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REVIEW

Intracellular checkpoints for NK cell cancer immunotherapy

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Abstract **Natural killer (NK) cells are key innate immune lymphocytes, which play important roles against tumors. However, tumor-infiltrating NK cells are always hypofunctional/exhaustive. On the one hand, this state is contributed by context-dependent interactions between inhibitory NK cell checkpoint receptors and their ligands, which usually vary in different tumor types and stages during tumor development. On the other hand, the inhibitory functions of intracellular checkpoint molecules of NK cells are more similar across different tumor types, representing common mechanisms limiting the potential of NK cell therapy. In this review, representative NK cell intracellular checkpoint molecules in different aspects of NK cell biology were reviewed, and therapeutic potentials were discussed by targeting these molecules to promote antitumor NK cell therapy.**

Keywords genomic editing; NK cell exhaustion; immune checkpoint; inhibitory molecules; immune tolerance

Introduction

Natural killer (NK) cells are cytotoxic innate lymphocytes, which play an important role in the body's first line of defense against cancers and viral infections [1–3]. Increased tumor infiltration of NK cells is associated with favorable tumor outcomes for patients with colon cancer [4], gastric cancer [5,6], and lung carcinoma [7,8], whereas decreased peripheral blood NK cell activity is associated with cancer progression, such as in invasive breast cancer [9], highlighting the essential role of NK cells in surveillance against cancers. However, NK cells are usually functionally exhaustive in the tumor microenvironment, mainly manifested by a decrease in the number of NK cells and a decline in function, which are attributed to an imbalance in immune regulatory signals in tumor-associated NK cells [10,11].

Immune checkpoint receptors are inhibitory cell surface receptors that maintain immune tolerance but might suppress antitumor immunity, which are usually upregulated upon activation and during exhaustion of immune cells [12,13]. Among them are HLA class I (HLA-A, -B, -C)-specific inhibitory KIR/CD158 family members whose ligands, HLA class I molecules, are expressed on the surface of almost all nucleated cells and usually downregulated in transformed cells. Inhibitory KIRs and their ligands are independently polymorphic. For example, KIR2DL1, KIR2DL2/KIR2DL3, and KIR3DL1 bind HLA class I C2, C1, and Bw4 alleles, respectively. KIR3DL2 also binds some HLA-A alleles in the context of certain peptides [14–17]. MHC class I molecules are constructed from two distinct components [18,19], namely, an α chain (also recognized as the heavy chain) and β 2 microglobulin (light chain), with the α chain traversing the cell membrane. This α chain includes three external domains (α 1–3, starting from α 1 at the N-terminal), coupled with a membrane-spanning domain and a cytoplasmic tail at the C terminus. The β 2 microglobulin, an extracellular chain, primarily interacts with the α 3 domain, playing a crucial role in the structural integrity of the MHC complex. Within the α chain, the α 1 and α 2 domains constitute the peptide

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recognition site, forming a pronounced groove that serves as the binding site for antigenic peptides. KIR inhibitory receptors utilize the immunoreceptor tyrosine-based inhibitory motif (ITIM) within their cytoplasmic tail to transmit signals. Upon ligand engagement, tyrosine residues within the ITIMs undergo phosphorylation, which subsequently recruits protein tyrosine phosphatases such as SHP-1 to transmit inhibitory signals to restrain NK cell activity [20]. In mouse, structurally divergent Ly49 receptors displayed similar functions to human KIRs [21–23]. In addition, the CD94/NKG2A (CD94/CD159a) heterodimer [24] and additional inhibitory receptors such as PD-1 [25], TIGIT [26], and TIM-3 [27], whose specific ligands are frequently expressed in tumor tissues, are identified. They serve as context detectors, in that any single inhibitory receptor is functional only when at least one of its ligands is present and expressed at sufficient levels, and that these ligands for different receptors are usually expressed with different patterns in different kinds of tumors. Therefore, the impact of each checkpoint receptor on antitumor immunity varies across different tumor types.

In addition, the intracellular checkpoint molecules of NK cells are distributed in the cytoplasm or nucleus of NK cells (Fig. 1), and they usually function via converged signaling from multiple surface receptors or are triggered by similar stimulus under common status. They can inhibit the differentiation, development, proliferation, metabolism, and cytotoxic activity of NK cells, as well as promote the apoptosis of NK cells [28–31]. The intrinsic mechanism of action of intracellular checkpoint molecules underlies their universal role in antitumor immunity across different contexts, making them ideal targets for designing universal strategies for tumor immunotherapy [32]. Emerging studies have revealed novel intracellular checkpoint molecules in different aspects of NK cell biology, which mediate NK cell exhaustion in tumors [33,34]. Targeting these molecules represents potential therapeutic opportunities for tumors (Fig. 2).

Intracellular checkpoint molecules

BIM (apoptosis mediator)

Apoptosis is a programmed form of cell death, representing a primary mechanism of cell death, which might limit the number of NK cells present in a tumor microenvironment [35]. Therefore, apoptotic mediators are potential checkpoint molecules for NK cell antitumor immunity. The Bcl-2 protein family is an important regulator of apoptosis. This family includes anti-apoptotic proteins (such as Mcl-1, Bcl-xl, Bcl-2, BCL-1, and Bclw) and pro-apoptotic proteins (such as Bax, Bak, Bok, Noxa, Puma, Hrk, BIM, Bad, Bid, and Bik), of which Bax, Bak, and Bok are pro-apoptotic effect proteins [36].

