Review

Immunometabolic Responses of Natural Killer Cells to Inhibitory Tumor Microenvironment Checkpoints

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ABSTRACT

NK cell trafficking and migration into solid tumors is often poor. Altered NK cell metabolism in the tumor microenvironment has been correlated with tumor invasiveness, metastasis and cellular dysfunction. Tumor-induced stresses correlate to limited numbers of functional NK cells in solid tumors and, consequently, limited survival. Metabolic impairment of NK cells in cancer is still not fully understood, but the involvement of inhibitory checkpoints—both tumor-associated as well as NK-specific—plays a significant role in driving these responses. The metabolic reprogramming events that NK cells undergo in the tumor microenvironment are linked to their ability to support energy metabolism in scenarios of impaired glycolytic fueling and low oxygen. The contributions of inhibitory checkpoints to these events are not entirely known, but are emerging as increasingly significant to the restoration of NK cell function. Here, we discuss our understanding of the role of inhibitory receptors and checkpoints in the metabolic dysfunction of NK cells in solid tumors, as a critical key to the development of new immunotherapies.

KEYWORDS: natural killer cells; tumor microenvironment; inhibitory NK receptors; immunometabolism

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INTRODUCTION

When a natural killer (NK) cell infiltrates a solid tumor, it is subjected to a variety of immunosuppressive and regulatory signals in the tumor microenvironment (TME) [1]. These signals originate from either soluble checkpoints or those expressed on cancer or immune cells, metabolites and the environment surrounding the cancer cells [2]. Nutrient and oxygen limitations characteristic of cancer microenvironments lead to dysfunction of NK cells. For instance, high rates of glycolysis inherent to cancer cells limit the ability of NK cells to participate in regular glycolytic fueling, severely impairing their effector functions, such as interferon-γ (IFN-γ) production and granzyme B expression [3]. This, in turn, drives

changes in the metabolic states of NK cells which results in impaired anti-tumor immunity (Figure 1).

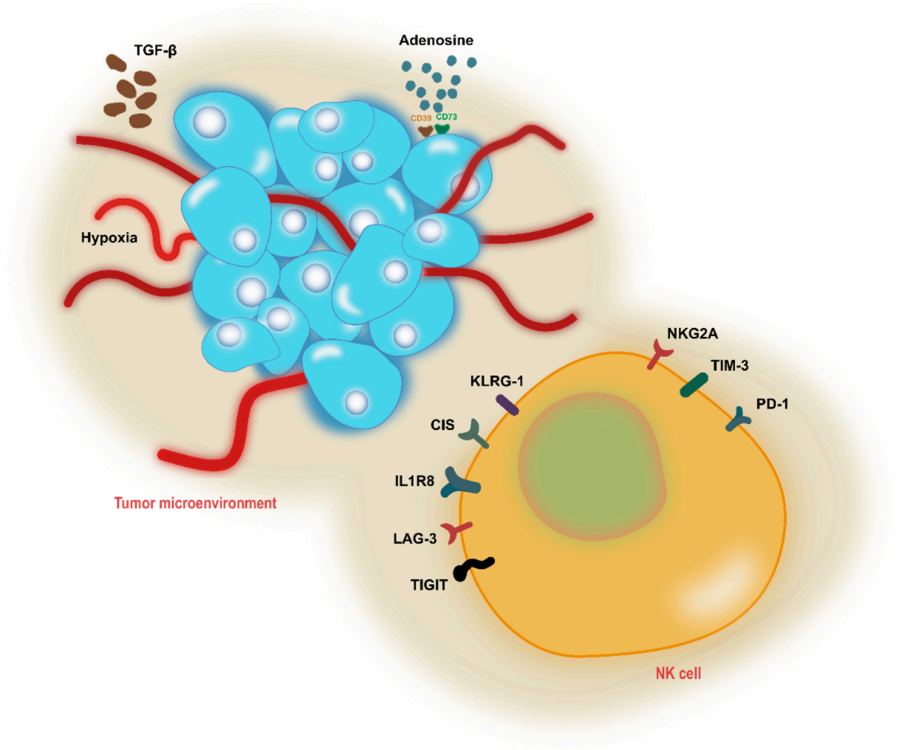

Figure 1. Tumor microenvironment checkpoints drive NK cell immunometabolic responses. NK cells which infiltrate a tumor are faced with a number of immunosuppressive checkpoints. These are either associated with NK cells directly, or are induced by the tumor microenvironment. Poor vascularization and impaired blood supply leads to adaptation of cancer cells to survival in environments of low oxygen, while causing immunometabolic reprogramming of NK cells. Inhibitory receptors expressed on NK cells further contribute to immune dysfunction, leading to pathologies characterized by tumor progression and metastasis.

Given the link between distinct metabolic profiles and NK cell effector functions, targeting immunometabolic reprogramming events that NK cells undergo in the TME is becoming a very attractive immunotherapeutic proposition.

However, NK-cell function is also regulated by the interaction between activating and inhibitory receptors expressed on their surface and their cognate human leukocyte antigen (HLA) class I ligands on target cells [4]. The signaling on these receptors occurs through immunoreceptor tyrosine-based activation motifs (ITAM) (via, for instance, FcRγ, CD3ζ and DAP12) as well as via ITAM-independent pathways [5]. These receptors thus utilize different signaling elements and may lead to divergent, target-specific responses. Questions remain, however, about how inhibitory signals translate to functional immunometabolic signatures of NK cells and how this drives their anti-tumor functions.

Here, we review the current knowledge on the metabolic responses triggered by engagement of NK inhibitory receptors—NKG2A, PD-1, TIM-3, TIGIT, KLRG-1, LAG-3, Interleukin-1 Receptor 8 and CIS—as well as broad tumor microenvironment immunosuppressors—adenosine, hypoxia, TGF-β and lactate—within the context of NK reprogramming events that can be targeted to improve adoptive NK-based transfer immunotherapy.

