Immunometabolism and Natural Killer cell responses.

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Abstract:

Natural killer (NK) cells are lymphocytes with important roles for innate and adaptive immune responses to tumours and viral infection. However, in certain chronic diseases, including obesity and cancer, NK cells are found to have impaired functional responses. Recently research has highlighted the importance of NK cell metabolism in facilitating robust NK cell effector functions. This article will discuss our current understanding of murine and human NK cell metabolism and the key signalling pathways that mediate metabolic responses in NK cells. Furthermore, it will explore how defects in metabolism can contribute to the generation of dysfunctional NK cells in chronic disease. Finally, the potential for new therapeutic strategies targeting cellular metabolism will be discussed.

Introduction

Immune responses can involve rapid and extensive changes in immune cell function and it has become clear that substantial alterations in cellular metabolism are required to support the activities of such cells[1]. Metabolic pathways can be configured to meet the particular demands of the cell for energy and/or biosynthesis (see Box 1). Cellular metabolism is emerging as a key factor in the control of immune responses[1,2].

Natural Killer (NK) cells are important effector lymphocytes best characterised for their anti-viral and anti-cancer activities[3,4]. They are potent producers of IFNγ cytokine and can directly kill target cells through cytotoxic mechanisms. NK cell responses can be detected very rapidly after infection and they have historically been considered as cells of the innate immune system. However, with the discovery of long-lived NK cells and the demonstration of intrinsic innate

immune memory it is now appreciated that NK cells have roles spanning both innate and adaptive immune responses[5-8]. NK cells can also have regulatory roles and can contribute to shaping T cell responses; depending on the context NK cells can either stimulate or inhibit T cell responses (reviewed in[9]). This review will discuss what is currently known about the metabolic pathways used by NK cells and how cellular metabolism shapes NK cell responses.

Metabolic responses in activated NK cells.

NK cells are traditionally considered part of the innate immune system with an important role in acute responses to viral infection and tumours. NK cells are poised and ready to kill target cells and produce cytokines like IFNγ rapidly upon activation. Resting murine NK cells have low basal metabolic rates, maintaining low levels of glycolysis and OXPHOS[10,11]. These low metabolic rates are sufficient to sustain acute NK cell responses, in terms of IFNγ production, and 4 hours cytokine stimulation (IL15, IL12/IL15 or IL12/IL18) or 6 hours receptor ligation (anti-NK1.1 or anti-Ly49D) does not result in any increase in rates of glycolysis or OXPHOS[10]. However, these low rates of metabolism are important for the acute NK functions such as IFNγ production. Inhibition of OXPHOS or glycolysis significantly impaired IFNγ production at these short timepoints but to varying degrees depending of on the activation stimuli; receptor stimulation is much more sensitive to metabolic inhibition compared to cytokine stimulation[10].

It is now well accepted that NK cells play an important role in immune responses over prolonged periods where they operate alongside the adaptive immune system. Interestingly, stimulation of NK cells for sustained periods results in robust metabolic changes that are required for NK cell effector functions. Overnight stimulation of murine or human NK cells with various cytokine combinations results in substantial increases in the rates of both glycolysis and OXPHOS[11-14]. These increased metabolic rates are facilitated by alterations in the metabolic machinery and the metabolic configurations of the NK cells. Activated NK cells increase glucose uptake and flux through glycolysis, aided by increased expression of glycolytic enzymes and associated nutrient transporters[12]. Increases in both basal OXPHOS rates and maximal respiratory capacity are accompanied by increased mitochondrial mass[15]. NK cells have been studied extensively in the context of CMV infection using the mouse cytomegalovirus (MCMV) model. During the course of MCMV infection NK cells undergo mitochondrial changes *in vivo* and have significantly increased mitochondrial mass at the peak of infection[16].

In human blood there are two NK cell subsets that are distinguished by the expression of CD56. $CD56^{bright}$ and $CD56^{dim}$ NK cells produce large quantities of cytokines such as IFNγ in response cytokine stimulation and receptor ligation, respectively. $CD56^{dim}$ NK cells are important for immediate cytotoxic killing of target cells, while $CD56^{bright}$ must upregulate the cytotoxic machinery before gaining cytotoxic potential. These two human NK cells subsets are also different in terms of a range of metabolic parameters. Cytokine stimulation, including IL2 and IL12/IL15, upregulates the expression of nutrient receptors, including glucose (Slc2a1) and amino acid transporters (Slc1a5, Slc7a5, Slc3a2/CD98) and the transferrin receptor (CD71), to a greater degree in $CD56^{bright}$ NK cells, arguing for greater metabolic responses in the $CD56^{bright}$ NK cells compared to $CD56^{dim}$ NK cells[13,17]. Metabolic analysis of total NK cells from human blood shows that resting NK cells have relatively low rates of glycolysis and OXPHOS, which then increase significantly after overnight stimulation with IL2 or IL12/15[13]. Interestingly, chronic exposure of human NK cells to IL15 cytokine *in vitro* results in reduced metabolic rates[18]. Tissue resident NK cells also increase the expression of nutrient receptors upon stimulation though the magnitude of these increases are less than for blood NK cells [19].

The importance of these metabolic changes for NK cell function has been investigated using a variety of approaches to limit the flux through either glycolysis or OXPHOS. Strategies that reduce metabolic rates in murine NK cells inhibit cytokine-induced proliferation and impair NK cell cytotoxicity against a range of cell lines *in vitro*[15,20]. Concentrations of the metabolic inhibitors 2-deoxyglucose (2DG) and oligomycin that limit but do not completely inhibit glycolysis and OXPHOS, respectively, are found to inhibit the production of IFNγ and the expression of granzyme b in both murine and human NK cells[12,13]. 2DG-treatment also decreases the ability of IL15 activated human NK cells to kill K562 target cells[20]. Culturing NK cells in galactose, a fuel that can only support low rates of glycolysis, rather than glucose also inhibits the effector function of both human and murine NK cells[12,13]. When 2DG is injected into mice it is preferentially taken up by highly glycolytic cells and will limit the rate of glycolysis in these cells. 2DG treatment in mice following poly I:C administration or MCMV infection results in reduced NK cell IFNγ production and in the case of MCMV infection, reduced clearance of virally infected target cells and increased viral loads[12,20]. In human NK cells inhibition of amino acid uptake through Slc1a5 and Slc7a5 prevented IFNγ production and degranulation following antibody crosslinking of the activating receptor NKG2D[17].

What fuels activated NK cells?