Among them, BIM stands at an essential point in the complex regulatory network of the apoptotic pathway. Normally, the expression and proapoptotic activity of BIM are negatively regulated at multiple levels, by

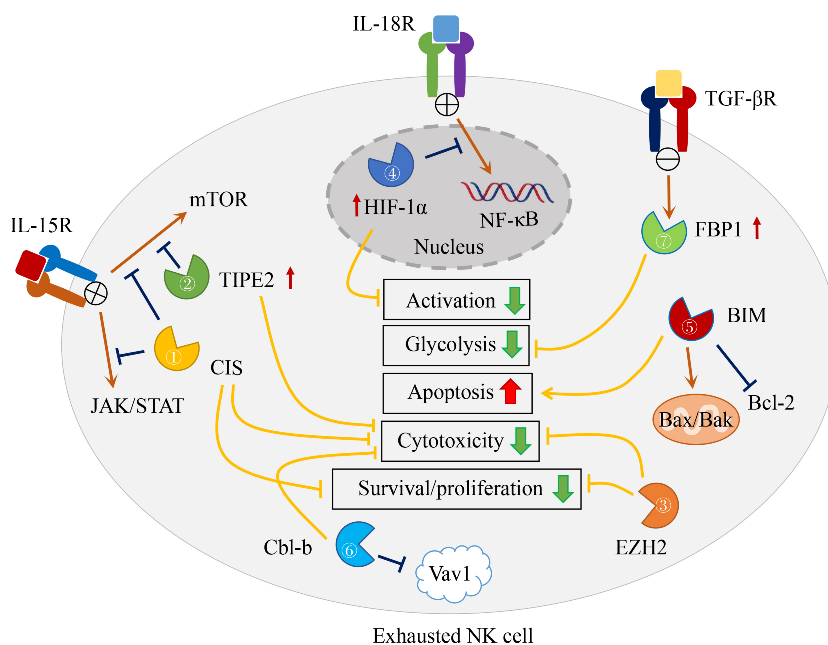

Fig. 1 Intracellular checkpoint molecules from different aspects of NK cell biology. Representative molecules are as follows: FBP-1 related to metabolism, EZH2 related to epigenetics, CIS and TIPE2 as negative IL-15 signaling regulators, HIF-1α associated with hypoxia, Cbl-b associated with protein ubiquitination, and BIM related to apoptosis. These intracellular checkpoints contributed to NK cell exhaustion by different aspects.

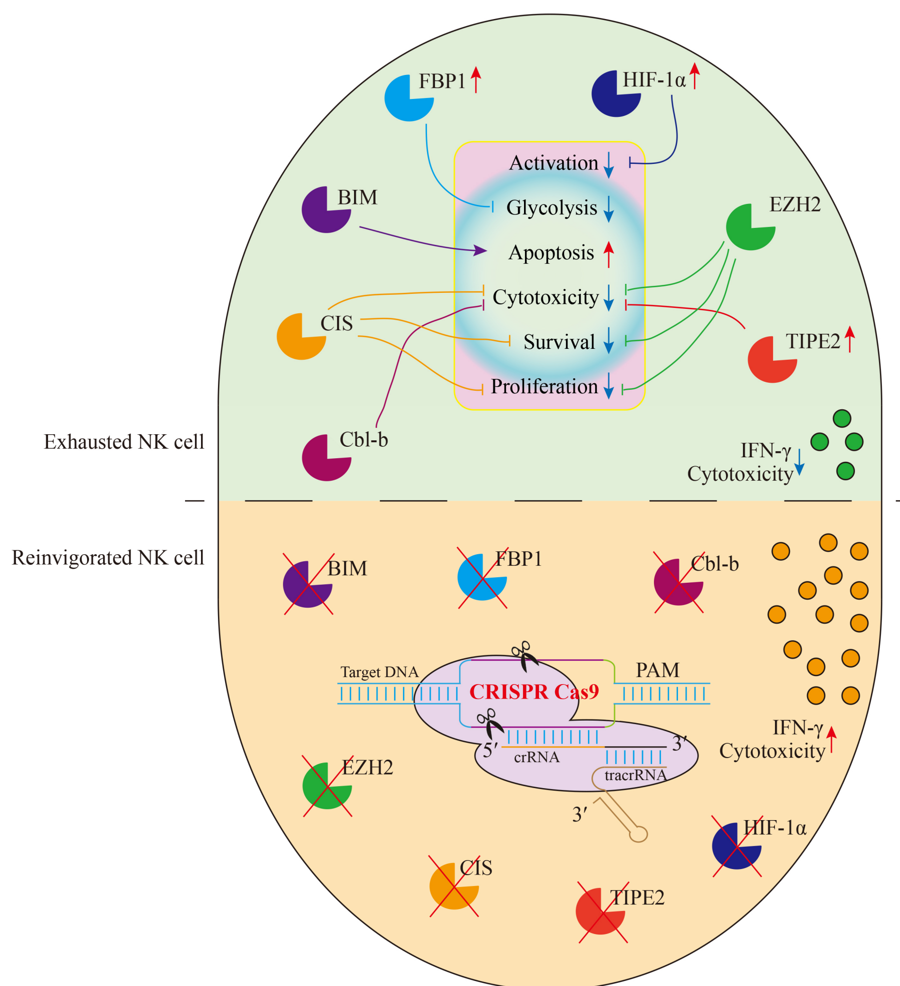

Fig. 2 Several known intracellular checkpoints of NK cells described in this article (e.g., BIM, FBP1, Cbl-b, EZH2, CIS, TIPE2, and HIF-1α) mediate the depletion state of NK cells (upper part of the figure), which is manifested by an overall decline in the activation function, glycolysis, cytotoxicity, survival, and proliferation of NK cells. The level of apoptosis increased, and the amount of IFN-γ produced decreased sharply and finally lost the antitumor ability. By knocking out one or more intracellular checkpoints in NK cells by CRISPR Cas9 technology (bottom half of the figure), the depletion state of NK cells can be reversed, and the cytotoxic effect of NK cells to produce high levels of IFN-γ can be restored. Consequently, NK cells can regain antitumor ability.

