

Adaptation of Natural Killer Cells to Hypoxia: A Review of the Transcriptional, Translational, and Metabolic Processes

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Abstract: As important innate immune cells, natural killer (NK) cells play an essential role in resisting pathogen invasion and eliminating transformed cells. However, the hypoxic microenvironment caused by disease conditions is an important physicochemical factor that impairs NK cell function. With the increasing prominence of NK cells in immunotherapy, there has been a surge of interest in developing biological means through which NK cells may overcome the inhibition caused by hypoxia in disease conditions. Although the effects of hypoxic conditions in shaping the functions of NK cells have been increasingly recognized and investigated, reviews have been scanty. A comprehensive understanding of how NK cells adapt to hypoxia can provide valuable insights into how the functional capacity of NK cells may be restored. This review focuses on the functional alterations of NK cells in response to hypoxia. It delineates the mechanisms by which NK cells adapt to hypoxia at the transcriptional, metabolic, translational levels. Furthermore, given the complexity of the hypoxic microenvironment, we also elucidated the effects of key hypoxic metabolites on NK cells. Finally, this review discusses the current clinical therapies derived from targeting hypoxic NK cells. The study of NK cell adaptation to hypoxia has yielded new insights into immunotherapy. These insights may lead to development of novel strategies to improve the treatment of infectious diseases and cancer.

Keywords: NK cells, hypoxia, immunotherapy, innate immunity, metabolism

Introduction

Natural killer (NK) cells are recognized as indispensable and important innate immune cells.^{1,2} NK cells develop from bone marrow progenitor cells and are distributed throughout the body in liver, bone marrow and blood.^{3–8} The number of NK cells has been estimated to be 2×10^6 , comprising 1% of the total immune cell population, 2% of the lymphocyte population, and 10% of the peripheral lymphocyte population.⁹ As a family of innate lymphocyte cells (ILCs), NK cells are equipped with a repertoire of inhibitory and activating receptors.³ The combined action of these two types of receptors can selectively defend against infected and transformed cells while sparing normal cells.^{3–8} The sustained maintenance and enhancement of NK cell recognition and functionality have emerged as pivotal to NK cell-based immunotherapies over the past five years.³

However, hypoxia challenges the maintenance of normal NK cell function in various diseases. NK cells exert their functions through the combined action of surface germline-encoded activating and inhibitory receptors.¹⁰ The expression of NK cell surface receptors makes them susceptible to hypoxia.¹¹ The equilibrium between NK cell receptor activation

and inhibition is disrupted in the hypoxic environment, resulting in a series of hypoxia-induced adaptation in NK cells. The predominant trend is the downregulation of activating receptors, with minimal effects observed on inhibitory receptors (Box 1).¹¹ This imbalance between activating and inhibitory receptors ultimately results in impaired cytotoxicity and cytokine secretion of NK cells in hypoxic conditions.¹² The expression of perforin and granzyme in NK cells has been reported to decrease in hypoxia conditions.¹³ In experiments involving co-cultures of NK cell and tumors, the cytotoxic effect of NK cells on tumor cells was found to be significantly impaired under hypoxic conditions.¹⁴ In addition to cytotoxicity, the capacity of NK cells to secrete proinflammatory cytokines (eg, IFN γ and TNF α) and chemokines (eg, GM-CSF, CCL3, CCL5) is also impaired under hypoxic conditions (Figure 1A).¹⁵

A multitude of factors contribute to the reduction of NK cell function in hypoxia (Figure 1B). Many researchers hypothesize that this is a self-protective mechanism of NK cells after oxygen deprivation, whereby energy-expensive processes, including the production and secretion of cytokines, perforin and granzyme, are curtailed.²¹ Although the effects of hypoxic conditions in shaping the functions of NK cells have been increasingly recognized and investigated, reviews have been scanty, and have largely focused on the tumor microenvironment.^{22,23} This review provides a comprehensive analysis of the mechanisms by which NK cells adapt to hypoxia. It presents a summary of the relevant transcriptional, translational, and metabolic processes of hypoxic NK cells. Ultimately, it suggests a basis for the pathophysiological mechanisms that may be employed to overcome NK cell inhibition under hypoxic conditions. The methodology of the literature search is partially delineated in Box 2.

Transcriptional Adaptation to Hypoxia

HIF-1 Mediates NK Cell Adaptation Under Hypoxia

Hypoxia-inducible factors (HIF) are heterodimeric transcription factors that are stabilized under conditions of hypoxia.²⁴ The level of HIF-1 α is regulated by oxygen levels, whereas HIF-1 β is constitutively expressed in cells.²⁵ Under normoxia, prolyl

Box 1 Imbalanced Receptors of NK Cells Under Hypoxia

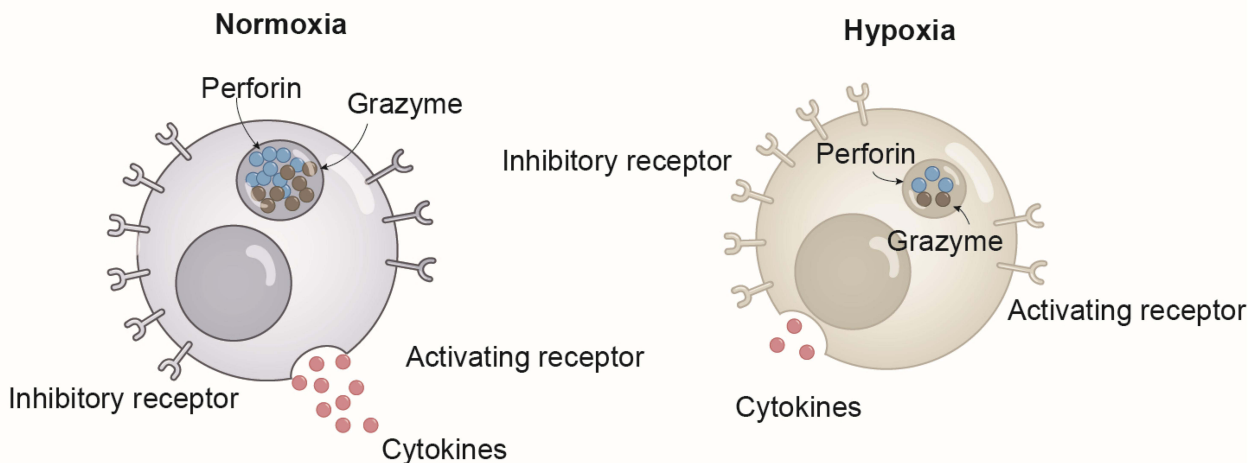
NK cell surface receptors are classified into two categories: activating receptors and inhibitory receptors.¹⁶ A multitude of activating receptors of NK cells are founded. Although diverse, activated receptors share similar biochemically signaling pathways.¹⁶ This section will focus on activating receptors that activate two biochemically distinct signaling pathways, include immunoreceptor tyrosine-based activating motif (ITAM) bearing NK receptor complexes and the DAP10 associated NKG2D receptor complexes. Inhibitory NK receptors contains the killer cell immunoglobulin-like receptors¹⁷ and the leukocyte immunoglobulin-like receptors (LIRs) and the CD94-NKG2A heterodimers in humans and mouse.¹⁸ NK cells exert their functions through the combined action of surface germline- encoded activating and inhibitory receptors.¹⁸ The coordinated action of these receptors enables NK cells to function in an exquisite balance, maintaining their ability to kill tumor- transformed and infected cells while preventing cytotoxic effects on their own cells.¹⁸

Natural cytotoxicity receptors (NCRs) belong ITAM-bearing NK activating receptors, which contains NKp30, NKp44, and NKp46.¹⁰ These receptors recognize corresponding tumor- transformed and pathogen-infected cell surface ligands, thereby triggering cytotoxicity.¹⁶ The expression of the NCRs is impaired under hypoxic conditions. In NK cells co-cultured with IL-2 at 1% oxygen concentration for 96 hours, the expression of both NCRs and NKG2D on the surface of NK cells was significantly decreased.¹¹

As with NCRs, another NK cell activating receptor, NKG2D, exhibits decreased expression under hypoxic conditions.¹¹ NKG2D is a specific type of NK activating receptor that is related to the DAP10.¹⁹ It is capable of recognizing ligands that are distant relatives of MHC class I proteins.¹⁸ Activated NKG2D will cause downstream activation signaling by activating the ITAM structural domain of the junction protein DAP10.¹⁹ Specifically, DAP10 also contains distal motifs (YINM) that can recruit PI3K and promote NK cell activation.¹⁹ Nevertheless, the absence of NKG2D due to hypoxia renders NK cells incapable of receiving this potent activation signal.

Another special activating receptor, CD16, which mediates the antibody-dependent cell- mediated cytotoxicity (ADCC) of NK cells, is down-regulated in expression under extreme hypoxia.¹¹ CD16 is capable of recognizing the Fc segment of antibody and subsequently mediating the cleavage of antibody-bound target cells by NK cells.²⁰ The decline in CD16 appeared to correlate with the degree of hypoxia, with a non-significant decline in CD16 at 1% oxygen concentration and a significant impairment of CD16 expression on NK cells with a plunge to 0% oxygen concentration.¹¹

Intriguingly, the effect of hypoxia on inhibitory receptors was not significant. Hypoxia- specific inhibition of NK cell activating receptors results in a disruption of the equilibrium between NK cell activating and inhibitory signaling. In hypoxia conditions, the disruption of this equilibrium is inextricably linked to a decrease in the cytotoxicity and IFN- γ secretion of NK cells.

A

The effect of hypoxia on NK cells

No effect on inhibitory receptors

Reduced activating receptors, including NKp46, NKp33, NKp44, NKG2D, CD16.

Reduced cytokines and chemokines secretion, including IFN γ , TNF α , GM-CSF, CCL3, CCL5...

Reduced granzyme and perforin secretion.

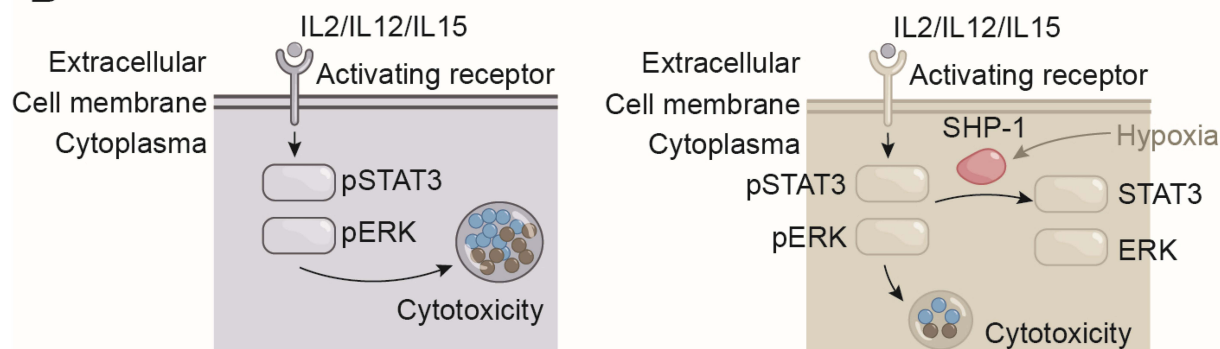
B

Figure 1 Effects of hypoxia on NK cells. **(A)** The expression of NK cell activating receptors is decreased under hypoxic conditions, whereas inhibitory receptors remain unaffected. Inhibition of activating receptors is associated with a reduction in the secretion of cytokines and chemokines, as well as impaired cytotoxicity in hypoxic NK cells. **(B)** Hypoxia impairs the cytotoxicity of NK cells by inducing the expression of SHP-1, which dephosphorylates STAT3 and ERK.