Metabolism of NK Cells in Cancer

Much of what is known about metabolism in lymphocytes comes from studies of T cells. While NK cells share a number of similarities with $CD8^+$ T cells, their metabolic statuses differ in terms of signaling output, and the lack of insight into innate cell function limits their use as effective cancer immunotherapies. Recently, insights into metabolic responses of NK cells in the TME have started to emerge [6].

NK cell metabolism is regulated by mammalian target of rapamycin (mTOR), particularly its complex mTORC1. mTOR is a protein kinase which responds to glucose availability and, in response, upregulates glycolytic metabolism. Recent evidence has shown that mTORC2, though related to mTORC1, possesses independent and distinct roles in the context of NK cytotoxicity, and is responsible for control of NK development [7]. On NK cells, mTOR is responsible in controlling the production of the effector molecules granzyme B and perforin, which are involved in NK-mediated cytotoxicity. Cytokines, particularly interleukin (IL)-15, but also IL-2, IL-12, IL-18 [8], can strongly activate mTOR. One of the pathways by which IL-15 mediates activation of mTOR involves signaling via phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt), which uses mTOR as a downstream target [9]. On the other hand, metabolic and effector responses of NK cells to other cytokines, such as IL-2 and IL-12, can signal independently to PI3K/Akt. Indeed, IL-2/IL-12-primed NK cells were shown to induce mTORC1 signaling in the absence of Akt activation [10]. These cytokine-stimulated mouse NK cells mediate their metabolic responses via the expression of transcription factor cMyc, which is facilitated by upregulation of the expression of the amino acid transporter SLC7A5 [11]. Alongside cMyc, hypoxia-inducible factor 1α (HIF-1α) has been described as another important glycolytic regulator in various lymphocyte populations such as T cells. Interestingly, IL-2/IL-12-stimulated NK cells were shown to require only cMyc, but not HIF-1α, to drive their metabolic responses and effector functions [10]. Cytokine priming of NK cells drives their glycolytic metabolism often in a dose-dependent manner. High-dose-IL-15-primed NK cells, for instance, upregulate glycolysis after just 20 hours of stimulation, and show enhanced IFN-γ production [12,13]. Extended stimulation with IL-15 renders NK cell metabolic programs highly dependent on mTOR.

Recently, the activity of sterol regulatory element binding protein (Srebp), a transcription factor known to be involved in the regulation of

lipid metabolism and cellular homeostasis, was linked to the control of NK cell glucose metabolism during NK cytotoxic activation. Cytokine-primed NK cells correlated elevated glycolysis and oxidative phosphorylation to the expression of Srebp1 targets *Acly* and *Slc25a1*, implicating the involvement of the citrate-malate shuttle in NK effector function [14]. Citrate shuttled to the cytosol by the *Slc25a1*-encoded mitochondrial citrate transporter during NK activation is converted to acetyl-CoA by ATP-citrate lyase, encoded by *Acly*. Though acetyl-CoA is a precursor for fatty acid metabolism and lipid biosynthesis, its supply through this metabolic circuit is thought to be linked to its use in NK-related histone acetylation reactions, which regulate expression of genes encoding NK cell activating receptors.

Glucose is considered essential in supporting cellular and metabolic functions of NK cells. Evidence suggests that in response to diminishing glucose supplies, a feature common to the TME, NK cells might shift to killer-cell immunoglobulin-like receptor (KIR) dependence in humans in favor of cytokine-primed responses [15]. However, the metabolic reprogramming of failed NK metabolism in a tumor setting is still not fully understood. Further complicating the picture is the knowledge that the two main human NK subsets, $CD56^{dim}$ and $CD56^{bright}$, differ in their effector functions and could be differentially regulating their metabolic responses. $CD56^{bright}$ cells are less cytotoxic, though they are superior activators of mTORC1 and were shown to have higher rates of glycolysis based on *ex vivo* glucose uptake rates compared to $CD56^{dim}$ cells, which have more potent lytic activity. And despite the high responsiveness of NK cells to cytokine stimulation, priming with only IL-2, but not a combination of IL-12/IL-15, rendered NK cells responsive to mTOR inhibition [16]. Such cytokine-stimulated NK cells are capable of producing IFN-γ independently of glycolysis or oxidative phosphorylation. In contrast, when NK cells are stimulated with activating receptors, they require oxidative phosphorylation for IFN-γ production [12]. A caveat of many of these studies is that they are either carried out *ex vivo* or they use mouse NK cells, which differ from human NK subsets in functional, phenotypic and metabolic programs, and therefore preclude analysis in environments mimicking the highly immunosuppressive tumor milieu.

Though, as mentioned, metabolic responses of NK cell function in specific cancer scenarios are less known, insights have started to emerge. Fructose 1,6-bisphosphatase 1 (FBP-1), a key regulating enzyme of gluconeogenesis which inhibits glycolysis, was shown to drive NK cell dysfunction toward complete loss of anti-tumor activity in a *KRAS*-driven model of lung cancer [17]. This was associated with impaired glycolysis and a diminished NK cell viability, suggesting that targeting FBP could be a potentially promising approach in restoring NK cell cytotoxicity in lung cancer. More insights, however, from other cancers still needs to be elucidated.