transcription factors (e.g., YY1, HoxB8, Spi-1/PU.1, PINCE-1, and Pokeman) [37–41], by microRNAs (e.g., miR-17-92, -25, -32, and -301a) [38,42–44], by RNA-binding proteins (e.g. Hsp27) [45], and by being sequestered in its inactive form to the microtubular cytoskeleton [46] or with anti-apoptotic Bcl family members sequestered to the mitochondria [47]. Upon apoptosis induction, BIM promotes apoptosis by binding to and inhibiting anti-apoptotic Bcl-2 proteins (such as Mcl-1, Bcl-2, Bcl-xL, Bcl-w, Bfl-1, and Epstein–Barr virus BHRF-1), as well as by binding to activate the pro-apoptotic proteins Bax and Bak, leading to the permeabilization of the outer mitochondrial membrane.

BIM plays a critical role in maintaining immune homeostasis. The loss of BIM alone renders lymphocyte resistance to apoptotic stimuli such as cytokine deprivation, calcium ion flux, and microtubule perturbation [48]. In addition, the loss of BIM and another proapoptotic protein, Puma, leads to hyperplasia of lymphatic organs [49]. T cell receptor (TCR) stimulation induces the expression of BIM [50]. BIM inhibits the survival of memory precursor cells of $CD8^+$ T cells, leading to the restrained memory potential of $CD8^+$ effector T cells [51]. BIM also depletes some virus-specific $CD8^+$ T cell clones essential for $CD8^+$ T cell antiviral activity [52]. The expression level of BIM in naïve $CD4^+$ T cells decreases with age, which extends persistence but leads to the dysfunction of $CD4^+$ T cells [53]. For B cells, BIM limits the survival of mature B cells [54] and promotes the programmed death of memory cells lacking affinity-enhancing mutations in their immunoglobulin genes and antibody-forming cells

secreting low-affinity antibodies [55,56].

In NK cells, BIM mediates apoptosis upon cytokine deprivation, and BIM-deficient NK cells are resistant to apoptosis caused by IL-15 deficiency. In steady state, BIM-deficient NK cells accumulate in the late stage of maturation *in vivo* [31]. In MCMV infection, BIM deficiency results in higher levels of antigen-specific Ly49H$^+$ NK cells [57]. On the contrary, BIM is inhibited by IL-15, which causes the proteasomal degradation of BIM by activating kinases Erk1 and Erk2 and by inactivating the transcription factor Foxo3a. The absence of IL-15 led to increased levels of BIM. Meanwhile, BIM-deficient NK cells have no defects in cytotoxicity or cytokine production [31].

Although BIM does not seem to directly regulate NK cell effector functions, BIM deficiency might increase the number of available NK cells for antitumor response. Therefore, BIM might be a potential checkpoint molecule in NK cell antitumor immunity. Future studies must investigate the therapeutic potential of BIM-deficient NK cells for adoptive therapy against cancers.

Cbl-b (protein post-translational modification)

The casitas B-lineage lymphoma proto-oncogene-b (Cbl-b) is an E3 ubiquitin protein ligase that plays various physiologic roles. Cbl-b is the widely studied member of the Cbl ubiquitin ligase family in the immune system. This family is a characteristic of the spiral connector, N-terminal tyrosine kinase binding (TKB) domain, RING finger domain, C-terminal leucine zipper/ubiquitin-related (UBA) domain, and proline-rich (PR) motif. Cbl-b functions by physically interacting with other proteins via the TKB and UBA domains or the PR region or by ubiquitinating signaling proteins [58]. For example, Cbl-b targets the p85 subunit of PI3K for ubiquitination to downregulate Vav1 activation [59], which might compromise NK cell cytolytic activity. Cbl-b also partners with Stub1 to target Foxp3 for ubiquitination to regulate thymic Tregs [60]. Furthermore, Cbl-b ubiquitinates Dectin-1, -2, -3, and Syk for degradation to dampen pro-inflammatory cytokine production and ROS release in response to systemic *Candida albicans* infection [61–63].

Evidence also indicates the role of Cbl-b in NK cell biology. By using E3 ligase-defective *C373A*$^{KI/KI}$ and the loss of Cbl-b expression (*Cbl-b*$^{-/-}$) NK cells and mice, Paolino *et al*. [64] found that E3 ubiquitin ligase Cbl-b gene deficiency or the loss of Cbl-b's E3 ligase activity promotes NK cell-mediated surveillance of tumor metastasis. NK cells treated with TAM kinase inhibitors displayed enhanced activity of metastasis control *in vivo*. The ablation of Cbl-b increased NK cell cytotoxic activity, IFN-γ production, and perforin release, leading to increased cancer cell killing. These studies indicate that Cbl-b and other ubiquitin ligase with similar mechanisms of actions might be attractive candidate targets for improving NK cell antitumor immunotherapy.