While IL2/IL12-activated murine NK cells metabolise glucose primarily to pyruvate and then lactate through aerobic glycolysis, the pyruvate that does enters the mitochondria is not metabolised through the TCA cycle as is the case in other lymphocytes[15]. Instead, pyruvate is converted to mitochondrial citrate which is metabolised by the citrate malate shuttle (CMS, Figure 1)[15]. The CMS provides an alternate mode of generating NADH in the mitochondria to fuel OXPHOS and ATP synthesis via the export of mitochondrial citrate in exchange for cytosolic malate. This CMS also provides cytosolic NAD+ an important co-factor for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), thus facilitating elevated rates of glycolysis[15]. Unlike the TCA cycle, which can receive inputs from multiple fuels (see Box 1), the CMS is fuelled exclusively by glucose. Indeed, NK cells do not use glutamine as a fuel for driving OXPHOS; inhibition of glutaminase, the first enzyme in glutaminolysis, does not inhibit OXPHOS and does not affect NK cell effector functions[10,21]. Whether NK cells use fatty acids as a fuel source has not been extensively studied, partly due to the fact that there is a lack of specific tools for examining fatty acid oxidation. Etomoxir, an inhibitor commonly used to block fatty acid oxidation has recently been found to have a number of off-target effects[22-24]. However, the fact that etomoxir treatment had no effect on IFNγ production by receptor or cytokine-activated NK cells does suggest that fatty acids are not an important fuel for NK cells[10]. Interestingly, the accumulation of excess fatty acids within NK cells is found to be detrimental to NK cell metabolism and function[25]. Therefore, NK cells are primarily fuelled by glucose, which is metabolised by aerobic glycolysis in the cytosol and in the mitochondria via the citrate malate shuttle to drive OXPHOS and ATP production[15,21].

Metabolism during NK cell development

While metabolism has been well studied in mature NK cells, cellular metabolism during NK cell development has not yet been directly investigated. Developing murine NK cells undergo several stages of maturation in the bone marrow that can be identified based on expression levels of CD11b and CD27[26]. Pre-NK cells ($CD11b^{lo}$-$CD27^{hi}$) undergo a burst of proliferation that correlates with expression of the nutrient transporters CD71 and CD98[11,26]. As they terminally differentiate from $CD11b^{lo}$-$CD27^{hi}$ cells to $CD11b^{hi}$-$CD27^{hi}$ cells and then into mature $CD11b^{hi}$-$CD27^{lo}$ NK cells they quiesce to a resting state and co-ordinately they reduce expression of nutrient transporters[11]. Interrogation of the bone marrow scRNAseq data set

recently generated by the Tabula Muris consortium reveals that the CD11b^{lo}-CD27^{hi} pre-NK cells express higher mRNA levels for a range of metabolic enzymes and nutrient transporters compared to the immature (CD11b^{hi}-CD27^{hi}) and mature (CD11b^{hi}-CD27^{lo}) NK cell subsets[27]. For example pre-NK cells express higher mRNA levels of amino acid transporters *Slc7a5* and *Slc1a5*, glycolytic enzymes including hexokinase (*Hexa* and *Hexb*) and phosphofructose kinase (*Pfkl*), and enzymes involved in lipid synthesis such as Fatty acid synthase (*Fasn*) and ATP citrate lyase (*Acly*). Therefore, the data suggest that during NK cell development in the bone marrow there is increased metabolic activity in pre-NK cells to support the demands placed on them by cellular proliferation.

NK cell education (also called NK cell licencing) is a process for achieving functional maturation and self-tolerance. There is evidence that changes in cellular metabolism are associated with this NK cell education process. Educated human NK cells have been described to have increased expression of glucose transporters and elevated rates of glycolysis, but not of OXPHOS, compared to uneducated NK cells[28,29].

Regulation of NK cell metabolism

A number of key metabolic regulators have now been identified in NK cells. The mammalian target of rapamycin complex 1 (mTORC1) is well established as a regulator of various metabolic processes in diverse cell types. In NK cells, mTORC1 activity is important for normal murine NK cell development and for activation-induced metabolic and functional responses in mature NK cells. mTORC1 activity is highest in CD11b^{lo}-CD27^{hi} pre-NK cells and decreases as NK cell mature, which correlates with the expression pattern of nutrient transporters and metabolic enzymes[11,27]. This data suggests that mTORC1 is important for increased metabolism in pre-NK cells to support proliferative expansion[11,26]. Indeed, deletion of the mTOR kinase subunit or Raptor, an adaptor subunit of mTORC1, using the NCR1-Cre that is expressed in CD11b^{lo}-CD27^{hi} pre-NK cells resulted in a substantial block in differentiation in the bone marrow at the CD11b^{lo} to CD11b^{hi} stage that was attributed to decreased pre-NK cell proliferation[11,30].

While resting NK cells have low mTORC1 activity, cytokine stimulation induces robust increases in mTORC1 signalling in both human and murine NK cells[11-13,17]. Consistent with the increased metabolic readouts in CD56^{bright} NK cells, cytokine-induced mTORC1 signalling is much greater in CD56^{bright} NK cells compared to CD56^{dim} NK cells[13,17]. Activation of murine NK cells *in vitro* or *in vivo* leads to increased mTORC1 activity that is required for increased expression of nutrient transporters and glycolytic enzymes, for increased mitochondrial mass

and for the expression of effector molecules including IFNγ and granzyme B[11,12,31]. Inhibition of mTORC1 using the pharmacological inhibitor rapamycin results in decreased glycolysis in murine NK cells stimulated with either IL2/IL12 or high dose IL-15[12,14]. In mice treated with rapamycin during infection with MCMV, reduced mTORC1 activity in NK cells was associated with reduced NK cell proliferation, IFNγ production and cytotoxicity leading to elevated viral burdens[31]. In human NK cells activated overnight with cytokine, mTORC1 activity is only required for increased NK cell metabolism in response to certain cytokine stimulations. IL2-stimulated NK cells require mTORC1 activity for increased levels of glycolysis whereas increased glycolysis with IL12/15-stimulated NK cells is mTORC1-independent[13]. IFNγ production and CD98 expression following ligation of the NKG2D receptor is also mTORC1 dependent[32].

The functions of the other mTOR complex in NK cells, mTOR complex 2 (mTORC2), are less well defined. Unlike mTORC1, mTORC2 is not considered a major metabolic regulator. However, mTORC2 is important for murine NK cell development and function and can impact upon NK cell metabolism because it negatively regulates the activity of mTORC1[30,33]. mTORC2 limits mTORC1 activity by inhibiting STAT5-mediated expression of the amino acid transporter SLC7A5 (Figure 2)[33]. It is also worth noting that mTORC1 activity in murine NK cells, and also in murine CD8 cytotoxic T lymphocytes (CTL), is not downstream of PI3K/Akt signalling axis but is acutely sensitive to the levels of the amino acids leucine and glutamine (Figure 2)[21,34,35].