NK Cell-Specific Inhibitory Checkpoints

NKG2A

Natural killer group 2A (NKG2A)/CD94 is an immunoreceptor tyrosine-based inhibition (ITIM)-bearing inhibitory receptor, which is expressed on approximately half of peripheral blood NK cells [18]. Engagement of NKG2A with its ligand HLA-E on target cells suppresses NK cell cytotoxic signaling events [19] via interaction of the phospho-ITIM domain with Src homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2 [20]. This binding affinity is higher than the affinity between the NK cell activating receptor natural killer group 2C (NKG2C) and HLA-E [21,22]. Expression of NKG2A on NK cells was found to be inducible upon stimulation with either irradiated peripheral blood mononuclear cells or exogenous cytokines, such as IL-2, IL-15 or IL-21 [23–25]. The roles of the reversible expression of NKG2A on cells were further described by Björkström *et al.* [26], who found that loss of NKG2A from NK cells was associated with a terminal differentiation pattern which is accompanied by the sequential acquisition of inhibitory KIRs and the appearance of CD57.

Monalizumab is a recently-developed antibody against NKG2A. Blockade of NKG2A on NK cells with monalizumab was shown to result in significant NK cell-mediated immune response *in vitro* and *in vivo*, both alone and in combination with other agents such as programmed cell death protein 1 (PD-1) or epidermal growth factor receptor (EGFR) blockade antibodies against various tumors [27–29]. Monalizumab is currently in clinical trials. To achieve sustained anti-tumor responses with NKG2A blockade, Kamiya and colleagues developed a protein expression blocker-based construct which encodes expression of NKG2A single-chain variable fragment linked to endoplasmic reticulum-retention domains [30]. NKG2A^{null} NK cells transduced with this construct showed more potent cytotoxicity against HLA-E–expressing tumors *in vivo*.

The metabolic responses of NK cells in the context of NKG2A signaling have also been described. NK cells, educated via NKG2A, were shown to exhibit higher rates of glycolysis compared to uneducated cells, though comparable oxidative phosphorylation rates. NKG2A-educated NK cells expressed, however, lower glucose transporter 1 (Glut1) levels compared to KIR-educated cells, but took up more glucose analog 2-NBDG and remained functionally active following blockade of glycolysis with 2-deoxyglucose in the absence of glucose, unlike uneducated or KIR-educated cells. This suggests that NKG2A-educated cells are endowed with a metabolic behavior supporting adaptation to low glucose. These findings also hint at the involvement of glucose transporters other than Glut1 in the metabolic responses of NKG2A-educated NK cells [31]. High NKG2A expression on NK cells was also associated with functional exhaustion and impaired IFN-γ production [32]. mTOR is required for these cells' functional

responsiveness, while the degranulation capacity of NKG2A$^+$ NK cells was shown to be superior to that of uneducated subsets [33].

TIM-3

T-cell immunoglobulin- and mucin domain-containing (Tim)-3 belongs to the Tim family of receptors. There are three Tims in humans (Tim-1, Tim-3, and Tim-4). Among them, Tim-3 was described as a maturation marker of NK cells based on evidence that it can be induced on NK cells *in vitro* following stimulation with IL-12, IL-15 and IL-18 [34]. Expression of Tim-3 on NK cells was shown to correlate to pathology of a variety of cancers, including lung adenocarcinoma [35] and melanoma [36]. Its ligands include galectin-9, as well as high mobility group box 1 (HMGB1), phosphatidylserine, and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) [37]. Interestingly, in the presence of either soluble galectin-9 or cell targets expressing galectin-9, Tim-3 was shown to induce expression of IFN-γ on NK cells [38], though it also inhibited NK cell degranulation and, in turn, their cytotoxicity [39]. Statins could, at least in models of atherosclerosis, reduce expression of Tim-3 on NK cells [40]. Within the context of cancer, Tim-3 blockade is being explored as an immunotherapeutic approach to rescue NK cell dysfunction caused by overexpression of Tim-3.

It was suggested that Tim-3 could upregulate mTOR, via the upregulation of ribosomal protein S6, downstream of the PI3K/Akt/mTOR pathway [41]. Though the exact mechanisms are not known, this was corroborated by studies showing that Tim-3 expression activates hypoxic signaling and upregulates glycolysis in myeloid leukemia cells [42,43]. More recently, Tim-3 expression was correlated with the generation of short-lived effector T cells in favor of impairment of the establishment of memory T cells [44]. Though the exact effect of Tim-3 on immunometabolic responses of NK cells are still unknown, collectively, these findings implicate Tim-3 to have potentially significant roles in the metabolic control of immune cell maturation and function [45].

PD-1

Expression of PD-1 on immune cells impairs their immune response against various malignancies through interaction with cognate ligands such as programmed death-ligand 1 (PD-L1) on tumor cells. Early studies have shown that PD-1 expression on NK cells from cancer patients is involved in their immune response in multiple myeloma [46]. Recently, interest in better defining the role of PD-1 on NK cells has been reinvigorated. Evidence that blockade of PD-1 triggers a strong antitumor NK cell response was recently given by Hsu *et al.* [47], who ultimately, however, concluded that mechanistically, the exact effect of blocking PD-1 on NK cells is not fully known—the authors suggested that NK cells could either be directly killing tumors or merely recruiting T cells to the tumor niche. NK cells were also recently shown to upregulate PD-1 in

response to regulatory T cell (Treg)-mediated suppression of their effector functions [48], and display impaired lytic granule polarization due to PD-1/PD-L1 engagement [49].

Elsewhere, more nuanced approaches to PD-1 signaling in NK cells were proposed. In one such study, cetuximab-activated NK cells were shown to express elevated PD-1, blockade of which increased NK cell cytotoxicity against PD-L1$^+$ head and neck carcinoma targets [50]. Expression of PD-L1 on tumor cells *in vivo* was shown to be induced by IFN-γ secreted by activated NK cells, thus requiring PD-L1 blockade to be administered alongside adoptively-transferred, IL-21-activated NK cells [51]. This infers that blockade of PD-L1 could be effective, and perhaps required, in settings independent on expression of PD-L1 on patient cancers.