CIS (negative regulator of IL-15 signaling)

IL-15 signaling is critical for NK cell survival [65,66], proliferation, and cytolytic activity. IL-15 is mainly trans-presented by IL-15Rα-expressing hematopoietic cells (monocytes, macrophages, and dendritic cells) or parenchymal cells (epithelial cells, fibroblasts, and keratinocytes) to IL-15Rβ/γ$^+$ NK cells [67–69] or in the soluble IL-15/IL-15Rα complex in the circulation [70]. Therefore, IL-15α is essential for IL-15 availability to NK cells. When IL-15 binds to IL-15Rβ/γ, receptor-associated JAK1 and JAK3, as well as the receptor itself, are phosphorylated, recruiting STAT5 to be phosphorylated and to form dimer or tetramer for the induction of target gene transcription [71]. JAK-STAT5 signaling downstream of IL-15R induces the expression of Mcl1, which mediates the survival of NK cells [72]. In addition to JAK-STAT5, mTOR activity is induced by IL-15 but at higher concentrations, and it mediates NK cell proliferation, metabolism, and differentiation to fully functional effector cells during infections [73].

Cytokine-inducible SH2-containing protein (CIS), together with SOCS1-7 (the suppressor of cytokine signaling), belongs to the SOCS family, which are induced by IL-15 signaling as a negative feedback mechanism to the JAK-STAT5 pathway. SOCS family proteins contain a variable N-terminal region, a central SH2 domain, and a conservative C-terminal SOCS box [74,75]. SOCS proteins bind to an E3 ubiquitin ligase complex, which is formed by RING box protein 2 (RBX2), Elongins B and C, and scaffold Cullin-5. The SOCS box ubiquitinates target proteins for degradation in the proteosome. The expression level of SOCSs is upregulated by cytokines, thereby suppressing the signaling transduction downstream of these cytokine receptors [76].

CIS interacts with PLC-γ1 downstream of TCR for proteasomal degradation after TCR stimulation to suppress CD8$^+$ T cell expansion and function [77]. CAR-T cells with *CISH* deletion displayed decreased expression of PD-1, longer survival, enhanced cytokine production, and increased antitumor activity [78]. Although CIS inhibits proliferation and promotes apoptosis, it maintains the expression level of MHC I, co-stimulatory molecules, and pro-inflammatory cytokine production in DCs for T cell activation [79]. CIS could also be induced in macrophages in response to steady-state GM-CSF signaling to restrain the activation of STAT5, AKT, and ERK, as well as the expression of the transcription factor GATA2, which maintains macrophage identity and function [80].

CIS is a negative regulator of IL-15 signaling in NK cells. IL-15 stimulates the expression of CIS in NK cells. The absence of CIS enhances NK cell sensitivity to IL-15, proliferation, survival, IFN-γ production, and cytotoxicity against tumor cells, along with improved control of tumor metastasis [81]. Mechanistically, CIS interacts with and targets the tyrosine kinase JAK for proteasomal degradation; therefore, the absence of CIS promoted the JAK-STAT signaling pathway in *Cish*$^{-/-}$ NK cells. CIS inhibition also synergizes with TGF-β inhibition to further promote NK cell antitumor immunity [82]. Therefore, CIS should represent an important intracellular checkpoint for NK cell-mediated tumor immunity.

In addition, the therapeutic potential of targeting *CISH* in human NK cells was demonstrated by several studies. *CISH*-deleted human NK cells derived from induced pluripotent stem cells (iPSCs) showed increased cytotoxicity and improved metabolic fitness against tumor cells [83]. *CISH*-deleted iPSC NK cells displayed improved mTOR-dependent basic glycolysis, glycolytic capacity, maximum mitochondrial respiration, ATP-linked respiration, and standby respiratory capacity, which accounts for enhanced NK cell function. In addition, *CISH* deletion promotes cord blood (CB)-derived NK cell antitumor immunity [84]. Combining IL-15 overexpression and *CISH* KO improves the metabolic "fitness" of CB-derived CAR-NK cells, resulting in improved *in vivo* persistence and cytotoxic activity of NK cells. Finally, *CISH* deletion also improves primary NK cells. The deletion of *CISH* in primary human NK cells increased IFN-γ and TNF-α production, as well as enhanced cytotoxicity against allogeneic GBM cells and spheroids [85]. The intracranial injection of *CISH*-deleted allogeneic NK cells prolonged the overall survival of xenograft brain tumor mice. The deletion of *CISH* could also be combined with the deletion of other NK cell checkpoint molecules to further promote the antitumor potential of NK cells, such as with *TIPE2* deletion [86]. Collectively, these studies indicate that targeting *CISH* represents a potential strategy for improving the antitumor effects of adoptively transferred NK cells.

EZH2 (epigenetic regulator)

The polycomb-group protein enhancer of zeste homolog 2 (EZH2), together with the suppressor of zeste 12 protein homolog (SUZ12), embryonic ectoderm development (EED), and histone binding retinoblastoma-binding proteins 4 and 7 (RBBP4/7), formed the core of the polycomb repressive complex 2 (PRC2) [87,88]. EZH2 comprises of a SET domain at the C-terminal, which is a conservative feature of histone methyltransferase (HMTase), and specifically mediates the catalytic activity of PRC2 on H3K27 (lysine 27 of histone H3) [89], resulting in the chromatin compaction of target genes and epigenetic silencing. Most of the genome undergoes methylation on H3K27 through PRC2 [90], which is a gradual process, including transferring methyl groups to form the mono-, di-, and trimethylation of histone H3 at lysine 27 (H3K27me1, H3K27me2, and H3K27me3 [H3K27me1/me2/me3]). Although EZH2 has a SET domain with histone HMTase activity, the HMTase activity is low [91]. The gene silencing activity of PRC2 is mediated by EZH2's catalytic SET domain and at least two other PRC2 subunits, including SUZ12 and EED [92,93]. In particular, the C-terminal domain of EED interacts with the tail of histones carrying trimethyl-lysine residues associated with inhibitory chromatin labeling, which in turn activates the methyltransferase activity of PRC2 [92].