Two transcription factors have also been shown to be crucial for metabolic responses in cytokine activated NK cells, Sterol regulatory element binding protein (Srebp) and cMyc. Srebp is classically considered the master regulator of fatty acid and cholesterol synthesis as they induce the expression of the majority of genes in these biosynthetic pathways[36]. Cytokine induces the activity of the Srebp in murine NK cells and mTORC1 is required for maximal Srebp activity. While Srebp-regulated fatty acid and cholesterol synthesis is not important for cytokine-induced responses in murine NK cells, a completely novel role for Srebp has been described. Srebp activity is essential for increased rates of glycolysis and OXPHOS in cytokine-stimulated NK cells because Srebp controls the expression of two of the key factors required for the citrate malate shuttle, the citrate-malate anti-porter Slc25a1 and ATP citrate lyase (ACLY)[15]. Therefore, Srebp controls the metabolic configuration engaged by NK cells (Figure 2). Inhibition of Srebp activation using both pharmacological and genetic approaches

results in a dramatic reduction in glycolysis and OXPHOS, impaired NK cell IFNγ production and reduced cytotoxicity against tumour cells both *in vitro* and *in vivo*[15]. Srebp transcription factors are also important for the effector functions of human NK cells; inhibition of Srebp activation results in impaired IFNγ production and decreased expression of effector molecules such as granzyme b[15].

cMyc is required for the metabolism of activated NK cells because it controls the expression of the metabolic machinery in NK cells. cMyc is required for the increased expression of glucose transporters and glycolytic enzymes to support glycolysis and for mitogenesis to provide the increased mitochondrial mass required to support high rates of OXPHOS (Figure 2)[21]. Interestingly, while in CD8 CTL the transcription factor HIF1α is the key transcription factor required to support elevated glycolysis, this is not the case in NK cells (for a comparison of NK and T cell metabolism see Table 1)[21,34]. HIF1α deficient murine NK cells have normal levels of both glycolysis and OXPHOS in response to cytokine stimulation[21]. While HIF1α does not appear to be important in this context, it might be predicted that HIF1α may have a role in controlling NK cell metabolism in other contexts such as under conditions of hypoxia.

While cMyc is often considered to be controlled downstream of mTORC1 this is not necessarily the case in NK cells, or indeed in CD8 CTL[21,34,35]. The initial upregulation of cMyc expression in NK cells that occurs within minutes of IL2/IL12 stimulation is partially dependent on mTORC1 activity, but cMyc expression is completely independent of mTORC1 at all timepoints after a few hours. Instead, cMyc expression is dependent on the availability of the amino acids that are required to maintain high rates of cMyc translation to offset continuous cMyc degradation[21]. While glutamine is not an important fuel for NK cells (discussed above), glutamine is important for cMyc signalling as it facilitates amino acid uptake through the system L-amino acid transporter Slc7a5 (Figure 2). Withdrawal of glutamine or inhibition of amino acid uptake through Slc7a5 results in the rapid loss of cMyc protein within minutes[21].

The transcription factor Rfx7 has been identified as a negative regulator of murine NK cell metabolism. NK cells lacking Rfx7 are increased for cell size and mTORC1 activity and have elevated rates of glycolysis and OXPHOS. Interestingly, in $Rfx7^{flox/flox}$ x Vav-Cre mice there are reduced NK cells due to impaired survival of mature NK cells, indicating the importance of controlled metabolism for NK cell homeostasis[37].

Metabolism and NK cell memory

It is now well accepted that NK cells have long-term functions acting alongside the adaptive immune system and can have features of immunological memory[7]. Depending on the context these NK cells have been described as memory-like NK cells, trained NK cells, or adaptive NK cells. A number of studies have investigated whether metabolic processes are important in the formation and enhanced responses of these memory-like NK cells.

In mice, NK cells expressing the activating receptor Ly49H, which recognises a MCMV-specific viral ligand m157, undergo clonal expansion in response to MCMV infection[6,38]. This rapid proliferation leads to reduced mitochondrial fitness characterised by decreased mitochondrial membrane potential and increased levels of mitochondrial reactive oxygen species (mtROS) [16]. Following a contraction phase these virus-specific NK cells persist for months as a self-renewing pool of memory-like NK cells with enhanced responsiveness upon reactivation[6]. An important process in the formation of these memory-like cells is restoring mitochondrial fitness, achieved by removing damaged mitochondria through autophagy, called mitophagy[16]. Pharmacological approaches to induce autophagy during the peak of MCMV infection, such as inhibition of mTORC1 with rapamycin or the activation of AMPK with metformin, lead to increased numbers of memory NK cells[16]. Interestingly, similar roles have been described for mTORC1 and AMPK in the control of murine CD8 T cell memory[39-41].

In humans, HCMV infection results in long term changes in a subset of NK cells, called in this instance adaptive NK cells, that have distinct metabolism and function that have been linked to epigenetic alterations[42-44]. In terms of function, adaptive NK cells in HMCV seropositive donors are less sensitive to innate cytokines, such as IL12 and IL18, but have increased responses through CD16-dependent recognition of antibody-coated target cells[42,43]. CD16 crosslinking on adaptive NK cells induces greater mTORC1 activity compared to non-adaptive NK cells[45]. Adaptive NK cells also have altered metabolism with increased mitochondrial mass and mitochondrial membrane potential along with higher rates of OXPHOS when compared to canonical NK cells [44]. The chromatin-modifying transcriptional regulator, AT-rich interaction domain 5B (ARID5B) has been implicated in the control of enhanced mitochondrial metabolism in these adaptive NK cells[44].

NK cell memory-like responses have also been described in response to cytokine stimulation. Cytokine-induced memory-like (CIML) murine and human NK cells have increased levels of IFNγ production upon restimulation and enhanced cytotoxicity towards tumour cells for periods of weeks to months following initial stimulation[46-49]. Similarly, human NK cells stimulated through the CD16 receptor show memory-like attributes with increased cytotoxicity

towards tumour cells [50]. However, the importance for NK cell metabolism in the generation of these memory-like NK cells or for the enhanced functionality these cell possess has still to be determined.