Mechanisms of involvement of PD-1 in the regulation of lymphocyte metabolism have been described for T cells, with much less known on the effects of PD-1 on NK cell metabolism. Recent evidence suggests that PD-1 controls NK cell activation upon chronic stimulation with cells expressing activating ligands to ultimately induce functional NK exhaustion marked by upregulation of PD-1 [52]. Functionally-compromised, PD-1-expressing NK cells may be characterized by altered metabolic states in response to PD-1 signaling. More is known, on the other hand, on the role of PD-1 in controlling T cell metabolism. PD-1 was shown to shift T cell metabolism from glycolysis to autophagy and lipid oxidation by inhibiting PI3K/Akt and activating adenosine monophosphate-activated protein kinase (AMPK) [53], and to regulate T cells' oxygen consumption rates [54]. T cells engaged in PD-1 signaling were shown to be deficient in their ability to undergo glycolysis, glutaminolysis or metabolism of branched-chain amino acids. These cells, however, display enhanced fatty acid oxidation, promoting their survival despite metabolic impairment [55]. Though this suggested that PD-1/PD-L1 engagement promoted a state of T cell exhaustion, these findings have not been confirmed on NK cells and more work is needed to confirm the role of PD-1 on NK cell metabolism.

TIGIT

T cell immunoglobulin and ITIM domain (TIGIT) is an inhibitory receptor expressed on NK cells, but also on CD4$^+$, CD8$^+$ and regulatory T cells. It bears an immunoglobulin tail tyrosine (ITT)-like phosphorylation motif and an ITIM motif in its cytoplasmic tail [56]. Its ligands include poliovirus receptor (PVR or CD155), CD112 and CD96 [57,58], though it also competes with the activating NK receptor DNAM-1 for its ligands. Once bound to its ligand, TIGIT was shown to inhibit human NK cell cytotoxicity by impairing NK cell degranulation [59,60]. It was also described as being required for functional maturation of NK cells [61]. Inhibitory signaling of TIGIT on NK cells was described mechanistically: the ITT-like motif of TIGIT becomes phosphorylated at Tyr225 and binds to cytosolic adapter

growth factor receptor-bound protein 2 (Grb2) after ligand engagement. In turn, this recruits Src homology 2 (SH2)-domain containing inositol polyphosphate 5-phosphatase 1 (SHIP1) to stunt PI3K and mitogen-activated protein kinase (MAPK) signaling [62], triggering NK cell immunosuppression. More recently, Li *et al.* [63] showed that binding of TIGIT to PVR also inhibits IFN-γ production by NK cells. This occurs upon the association of TIGIT with β-arrestin 2 and the subsequent ITT-like motif-mediated recruitment of SHIP1. TIGIT blockade has emerged as a potential supplement to other immunotherapies, such as PD-1 blockade. This was further promoted by recent reports which have indicated that TIGIT blockade is able to reverse functional exhaustion of tumor-infiltrating NK cells induced by TIGIT expression, ultimately leading to suppression of tumor growth alone [64] or in combination with trastuzumab [65]. TIGIT blockade was also shown to abrogate suppression of $TIGIT^+$ NK cells by myeloid-derived suppressor cells (MDSCs) in co-culture experiments with patient-derived MDSCs. Conversely to conventional NK cells, adaptive NK cells, which express lower levels of TIGIT, were found to be resistant to MDSC suppression [66].

The involvement of TIGIT engagement in the suppression of PI3k/Akt/mTOR signaling suggests impaired metabolic activity of tumor-infiltrating NK cells. Moreover, we know that upstream inhibition of PI3K by TIGIT results in inhibition of mTOR [67]. In Tregs, production of TIGIT-controlled fibrinogen-like protein 2 (Fgl-2) was reported as driving the acquisition of tolerance to allografts following treatment of mice with the mTOR inhibitor rapamycin [68], thus suggesting a link between TIGIT and immune metabolic signaling. Elsewhere, He *et al.* [69] showed that engagement of TIGIT with CD155 on gastric cancer cells impaired $CD8^+$ T cell metabolism by downregulating the Akt/mTOR pathway and inhibiting glucose uptake in $CD8^+$ T cells. However, the direct regulation of mTOR and, in turn, metabolic fitness of NK cells due to TIGIT, is still largely unknown.

Interleukin-1 receptor 8

A member of the IL-1 receptor family, interleukin-1 receptor 8 (IL-1R8 or also known as single immunoglobulin IL-1R-related receptor, SIGRR or toll interleukin-1 receptor 8, TIR8), is an inhibitor of Toll-like receptor (TLR)/IL-1R signaling [70]. Structurally, IL-1R8 bears an intracellular signaling, toll-IL-1 resistance (TIR), domain, and Ig-like, leucine-rich repeats in their extracellular portion [71]. Though its roles on NK cells have only begun to be understood, IL-1R8 was earlier shown to be involved in the modulation of a variety of cellular functions, including metabolism and effector functions, of T-helper 17 (Th17) cells by, among others, downregulating mTOR [72].

IL-1R8 was found to be highly expressed on NK cells. Functionally, IL-1R8 acts intracellularly and has roles in responding to IL-37, an anti-inflammatory cytokine that acts as a ligand for IL-1R8 [73]. IL-1R8,

alongside PD-1, is upregulated in response to Treg-mediated suppression of NK cells and the overexpression of its ligand IL-37. Blockade of either PD-1, IL-1R8, or IL-37 was able to reverse Treg suppression of NK cells effector functions [48].