EZH2 regulates the differentiation and functions of different populations of immune cells. For example, EZH2 is required for the differentiation of effector CD8$^+$ T cells and precursors of central memory CD8$^+$ T cells, as well as the recall response of memory CD8$^+$ T cells upon antigen stimulation [94,95]. EZH2 also plays a critical role in Treg stability [96,97] and promotes Tfh differentiation during early cell commitment [98,99]. Moreover, EZH2 is required for pro-inflammatory cytokine production in macrophages [100] and for plasma cell differentiation [101]. EZH2 promotes GC B cell proliferation and inhibits its terminal differentiation [102–105]. On the one hand, EZH2 functions as a transcriptional repressor of lineage-specific genes in CD4$^+$ T cells and inhibits the differentiation and plasticity of T helper cells [106]. On the other hand, EZH2 suppresses MDSC differentiation [107].

For NK cells, EZH2 represents a negative regulator of NK cell effector functions. By investigating the impact of a histone methylation repressive marker (H3K27me3) on early NK cell differentiation, Yin *et al*. [108] observed enhanced NK cell lineage commitment, improved NK survival, and NKG2D-dependent NK cell cytolytic activity in NK-specific *Ezh2* KO mice or mice with inactivated EZH2 activity, suggesting that the epigenetic regulator EZH2 should be a potential checkpoint molecule for NK cell antitumor immunity, and targeting EZH2 might promote NK cell immunotherapy.

FBP1 (metabolic correlation)

Fructose-1,6-diphosphatase (FBP1) is a rate-limiting enzyme involved in gluconeogenesis, which mainly promotes gluconeogenesis and suppresses glycolysis [109]. Structurally, FBP1 is a square plane tetramer with the same subunit, which exists in two different quaternary conformations (T and R) and catalyzes the dephosphorylation of fructose 1,6-diphosphate to fructose 6-phosphate [110–112]. FBP1 deficiency is accompanied

with lactic acid acidosis and hypoglycemia, which could lead to accidental infant death [113]. Apart from its role in glucose metabolism, FBP1 has a non-enzymatic function in the nucleus, binding and regulating various transcription factors, such as hypoxia-inducible factor [114]. In addition, FBP1 induces reactive oxygen species (ROS) production and suppresses cell growth [115].

The tumor microenvironment partly induces NK cell exhaustion by suppressing glycolysis metabolism, during which FBP1 plays a key role. TGF-β upregulates the expression level of FBP1 in tumor-associated NK cells, thereby decreasing the glycolysis of NK cells. Thus, NK cell function would be inhibited by the low level of metabolic function (Fig. 3). By using the *Kras*-driven model of spontaneous lung cancer, Cong *et al.* [29] confirmed that NK cells gradually transited into exhausted states along tumor development. The compromised functions resulted from glycolysis inhibition by FBP1, which decreased NK cell viability. Furthermore, inhibiting FBP1 activity partly recovered NK cell activity, suggesting that the metabolism regulator FBP1 might represent a checkpoint for NK cells, and that targeting FBP1 represents a novel strategy for promoting NK cell antitumor immunity.

TIPE2 (negative regulator of IL-15 signaling)

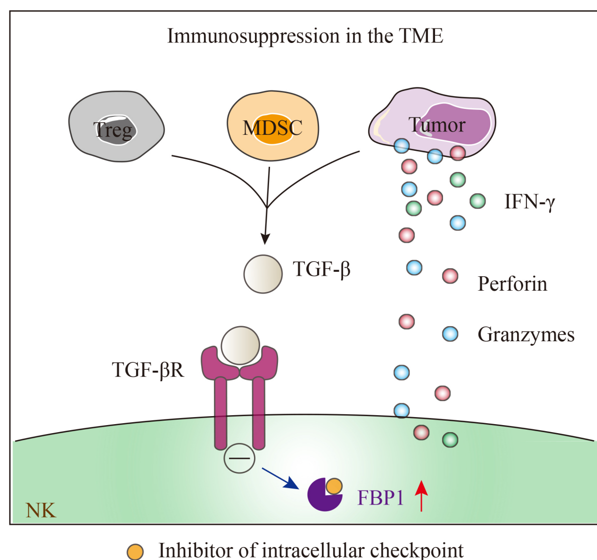

Fig. 3 Tumor-infiltrating NK cells are immunosuppressed by various factors and are often in a state of immune exhaustion. Various cells in the tumor microenvironment (such as Treg, MDSC, and tumor cells) secrete the immunosuppressive factor TGF-β. TGF-β transmits inhibitory signals downward by acting on the corresponding receptors on the surface of NK cells. Simultaneously, the level of the intracellular checkpoint molecule FBP1 increases. By blocking this process with inhibitors of FBP1, the depletion state of NK cells can be reversed, and the secretion of cytotoxic cytokines (such as IFN-γ, perforin, and granzymes) by NK cells can be increased to promote the antitumor effect of NK cells.