Abbreviations: IFN γ , Interferon gamma; TNF α , Tumor necrosis factor alpha; GM-CSF, Granulocyte-macrophage colony-stimulating factor; CCL, Chemokine (C-C motif) ligands; STAT3, Signal transducer and activator of transcription 3; ERK, Extracellular signal-regulated kinase; SHP-1, Src homology region 2 domain-containing phosphatase-1; IL, Interleukin.

hydroxylase (PHD) hydroxylates the proline residues (P402 and P564) in the HIF-1 α , in conjunction with oxygen (O $_2$), iron (Fe $^{2+}$) and α -ketoglutarate.²⁶ Hydroxylated HIF-1 α is recognized and polyubiquitylated by the pVHL–elongin BC–CUL2 complex (referred to as the VHL complex), which then targeted it for proteasomal degradation.²⁷ In the context of hypoxia, the process of hydroxylation mediated by PHD is inhibited, resulting in the failure of HIF-1 α to undergo degradation.²⁶ Stabilized HIF-1 α binds to HIF-1 β to form the heterodimer HIF-1. HIF-1 translocates into the nucleus and binds to hypoxia-responsive elements (HRE), thereby initiating transcription of a range of genes, including those involved in cell survival, angiogenesis, glycolysis, and invasion/metastasis (Figure 2A).²¹ In NK cells, the stabilization of HIF-1 α is not solely contingent on the dysfunction of PHD due to hypoxia. It is also dependent on cytokine induced HIF-1 α expression.^{28,29} The IL-2 and IL-15

Box 2 The Methodology of the Literature Search

This review encompasses all literature related to NK cells and hypoxia until May 2024. The search was conducted using the PubMed and Google Scholar engines. The search keywords included “NK cells and hypoxia”, “hypoxia and immune cells”, “oxygen therapy”, “transcription factors and NK cells” “translation and NK cells”, “immunometabolism and NK cells”, “metabolite and NK cells”. After examining the abstracts of each keyword in isolation, the articles were filtered to identify those related to NK cells and hypoxia. A total of 185 articles were identified as relevant. These articles were then reviewed individually to identify the logical relationships between them. The 187 articles were then rearranged and organized into this review. The selection of articles was guided by specific inclusion criteria, meticulously designed to identify relevant literature pertaining to NK cells and hypoxia. Inclusion criteria included: articles in English, Reviews and Articles related to NK cells and hypoxia. We will focus on reviewing literature that fits the framework and include it in the review. Literature that does not fit the overall framework will also be organized and included in Box. During the screening of articles, the authors primarily screened by reading the title and abstract. The exclusion criteria for articles included the following: articles not related to either NK cells or hypoxia, articles that were not subject to peer review, preprint articles, non-English articles, and duplicate articles.

signaling pathways promote the translation of HIF-1 α through the phosphoinositide 3-kinases / mechanistic target of rapamycin complex 1 (PI3K/mTORC1) signaling pathway.^{28,29} Cytokines initiate the synthesis of HIF-1 α , whereas hypoxia stabilizes the HIF-1 α protein, ultimately allowing for the accumulation of HIF-1 α in NK cells.

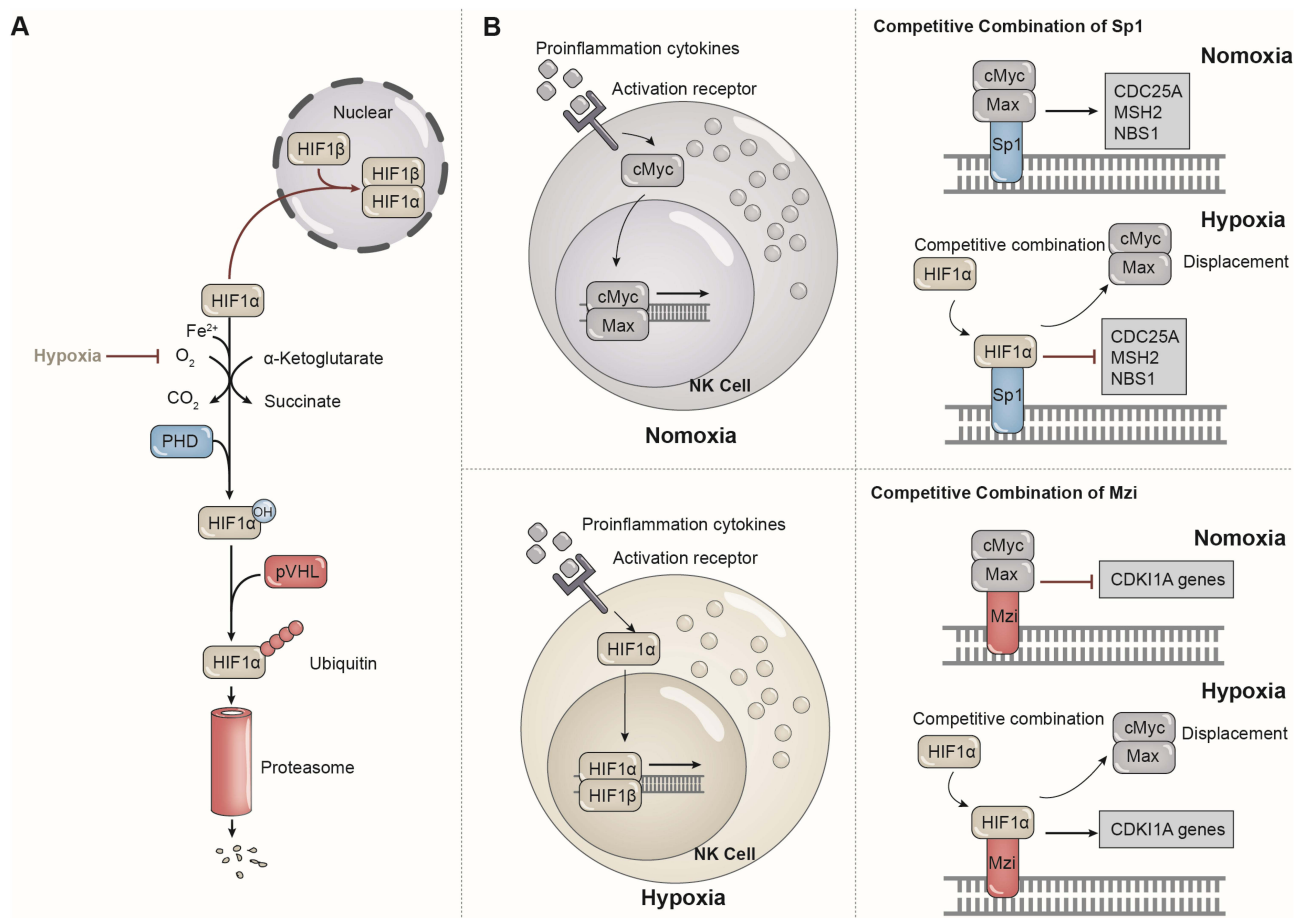


Figure 2 Transcriptional alterations in NK cells under hypoxic conditions. **(A)** PHD hydroxylates HIF-1 α in the presence of oxygen, iron ions, and α -ketoglutarate. Hydroxylated HIF-1 α is polyubiquitinated by pVHL. Ubiquitinated HIF-1 α is degraded by the proteasome. In contrast, under hypoxic conditions, the deprivation of oxygen results in the inhibition of the hydroxylation of HIF-1 α . HIF-1 α is stabilized and translocates to the nucleus, where it forms a heterodimer with HIF-1 β . **(B)** Under normoxia, NK cells promote the expression of cMyc and HIF-1 α following cytokine activation. However, because HIF-1 α is degraded by the proteasome, NK cells effector functions, primarily through cMyc. Conversely, under hypoxic conditions, stabilized HIF-1 α competes with cMyc for binding to target genes. Specifically, in the context of normoxia, the cMyc/Max complex binds to Sp1, thereby promoting the expression of CDC25A, MSH2, and NBS1. In hypoxic conditions, HIF-1 α competitively binds to Sp1, thereby inhibiting the expression of these genes. The cMyc/Max complex is able to repress transcription of the CDK1A gene via Mzi. Nevertheless, this inhibition can be overcome by the competitive binding of HIF-1 α to Mzi. **Abbreviations:** HIF, Hypoxia inducible factor; PHD, Prolyl hydroxylases; pVHL, Hippel-Lindau tumor suppressor protein. CDC25, Cell division cycle 25; MSH2, MutS Homolog 2; NBS1, Nibrin; CDK1A, Cyclin-dependent kinase inhibitor gene.

cMyc Mediate NK Cell Function Under Normoxia

Among the numerous transcription factors that influence NK cells, cMyc is the most intricately and closely associated with HIF-1. The two transcription factors share common target genes. cMyc is an important transcription factor whose function in normoxia is very similar to that of HIF-1 in hypoxia. Both transcription factors ultimately modulate NK cell function by regulating NK cell metabolism and protein synthesis.³⁰ The cMyc to HIF1 α transition reprograms NK cell metabolism, protein synthesis, and cell cycle progression at the transcriptional level, thereby fine-tuning the adaptive response of NK cells to hypoxic environments.³¹

cMyc is a pivotal transcription factor that plays a crucial role in regulating a multitude of cellular processes, including cell cycle progression, cell proliferation, apoptosis, cell metabolism, and cell transformation.^{32,33} cMyc inhibits the expression of genes that suppress cell proliferation, thereby facilitating the proliferation of cells.^{34,35} For example, cyclin-dependent kinase inhibitor gene (CDKN1A), as a pivotal cell cycle inhibitory gene, encodes the expression of cell-cycle inhibitor p21.^{cip1} CDKN1A is repressed by cMyc through the formation of a ternary repressive complex with Max and Mzi, which binds to the initiator element (INR) of the growth-suppressor gene.^{34,35} In addition, cMyc plays a role in promoting cell proliferation by increasing the expression of genes involved in DNA damage repair and cell cycling. In the absence of Mzi, the cMyc/Max complex binds directly to Sp1 to form a heterotrimer. This heterotrimer relies on Sp1 subunit to bind to the GC-rich regions of DNA damage repair and cell cycling genes, thereby promoting their expression.³⁶ Furthermore, cMyc/Max directly binds to the E-box region of these genes, thereby promoting their expression.³⁶ The transcription factor cMyc promote cell proliferation by inhibiting the expression of genes that inhibit cell cycle progression and by facilitating the transcription of genes that involved in cellular proliferation, mitochondrial biosynthesis, rRNA and protein biosynthesis, glycolytic and oxidative phosphorylation (Figure 2B).³²