IL-1R8 was recently described as a checkpoint for NK cell maturation and effector functions [74]. Molgora *et al.* [74] found that activating NK receptors NKG2D, DNAX accessory molecule 1 (DNAM-1), Ly49H and Fas ligand (FasL) were upregulated in peripheral blood IL-1R8-deficient NK cells. These cells also produced higher levels of IFN-γ and granzyme B upon *ex-vivo* stimulation with IL-18. IL-1R8-mediated control of NK cell function was dependent on the IL-18-myeloid differentiation primary response 88 (MyD88) pathway. *Il1r8*$^{-/-}$ NK cells also showed enhanced maturation, which could be abolished by IL-18 blockade. In the study, Molgora and colleagues also hinted at a role for IL-1R8 in the control of NK cell metabolism. After treating NK cells from IL-1R8-deficient mice with IL-18 for either 15 or 30 min, the authors observed increased phosphorylation of S6, a downstream target of mTOR, and c-Jun N-terminal kinase (JNK). This led them to speculate that IL-1R8 inhibited the signaling of the mTOR and JNK pathways, implicating impaired NK cell metabolism, differentiation and activation in response to IL-1R8 signaling.

LAG-3

Lymphocyte activation gene-3 (LAG-3 or CD233) is an inhibitory, CD34-related receptor expressed on NK cells, in addition to other immune cells [75–77]. It binds to major histocompatibility complex (MHC) class II molecules via its D1 IgG-like domain and regulates immune cell function [78,79]. Though structurally-related in that they both express four IgG domains, LAG-3 is only 20% homologous to CD4 at the amino acid level [80]. Most of the knowledge on the effects of LAG-3 on immune cell function come from studies on T cells, where it was shown to negatively regulate T cell cytotoxicity upon ligand engagement [81,82]. The knowledge of the exact effects of LAG-3 on NK cells remains incomplete. Early reports showed that LAG-3-deficient mice exhibited impaired ability to kill tumor targets [83], though antibody-mediated blockade of LAG-3 had no measurable effect on NK cell cytotoxicity [75]. Moreover, expression of LAG-3 on NK cells was associated with an exhausted NK state [84] and was powerfully induced by stimulation with IL-12 [85]. Cytokine-induced memory-like KIR^{+} NKG2C^{+} NK cells were also shown to express elevated LAG-3 [86], largely in response to stimulation by IL-12 and, to a lesser extent, IL-18. Recent evidence has indicated that LAG-3 is highly expressed on NK cells infiltrating renal cell carcinomas [87]. However, no correlation between the expression of LAG-3 on tumor-infiltrating and peripheral blood NK cells could be established. Though therapeutic antibodies against LAG-3 are being evaluated in combination with antibody-mediated blockade of PD-1 in the context of T cell immunotherapy [88,89], more

work is required to understand the effects of LAG-3 on NK cell anti-tumor immunity.

Metabolically, little is known about the effects of LAG-3 on NK cells. A recent study by our lab showed that mRNA expression of *LAG3* was upregulated on IL-12 and IL-15-co-stimulated human NK cells in the presence of the metabolite adenosine, which also inhibited NK cell glycolysis and cytotoxicity [90]. Most of the knowledge on the immunometabolic effects of LAG-3, however, comes from studies on T lymphocytes. LAG-3 was shown to modulate bioenergetics of T cells. Specifically, expression of LAG-3 on $CD4^+$ T cells inhibited mitochondrial biogenesis and metabolism [91]. LAG-3-deficient T cells were also more glycolytically active than wild-type T cells. Furthermore, LAG-3 expression was also correlated to terminal exhaustion of tumor-infiltrating T cells, as well as elevated chronic Akt signaling [92]. LAG-3 may be playing additional roles in regulating immune cell metabolism that have yet to be elucidated. It is also possible that the metabolic effects of LAG-3 on NK cells differ to those that the receptor exerts on T cells due to its inherently different functional roles on both cell types.

KLRG-1

Inhibitory Killer cell lectin-like receptor G1 (KLRG1) is a transmembrane protein highly expressed on $CD56^+$ NK cells, though preferentially the $CD56^{dim}$ subset [93,94]. It binds to E-, N- and R-cadherins on target cells. Its binding to E-cadherin was shown to only occur when E-cadherin is in its monomeric form, a characteristic of impaired or diseased cells [95]. KLRG-1 has long been identified as an inhibitory receptor of NK cell function [96], and a marker of terminal differentiation of NK cells [97]. Its expression was also shown to be correlated to impaired proliferative capacity and responsiveness to IL-15, lower IFN-γ production and increased apoptosis of NK cells [93,98]. In addition to suppressing NK cell cytotoxicity, KLRG-1 was suggested to regulate NK cell homeostasis [99]. Loss of KLRG-1 did not, however, affect NK cell development [100]. Immunotherapy with anti-KLRG-1 antibodies has demonstrated efficacy in inhibiting metastases in a murine 4T1 breast cancer model in combination with anti-PD-1 blockade [101]. KLRG-1 was identified to be involved in mediating cytotoxicity of NK cells via E-cadherin signaling against A549 cells undergoing epithelial-to-mesenchymal (EMT) transition, a process that leads to the downregulation of E-cadherin expression [102]. While siRNA silencing of KLRG-1 on NK cells enhanced the cytotoxicity of NK cells against A549 EMT and non-EMT tumor targets, inhibition of E-cadherin/KLRG-1 ligation did not fully restore NK cytotoxicity against A549 cells undergoing EMT, suggesting the involvement of an activating molecule. This was identified to be cell adhesion molecule 1 (CADM1), an activating ligand for cytotoxic and regulatory T cell–associated molecule (CRTAM), a receptor expressed on NK cells. The authors showed that CADM1-KO A549 cells displayed

significantly reduced EMT-induced susceptibility to killing by NK cells *in vitro* and increased tumor metastasis *in vivo*.