Tumor necrosis factor-α induced protein-8 like-2 (TIPE2) belongs to the TIPE protein family [116–118]. The aberrant expression of this protein family has been reported in many human diseases [119–124]. Among the family members, TIPE2 is mainly expressed by leukocytes [125], and it could be upregulated by TNF-α [125], IL-10 [86], ROS, IL-6, and L-arginine [126]. Structurally, TIPE2 consists of six reverse parallel α-helical structures, with the entire structure as a mirror of the death effector domain [127]. TIPE2 could bind to and activate caspase-8 to promote apoptosis [125]. It could also bind to and inhibit Rac to suppress downstream AKT and Ral activation [128,129]. Furthermore, TIPE2 possesses a hydrophobic pocket that could bind phospholipids, which makes TIPE2 a potential transfer protein of lipid second messenger [119].

TIPE2 was first identified as a negative regulator of innate and adaptive immunity. *Tipe2* KO immune cells displayed hypersensitivity to Toll-like receptor and TCR stimulation [125,130], as well as enhanced phagocytosis, oxidative burst, and cytokine production during infection [131,132]. Later, TIPE2 was shown to be a negative regulator for antitumor immunity. TIPE2 is essential for the tumor-promoting polarization of bone marrow-derived suppressor cells [126]. TIPE2 also facilitates immunosuppressive M2 macrophage differentiation [133] and plays an important role in the immunosuppressive function of $CD4^+CD25^+$ regulatory T cells [134].

Recently, we further showed that TIPE2 is also a negative regulator for NK cell antitumor immunity. *Tipe2*-deleted NK cells displayed increased functional maturation with enhanced mTOR response to IL-15 for human and mouse NK cells [135]. *Tipe2* KO NK cells were more mature and more capable of cytotoxicity and IFN-γ production upon stimulation in the steady state and in the tumor microenvironment [86,136]. The absence of TIPE2 in mouse or human NK cells enables these NK cells to effectively control the growth of solid tumors *in vivo* after adoptive transfer to tumor-bearing mice [86]. In addition, the absence of TIPE2 in NK cells directly enhances the intrinsic antitumor activity of NK and indirectly improves antitumor $CD8^+$ T cell response by promoting T-bet/Eome-dependent NK cell effector function [136]. Therefore, TIPE2 represents a checkpoint molecule for NK cells. Targeted deletion of the checkpoint molecule TIPE2 may be a promising approach for enhanced adoptive NK cell antitumor therapy. Moreover, TIPE2 and CIS serve as negative regulators for IL-15 downstream mTOR pathway and JAK-STAT pathway, respectively. They are also expressed by tumor-infiltrating NK cells of functionally distinct subsets, with *CISH* preferentially expressed by a more functional subset and *TIPE2* expressed by a more exhausted subset [86]. *TIPE2* deletion synergizes with *CISH* deletion to promote adoptive NK cell therapy [86], further

highlighting the essential role of this checkpoint molecule in NK cell antitumor immunity.

Hypoxia-inducible factor-1α (transcription factor and hypoxic response)

Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that plays an important role in cellular and developmental response to hypoxia. HIF-1α is composed of α and β subunits, and they co-regulate transcription. The α and β subunits have similar structures to the N-terminal bHLH domain for binding to DNA, a PAS domain that promotes the formation of heterodimers, as well as a C-terminal for recruiting transcription co-regulatory factors [137–139].

HIF-1α plays an important role in immune regulation. HIF-1α is required for glycolysis and optimal macrophage functions, such as migration, bacterial killing, and pro-inflammatory cytokine production [140–142]. HIF-1α is also important for glycolysis, MHC class II expression, IL-22 production, and CCR7-mediated migration of dendritic cells [143,144]. For neutrophils, HIF-1α promotes glycolysis and inhibits apoptosis, and it is required for NET formation [145–147]. In addition, HIF-1α is critical for T cell glycolysis, Th17 differentiation, and Tfh cell CD40L expression, and it inhibits Treg differentiation [148–150]. Finally, HIF-1α is required for normal B cell development, IL-10 production in B cells, and B cell-mediated protection in collagen-induced arthritis and experimental autoimmune encephalopathy [151–154].

A recent study has revealed that HIF-1α also represents a negative regulator of NK cells [155]. HIF-1α inhibits IL-18-driven NF-κB signaling and antitumor activity of tumor-infiltrating NK cells. In tumor-infiltrating NK cells, the expression of *Hif1α* and its target genes is negatively correlated with the expression of the *Ifng* gene, suggesting the negative effect of HIF-1α on the activity of tumor-infiltrating NK cells. The high expression level of *HIF1A* and its target genes was also observed in tumor-infiltrating NK cells of patients with non-small cell lung carcinoma, which is negatively correlated with the abundance of *IFNG* transcripts, whereas the enriched *NK-IL18-IFNG* signal in solid tumors is associated with an increase in overall patient survival. By single-cell transcriptomic analysis, an additional subpopulation among *Hif1α*$^{-/-}$ over wild-type tumor-infiltrating NK cells was identified, which expressed high levels of *Cd69* and *Ifng*. This NK cell subpopulation also expressed high levels of *Gzma* and *Gzmb*, moderate level of *Prf1*, and low levels of the checkpoint receptor genes *Pdcd1*, *Tigit*, and *Ctla4*. The conditional knockout of *Hif1α* in NK cells remarkably inhibited tumor growth, along with higher expression of activation markers and effector molecules, as well as NF-κB pathway enrichment in tumor-infiltrating NK cells. In addition, HIF-1α inhibitors increased the production of IFN-γ in human NK cells. These data indicated that HIF-1α negatively regulated NF-κB signaling in NK cells.