In the context of normoxia, cMyc, but not HIF-1 α , is essential for the activation of NK cells induced by cytokines. The expression of cMyc promotes the secretion of IFN γ , perforin and granzyme by NK cells.^{37,38} cMyc is a pivotal transcription factor in the cytokine-induced metabolic reprogramming of NK cells.³⁸ The level of cMyc is found to increase during the stimulation of NK cells with IL2/IL12.³⁸ Elevated cMyc levels stimulate glycolysis and oxidative phosphorylation in NK cells, thereby orchestrating metabolic reprogramming of NK cells under normoxia.³⁸ This metabolic reprogramming would prompt NK cells to exert effective cytotoxic effects and IFN γ secretion.³⁸ In contrast, the absence of HIF-1 α under normoxia did not affect the expression of IFN γ and the secretion of perforin and granzyme B in NK cells.³⁸ This indicates that cMyc is the dominant factor under normoxic conditions. However, under hypoxic conditions, HIF-1 α interacts with cMyc, gradually gaining dominance over NK cells under hypoxia.^{31,39}

Transcriptional Adaptation of NK Cells to Hypoxia is Manifested in the Interaction of HIF-1 with cMyc

Although cMyc is a transcriptional center that controls NK cell proliferation and development under normoxic conditions, its activity is progressively replaced by HIF-1 α under hypoxic conditions.^{31,39} HIF-1 α has been demonstrated to play a pivotal role in the regulation of NK cell survival, metabolism, and adaptation.^{31,39} HIF-1 α exerts its inhibitory effect on cMyc through various distinct pathways.^{31,39} For instance, HIF-1 α inhibits the transcription of cMyc target genes by competitively binding to Sp1, thereby displacing the cMyc/Max complex from Sp1.⁴⁰ HIF-1 α is also capable of binding to Mzi1, which results in the displacement of the cMyc/Max complex and the removal of the inhibition of cell cycle inhibitory proteins (CDKN1A). This shift ultimately contributes to the slowed proliferation of NK cells and cell cycle arrest under hypoxic conditions. The HIF-1 α target gene Mix also inhibits cMyc.⁴¹ In response to prolonged hypoxia, HIF-1 induces the production of Mix, which forms a complex with Max and binds to the E-Box, the binding site of cMyc on DNA. This blocks the binding of cMyc to the E-box, inhibiting the transcriptional activity of cMyc.⁴¹⁻⁴³ Notably, this inhibitory effect of HIF-1 α on cMyc is reversible. Upon restoration of oxygen levels to normal, cMyc function will also gradually recover.³¹ The interaction between cMyc and HIF that contributes to transcriptional adaptation of NK cells under hypoxic conditions (Figure 2B).

After dominance, HIF-1, which is primarily involved in transcriptional processes, is responsible for the induction of a multitude of hypoxic adaptive responses in NK cells. The impairment of NK cell cytotoxicity and antimicrobial efficacy induced by hypoxia is correlated with the activity of HIF-1. In response to IL-18 stimulation, NK cells are

activated to exert cytotoxicity. However, hypoxia-induced accumulation of HIF-1 impairs the ability of NK cells to secrete perforin, granzyme, and IFN γ .⁴⁴ HIF-1 α -deficient NK cells unleash cytokine secretion function and cytotoxicity in response to IL-18, thereby limiting tumor volume.⁴⁴ Nevertheless, the impact of HIF-1 on NK cells varied across different models. In the wound model, accumulation of HIF-1 under hypoxic conditions is a key driver of IFN γ secretion by NK cells.⁴⁵ This strikingly disparate outcome may be linked to the pathophysiological processes underlying wound and tumorigenesis. This suggests the presence of an unknown factor that can reverse the inhibitory effect of HIF-1 on NK cells under hypoxic conditions.

In addition to their role in tumor cell cytotoxicity, NK cells affect tumor volume by interfering with angiogenesis in solid tumors. Research indicates that NK cells represent a significant source of secreted vascular endothelial growth factor receptor1 (sVEGFR1) in solid tumors.^{45,46} sVEGFR1 binds to VEGF, thereby exerting a negative regulatory effect on VEGF bioavailability.^{47–49} Such inhibition disrupts the balance between angiogenic and angiostatic factors during tumor cell induced angiogenesis.⁴⁶ Imbalanced angiogenesis results in the formation of a large network of non-functional blood vessels within the tumor.⁴⁶ Non-productive angiogenesis leads to an inadequate oxygen supply to tumor cells, which in turn limits tumor volume.⁴⁶ HIF-1 α promotes sVEGFR1 secretion by NK cells under hypoxic conditions.^{45,46} The absence of HIF-1 α will impair the secretion function of NK cells, resulting in a reduction in sVEGFR1.⁴⁶

In addition to its impact on NK cell functionality, HIF-1 α serves as a vital transcription factor that protects NK cells from apoptosis during infection.⁵⁰ HIF-1 α is specifically elevated in NK cells during infection. The target genes of HIF-1 α inhibit the expression of the pro-apoptotic protein BCL2/adenovirus E1B 19 kd-interacting protein (BNIP), thereby inhibiting apoptosis.⁵⁰ Despite the substantial proliferation of NK cells observed in the presence of HIF-1 α deficiency, the number of NK cells still declines.⁵⁰ This decline is closely associated with unrestrained cell death.⁵⁰

In summary, the transcriptional levels of NK cells under hypoxic conditions undergo adaptive changes. HIF-1 replaced cMyc as the major transcription factor for mobilizing metabolism and protein synthesis after NK cell activation. However, the contrasting results prompt the complexity of the effects of HIF-1 α on NK cells. Further investigation of HIF-1 α -mediated adaptation of NK cells to hypoxia becomes imperative.

Immunometabolism Reprogramming in Hypoxia

Immunometabolism in NK Cell

Immunometabolism describes changes in intracellular metabolic pathways during immune cell activation.⁵¹ NK cell functions are closely correlated with intrinsic cellular Immunometabolism.³⁰ In resting NK cells, the levels of oxidative phosphorylation (OXPHOS) and glycolysis were maintained at baseline.⁵² This baseline rate of OXPHOS and glycolysis was adequate for the acute phase response of NK cells.³⁰ Rates of OXPHOS or glycolysis were not elevated in NK cells after 4 hours of cytokine stimulation (IL15/IL15+IL12/IL15+IL18) or after 6 hours of receptor ligation (anti-NK1.1/anti-Ly49D).⁵² However, although stimulation did not increase metabolic activity, inhibition of glucose/glycolysis or overall OXPHOS inhibition resulted in almost complete elimination of receptor-stimulated IFN- γ production.⁵² This demonstrates the importance of the baseline metabolic levels in sustaining the acute response of NK cells. Growing evidence indicates that NK cells not only function in the acute phase, but also play an essential role in the prolonged immune response. In the overnight cytokine stimulation assay *in vitro* and the overnight pathogen infection assay *in vivo*, basal OXPHOS and glycolysis were significantly increased in NK cells.⁵³ In addition, overnight activated NK cells exhibited higher expression of nutrient transporter receptors (amino acid transporter CD98 and transferrin receptor CD71) and higher glucose uptake than resting NK cells.^{53,54} Overall, the immune metabolism of NK cells is intricately associated with their function.

Suppression of OXPHOS by Hypoxia Impairs NK Cell Function

Given the irreplaceable position of oxygen in the production of intracellular ATP and as an electron acceptor in various biochemical reactions serving, it is not surprising that the metabolic reprogramming of NK cells is intricately related to the oxygen supply. The main metabolic approaches of NK cells are OXPHOS and glycolysis, both of which are closely related to hypoxia.⁵³ OXPHOS represents the most efficient metabolic approach to producing ATP. There are two distinct

patterns of metabolism that differ from glycolysis: the tricarboxylic acid cycle (TAC) and the electron transport chain,⁵⁵ both of which are dependent on the normal function of mitochondria.

Hypoxia impairs OXPHOS through the inhibition of the TAC. The conversion of pyruvate to acetyl coenzyme A (acetyl-CoA) by pyruvate dehydrogenase (PDH) represents the initiation of TAC.^{21,56} HIF-1-induced pyruvate dehydrogenase kinase 1 (PDK1) impairs the pyruvate-acetyl-CoA transition by phosphorylating and inactivating the catalytic subunit of PDH.^{21,56} Furthermore, the HIF-1 protein was found to induce an increase in the activity of lactate dehydrogenase A.^{21,57–60} LDHA catalyzes the conversion of pyruvate to lactate, which subsequently impairs the conversion of pyruvate to acetyl-CoA. HIF-1 induced the expression of PDK1 and LDHA, which have ability to inhibit OXPHOS and promote glycolysis. Glycolysis is another important metabolic pathway for energy replenishment in the event of an imbalance between cellular oxygen supplies and metabolic demands.^{21,58–60} In the hypoxic environment, the inhibited OXPHOS will result in a reduction in the secretion of cytokines and cytotoxicity (Figure 3A).⁵²

At an intracellular oxygen level of 0.3%, ETC activity begins to be impaired.⁶¹ Complex 4 (also known as cytochrome c oxidase or COX) is most intimately associated with oxygen within ETC. It contributes 4 electrons to oxygen and produces 2 molecules of water.⁶² It is noteworthy that the suppressive impact of hypoxia on ETC did not result from a decrease in the level of oxygen as a substrate. Rather, it was mediated by a HIF-1-dependent mechanism.⁶³ Specifically, hypoxia induces the up-regulation of the mitochondrial NADH dehydrogenase (ubiquinone) 1 α subcomplex, 4-like 2 (NDUFA4L2) gene expression via HIF1. It has been demonstrated that NDUFA4L2 suppresses complex I activity.⁶⁴ In addition, hypoxia induces miRNA (*mir-210*) expression through HIF-1, which represses iron-sulfur cluster assembly enzyme 1 (ISCU1) and ISCU2. These factors are pivotal in the assembly of iron-sulfur clusters within Complex I, II and III.⁶⁵ Additionally, *mir-210* has been demonstrated to suppress the expression of NDUFA4, succinate dehydrogenase complex subunit D (SDHD), and COX10, which are crucial components in the formation and assembly of ETC-associated complexes (Figure 3B).^{66,67}

Hypoxia-Induced Mitochondrial Fragmentation Limits NK Cell Function

Mitochondria, as the primary organelles involved in metabolic processes, play a pivotal role in the functionality of NK cells. Normoxic NK cells exhibit large, tubular, and densely packed mitochondrial, whereas hypoxic NK cells display small, fragmented, and distinct mitochondrial within the cytoplasm.⁶⁸ The morphology of mitochondrial fragmentation has been demonstrated to limit the secretion of granzyme B by NK cells, as well as anti-tumor function and survival.⁶⁸ The oxygen consumption rate (OCR) of mitochondrial respiration is standard method for assessing the level of OXPHOS.⁶⁹ In hypoxic NK cells with fragmented mitochondria, the basal OCR level is observed to be lower than that of normal NK cells.⁶⁸ The basal and maximal OCR of hypoxic NK cells can be restored by the use of the mitochondrial fragmentation inhibitors, such as mdivi-1 or Dyn.⁶⁸ Furthermore, unbiased metabolomics profiling of purified hypoxic and normoxic cultured NK cells revealed that the inhibition of TAC-related metabolic intermediates under hypoxia could be alleviated by mitochondrial fragmentation inhibitors (Figure 3C).⁶⁸