Metabolism and dysfunctional NK cells in obesity and cancer

There is accumulating evidence that impaired cellular metabolism is a crucial factor leading to dysfunctional NK cells in a range of chronic diseases such as obesity and cancer. Murine and human studies show that obesity impairs NK cell functional responses. The use of diet induced obesity models or genetic obesity models, such as ob/ob mice, have shown that obese mice are more susceptible to mortality from influenza infection, due to lung inflammation. Splenic and lung NK cell numbers and cytotoxicity are substantially reduced in obese mice following infection as compared to lean mice[51]. In obese mice NK cells have impaired anti-tumour responses and fail to control tumour growth[25]. Obese humans, both children and adults, have reduced frequencies of circulating NK cells compared to their lean counter-parts and these NK cells are dysfunctional; they produce less IFNγ, express lower levels of granzyme B and perforin and have reduced cytotoxicity towards tumour target cells[25,52-54]. Recent research has linked this NK dysfunction to impaired cellular metabolism[25,54]. NK cells from obese mice or humans fail to engage a metabolic response when stimulated with cytokine and have substantially reduced metabolic rates compared to NK cells from lean mice or humans[25]. This metabolic dysfunction has been linked to peroxisome proliferator-activated receptor (PPAR)-driven lipid accumulation in NK cells leading to altered gene expression, the downregulation of cMyc and mTORC1 signalling and decreased rates of glycolysis and OXPHOS [25]. NK cells from obese mice and humans fail to kill tumour cells in part because they fail to correctly form a synapse with target cells; the cytotoxic machinery does not traffic to the NK cell–tumour synapse[25]. Indeed, there is evidence that the formation and maintenance of this NK cell–tumour synapse is an energy demanding process[55,56]. Human NK cells polarise mitochondria to the NK cell–tumour synapse and NK cell mitochondrial membrane potential rapidly drops following target cell engagement, consistent with rapid energy utilisation[56,57]. Therefore, altered signalling and defective metabolism are key aspects of NK cell dysfunction in obese individuals.

A reduction in NK cell number and function has also been described in cancer and altered NK cell metabolism is likely to be an important factor in this NK cell dysfunction[58,59]. Tumour

microenvironments are known to be metabolically restrictive and can contain very low concentrations of nutrients such as glucose, the key fuel for NK cells[60-63]. Tumours can also contain high concentrations of lactate and low pH, both of which can impair NK cell anti-tumour functions[64,65]. Indeed, a recent human study showed that lactate produced by colorectal liver metastasis' resulted in a decrease the intracellular pH of tumour infiltrating liver-resident NK cells leading to mitochondrial dysfunction and apoptosis[66]. Additionally, a range of molecules that can actively repress NK cell metabolism are made within tumours. Elevated levels of TGFβ are associated with various cancers[67,68] and TGFβ can directly represses NK cell metabolism through multiple mechanisms including the inhibition of mTORC1 signalling (Figure 3)[14,69]. A recent study showed that tumours in the lung can induce the expression of the gluconeogenesis enzyme fructose bisphosphatase 1 (FBP1) within tumour infiltrating murine NK cells, through a mechanism possibly involving TGFβ[58]. FBP1 directly counteracts flux through glycolysis and the study showed that pharmacological inhibition of FBP1 could achieve partial rescue of NK cell metabolism and effector functions[58]. Other molecules that can potentially repress NK cell metabolism include the oxysterols 25-hydroxycholesterol and 27-hydroxycholesterol, both of which inhibit the activation of Srebp transcription factors, key regulators of NK cell metabolism (Figure 2 and 3)[15,70,71]. Cholesterol is converted to 27-hydroxycholesterol by the enzyme Cyp27a1 that is expressed in most tissues but at high levels in macrophages[72,73]. Circulating levels of 27-hydroxycholesterol have been found to be elevated in breast cancer patients[74]. Cholesterol is converted to 25-hydroxycholesterol by the enzyme Ch25h that is expressed at high levels by inflammatory macrophages and is also expressed by certain tumours including glioblastoma[75-77]. The enzyme Indoleamine 2,3-dioxygenase (IDO) is often highly expressed in tumour cells or in tumour associated cells such as tolerogenic DCs[78,79]. IDO-mediated metabolism of tryptophan and the IDO-derived metabolite L-kynurenine can inhibit human NK cell proliferation and cytotoxicity[80,81]. The L-kynurenine transporter has recently been identified as Slc7a5, which is highly expressed on activated NK cells, suggesting that NK cells are susceptible to the inhibitory effects of L-kynurenine in tumours[21,82].

Targeting NK cell metabolism during cancer immunotherapy

Cellular immunotherapeutic strategies against solid tumours have been less successful than against haematological cancers[83,84]. Solid tumours suppresses anti-tumour immune responses through multiple mechanisms including creating a metabolically restrictive microenvironment.

To support high metabolic rates tumour cells consume large quantities of fuels including glucose and glutamine, which can lead to the depletion of these fuels from the tumour microenvironment[60-63,85-88]. Understanding the metabolic requirements of activated NK cells will lead to strategies to modulate NK cells to function more effectively within this nutrient restrictive tumour microenvironment. For instance, coordinated use of chemotherapy and/or radiotherapy may be an important mechanism to enhance NK cell anti-tumour responses. Inducing cell death pathways in a proportion of tumour cells will reduce the demand for glucose and therefore increase glucose levels in the tumour microenvironment, facilitating glycolysis and robust anti-tumour responses of adoptively transferred NK cells. CB-839, a drug targeting glutaminase, the first enzyme of glutaminolysis, is in multiple phase 1 clinical trials in patients with advanced solid tumours (NCT02861300, NCT02071862). Inhibition of glutaminase does not inhibit NK cells anti-tumour functions but this inhibitor will reduce glutamine consumption within tumours. Increased glutamine availability would support glycolytic metabolism in NK cells by sustaining cMyc signalling (see Figure 2)[21]. Inhibiting cMyc degradation through the inhibition of GSK3 could be another strategy to sustain NK cell metabolism and function within the tumour microenvironment[21]. Indeed, GSK3 inhibitors have been shown to increase anti-tumour NK cell responses in multiple studies, though any link to cellular metabolism was not investigated[89,90].

There are a number of cancer immunotherapies that involve engineering NK cells prior to adoptive transfer into patients. These include ‘off the shelf’ strategies where engineered NK cells are stored in cell banks ready for use[91]. For instance, the NK cell line NK92 and engineered induced pluripotent stem cells (iPSCs) are being developed for this purpose[92,93]. Here, there is the possibility to also engineer NK cell metabolism to be resistant to the metabolically restrictive tumour microenvironment and to the molecules generated by tumours that can supress NK cell metabolism and function (Figure 3).