WT and *Hif1α*$^{-/-}$ NK cells showed similar extracellular acidification rate in response to hypoxia or stimulatory cytokines (e.g., IL-12 and IL-18). However, *Hif1α* KO NK cells displayed increased oxygen consumption rate, along with increased IFN-γ production, which indicated improved metabolic regulation, thereby promoting effector functions in these NK cells. Therefore, in the absence of *Hif1α* NK cells, oxidative phosphorylation may be fully utilized under hypoxic conditions.

Collectively, these data revealed the potential application of HIF-1α as a checkpoint molecule for the responsiveness of human and mouse NK cells in solid tumors, suggesting that the selective inhibition of HIF-1α in NK cells may be a promising strategy to improve the treatment of solid tumors based on NK cells.

Perspectives

Therapeutic effects of targeting NK cell intracellular checkpoint molecules might be more universal

Checkpoint receptor inhibition plays an important role in the current immunotherapy against cancers, whose efficacy relies largely on the sufficient interactions between checkpoint receptors and their ligands [156]. However, these interactions are usually redundant and tumor type/stage specific, possibly underlying the currently limited clinical responses of these therapies. On the contrary, the intracellular checkpoint molecules of NK cells function independently of ligands expressed in the tumor microenvironment, but rather by intrinsic signaling events triggered similarly among tumors. Therefore, targeted inhibition/deletion of intracellular checkpoints might be a more universal strategy to boost NK cell immunotherapy, which could be further combined with tumor sensors (e.g., NKR or CAR), immune checkpoint blockade (e.g., KIR and TIGIT), and synthetic gene circuits for future NK cell immunotherapy [157].

More novel intracellular checkpoints of NK cells

In addition to the abovementioned intracellular checkpoint molecules (Table 1), several emerging intracellular checkpoint molecules play important roles in limiting NK cell antitumor activity. Although thorough discussions of all molecules are limited by the length of this review article, they represent potential targets for improving NK cell immunotherapy. Apart from obtaining comprehensive understanding of NK cell biology, more novel checkpoint molecules might be revealed.

Table 1 Intracellular checkpoint molecules involved in NK cells

Regulatory effects	Molecules	References
Cytotoxicity (−)	CIS, TIPE2, Cbl-b, EZH2	[28,30,64,158–162]
Survival (−)	CIS, EZH2	[30,158,159,161]
Proliferation (−)	CIS, EZH2,TIPE2	[30,86,158,161]
Metabolism (−)	FBP1, HIF-1α	[29,155]
Apoptosis (+)	BIM	[31,57]
Mechanisms of action	**Molecules**	**References**
Signaling transduction	CIS, TIPE2	[30,159,161,162]
Transcription	HIF-1α	[155]
Epigenetics	EZH2	[158]
Post-translational modifications	Cbl-b	[64]
Pro-apoptotic protein	BIM	[31,57]

Multiple strategies to target NK cell intracellular checkpoints

Another important issue for targeting these intracellular checkpoint molecules for NK cell therapy is the feasible gene/protein modulation strategy. Checkpoint receptor inhibition could use a blocking monoclonal antibody against the target checkpoint receptors, with the support of long-developed technologies and a mature industry. On the contrary, the strategies for targeting intracellular molecules, especially those expressed in NK cells, might be limited. Considering that intracellular molecules could not be reached by antibodies, alternative technologies such as CRISPR/Cas9, shRNA, and small-molecule inhibitors might be used (Fig. 4). The major difficulty for genetic editing of NK cells is the relatively low efficiency