Hypoxia-induced mitochondrial fragmentation represents a critical initiating event in the apoptotic process of NK cell. Mitochondrial release of sequestered pro-apoptotic proteins, which are stored in the intermembrane space, results in their translocation into the cytoplasm.⁷⁰ The release of pro-apoptotic proteins, including cytochrome c, Smac/DIABLO, and serine protease HtrA2/Omi, initiates the apoptotic process, ultimately leading to cell death.⁷⁰ Caspase 3 is the most significant executor of apoptosis. The intrinsic pathway, the extrinsic cellular pathway, and the perforin/granzyme pathway collectively induce apoptosis via caspase 3.⁷⁰ The number of NK cells was found to be significantly reduced in the hypoxic microenvironment of the tumor. The level of cleaved caspase 3 in hypoxic NK cells has been observed to increase.⁶⁸ In contrast, the number of NK cells in the hypoxic microenvironment of the tumor increased following the use of the mitochondrial fragmentation inhibitor mdivi-1.⁶⁸

Hypoxia-induced mitochondrial fragmentation is associated with the mitochondrial protein fission 1 (FIS1) signaling pathway.⁷¹ FIS1, which localizes to the outer mitochondrial membrane, is a pivotal receptor that promotes mitochondrial fragmentation.⁷² The GTPase dynamin-related protein 1 (DRP1) functions as a ligand for FIS1, thereby activating FIS1 to facilitate mitochondrial fragmentation.⁷² In the context of hypoxia, DRP1 is recruited to the periphery of the outer mitochondrial membrane by FIS1.⁷¹ DRP1's activating site, Ser616, is phosphorylated in hypoxic NK cells.⁷² The

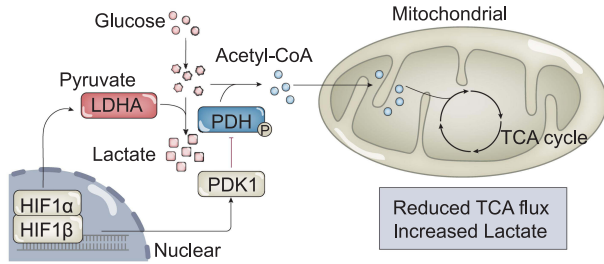
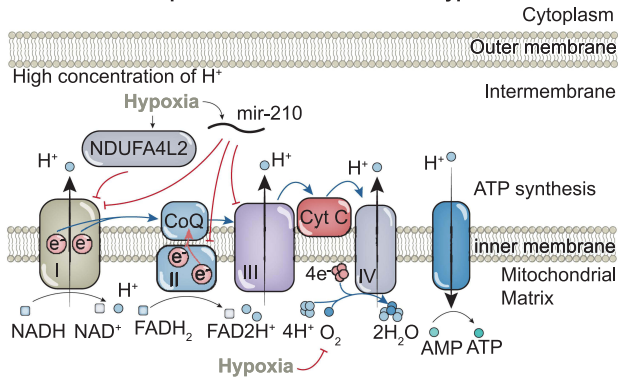
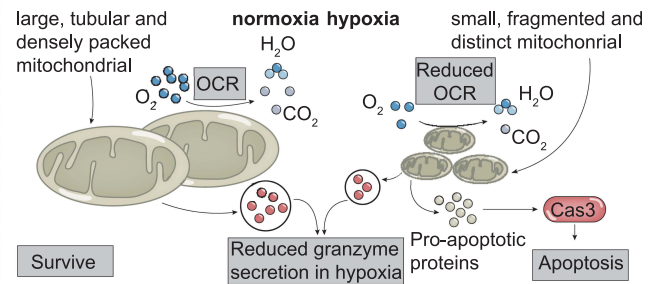
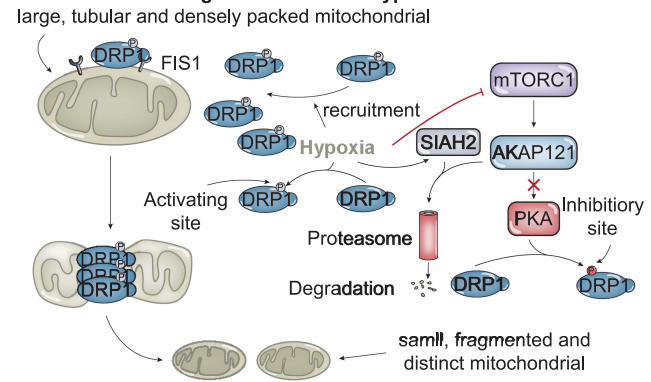
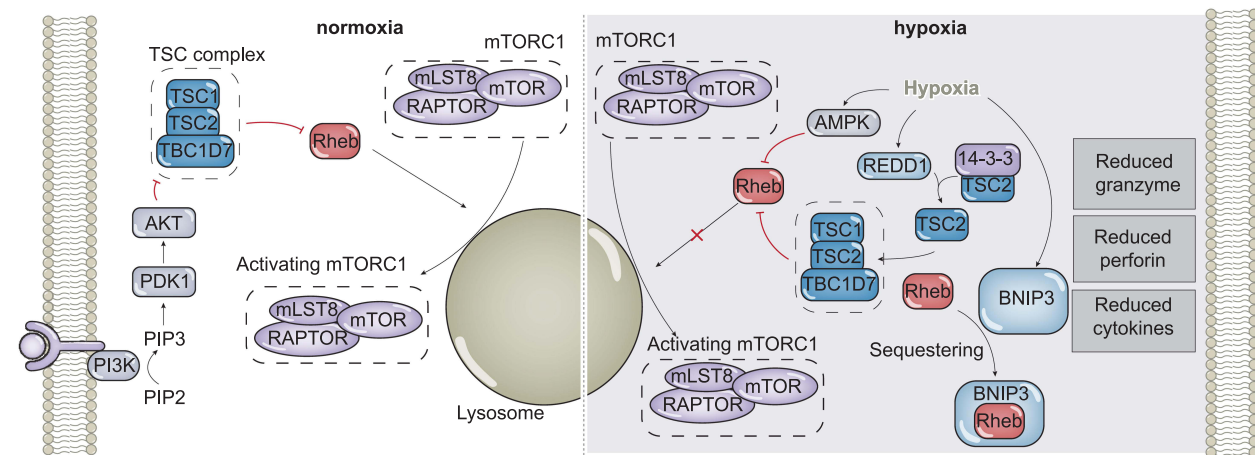
A Tricarboxylic acid in NK cells under hypoxia**B** Electron transport chain in NK cells under hypoxia**C** Mitochondrial morphology under normoxia and hypoxia**D** Mitochondrial fragmentation under hypoxia**E** The mechanism of inhibition of mTORC1 under hypoxia

Figure 3 The effects of hypoxia on the metabolism and mitochondrial morphology of NK cells. **(A and B)** Hypoxia inhibits the OXPHOS process from both the TAC cycle **(A)** and the ETC **(B)**. Hypoxia stabilizes HIF-1, which in turn transcribes PDK1 and LDHA. PDK1 dephosphorylates PDH, thereby reducing the conversion of pyruvate to Acetyl-CoA. The expression of LDHA facilitates the conversion of pyruvate to lactate, which subsequently impairs the production of Acetyl-CoA. In essence, hypoxia impairs the TAC cycle by reducing the production of Acetyl-CoA **(A)**. Oxygen is a crucial substrate for complex IV. In the context of hypoxia, electrons transferred to complex IV lack acceptor, resulting in an increased production of ROS. Hypoxia also induces the production of miR-210, which represses the transcription of complex I, II, and III. The inhibition of these three complexes has the effect of reducing the efficiency of electron transfer through the ETC **(B)**. **(C and D)** Hypoxia-induced mitochondrial fragmentation inhibits OXPHOS in NK cells. NK cells possess large, tubular and densely packed mitochondria. In contrast, the mitochondria of NK cells under hypoxia become smaller, fragmented and distinct. The fragmentation of mitochondria not only reduces the level of OXPHOS but also releases pro-apoptotic proteins that promote apoptosis in NK cells **(C)**. The mechanism by which hypoxia regulates mitochondrial fragmentation is related to the activity of the DRP1 and FIS1. The inhibition of DRP1 by mTORC1 was found to be deregulated under hypoxic conditions. Activated DRP1 is recruited to the mitochondrial periphery and binds to FIS1, thereby inducing mitochondrial fragmentation **(D)**. **(E)** Hypoxia inhibits mTORC1 through the protein Rheb. The lysosomal membrane translocation is a crucial step in the activation of mTORC1 and Rheb is a crucial protein that facilitates the translocation of mTORC1 to the surface of the lysosomal membrane. Inhibition of mTORC1 has a profound impact on multiple signaling pathways in NK cells, resulting in a reduction in their cytotoxicity and cytokine secretion.

Abbreviations: LDHA, lactate dehydrogenase A; PDH, pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; Acetyl-CoA, acetyl coenzyme A; TAC, tricarboxylic acid; OXPHOS, oxidative phosphorylation; HIF, hypoxia inducible factor; NDUFA4L2, NADH dehydrogenase (ubiquinone) 1 α subcomplex, 4-like 2; CoQ, coenzyme Q; NADH, Nicotinamide adenine dinucleotide; FADH₂, flavin adenine dinucleotide; ATP, Adenosine triphosphate; AMP, Adenosine monophosphate; Cyt C, cytochrome C; OCR, Oxygen consume rate; Cas3, Caspase 3; DRP1, dynamin-related protein 1; FIS1, fission 1; SIAH2, seven in absentia homolog 2; mTORC1, mammalian target of rapamycin complex 1; AKAP121, A kinase anchoring protein 121; PKA, Protein kinase A; TSC, Tuberous sclerosis; PDK1, pyruvate dehydrogenase kinase 1; PIP3, Phosphatidylinositol (3,4,5)-trisphosphate; PIP2, Phosphatidylinositol 4,5-bisphosphate; PI3K, Phosphoinositide 3-kinases; mLST8, mammalian lethal with sec-13 protein 8; RAPTOR, regulatory-associated protein of mTOR; AMPK, AMP-activated protein kinase; REDD1, Regulated in development and DNA damage-response 1; Rheb, Ras homolog enriched in brain; BNIP3, Bcl-2/adenovirus E1B 19 kDa-interacting protein 3.

phosphorylation of Ser616 in DRP1 has been demonstrated to be associated with the activation of mechanistic target of rapamycin (mTOR) signaling. Inhibition of the mTOR signaling pathway results in a significant reduction in the level of phosphorylated DRP1^{Ser616} in NK cells.^{21,68,72}

In addition to the facilitated activation of mTOR, the deregulation of the inhibitory effect on DRP1 is also a critical step in facilitating the binding of DRP1 to FIS1. In the presence of sufficient oxygen, Scaffolding protein A kinase anchoring protein 121 (AKAP121) inhibits DRP1 through a cAMP-regulated protein kinase A⁷³-mediated phosphorylation process.⁷² The inhibition is alleviated in hypoxic environments by seven in absentia homolog 2 (SIAH2).^{74,75} Hypoxia-induced SIAH2, an E3 ubiquitin ligase, mediates the ubiquitination and proteasome-mediated degradation of AKAP121.^{74,75} The degradation of AKAP121 results in the de-repression of DRP1, which in turn promotes the DRP1-FIS1-mediated mitochondrial fragmentation (Figure 3D).