Overall, it is now clear that cellular metabolism is crucial for the normal function of NK cells and future research is in this area is likely to reveal new and exciting therapeutic opportunities.

	NK cells	CTL	TCR-activated T cells
Metabolism			
Aerobic Glycolysis	High[12,15]	High[34]	High[34]
OXPHOS	High[12,15] Citrate-malate-shuttle	Low[94]	High[95] TCA cycle
Overall metabolic phenotype	Glycolytic[12,15]	Glycolytic[34]	Glycolytic[34,95]
Key fuels	Glucose [12,15,21]	Glucose [34]	Glucose and Glutamine [34,95]
Metabolic regulators			
mTORC1	Glycolysis and OXPHOS[12]	Glycolysis[34]	Glycolysis[34,95]
HIF1	Not required[21]	Glycolysis[34]	Not required[34,95]
cMyc	Glycolysis, OXPHOS, and mitogenesis[21]	Iron metabolism[96]	Glycolysis, Glutaminolysis but not OXPHOS[95,97]
Srebp	Glycolysis and OXPHOS[15]	Not studied	Glycolysis and OXPHOS[98]
Glutamine	Regulates mTORC1 and cMyc[21]	Regulates mTORC1 and cMyc[35,99]	Regulates mTORC1 and cMyc [35,95,99]

Table 1: Comparison of cellular metabolism and metabolic regulators in cytokine stimulated NK cells (IL2/IL12), TCR stimulated T cells and IL2-differentiated CD8 CTL.

Box 2: Important questions that remain in relation to NK cell metabolism

1. Why do activated NK cells use the citrate malate shuttle rather than the TCA cycle to drive OxPhos?

2. While research has advanced our understanding of the metabolism of circulating NK cells from the blood and spleen, nothing is known about the metabolic pathways used by tissue resident NK cells and the impact cellular metabolism has on their function.

3. NK cell metabolism has largely been studied *in vitro*. It will be important to investigate NK cell metabolism *in vivo*, especially in immune microenvironments where levels of nutrients may be limiting.

4. In CD8 T cells there is plasticity with metabolic pathways; under conditions of glucose limitation T cells adapt and use more glutamine. It will be interesting to determine how NK cell metabolic pathways adapt to metabolically restrictive environments.

5. Can NK cell metabolism be modulated to provide more robust NK cell based immunotherapy?
6. Are metabolic alterations important for NK cell memory-like responses?

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Box 1: Metabolism configured to support energy homeostasis and biosynthesis.

Cellular metabolism can be configured to efficiently generate energy in the form of ATP. Glucose is metabolised by glycolysis to pyruvate, which in turn can be used to fuel oxidative phosphorylation (OXPHOS). Pyruvate is converted to Acetyl-CoA in the mitochondria, which feeds into the tricarboxylic acid (TCA) cycle generating the reducing equivalents NADH and $FADH_2$ (not shown) that transfer electrons (e') to complex I and II of the electron transport chain (ETC) leading to a proton (H^+) gradient across the mitochondrial inner membrane that is used to drive the activity of ATP synthase (AS). Additional fuels can be used for OXPHOS and mitochondrial energy production. Fatty acids are broken down in the mitochondria through a process called β-oxidation that yields Acetyl-CoA. Amino acids, most notably glutamine, can also be metabolised for the purposes of ATP production. Glutamine is metabolised via glutaminolysis to a-ketoglutarate (αKG) that can feed into the TCA cycle. In addition to fuelling ATP synthesis glucose and glutamine can also be metabolised and used to support biosynthetic processes. Intermediates of glycolysis can be diverted into metabolic pathways to generate biosynthetic precursors important for the synthesis of lipids, nucleotides and proteins. Similarly, the TCA intermediate citrate can be exported from the mitochondria and metabolised to support the biosynthesis of these molecules. Cells will metabolise glucose to lactate when there is no oxygen available for OXPHOS (anaerobic glycolysis, not shown). Cells with high biosynthetic demands will also metabolise glucose to lactate in the presence of oxygen (aerobic glycolysis) while also feeding pyruvate into the mitochondria to support OXPHOS. This metabolic configuration is of benefit because it allows for high flux through glycolysis leading to elevated levels of glycolytic intermediates that can be diverted towards biosynthesis, while also generating large amounts of ATP.

Figure 1: The Citrate Malate Shuttle

Glucose is metabolised to pyruvate and then to mitochondrial Acetyl-CoA yielding reduced NADH. Acetyl-CoA combines with oxaloacetate to make mitochondrial citrate, which is then exported from the mitochondria through the SLC25A1 citrate/malate antiporter. In the cytosol, citrate is metabolised by ATP citrate lyase generating cytosolic Acetyl-CoA and OAA, which can be converted to malate in a reaction that oxidises NADH to yield NAD+. Maintaining NAD+ in the cytosol is essential for glycolysis to continue as it is an essential cofactor for the glycolytic enzyme GAPDH. Malate re-enters the mitochondria through SLC25A1 where it is converted back to OAA yielding a second NADH molecule in the mitochondria. OAA can then react with another glucose derived Acetyl-CoA to form another molecule of citrate, thus completing the cycle. In this way the citrate malate shuttle generates 2 molecules of NADH per molecule of pyruvate that enters the mitochondria, which feed into the electron transport chain (ETC) driving OXPHOS and ATP synthesis via ATP synthase (AS). In contrast, the TCA cycle makes 4 NADH plus 1 $FADH_2$ (not shown). However, the citrate malate shuttle has the additional output of cytosolic Acetyl-CoA that can be used as the substrate for acetylation reactions or lipid synthesis.