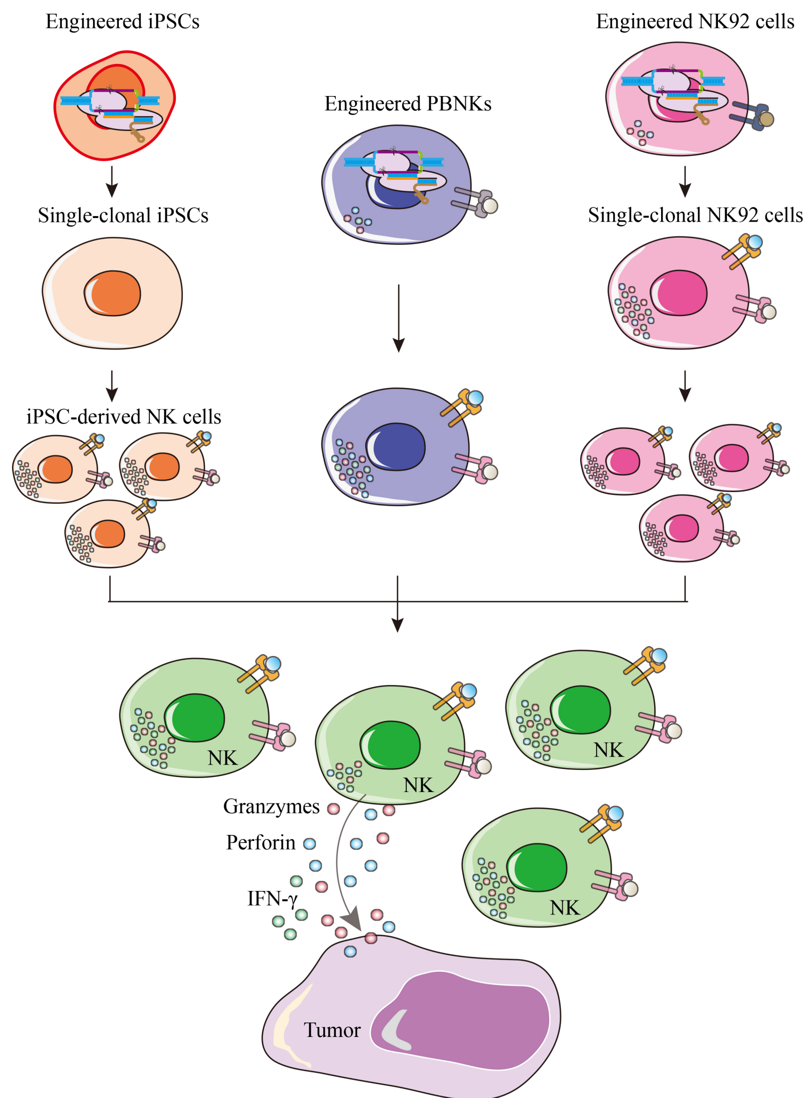

Fig. 4 NK cells of different origin are used in the immunotherapy of tumors. At present, three known methods can be used to obtain modified NK cells: (1) induced pluripotent stem cell (iPSC)-derived NK cells (iPSC NK) are genetically edited at the iPSC stage and are monoclonal, and such cells are differentiated into NK cells; (2) peripheral blood-derived NK cells (PBNK) are directly edited on NK cells; (3) the human NK cell line NK92 was edited and monoclonal on NK92.

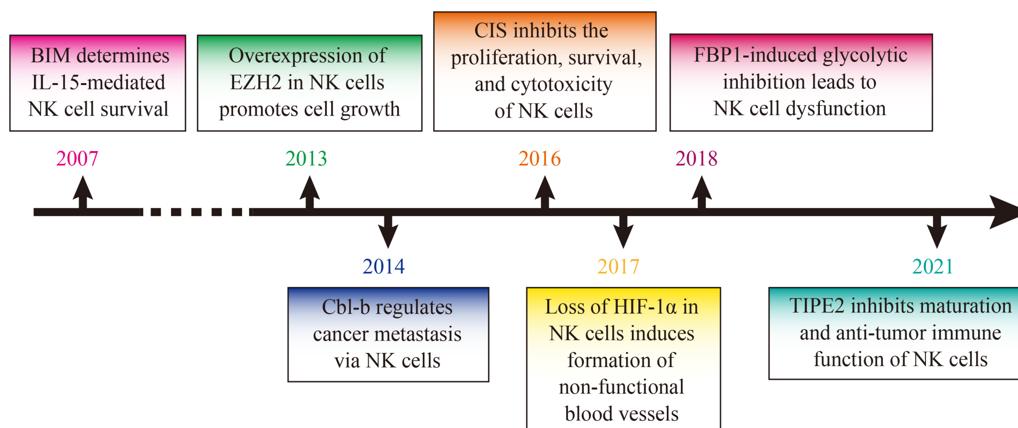

Fig. 5 Timeline of the intracellular checkpoints of NK cells. In recent years, with the gradual deepening of the research on NK cells, an increasing number of new intracellular checkpoints have been discovered. Intracellular checkpoints inhibit the growth, survival, proliferation, differentiation, cytotoxicity, and antitumor effects of NK cells, leading to the exhaustion of tumor-infiltrating NK cells.

for viral delivery of cas9/guide RNA components into NK cells, which was discussed elsewhere [163]. In addition, the application of this technology in the industry requires licensing of CRISPR-related patents. At present, the electroporation of a cas9/guide RNA complex displays the highest editing efficiency of NK cells [164,165], despite the high cost of GMP grade and amount of cas9 protein and guide RNA. Alternatively, lentivirus-expressed short-hairpin RNA (shRNA) represents another approach for checkpoint gene targeting, whose targetable sequences are more diverse, and shRNA is less likely to be affected by patents. However, the low transduction efficiency of lentiviral vectors limits the proportions of NK cells to be affected by shRNA. In addition, some abundantly expressed genes are difficult to be adequately silenced by a single shRNA. Finally, small-molecule inhibitors are available for some intracellular target molecules. However, this approach is limited by their relatively low specificity and potentially detrimental effects from targeting other cells than NK cells in the therapeutic setting.

Perspective of targeting checkpoint molecules for improving NK cell immunotherapy

Collectively, emerging checkpoint molecules limit NK cell antitumor immunity, representing potential targets for improving NK cell immunotherapy (Fig. 5). The non-redundancy of these molecules promotes the simultaneous targeting of more than one checkpoint molecules to boost NK cells more robustly. Thus, comprehensively understanding the NK cell biology and discovering novel checkpoint molecules are necessary to further identify targetable checkpoints. Furthermore, developing targeting technologies is of great importance to NK cells. Although the electroporation of a cas9/guide RNA complex represents a feasible and efficient approach for NK cell genetic editing, improving the NK cell-tailored viral transfection platform is not only an alternative for checkpoint editing/silencing of NK cells, but also the only available approach for stable gene overexpression in NK cells.

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Compliance with ethics guidelines

Conflicts of interest Yingying Huang, Zhigang Tian, and Jiacheng Bi declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Zhigang Tian is a member of the Editorial Board of Frontiers of Medicine, who was excluded from the peer-review process and all editorial decisions related to the acceptance and publication of this article. Peer-review was handled independently by the other editors to minimise bias.

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