Overall, hypoxia activates DRP1 through two distinct mechanisms: mTOR and SIAH2. The activation of DRP1 results in the fragmentation of mitochondria through binding of FIS1, which ultimately leads to a reduction in the functionality and an increase in the death of NK cells.

mTOR is an Important Molecule in Hypoxia-Regulated NK Cell Metabolism

The mammalian target of rapamycin (mTOR) is a vital cellular metabolic checkpoint kinase that primarily promotes anabolic metabolism, including ribosome, RNA, and protein synthesis. Conversely, it inhibits catabolic metabolism, such as autophagy.⁷⁶ mTOR is a component of two complexes, each with a unique structure and functions.⁷⁶ mTOR complex 1 (mTORC1) is a heterotrimeric complex comprising regulatory-associated protein of mTOR (RAPTOR), mammalian lethal with sec-13 protein 8 (mLST8), and mTOR. The mTOR complex 2 (mTORC2) is a heterotetramer comprising the mLST8 and mTOR subunits, as well as the rapamycin insensitive companion of mTOR (RICTOR) and the mammalian stress activated MAPK-interacting protein 1 (mSIN1).⁷⁷ mTORC1 rather than mTORC2, is responsible for regulating the development and activation-induced metabolic reprogramming of NK cells.

Coincidentally, hypoxia play a crucial role in the inhibition of mTORC1. mTORC1 activation is lysosome-dependent. Facilitating the translocation of mTORC1 to the lysosome necessitates the involvement of two classes of Ras-associated small G proteins: amino-acid-induced Rag GTPase and Rheb GTPase.⁷⁸ Hypoxia exerts its inhibitory effect on mTORC1 primarily through the inhibition of Rheb activity.²¹ Rheb were found to be inhibited by the Tuberous sclerosis (TSC) complex in quiescent cells.⁷⁸ The TSC complex is comprised of TSC1, TSC2, and TBC1 domain family member 7 (TBC1D7).⁷⁸ In activated cells, the phosphoinositide 3-kinases (PI3K) signaling pathway is activated, resulting in the phosphorylation of downstream AKT.⁷⁸ p-AKT then phosphorylates the TSC complex, thereby depriving the TSC complex's inhibition of Rheb.⁷⁸ In the absence of any inhibitory factors, Rheb will recruit mTORC1 for transfer to the lysosome, which ultimately leads to the activation of mTORC1.⁷⁸ AMP-activated protein kinase (AMPK) is activated under hypoxia.⁷⁹ The activation of AMPK will result in the inhibition of Rheb, rather than TSC.⁷⁹ In addition, hypoxia has been demonstrated to induce the up-regulation of REDD1.⁸⁰ Up-regulated REDD1 results in the release of TSC2 from its chaperone proteins 14-3-3.⁸⁰ TSC2 then recombines with TSC1 and TBC1D7 to form the TSC complex, which inhibits Rheb activity.⁸⁰ Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3), a protein induced by HIF-1, was also found to bind directly to Rheb, thereby sequestering Rheb away from mTORC1.⁸¹ In hypoxic cells, the increase in glycolytic metabolism results in an elevation of lactate levels and the formation of an acidic environment. In such conditions, perinuclear lysosomes undergo a redistribution.^{82,83} Perinuclear lysosomes move away from perinuclear Rheb, thereby rendering lysosome-bound mTORC1 inactivation.^{21,82,83} Collectively, hypoxia affected the activity of mTORC1 by inhibiting Rheb. Inhibition of mTORC1 will compromise NK cell function (Figure 3E).

Translation Adaptation to Hypoxia

Translation represents a pivotal stage in the process by which NK cells accumulate effector proteins and receptors. mRNA is responsible for assembling genetic information into proteins through translation, thereby enabling NK cells to perform immune activity. The overall translation process is divided into three distinct phases: initiation, elongation and termination. In the context of hypoxic stress, cells experience a lack of nutrients and disrupted signaling, which in turn leads to increase in the production of unfolded and misfolded proteins. This, in turn, triggers endoplasmic reticulum

stress. Hypoxia has been demonstrated to disrupt translation, resulting in imbalanced expression of NK cell receptors and inhibition of cytokine secretion.

Inhibition of Translation Initiation in NK Cells Under Hypoxic Conditions

The initiation of translation involves recruitment of the ribosomal 40S subunit, followed by the recognition of the mRNA's AUG promoter by tRNAs. Among them, Eukaryotic initiation factor 2 (eIF2) is a crucial factor that mediates tRNA-mRNA binding and is one of the targets of hypoxia-regulated translation initiation. eIF2 has been shown to correlate with the expression of NKG2D, an activating receptor for NK cells.⁸⁴ It has been demonstrated that the expression of NKG2D on the membrane of NK cells is upregulated following the activation of eIF2.⁸⁴ However, eIF2 is subject to extensive inhibition under hypoxic conditions. The main receptor of the unfolded protein response, protein kinase RNA-like endoplasmic reticulum kinase (PERK), acts as a kinase and phosphorylates eIF2 to prevent transcription initiation.^{85,86} In the context of hypoxia, the accumulation of unfolded proteins leads to the activation of PERK, which in turn phosphorylates and inhibits eIF2 activity.^{85,86}

In addition to the eIF2-mediated assembly of the 43S subunit binding complex, the recruitment of mRNA by the eukaryotic translation initiation factor 4F (eIF4F) complex is equally important for the initiation of protein translation.⁸⁷ The eIF4F complex plays a crucial role in cap-dependent translation.⁸⁷ eIF4F consists of the cap-binding protein eIF4E, the DEAD-box RNA helicase eIF4A, and the large scaffold protein eIF4G.⁸⁸ It has been demonstrated that the production of IFN γ in NK cells is correlated with eIF4F.⁸⁹ Hypoxia inhibits eIF4E by down-regulating the mTORC1 pathway, which ultimately affects eIF4F formation and inhibits the transcriptional initiation process.⁸⁸ The mTORC1 signaling pathway plays a pivotal role in the formation of the eIF4F complex. mTORC1 inactivates 4E-BPs by phosphorylating them, thereby deregulating the inhibitory effect of 4E-BPs on eIF4E.⁹⁰ In hypoxic conditions, mTORC1 is inhibited, which prevents eIF4E from dissociating from 4E-BPs.⁹⁰ This sustained inhibition of eIF4E results in the failure of the eIF4F complex assembly, which ultimately leads to the inhibition of translation initiation.⁹⁰ During prolonged hypoxia, the transporter 4-ET blocks eIF4E in the nucleus, which impedes the formation of eIF4F.⁹⁰ In essence, the failure of eIF4F formation impedes the secretion of IFN γ by NK cells, as evidenced by a reduction in IFN γ translation under hypoxic conditions (Figure 4A).

Inhibition of Translation Elongation in NK Cells Under Hypoxic Conditions

The elongation phase of translation is dependent upon the eukaryotic elongation factor 2 (eEF2). eEF2 plays an important role in the translocation of elongating ribosomal.^{91,92} The elevated expression of eEF2 in NK cells during the early stages of endotoxemia suggests a correlation between eEF2 and the functional capabilities of NK cells.⁹³ eEF2 kinase (eEF2K), an important kinase negatively regulating eEF2, phosphorylates Thr56 of eEF2 and inhibits the translation elongation process.^{91,92,94,95} eEF2K is known to be normally subject to two factors, mTORC1 and PHD2, which are themselves subject to hypoxia. mTORC1 is responsible for the degradation of eEF2K via the proteasomal pathway. However, in the context of hypoxia, mTORC1 is inhibited.⁹⁶ It has been demonstrated that PHD2 is capable of degrading eEF2K via a proteasomal degradation mechanism, in conjunction with the presence of Fe²⁺, O₂, and α -ketoglutarate.^{97,98} This process is analogous to the degradation of HIF-1 α . In the context of hypoxia, eEF2K is stabilized by the loss of PHD2-mediated proteasomal degradation, which occurs due to oxygen deprivation.²⁶ The stabilization of eEF2K results in the inactivation of eEF2, which ultimately impedes the elongation process of translation.

Intriguingly, eEF2K functions as a translation elongation repressor. However, for PD-L1, eEF2K emerged as a pivotal activator of its translation.⁹⁹ The discrepancy in the translation results in the overexpression of PD-L1 on the surface of tumor cells under hypoxic conditions, thereby rendering it challenging for NK cells to recognize and eliminate tumor cells (Figure 4B).

Inhibition of Translation Termination in NK Cells Under Hypoxic Conditions

The translation termination phase requires the involvement of eukaryotic translation termination factor 1 (eRF1).¹⁰⁰ eRF1 is the only class 1 eRF that recognizes all three stop codons.¹⁰¹ eRF1 recognizes stop codons and facilitates the hydrolysis of ester bonds in polypeptide chains with peptidyl-site tRNAs.¹⁰¹ This process of hydrolysis releases newly