Figure 2: Key regulators of NK cell metabolism

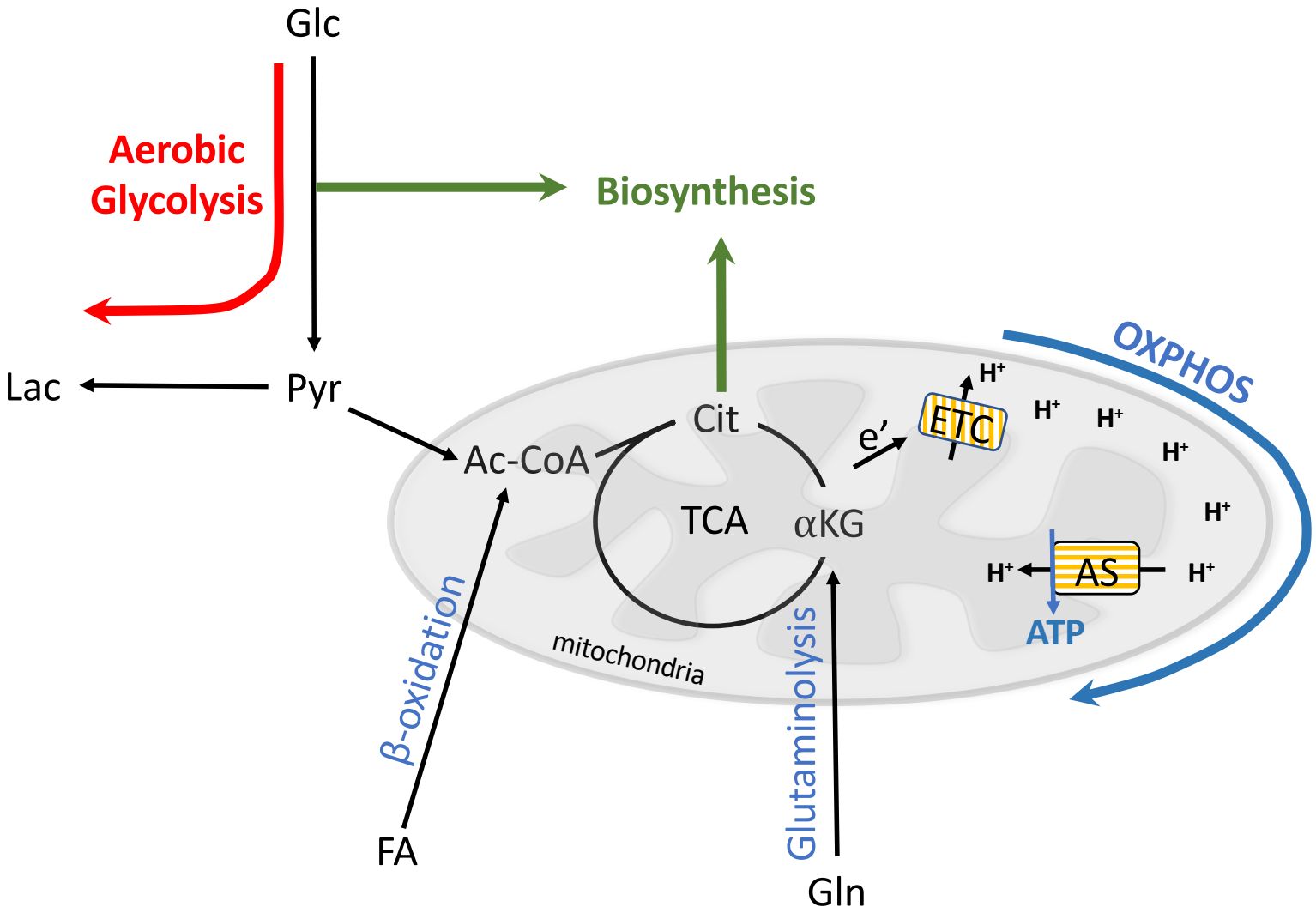
Two transcription factors have been identified as key metabolic regulators in murine NK cells, cMyc and Srebp. cMyc levels are controlled by the balance of cMyc protein synthesis and GSK3-targeted degradation of cMyc in the proteasome. At early time points following IL2/IL12 stimulation (minutes) cMyc protein levels accumulate in an mTORC1-dependent manner. mTORC1 is activated by IL2 signalling and can increase cMyc protein translation through promoting 5' cap-dependent translation. IL2/IL12 stimulation also induces the expression and activity of the amino acid transporter SLC7A5. At later time points following IL2/IL12 stimulation (hours), amino acid uptake through SLC7A5 is essential to sustain cMyc protein levels while mTORC1 activity is not required. Withdrawal of glutamine, which is exported through SLC7A5 to sustain SLC7A5-dependent amino acid import, or the direct inhibition of SLC7A5-mediated transport both result in rapid loss of cMyc protein expression

within minutes. While leucine uptake through SLC7A5 is required for mTORC1 activity at these later time points, leucine withdrawal is not sufficient to reduce cMyc expression. Therefore, the uptake of other amino acids, such as methionine, phenylalanine, tyrosine, arginine or tryptophan, through SLC7A5 is important in the regulation of cMyc. The activity of Srebp is greatly increased following IL2/IL12 stimulation in murine NK cells. While mTORC1 is required for optimal Srebp activation other mechanisms also contribute to Srebp activity in cytokine stimulated NK cells. cMyc is important for the expression of the metabolic machinery in NK cells and Srebp controls the metabolic configuration, glucose metabolism through the citrate malate shuttle.

Figure 3: Mechanisms disrupting NK cell metabolism in cancer and obesity.

In cancer, low levels of nutrients in the tumour microenvironment will inhibit NK cell metabolism either directly or through altering the activity of nutrient sensing signalling pathways. Limiting glucose, the key fuel for NK cells, will directly impact upon glycolysis and OXPHOS rates. Restricted availability of glucose, glutamine or amino acids, such as leucine, can impact upon the activity of the metabolic regulators mTORC1 and cMyc and so inhibit NK cell metabolism. TGFβ can inhibit NK cell metabolism through multiple mechanisms; the inhibition of mTORC1, inhibition of mitochondrial metabolism through canonical TGFβ signalling, and TGFβ has been linked to the induction of the gluconeogenesis enzyme FBP1 that directly counteracts glycolysis. The oxysterols 25HC and 27HC are elevated in certain cancers and can inhibit Srebp activation, which would lead to inhibition NK cell metabolism. In obesity, PPAR activation by lipid ligands leads to lipid accumulation in NK cells, the inhibition of mTORC1 signalling and a state of metabolic paralysis.