Recent work has shown that KLRG-1 activation has a likely role in the metabolic control of NK cell function. Müller-Durovic and colleagues [103] showed that KLRG1 internalizes upon ligation and significantly reduces protein phosphatase 2C (PP2C) binding to AMPK in human NK cells. This points to the fact that suppressed cytotoxic function of NK cells due to KLRG-1 may be linked to an altered metabolic state due to impaired metabolism. In another study, disruption of mTORC1 was found to result in reduction of KLRG-1-expressing NK cells in multiple organs [7]. Wang *et al.* [104] found that mice deficient in mothers against decapentaplegic homolog 4 (SMAD4), a protein which mediates signaling of transforming growth factor-β (TGF-β), had a significantly lower percentage of KLRG-1-expressing NK cells compared to $SMAD4^{+}$ mice. While this indicates that SMAD4 affects the maturation of NK cells, it also hints at impaired metabolism due to the regulation of NK metabolic functions by TGF-β.

CIS

Cytokine-inducible SH2-containing protein (CIS), which is encoded by the *CISH* gene, has emerged as a potent inhibitory checkpoint of NK cell function. Delconte *et al.* [105] have shown that deletion of *CISH*, which is induced by IL-15, led to enhanced NK cell cytotoxicity and IFN-γ production, and rendered $CISH^{-/-}$ mice resistant to metastasis of a number of solid tumors, including melanoma, prostate and breast cancer. *CISH* was also identified as a potentially potent inhibitor of NK cell function in ovarian cancer [106], while combination of *CISH* targeting with other checkpoint blockade or cytokine immunotherapies further ablated cancer metastasis [107].

Though induction of *CISH* appears related to that of mTOR, Delconte and colleagues found that IL-15-stimulated $CISH^{-/-}$ NK cells did bring about an increase in the phosphorylation of Akt, a key event in the mTOR activation cascade. This was confirmed by the fact that $CISH^{-/-}$ NK cells recorded regular mitochondrial respiration and glycolysis. The limited work on CIS/*CISH* and NK cells infers that more needs to be uncovered about its roles in the control of NK function and metabolism.

Global Tumor Microenvironment Immunosuppressors

Adenosine

Adenosine is a purine metabolite present in high concentration in the extracellular space of the microenvironment of many solid tumors [108,109]. We and others have shown that adenosine acts by altering a variety of NK cell functions, including activating receptor expression and cytotoxic functions against tumor targets, ultimately debilitating their anti-tumor effectiveness [86,110,111].

Driving the accumulation of adenosine is ecto-5'-nucleotidase (CD73), an ectoenzyme overexpressed on various tumors [112]. It does so alongside ectonucleoside triphosphate diphohydrolase-1 (CD39), which catalyzes the conversion of adenosine triphosphate (ATP), produced by fast-proliferating cancer cells, to adenosine monophosphate (AMP). CD73, in turn, catalyzes the dephosphorylation of extracellular AMP to adenosine [113]. Adenosine accumulation by CD73 in the TME causes profound immunosuppression by binding the purinergic adenosine receptors on infiltrating NK cells. Though NK cells possess four G protein-coupled adenosine receptors, it is thought that the A_{2A} receptor is the one most involved in the inhibitory signaling induced by adenosine. In turn, it is the receptor that has been most commonly studied. Blocking various components of the adenosinergic signaling cascade—such as CD39, CD73 or the A_{2A} receptor—is being evaluated within the context of immunotherapy of solid tumors. It is thought that the most sustained anti-tumor responses may come from combination therapy with other agents, such as PD-1 blockade. Co-blockade of the A_{2A} receptor and CD73 was shown to lead to significantly improved anti-tumor effects by inhibiting tumor initiation, growth and metastasis, and promote the recruitment and migration of NK cells to the tumor niche [113]. Reducing adenosine accumulation by blocking CD73 activity on cancer cells was also shown to enhance immunotherapy of engineered chimeric antigen receptor-NK cells against $CD73^+$ solid tumors *in vivo* [114].

Adenosine was reported as having profound effects on NK cell metabolism. Primary human NK cells primed with cytokines and exposed to adenosine were shown to be sensitive to mTOR signaling and exhibit impaired IL-15 signaling. Inhibition of the activity of adenosine deaminase by small molecule inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) led to a reduction in target cell cytolysis mediated by IL-12/IL-15-primed NK cells. Transcriptional gene expression analysis of IL-12- and IL-15-primed NK cells in the presence of adenosine showed downregulation of *ADCY3*, which encodes adenylyl cyclase 3, an enzyme which catalyzes the formation of cyclic adenosine monophosphate (cAMP). Glycolytic genes *GAPDH* and *LDHA* were also downregulated due to adenosine, suggesting altered cellular metabolism. This was confirmed by metabolic flux on a Seahorse XFP analyzer, which showed inhibited glycolysis and altered mitochondrial respiration in response to adenosine [86].

TGF-β

TGF-β is an important immunosuppressive cytokine that negatively regulates NK cell development and function [115,116]. It is produced by cancer cells, myeloid-derived suppressor cells and other stromal cells in the TME. Levels of TGF-β were measured to be many times higher (as high as 45 ng/mL in liver metastases) in the blood of cancer patients compared to that of healthy volunteers, which were measured to have less

than 1 ng/mL TGF-β [117,118]. The elevated expression of TGF-β in the blood of cancer patients was inversely correlated with the expression of activating receptor NKG2D on NK cells [119,120]. TGF-β also suppresses CD16-mediated production of IFN-γ and the engagement of ADCC by cytokine-stimulated NK cells via SMAD3 activation [121]. Inhibiting TGF-β signaling has thus emerged as a promising and potentially highly effective approach to enhance NK cell cytotoxicity in the context of adoptive transfer immunotherapy [122]. Various approaches at blocking TGF-β are being evaluated, including TGF-β neutralizing antibodies [123], small molecule inhibitors of TGF-β receptors [124], soluble TGF-β receptor traps and blockers [125,126], and fusion proteins of TGF-β receptor traps and other checkpoint inhibitors such as PD-1 [127,128].