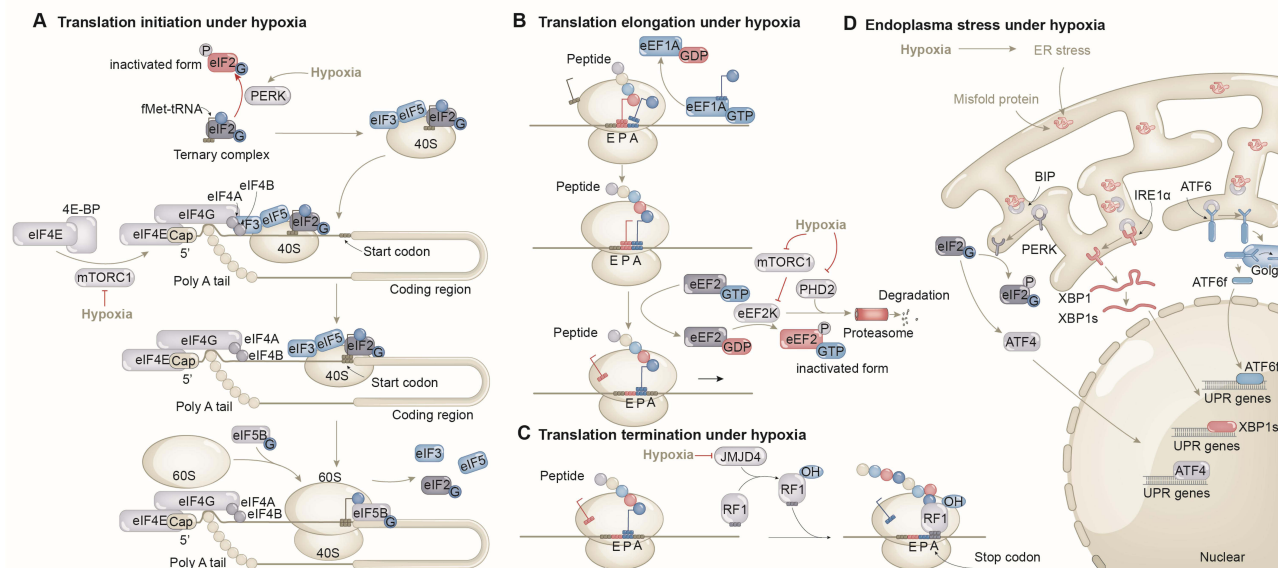


Figure 4 Effect of hypoxia on the translation process in NK cells. **(A)** During the initiation of translation, the inhibitory effect of hypoxia on mTORC1 prevents the release of eIF4E from its binding protein, 4E-BP. This results in the impaired formation of the eIF4F, which is important in translation initiation. In the context of hypoxia, the eIF2, which binds to tRNA, is inhibited by PERK. **(B)** During the transcription elongation phase, hypoxia invalids the inhibition of eEF2K by PHD2 and mTORC1. eEF2K inhibits eEF2, which is an important factor in transcription elongation. **(C)** eRF1 plays a pivotal role in the termination of transcription. It is inhibited under hypoxic conditions. **(D)** The production of large quantities of misfold proteins in response to hypoxia initiates the UPR pathway within the ER. The UPR comprises three receptors: The PERK, IRE1 α , and ATF6. All three types of receptors bind to BIP and prevent its signaling activity. BIP binds to misfold proteins when misfold protein loading is increased. This results in the release of three receptors. Activated PERK represses eEF2 and promotes ATF4 transcription factor expression. Upon activation, IRE1 α will cleave XBP1 to form XBP1s, which possesses transcriptional activity. Following its activation, ATF6 is transported to the Golgi apparatus. ATF6 in the Golgi is cleaved to form transcriptionally active ATF6f. The target genes of these transcription factors ultimately inhibit the translation process, thereby reducing the levels of endoplasmic reticulum misfold proteins.

Abbreviations: eIF, Eukaryotic initiation factor 2; eEF, Eukaryotic translation elongation factor; RF, Release factor; A, Aminoacyl site; P, Peptidyl site; E, Exit site; eEF2K, Eukaryotic elongation factor 2 kinase; mTORC1, Mammalian target of rapamycin complex 1; PHD2, pyruvate dehydrogenase 2; JMJD4, Jumonji domain-containing 4; PERK, Protein Kinase RNA (PKR) -like Endoplasmic Reticulum Kinase; IRE1 α , Inositol Requiring Enzyme 1; ATF, Activating Transcription Factor 6; UPR, Unfolded protein responses. ER, Endoplasmic reticulum.

synthesized proteins from the ribosome and dissociates the ribosome and mRNA complex.¹⁰¹ Hydroxylation of eRF1 is a crucial step in the process of translation termination.¹⁰² Jumonji domain-containing 4 (JMJD4) is a crucial oxidase responsible for hydroxylating eRF1.¹⁰² Under hypoxic conditions, JMJD4 is inhibited, and eRF1 fails to recognize the stop codon, resulting in protein termination failure and increased production of mismatched proteins.¹⁰² Despite the absence of definitive evidence regarding the impact of hypoxia on the translation termination process in NK cells, it cannot be ruled out that hypoxia may inhibit the translation termination process in NK cells by affecting eRF1, which in turn affects NK cell protein synthesis (Figure 4C).

Hypoxia Induced Endoplasmic Reticulum Stress in NK Cells

Endoplasmic reticulum (ER) is of critical importance for the regulation of multitude of intracellular physiological process including protein folding and calcium homeostasis.^{103,104} Hypoxia and other cell stress results in a multitude of protein modifications, including protein oxidation, nitrosylation, and carbonylation.¹⁰⁵ This exceeds the capacity of the ER for protein synthesis, resulting in an accumulation of unfolded proteins.¹⁰⁶ In hypoxic conditions, the accumulation of misfolded and unfolded proteins can trigger the unfolded protein responses (UPR), a cellular mechanism that alleviates ER stress.¹⁰⁵ The activation of the UPR has been demonstrated to alleviate ER stress through the attenuation of overall protein synthesis, ER-associated degradation pathway and autophagy mediated by PERK (Protein Kinase RNA (PKR) -like Endoplasmic Reticulum Kinase), IRE1 α (Inositol Requiring Enzyme 1), and ATF6 (Activating Transcription Factor 6) (Figure 4D).¹⁰⁷

The activation of IRE1 is a hallmark of NK cells that must constitutively deal with a high demand for protein synthesis, folding and secretion.^{37,108} The substrate transcription factor for IRE1 is XBP1.^{109,110} Upon activation of IRE1, XBP1 is cleaved to transcriptionally active XBP1s.^{109,110} XBP1s translocates to the nucleus and promotes NK cell

proliferation and expansion.^{109,110} The proliferation of NK cells induced by XBP1s appears to be partially correlated with cMyc.³⁷ However, as previously described, cMyc is antagonized by HIF1 under hypoxic conditions. This antagonism may have partially impaired the proliferation of NK cells induced by XBP1s. In addition to its role in NK cell proliferation, the IRE1 α and its substrate XBP1 signaling pathway is also crucial for maintaining the migration, immune synapse formation and cytokine release of NK cells (Figure 4D).¹¹¹

Upon an increase in the concentration of misfolded proteins, PERK is activated by oligomerization and autophosphorylation, which is due to the dissociation of immunoglobulin binding protein (BiP).¹¹² Activated PERK phosphorylates eIF2 α , which results in inhibition of translation.¹¹³ In the context of translational repression, the level of PERK-induced activating transcription factor 4 (ATF4) remains elevated.¹¹⁴ This phenomenon can be attributed to the presence of an internal ribosome entry site (IRES) within the ATF4 gene.^{115,116} Genes containing IRES are subject to selective enhancement upon eIF2 α phosphorylation.¹¹⁷

The activation of ATF6 is initiated by the accumulation of unfolded peptide fragments within the cell, which subsequently leads to the translocation of ATF6 to the Golgi apparatus.¹¹⁸ Within the Golgi apparatus, ATF6 is cleaved by proteases to form the active ATF6f form.¹¹² The N-terminal structural domain of ATF6f contains an alkaline leucine zipper structural domain that binds to DNA and promotes the expression of target genes in response to unfolded proteins.¹¹⁹

Although the direct effects of ER stress on NK cells remain poorly understood, it is clear that ER stress plays an important role in regulating NK cell responsiveness.¹²⁰ NK cells exhibited a selective recognition of infected cells that had induced ER stress, while ignoring infected cells without ER stress.^{120,121} The cytolytic activity of NK cells is driven through ER stress and is particularly dependent on the activation of ATF6 and PERK downstream of ER stress.¹²¹ ATF6 is responsible for the up-regulation of key hub proteins in the ER proteostasis network, including BiP and calreticulin.¹²² Calreticulin, an ER lectin chaperone, has been demonstrated to bind to NKp46 and to promote cytotoxicity in NK cells.¹²⁰ In order to make contact with NK cells, calreticulin needs to be externalized to the cell surface. The stimulation of ER chaperone externalization is correlated with PERK.¹²³ In conclusion, the activation of ATF6 and PERK is responsible for the presentation of calreticulin at the cell surface. The presentation of calreticulin on the cell surfaces activates NKp46 and promotes NK cytotoxicity.

Hypoxic Metabolites Promote NK Cell Adaptation

In addition to the effects of hypoxia on NK cells, it is important to consider the impact of hypoxic metabolites. Lactic acid, adenosine, ROS, and other by-products of cellular metabolism in hypoxia can significantly affect NK cells and promote their adaptation to hypoxia (Figure 5).

Lactate

In the context of hypoxia, the metabolic process of NK cells undergoes a shift from OXPHOS to anaerobic glycolysis.³⁰ This shift is accompanied by the conversion of pyruvate to large quantities of lactate, a process that is catalyzed by the LDHA.^{30,51,57} Lactate is intimately associated with a reduction in the function of NK cells. In the tumor microenvironment, lactate accumulation is associated with a reduction in IFN γ production by NK cells.¹²⁴ The apoptosis of NK cells is contingent upon the concentration of lactate exceeding 20 mM.¹²⁴ This explains the observed reduction in the population of NK cells in tumors when high concentrations of lactate are present.¹²⁴ The inhibition of lactate in tumor cells resulted in enhanced cytotoxicity and increased expression of activating receptors in NK cells.¹²⁵

There are numerous subsets of NK cells, each exhibiting varying sensitivities to lactate. Two distinct populations of NK cells have been identified in the liver: tissue-resident NK (trNK) cells and conventional NK (cNK) cells.^{126–128} These NK cells exhibit disparate sensitivities to inflammation-induced lactate accumulation.¹²⁹ Specifically, inflammation-induced lactate promoted apoptosis of trNK cells, resulting in a significant decrease in their number. Conversely, the number of cNK cells at the same lactate concentration was not significantly altered.¹²⁹ With regard to the effector function of NK cells, it has been demonstrated that trNK cells are capable of producing IFN γ prior to cNK cells and that they play a protective role in the liver at the early stage of mouse cytomegalovirus (MCMV) infection.¹³⁰ The disparity in lactate sensitivity is the underlying cause of the diminished functionality observed in trNK cells, which are expected to