Box 1


Glc
Aerobic Glycolysis
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Lac
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Cit
Ac-CoA
TCA
αKG
e'
ETC
OXPHOS
H+
AS
ATP
mitochondria
β-oxidation
FA
Glutaminolysis
Gln

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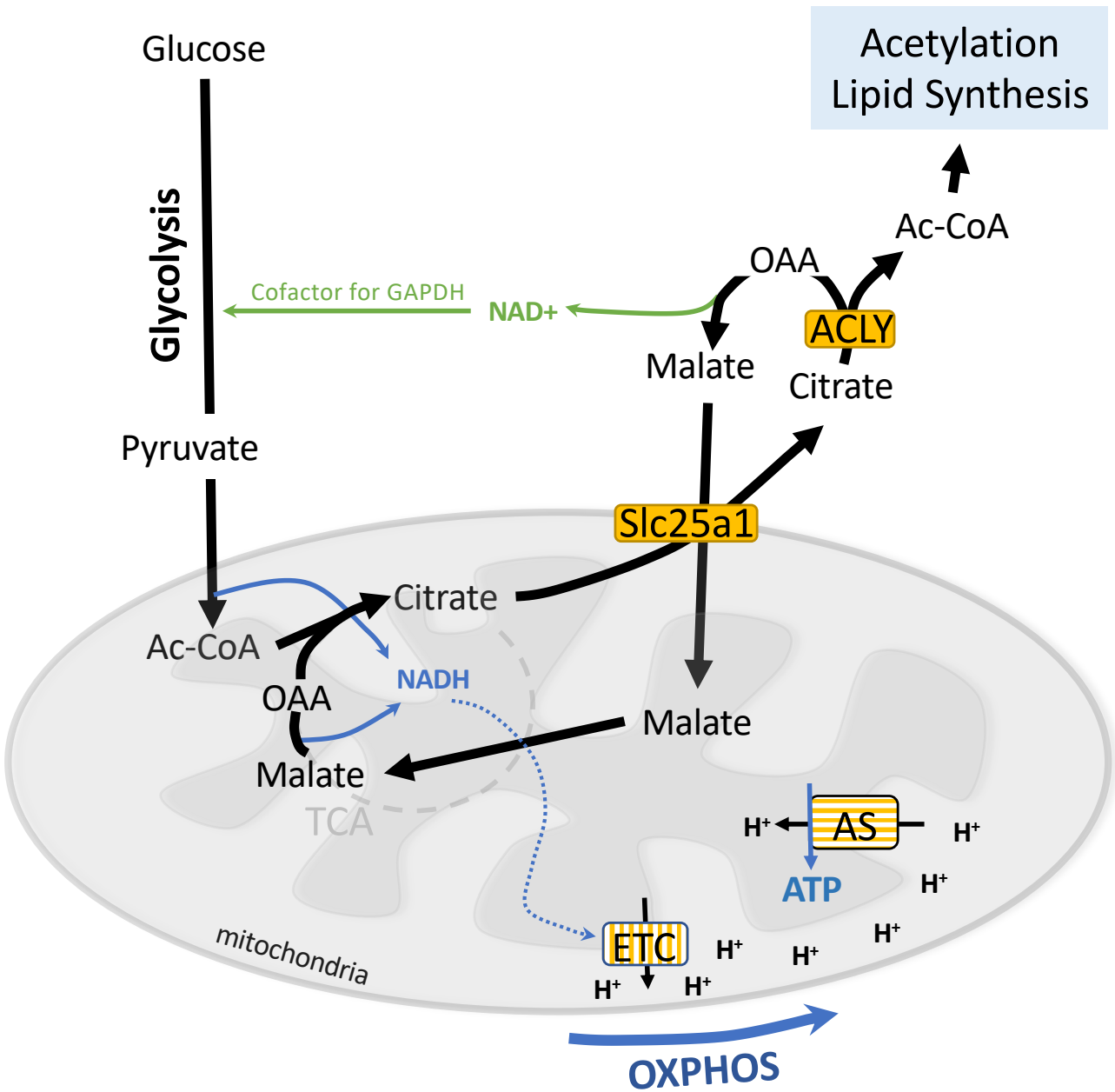
Figure 1


Glucose
Acetylation
Lipid Synthesis
Glycolysis
Ac-CoA
OAA
Cofactor for GAPDH
NAD+
ACLY
Malate
Citrate
Pyruvate
Slc25a1
Citrate
Ac-CoA
NADH
OAA
Malate
Malate
TCA
H+
AS
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H+
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mitochondria
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OXPHOS

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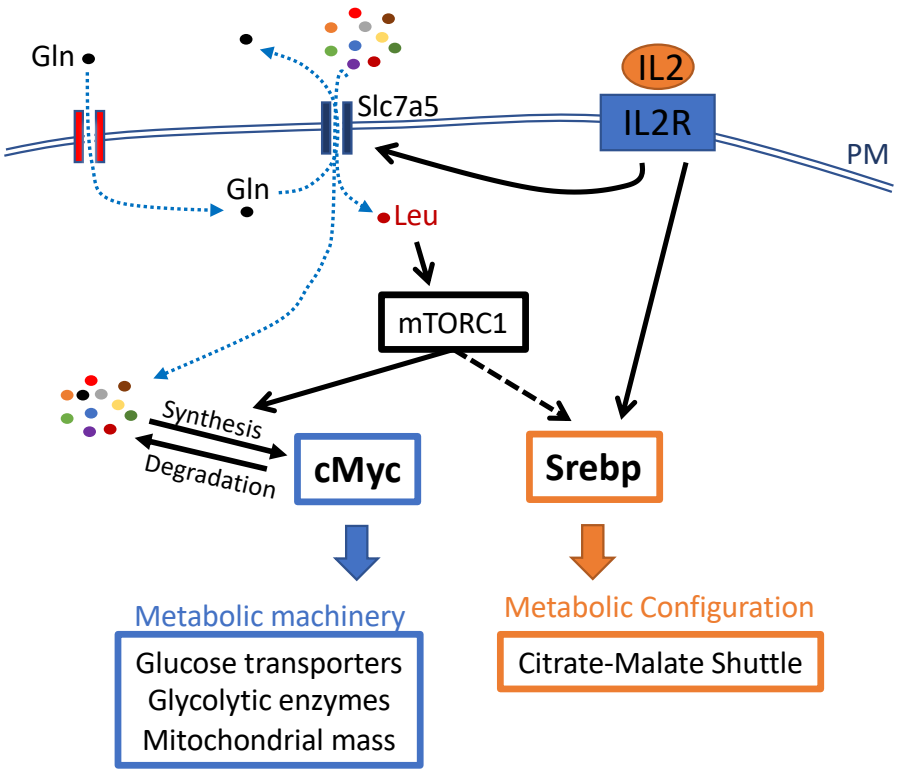
Figure 2


Gln
Slc7a5
IL2
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PM
Gln
Leu
mTORC1
Synthesis
Degradation
cMyc
Srebp
Metabolic machinery
Glucose transporters
Glycolytic enzymes
Mitochondrial mass
Metabolic Configuration
Citrate-Malate Shuttle