NK cells engineered with a dominant negative TGF-β receptor II (DNRII) mediated enhanced killing of glioblastoma tumor targets and maintained expression of NKG2D and DNAM-1 in the presence of TGF-β [129]. In another study, galunisertib (LY2157299), a clinical TGF-β receptor I inhibitor, rescued NK cell dysfunction in models of leukemia and colon cancer *in vivo* by maintaining the expression of NKG2A and CD16 on NK cells and delaying tumor growth [130].

TGF-β was shown to profoundly affect NK cell metabolism. Viel *et al.* [13] found that TGF-β can inhibit the activity of mTOR in IL-5-stimulated NK cells. The deletion of mTOR, or its constitutive signaling independent of cytokine priming, severely impaired both the development and the differentiation of NK cells *in vivo*. In another study, Zaiatz-Bittencourt *et al.* [131] showed that TGF-β impaired oxidative phosphorylation, glycolytic and respiratory capacity and expression of the transferrin receptor CD71 by human NK cells. This occurs via mechanisms that do not involve mTORC1 and likely implicate canonical TGF-β signaling.

Hypoxia

Oxygen availability is a key driver of immune cell proliferation and function. Most solid tumors are characterized by regions of hypoxia due to poor vascularization and disrupted blood supply [132]. Hypoxia results in dysfunction of surrounding immune cells, including NK cells [133]. The responses of NK cells to hypoxic environments have been discussed previously, yet despite the available knowledge, the specific role of hypoxia on NK cells is still not fully clear. Hypoxia was shown to affect functions of NK cells by impairing their cytotoxicity [134–136] and altering their metabolic signatures [137]. Expression of activating receptors NKp46, NKp30, NKp44, and NKG2D [138] as well as perforin and granzyme B [138] are all downregulated on NK cells in hypoxia. The negative effects of hypoxia on NK cells could be partially restored by treatment with IL-2, which was able to increase expression of NKG2D and restore cytotoxicity against multiple myeloma [139]. Reports have indicated that NK cell responses to hypoxia are shaped by the specific hypoxic conditions—such as degree and duration of hypoxic conditioning—as well as by the

presence (or absence) of cytokines. For instance, Velásquez *et al.* [140] showed that conditioning NK cells in 1% oxygen for 22 h in the presence of IL-15 did not alter glycolytic flux and glucose consumption by NK cells, but it did augment glycolytic gene expression. Moreover, these hypoxia-cultured NK cells lead to no appreciable difference in the numbers of viable K562 cells following a killing assay compared to NK cells under normoxia. Similarly, Mahaweni *et al.* [141] reported that only when combined with lactate did hypoxia (0.6%) show impaired killing of cancer targets *in vitro*. Collectively, our understanding of the exact effects of hypoxia on NK cell function and metabolism remains inconclusive.

Immune cells respond to hypoxia via a family of hypoxia-inducible transcription factors (HIFs)—HIF-1, HIF-2 and HIF-3 [142], which enable cellular adaptation to low oxygen and of which HIF-1α and HIF-2α are most commonly used by NK cells. Tumor-infiltrating NK cells are characterized by upregulated expression of HIF1-α. Mouse models with HIF1-α-deficient $NKp46^+$ NK cells show reduced tumor growth and enhanced production of IFN-γ by NK cells [143]. These cells utilize oxidative phosphorylation in preference to glycolysis for energy metabolism. HIF-1α was recently shown to also affect angiogenesis, by inhibiting the intratumoral infiltration of vascular endothelial growth factor receptor (VEGFR)-expressing NK cells [144]. As a result, targeting HIF-1α has emerged as a potentially relevant immune checkpoint target [143,144], though more work on the understanding of the complex roles of HIFs on NK cells are needed.

Lactate

Elevated expression of lactate dehydrogenase A (LDHA) on cancer cells has been correlated with poor prognosis in tumor patients [145]. LDHA is involved in the conversion of excess pyruvate and NADH into lactate and NAD^+, which in turn fuel ATP generation via glycolysis. As a result, the TME is characterized by the accumulation of lactate and protons (to generate lactic acid). Elevated lactate concentrations have been associated with tumor progression, metastasis and recurrence [146], while inhibition of LDHA was shown to impair tumorigenesis and tumor progression in mouse models of various cancers [147,148].

Brand and colleagues [149] recently reported that lactic acid severely impairs NK cell activation and function. Using immunocompetent C57BL/6 mice, they showed that low lactic acid-producing tumors developed significantly slower and were characterized by increased infiltration of NK cells. On the other hand, lactic acid concentrations above 20 mM caused NK cell apoptosis. Intracellular acidification associated with the uptake of lactic acid by NK cells was shown to interfere with the regulation of NFAT and, in turn, expression of IFN-γ. Elsewhere, Harmon *et al.* [150] reported that acidification due to lactate production induced apoptosis of liver-resident NK cells in colorectal liver metastasis tumors. In the study, lactate accumulation brought about a reduction of the pH in the TME. The

acidic environment resulted in impairment of the NK cells' ability to regulate the acidic intracellular pH, resulting in mitochondrial stress and apoptosis. Impaired IFN-γ production due to lactate-induced acidosis was also shown to lead to loss of NK cell functionality and IFN-γ production in a model of B-cell lymphoma [151]. Though IFN-γ production could be restored by transferring NK cells to a normal, non-acidic environment, NK cell cytotoxicity remained impaired even upon change of environment. Reversal of acidification by inhibition of monocarboxylate transporter-4, which is involved in lactate transport and efflux, also resulted in restoration of NK cell cytotoxicity, elevated expression of activating marker NKG2D, improved intratumoral infiltration of NK cells and attenuation of breast carcinoma tumor growth *in vivo* [152].