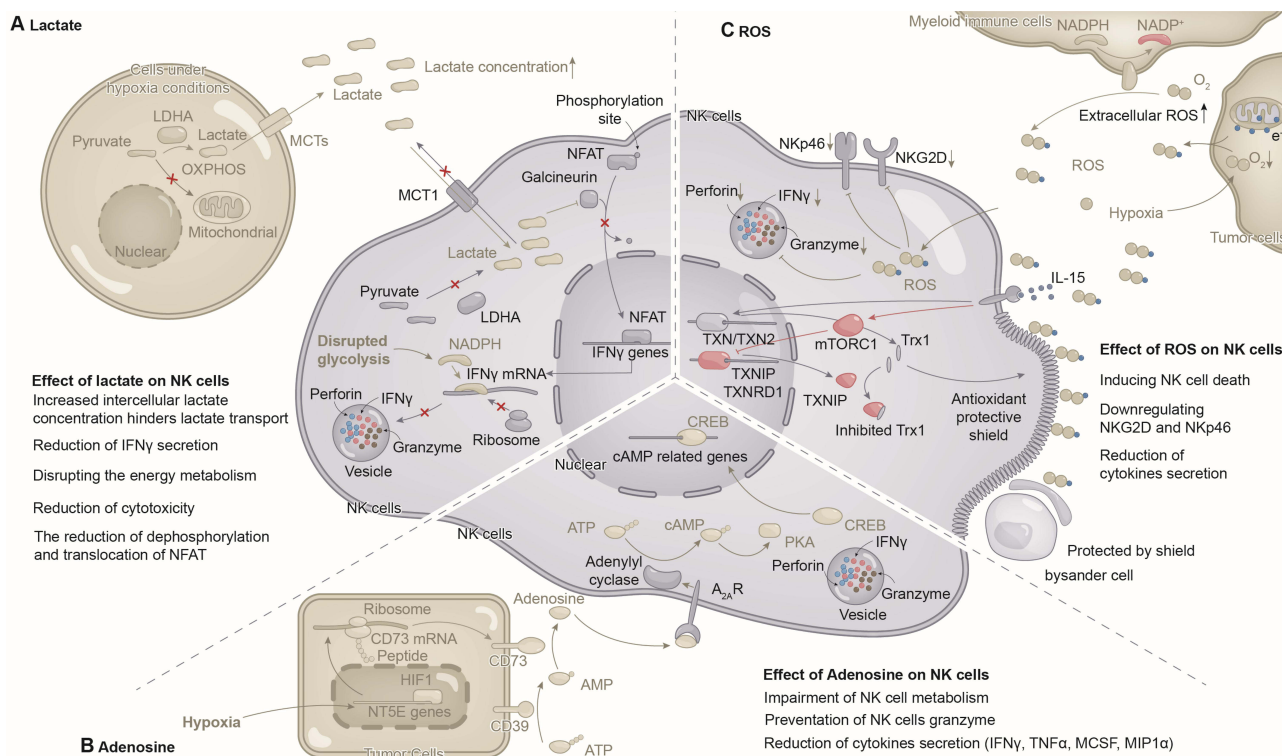


Figure 5 Effects of hypoxic metabolites on NK cells. In the context of hypoxia, there is a shift in cellular metabolism from oxidative phosphorylation to glycolysis. **(A)** This is accompanied by an increase in intracellular lactate levels. Normal lactate efflux from NK cells is inhibited by elevated lactate concentrations in the intracellular compartment. The accumulation of lactate in the cytosol results in the inhibition of glycolysis in NK cells. Inhibition of glycolysis results in an elevation of NADPH, which can bind to IFN_γ mRNA, thereby preventing IFN_γ transcription. Additionally, elevated lactic acid levels result in cellular acidification. The acidification of cell inhibits the calcineurin, which impairs the activity of NFAT, a transcription factor that regulates IFN_γ. **(B)** Adenosine is another hypoxia induced immunosuppressive metabolite. HIF1 has been demonstrated to promote the expression of CD73. The enzymes CD73 and CD39 work in concert to convert ATP into adenosine. Adenosine binds to NK cell receptors, thereby inhibiting NK cell function through the cAMP-PKA signaling. **(C)** In the context of hypoxia, tumor cells are known to produce elevated levels of reactive oxygen species (ROS) due to oxygen deprivation. This phenomenon has been observed to elicit a similar response in myeloid immune cells, which are known to produce ROS in response to the enzyme NOX. A significant extracellular accumulation of reactive oxygen species (ROS) has been demonstrated to inhibit the expression of natural killer (NK) cell activation receptors, including NKp46 and NKG2D. Furthermore, the secretion of NK cell cytotoxicity mediators, including perforin, granzyme, and IFN_γ, has also been shown to be impaired. In the meantime, NK cells have developed a defensive mechanism against ROS. Following IL-15 activation, the expression of the anti-ROS substance Trx1 was observed to increase. Conversely, the expression of the protein that inhibits Trx1 was found to decrease. Trx1 forms an antioxidant shield on the surface of NK cells, thereby protecting them from ROS. Moreover, the antioxidant protective shield afforded protection to the peripheral immune cells of NK cells, thereby maintaining optimal immune responsiveness.

Abbreviations: OXPHOS, oxidative phosphorylation; LDHA, lactate dehydrogenase A; MCTs, monocarboxylate transporters; NFAT, Nuclear factor of activated T cells; IFN_γ, Interferon gamma; HIF, Hypoxia-inducible factor; NT5E, ecto-5'-nucleotidase; ATP, Adenosine triphosphate; AMP, Adenosine monophosphate; PKA, Protein kinase A; CREB, cAMP response element-binding protein; TNF_α, Tumor necrosis factor alpha; M-CSF, Macrophage colony-stimulating factor; MIP1_α, Macrophage inflammatory protein-1 alpha; NADP, Nicotinamide adenine dinucleotide phosphate; ROS, Reactive oxygen species; IL, Interleukin; Trx1, Thioredoxin; TXN, Thioredoxin; TXNIP, Thioredoxin-interacting protein; TXNRD1, Thioredoxin Reductase I.

express IFN_γ with greater rapidity.¹²⁹ Nevertheless, impaired IFN_γ expression of trNK cells permitted cNK cells to become the principal cells secreting IFN_γ during MCMV infection.¹²⁹ The different sensitivity of NK cells to lactate is inextricably linked to mitochondrial function. Sensitivity of trNK cells to lactate correlates with impairment of their mitochondrial function and the carbonic anhydrase 5B (Car5b) expression.¹²⁹ The mitochondrial enzyme carbonic anhydrase is essential for the conversion of CO₂ to bicarbonate.¹³¹ This is of critical important for NK cells in resisting intracellular pH changes in an acidic microenvironment.¹³¹

The inhibitory effect of lactate on NK cells is a topic of ongoing research. When NK cells are activated under normoxic conditions, they require a substantial amount of ATP for a brief period of time, which leads to aerobic glycolysis.¹³² This accelerated glucose metabolism in NK cells, so-called “Warburg effect”, is accompanied by the production of a considerable amount of lactate. In order to maintain the glycolysis, lactate needs to be exported via monocarboxylate transporters 1 (MCT1), which depends on a concentration gradient from cytoplasmic to extracellular lactate.¹³³ Conversely, in the context of hypoxia, an increase in the extracellular lactate concentration results in a diminished concentration gradient, thereby impeding the transport of lactate from NK cells to extracellular

compartment. The accumulation of intracellular lactate disrupts the energy metabolism of NK cell, resulting in a reduction in ATP levels.¹²⁴ Furthermore, NADPH, which released by the lactate induced disruption of glycolysis, binds to IFN γ mRNA, thereby impeding the translation of IFN γ .¹³⁴ The nuclear translocation of nuclear factor of activated T cells (NFAT), the transcription factor for IFN γ , is also disturbed by intracellular acidification due to lactate accumulation. The translocation of NFAT is mediated through calcineurin, a phosphatase that dephosphorylates NFAT. In low pH cells, calcineurin is suppressed, leading to the inhibition of NFAT translocation.¹³⁵

Lactate has a profound effect on NK cells, but the exact mechanism is still a mystery. The extent to which lactate is important in hypoxia-induced NK cell inhibition and whether inhibition of lactate production can reverse the inhibitory effect of hypoxia on NK cells remains unexplored. The immunosuppressive effects of lactate make it an important clinical target for manipulation of NK cell function. The study of specific mechanisms for targeting lactate has profound implications for a wide range of diseases (Figure 5A).

Adenosine

Tumors employ a series of subterfuges to evade immune escape, with the accumulation of extracellular adenosine being a common tactic.¹³⁶ Adenosine, a ubiquitous immunosuppressive metabolite, impairs the infiltration, cytotoxicity and cytokines production of numerous immune cells, including NK cells and cytotoxic T cells.^{136–138} Extracellular adenosine triphosphate (ATP) is hydrolyzed to adenosine by nucleoside triphosphate dephosphorylase (CD39) and exto-5'-nucleotidase (CD73). Despite the identification of multiple mechanisms underlying ATP leakage to the extracellular compartment in various cell types, the exact mechanism remains elusive.^{139,140} In healthy tissues, the extracellular ATP concentration is typically low, in the nanomolar (nmol/l) range. In contrast, in diseased or stressed tissues, the extracellular ATP concentration increased to hundreds of micromolar (μ mol/l).^{139,141,142} Extracellular ATP is initially hydrolyzed to adenosine monophosphate (AMP) by CD39, which is expressed on the cell membrane. Subsequently, AMP is recognized by CD73 on the cell membrane and hydrolyzed to adenosine.¹⁴³ It is noteworthy that extracellular ATP signaling may have originally promoted antitumor immunity via P2 purinergic receptors.¹⁴⁴ Following the catalysis by CD39 and CD73, the immunopromoting ATP was hydrolyzed to the immunosuppressive adenosine.¹⁴⁵ As with ATP, extracellular adenosine concentrations elevate from nanomolar to micromolar range in the setting of disease and stress.^{146–148} The CD39-CD73-adenosine axis has emerged as a crucial regulatory pathway due to its potent immunosuppressive effects.

Tumor-induced hypoxic microenvironment is an important trigger for adenosine production. The deprivation of oxygen results in a reduction in the availability of energy and an increase in the concentration of extracellular ATP, which provides the material basis for the increase in adenosine.¹⁴⁴ Moreover, hypoxia is a potent inducer of CD39 and CD73 on the cell membrane.¹³⁶ HIF-1 has been demonstrated to promote the transcription of CD73 by binding to the promotor located in front of NT5E, the gene encoding CD73.¹⁴⁹ Hypoxia is also associated with the inhibition of adenosine metabolism, which is accompanied by the accumulation of adenosine. Adenosine can be reconverted to AMP by adenosine kinase, but under hypoxic conditions, adenosine kinase is inhibited, which accelerates the accumulation of extracellular adenosine concentrations.¹⁵⁰ The combined effects contribute to a significant increase in extracellular adenosine concentration in hypoxic micro-environments, which in turn leads to a profound suppression of immune cells, particularly NK cells.

