patients, which could explain AML-NK dysfunction [32]. Furthermore, GSK-3 silencing by pharmacologic manipulation (SB415286, LY-2090314, or Tidegusib) or genetic inhibition of either GSK-3 isofrom restores AML-NK cell cytotoxicity, indicating that targeted regulation of this intracellular checkpoint could be a feasible approach for NK cell-based cancer treatment [32].

In the last a few years, a subpopulation of NK cells characterized by NKG2D and high CD57 expression has attracted growing attention among researchers within the field of NK-based cancer immunotherapy. These NKG2D+CD57+ cells are often referred to as adaptive NK cells because they possess some memory B and T cells characteristics, including clonal-like expansion, rapid recall immune response, and viral antigen specificity [33]. The number of these adaptive NK cells in the tumor microenvironment has been found to correlate with better survival and tumor regression in patients with solid tumors, implying that promoting the differentiation of adaptive NK cells may be clinically beneficial [33]. Accordingly, Chichocki et al. have demonstrated that NK cells expanded ex vivo in media containing a GSK-3 inhibitor (CHIR99021) have increased CD57 acquisition and

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maturation [34]. More importantly, the GSK-3 inhibitor enhanced the ability of NK cells to secrete TNF-α and IFN-γ upon target recognition, improving their anti-tumor efficacy [34]. Motivated by these promising results, three clinical trials are evaluating the potential of incorporating CHIR99021 and IL-15 during the ex vivo expansion of NK cells. The expanded NK cells, as well as IL-2, Herceptin, or Erbitux, are reinfused into patients with AML, ovarian cancer, and solid tumors (NCT03081780, NCT03213964, NCT03319459).

4.3. SHP-1

SHP-1 is activated when inhibitory NK cell receptors, such as KIRs, are engaged by cognate MHC-I ligands [35]. The activation of SHP-1 ultimately results in the suppression of NK cell activity via Vav1 dephosphorylation, suggesting SHP-1 may serve as an intracellular immune checkpoint [35]. Consistent with these findings, an inactivated, mutant form of SHP-1 has been demonstrated to reduce the activation thresholds of NK cells and enhance their anti-tumor response [36]. Even though the strategy employed by SHP-1 to negatively regulate NK cell activation has been investigated for a relatively long period of time, it was not until very recently that how the regulatory pathway of SHP-1 itself was discovered. Ben-Shmuel et al. demonstrated that protein kinase c theta (PKC-θ) phosphorylates the S591 residue of SHP-1, maintaining SHP-1 in the inactivated, “dormant” state [37]. Accordingly, silencing PKC-θ reactivates SHP-1, consequently reducing NK cell cytotoxicity and promoting cancer progression [37]. These results reinforced the tumor-suppressive impact of blocking SHP-1 activity. Furthermore, as previously mentioned, a nanoparticle-based delivery system of siRNAs silences SHP-1 in mice NK cells, significantly improving NK cytotoxicity [28]. Although these early results obtained in mice demonstrate the potential of SHP modulation as an emerging cancer immunotherapy, extensive research is still in need to further explore the anti-tumor potential of SHP blockade in humans.

4.4. CIS

CIS is a critical negative regulator of cytokine signaling that has been previously demonstrated to have a role in regulating NK cell activity and development [38]. CIS suppresses the IL-15 signaling pathway in NK cells as a negative feedback regulation, making it a novel NK intracellular checkpoint [38]. Accordingly, the knockout of cish gene, which encodes CIS, lowered the activation threshold of NK cells upon IL-15 signaling [39]. Cish-deficient NK cells also showed improved proliferation, enhanced cytotoxicity against tumor cells, and increased IFN-γ production [39]. As a consequence, Cish knockout mice have less metastasis in multiple different tumor models, which is potentially owing to increased JAK-STAT signaling in activated NK cells [39]. Unfortunately, although blocking CIS via genetic inactivation has led to favorable outcomes, the development of pharmacological agents targeting CIS remains challenging. The primary structure of CIS is highly similar to that of other members of the suppressor of cytokine signaling (SOCS) family, so any potential CIS inhibitor must find a way to achieve high target-selectivity and minimize off-target effects [38].

5. Conclusion

NK cells are key innate lymphocytes that suppress cancer progression primarily by direct cytotoxicity and the production of pro-inflammatory cytokines. The activation and effector functions of NK cells are regulated by diverse intracellular signaling pathways, which are, in turn, controlled by specific checkpoint molecules such as Cbl proteins, GSK-3β, and SHP-1. Although growing evidence suggests that these intracellular checkpoints are attractive targets for immune checkpoint blockade therapies, they are still new to the field of cancer immunotherapy and have received insufficient attention.
References


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