Strategies to Target Immunometabolic Reprogramming of NK Cells in Cancer Immunotherapy

As evidenced by multiple studies, the TME induces profound immunometabolic reprogramming of NK cells. Some of these effects are due to global immunosuppressive cues, such as hypoxia, adenosine, lactate or TGF-β, while others signal on specific NK cell inhibitory receptors which act as checkpoints to their anti-tumor immunity (Table 1).

Targeted blockade of checkpoint inhibitors is a viable, and seemingly obvious, strategy currently being evaluated clinically. Remarkable anti-tumor responses have been observed with checkpoint inhibitor immunotherapy. Understanding which metabolic pathways are particularly involved in the engagement of NK cell inhibitory receptors could inform optimal co-treatments that could, at once, enable anti-tumor cytotoxicity as well as rescue impaired NK cell metabolism. As an alternative to checkpoint blockade, engineering of NK cells to express ligands against inhibitory receptors on cancer cells is an emerging approach that can redirect inhibitory cues into stimulatory signals [153]. The attractiveness of genetic engineering also stems from the fact that it can be combined to include silencing and/or engineering of specifically-targeted metabolic response elements within a "single agent" formulation. For NK cells to effectively target solid tumors, the involvement of chemokines will likely be required in order to promote trafficking of cells to solid tumor niches [154].

Because NK cells are profound responders to cytokine stimulation, it is no surprise that cytokines are being explored as a way to enhanced NK cell anti-tumor function. Cytokines have been shown to strongly affect NK cell metabolism [155]. Among them, IL-15 is the most widely studied [156], though combinations of IL-12, IL-15 and IL-18 [157]. or IL-15, IL-18 and IL-21 [158] were shown to result in highly active NK cells *in vivo*. Though is it largely thought that cytokines activate NK cell metabolism, evidence shows that prolonged stimulation of NK cells with IL-15 leads to metabolic deficiency, ultimately impairing the cells' cytotoxicity [159].

Table 1. Effects of tumor microenvironment inhibitory checkpoints on NK cell function and metabolism.

Checkpoint	Effect on cell metabolism and function	Refs.
NK Cell-associated		
NKG2A	NKG2A-educated cells have lower Glut1 expression compared to KIR-educated cells; are mTOR-dependent and have higher glycolysis compared to uneducated cells; high NKG2A expression correlates to exhaustion and impaired IFN-γ production	[31–33]
TIGIT	Inhibits NK cell degranulation; inhibits mTOR; impairs glycolysis in $CD8^+$ T cells	[59–61,67]
TIM-3	Inhibits NK cell degranulation; upregulates mTOR; enhances glycolysis in myeloid leukemia cells	[39,41,42]
KLRG-1	Inhibits NK cytotoxicity, regulates homeostasis; is dependent on mTORC1 signaling	[7,93,98]
Interleukin-1 Receptor 8	Promotes Treg-mediated suppression of NK cells; inhibits NK cell maturation and expression of activating receptors; impairs mTOR signaling	[48,71]
LAG-3	LAG-3 expression defines an exhausted NK state; might impair cytotoxicity; expression is induced by cytokines (IL-12, IL-18). On T cells, it inhibits mitochondrial biogenesis and metabolism.	[83,84,91]
CIS	*CISH*$^-$ NK cells show impaired cytotoxicity but normal glycolysis and mitochondrial respiration; activation is related to Mtor	[106]
PD-1	Impairs lytic granule polarization; likely contributes to impaired NK anti-tumor immunity; in T cells PD-1 blocks glycolysis and glutaminolysis	[47,49,54]
Tumor microenvironment		
Adenosine	Impairs cytotoxicity, glycolysis and alters mitochondrial respiration	[86]
TGF-β	Inhibits mTOR in NK cells; impairs oxidative phosphorylation, glycolytic capacity and capacity and expression of CD71	[13,131]
Hypoxia	Impairs function and metabolism of NK cells; effects of NK cells are condition specific and somewhat inconclusive	[137,140]
Lactate	Impairs NK cell activation, function and IFN-γ production	[149–151]

Despite the growing body of work that continues to uncover new mechanisms which tumors use to evade NK cell-mediated recognition and killing our understanding of NK cell metabolism in pathological settings lacks significant knowledge. For example, many of the studies on NK cell metabolism are carried out in *ex vivo* environments not representative of TME conditions, precluding accurate mechanistic insights to be elucidated. While the use of murine NK cells is common and beneficial, it also does

not fully reflect the complexity of human NK subsets. Similarly, emerging insights into novel pathways which regulate NK cell metabolic responses remain to be validated. For example, if, as recent reports suggest, Srebp directly regulates NK effector function by regulating glucose metabolism independent of lipid synthesis, are non-functional NK cells deficient in Srebp activity? Another unknown question is whether licensed NK cells display altered metabolic responses compared to unlicensed cells, and whether altered NK phenotypes associated with *in vivo* NK exhaustion correlate to altered metabolic programs. Moreover, the role of mTOR-mediated regulation of these processes in specific cancers is entirely unknown, as are NK cell effector functions.

It is clear then, that despite the remarkable progress in understanding that metabolism drives functional behavior of NK cells in tumors settings, significantly more work is needed in understanding how these basic discoveries can be translated into potential cures.

CONCLUSIONS

The interactions between NK cells and tumors are varied and complex. Though inhibitory NK receptors transmit signals which impair NK cell anti-tumor immunity, increasing evidence implicates their engagement to be linked to distinct metabolic responses on NK cells. Our discussion has shown, however, that many of those responses are not known. In order to advance NK cells as effective adoptive immunotherapies of solid tumors, it is imperative to understand their metabolic signatures in the context of TME-induced inhibition, particularly with regard to functional inhibitory markers unique to these cells and their distinct phenotypic and functional subsets.

AUTHOR CONTRIBUTIONS

T.N.D. and S.M. performed literature review and wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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