The precise mechanism by which adenosine exerts an inhibitory effect on NK cells remains elusive. It has been demonstrated that the primary mode of inhibition exerted by adenosine is through the NK cell surface G-protein-coupled adenosine 2A receptors (A_{2A}R).¹⁵¹ Adenosine inhibits IL-2 mediated TNF α release from NK cells.¹⁵² Activation of A_{2A}R up-regulates the concentration of cAMP in NK cells, which in turn activates protein kinase A.⁷³ Activated PKA inhibits cytotoxicity, cytokine secretion and immunometabolism in NK cells.¹⁵³ A₃R, another G-protein-coupled adenosine receptor expressed on NK cells, appears to antagonize A_{2A}R signaling and promotes NK cell function by inhibiting cAMP activity.¹⁵⁴ Nevertheless, this receptor is currently understudied. Activated PKA plays a pivotal role in adenosine-mediated inhibition of NK cells. In IL-2 primed NK cells, adenosine was found to inhibit cytotoxicity and cytokines production via PKA.^{151,155} Due to the metabolic instability of adenosine itself, some studies have employed an alternative, metabolically stable analog 2-chloroadenosine¹⁵⁶ in lieu of adenosine to stimulate NK cells. As anticipated, CADO suppressed lymphocyte activation, Ly49D-crosslinking-stimulated NK cell cytotoxicity and cytokines secretion, including IFN γ and TNF α .¹⁵⁷ In contrast to IL-2 stimulation, IL-12 and IL-15-primed NK cells exhibited the ability to overcome the inhibitory effects of adenosine, as

evidenced by an increase in IFN γ expression in primed NK cells.¹⁵⁸ Despite the observed increase in IFN γ expression in IL-12 and IL-15 primed NK cells, the observed enhancement in the lytic effect on tumor cells was not observed. This phenomenon indicates that adenosine may impede the exocytosis of IFN γ (Figure 5B).¹⁵⁸

The substantial inhibitory of adenosine on NK cell function has prompted the development of therapeutic strategies that aim at inhibiting this pathway. One of the most interesting therapies is the application of engineered human NK cells.^{159,160} Engineered cell therapy is a process whereby the CD73 antibody gene is integrated into the NK cell specific genome. This results in the NK cell becoming activated upon encountering a tumor cell, and the NK cell itself secreting anti-CD73 antibodies.^{159,160} The antibody inhibits CD73, thereby reducing the accumulation of adenosine and restoring NK cell function.^{159,160} Hypoxia-induced adenosine has been demonstrated to exert potent immunosuppressive effects. Consequently, inhibition of adenosine production is expected to be the key to reversing the abnormal NK cell function caused by hypoxia.

ROS

Reactive oxidative species (ROS) exerts profound inhibitory effects on NK cells. In the hypoxic microenvironment, as the ETC is disrupted by oxygen deprivation, tumor cells and a variety of infiltrating myeloid immune cells, such as macrophages, myeloid derived suppressor cells (MDSC) and granulocytes, release large amounts for ROS into the intercellular space.¹⁶¹ NK cells that are exposed to ROS exhibit apoptosis at an earlier stage than T cells. Chronic myeloid leukemia cells contribute to the premature apoptosis of NK cells through the generation of ROS.¹⁶² This inhibitory effect can be alleviated by the use of ROS scavengers.¹⁶² Monocyte derived and macrophage derived hydrogen peroxide has been demonstrated to significantly inhibit effector function of NK cells, resulting in a decrease in CD3 and CD16 on the surface of NK cells. This decrease is one of the mechanisms by which monocytes and macrophages exhibit their immunosuppressive effects.^{163,164} Furthermore, ROS suppressed the expression of NK cell surface activation receptors NKG2D and NKp46, thereby impeding the cytotoxicity and cytokine secretion functions of NK cells.^{165–167}

To resist the inhibitory effects of ROS, NK cells develop the antioxidant thioredoxin to counteract elevated extracellular ROS.¹⁶⁸ Thioredoxin acts as a scavenger of ROS by reducing oxidized cysteine residues to the reduced state and cleaving disulfide bonds.^{169,170} In response to IL-15 stimulation, NK cells up-regulate the expression of thioredoxin genes (TXN and TXN2). In addition, IL-15 primed NK cells also inhibit the gene expression of thioredoxin interacting protein by up-regulating mTORC1. This protein acts as an inhibitory counterpart of thioredoxin and exerts negative regulation of thioredoxin.¹⁶⁸ The up-regulation of thioredoxin results in an increased density of thiols on the surface of NK cells. This high density of thiols on the cell membrane serves to form an antioxidant protective shield against high extracellular concentrations of ROS.¹⁶⁸ The antioxidant effect of NK cells allows NK cells located in the high ROS region of the tumor core to maintain higher cell cytotoxicity and IFN γ secretion than peripheral NK cells. Additionally, the level of ROS in NK cells in the tumor core region was found to be lower than that in peripheral NK cells.¹⁶⁸ It is noteworthy that the antioxidant shield formed by NK cells not only preserves the functionality of themselves in high ROS environment but also protects bystander lymphocytes, such as T cells, from ROS.¹⁶⁸

The precise mechanism by which ROS inhibit NK cells remains elusive, and further research is necessary to elucidate the manner in which ROS attack NK cells, resulting in a reduction in their cytotoxicity, cytokines secretion, and ultimately in their apoptosis (Figure 5C).

Targeting Hypoxic NK Cells Immunotherapy

The inhibitory effects of hypoxia on NK cells are complex, involving not only the adaptation of NK cells to hypoxia and its metabolites, but also interactions with other cells under hypoxic conditions. These alterations collectively act on the NK cells to cause a decrease in their cytotoxicity and cytokine secretion. For example, tumor cells undergo adaptive changes in response to hypoxia, which enables them to evade the surveillance of NK cells.¹⁷¹ The expression of MICA, the NK-specific recognition receptor on the surface of tumor cells under hypoxia, is decreased. A reduction in MHC class I-related chain molecules A¹⁷ results in the inability of NKG2D receptors to recognize tumor cells, which ultimately leads to their immune escape.¹⁷¹ Furthermore, hypoxia results in the upregulation of autophagy in tumor cells. Autophagy degrades perforin and granzyme released by NK cells into tumor cells, rendering tumor cells resistant to

their cytotoxicity.¹⁷² The complexity and variability of hypoxia inhibition of NK cell function is further compounded by the cell-cell interactions involved.

The complexity of the hypoxia microenvironment has hindered the advancement of NK cell therapy targeting hypoxia. It can be postulated that enhancing the oxygen concentration within the microenvironment may assist in alleviating the impaired cytotoxicity function and decreased cytokine secretion of NK cells under hypoxia.^{123,173–175} One of the most significant challenges in tumor therapy has been the development of effective treatments for hypoxic microenvironment, particularly those found in solid tumors. It has been demonstrated that exercise can induce oxygenation in the tumor microenvironment, which in turn restores the functionality of NK cells.¹⁷⁶ However, the efficacy of exercise remains to be demonstrated.¹⁷⁷ Myo-inositol-trispyrophosphate (ITPP) enhances oxygen tension within solid tumors by stabilizing vascular normalization through the activation of endothelial Phosphatase-and-Tensin-homologue (PTEN).^{178,179} The ITPP-induced increase in intra-tumoral oxygen resulted in an increase in the number of NK cells and a decrease in the number of immunosuppressive cells.¹⁸⁰ Similar to the ITPP mechanism, manganese dioxide nanoparticles, which were encapsulated into poly(lactic-co-glycolic), catalyze the degradation of tumor-produced hydrogen peroxide, resulting in the generation of oxygen.¹⁸¹ The therapeutic measures of manganese dioxide nanoparticles not only elevated oxygen in the tumor microenvironment, but also consumed ROS, adenosine and lactic acid.¹⁸¹ In addition to the tumor microenvironment, sepsis and severe trauma can result in the body undergoing hypoxia.^{182,183} The administration of oxygen therapy at this time will, in fact, serve to exacerbate the poor prognosis of septic and trauma patients through the mechanism of ischemia-reperfusion injury.¹⁸⁴ Our recent research has also demonstrated that oxygen therapy following severe trauma is associated with a reduction in NK cell numbers. Further research is required to elucidate the correlation between oxygen therapy and the immune system in other diseases.

In addition to alleviating hypoxia in the microenvironment, an additional therapeutic approach is to enhance the tolerance of NK cells to hypoxia. It has been demonstrated that the stimulation of NK cells by cytokines enhances their tolerance to hypoxia. IL-2 primed NK cells prevent NKG2D down-regulation in hypoxic environment.¹³ A high-affinity NK cell that is capable of expressing the high-affinity CD16 receptor and internal IL-2 is able to maintain cytotoxicity under hypoxic conditions.¹⁸⁵ Hypoxia has been demonstrated to reduce the phosphorylation levels of extracellular signal-regulated kinase (ERK) and signal transducer and activator of transcription 3 (STAT3) in an Src homology region 2 domain-containing phosphatase-1 (SHP-1) dependent manner. Pharmacological blockade of SHP-1 activity has been shown to partially restore the cytotoxicity caused by hypoxia.¹⁵ However, the promotion of STAT3 and JAK signaling pathways has the unintended consequence of inducing PD-L1 expression in tumor cells, which impairs the cytotoxicity of NK cells.¹⁸⁶ As previously stated, tumor cells are able to resist the cytotoxic effects of NK cells through autophagy. Inhibition of autophagy has been demonstrated to enhance the cytotoxicity of NK cells under hypoxic conditions.¹⁷² However, the development of NK cells is heavily dependent on autophagy, and selective inhibition of autophagy in tumor cells represents a key objective of this therapeutic approach.¹⁸⁷ It can be posited that increasing the oxygen content of the microenvironment and increasing the tolerance of NK cells to hypoxia represent prospective potential treatments.

Conclusion and Perspective

In conclusion, the functional impairment of NK cells in a hypoxic environment is the result of a series of adaptive responses. These responses result in NK cells downregulating their own high-energy processes and avoiding potential damage caused by hypoxia. In the context of hypoxia, the transcription factor of NK cells undergoes a shift from cMyc to HIF-1, resulting in an altered mRNA profile at the transcriptional level. Furthermore, the energy metabolism of NK cells undergoes a transition from aerobic respiration to anaerobic glycolysis, thereby providing a rapid and continuous energy supply for NK cell function in the short term. Nevertheless, this metabolic shift is insufficient to maintain cytotoxicity and cellular function over an extended term. The long-term cytotoxicity and cellular function of NK cells are affected by mitochondrial fragmentation under conditions of hypoxia, which leads to an energy crisis. Hypoxia induced misfolding protein causes ER stress, resulting in the downregulating of activating receptors in NK cells. In addition to adapting to hypoxia, NK cells are affected by a variety of metabolites produced under such conditions, including lactate, adenosine, and ROS. These byproducts are immunosuppressive and collectively inhibit NK cell activity.

Box 3 Important Outstanding Questions in Relation to the Effects of NK Cells Under Hypoxic Conditions

- Hypoxia has been demonstrated to inhibit the activating receptors on NK cells, while the inhibitory receptors remain unaffected. Further investigation is warranted to elucidate the underlying mechanisms responsible for the selective inhibition of activating receptors by hypoxia.
- The effects of HIF-1 on NK cells were found to be diametrically opposed in different disease models. The impact of HIF-1 on NK cells remains inconclusive.
- The effects of hypoxia on NK cells were observed to be limited to splenic and circulating NK cells. The effects of hypoxia on other tissue-resident NK cells remain understudied. Further investigation is required to elucidate the mechanism by which hypoxia affects tissue-resident NK cells.
- Current studies are limited to pathologic hypoxia, but the effects of physiologic hypoxia on NK cells are equally important. How physiological hypoxia affects NK cell proliferation, differentiation and expansion?
- What is the role of hypoxia in NK cell memory-like response?

Overall, it is now well established that hypoxia plays an important role in the effector function of NK cells. Despite the numerous outstanding questions (Box 3), future research in this area will undoubtedly reveal more exciting and intriguing mechanisms and therapeutic opportunities.

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Disclosure

The authors declare no competing interests.

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