Figure 2: Key regulators of NK cell metabolism

Two transcription factors have been identified as key metabolic regulators in NK cells, cMyc and Srebp. cMyc levels are controlled by the balance of cMyc protein synthesis and GSK3-targeted degradation of cMyc in the proteasome. At early time points following IL2/IL12 stimulation (minutes) cMyc protein levels accumulated in an mTORC1 dependent manner. mTORC1 is activated by IL2 signalling and can increase cMyc protein translation through promoting 5' cap-dependent translation. IL2/IL12 stimulation also induces the expression and activity of the amino acid transporter SLC7A5. At later time points following IL2/IL12 stimulation (hours), amino acid uptake through SLC7A5 is essential to sustain cMyc protein levels while mTORC1 activity is not required. Withdrawal of glutamine, which is essential for SLC7A5 activity, or the direct inhibition of SLC7A5 mediated transport both result in rapid loss of cMyc protein expression (minutes). While leucine uptake through SLC7A5 is required for mTORC1 activity, at these later time points, leucine withdrawal is not sufficient to reduce cMyc expression. Therefore, the uptake of other amino acids through SLC7A5 are important in the regulation of cMyc, such as methionine, phenylalanine, tyrosine, arginine or tryptophan. The activity of Srebp is greatly increased following IL2/IL12 stimulation in murine NK cells. While mTORC1 is required for optimal Srebp activation other mechansims also contribute to Srebp activity in cytokine stimulated NK cells. cMyc is important for the expression of the metabolic machinery in NK cells and Srebp controls the metabolic configuration, glucose metabolism through the citrate malate shuttle.

Figure 3

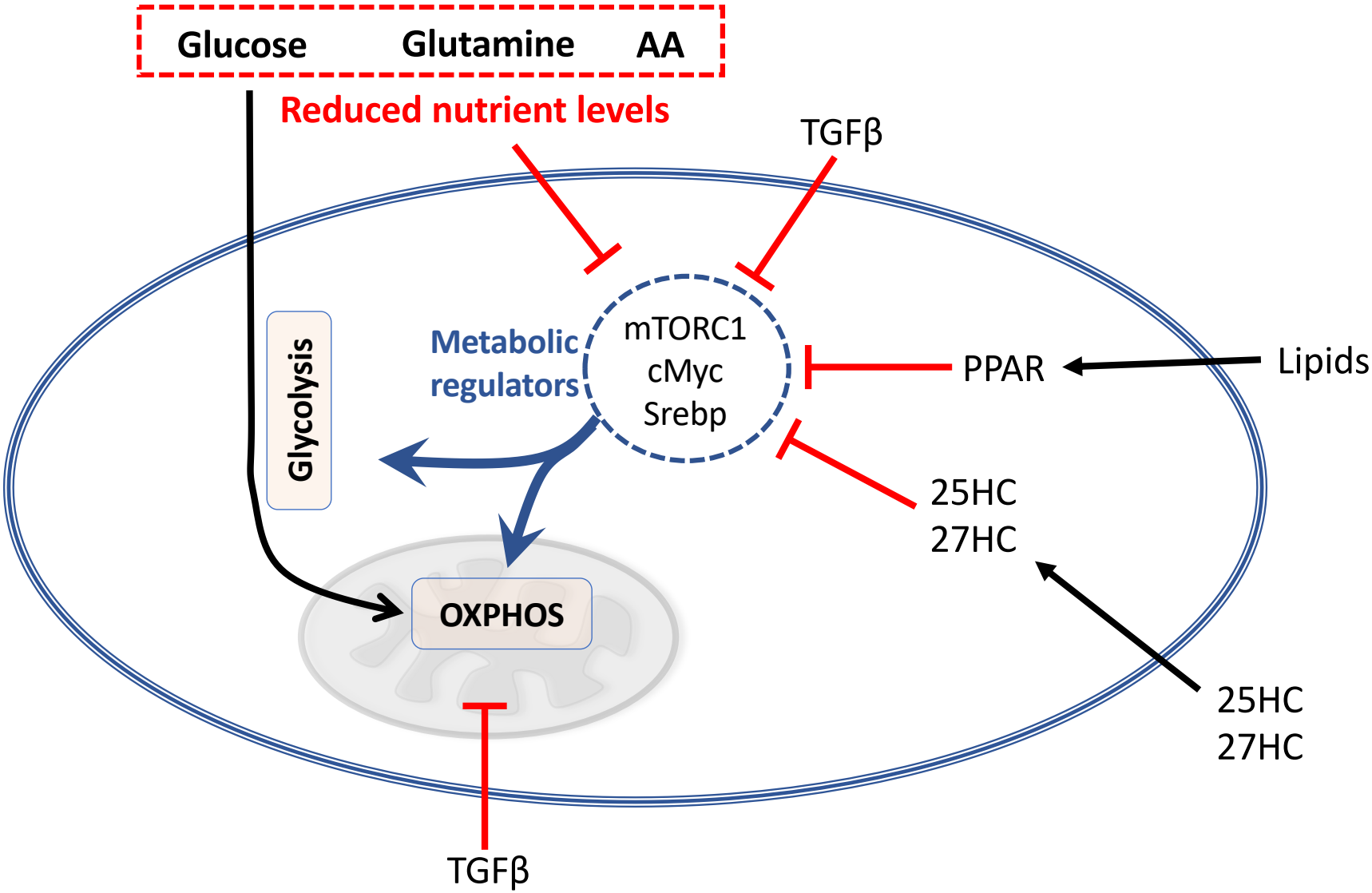
Glucose
Glutamine
AA
Reduced nutrient levels
TGFβ
Glycolysis
Metabolic regulators
mTORC1
cMyc
Srebp
PPAR
Lipids
25HC
27HC
OXPHOS
25HC
27HC
TGFβ

Figure 3: Mechanisms disrupting NK cell metabolism in cancer and obesity.
In cancer, low levels of nutrients (red box) in the tumour microenvironment will inhibit NK cell metabolism. Limiting glucose, the key fuel for NK cells, will directly impact upon glycolysis and OXPHOS rates. Restricted availability of glucose, glutamine or amino acids (AA), such as leucine, can impact upon the activity metabolic regulators mTORC1 and cMyc and so inhibit NK cell metabolism. TGFβ can inhibit NK cell metabolism through multiple mechanisms; the inhibition of mTORC1, inhibition of mitochondrial metabolism through canonical TGFβ signalling, TGFβ has been linked to the induction of the gluconeogenesis enzyme FBP1 that directly counteracts glycolysis. The oxysterols 25HC and 27HC are elevated in certain cancers and can inhibit Srebp activation which would lead to inhibition NK cell metabolism. In obesity, PPAR activation by lipid ligands leads to lipid accumulation in NK cells, the inhibition of mTORC1 signalling and a state of metabolic paralysis.