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mTORC1-dependent metabolic reprogramming is a prerequisite for Natural Killer cell effector function

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Abstract

The mammalian target of rapamycin complex 1 (mTORC1) is a key regulator of cellular metabolism and also has fundamental roles in controlling immune responses. Emerging evidence suggests that these two functions of mTORC1 are integrally linked. However, little is known regarding mTORC1 function in controlling the metabolism and function of natural killer (NK) cells, lymphocytes that play key roles in anti-viral and anti-tumour immunity. This study investigated the hypothesis that mTORC1-controlled metabolism underpins normal NK cell pro-inflammatory function. We demonstrate that mTORC1 is robustly stimulated in NK cells activated *in vivo* and *in vitro*. This mTORC1 activity is required for the production of the key NK cell effector molecules IFN γ , important in delivering antimicrobial and immunoregulatory functions, and granzyme B, a critical component of NK cell cytotoxic granules. The data reveal that NK cells

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²Abbreviations

mTORC1 mammalian Target of Rapamycin complex 1

NK Natural Killer

OxPhos oxidative phosphorylation

Gnzb Granzyme B

2DG 2-Deoxy-glucose

ECAR extracellular acidification rate

OCR oxygen consumption rate

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undergo dramatic metabolic reprogramming upon activation, up-regulating rates of glucose uptake and glycolysis, and that mTORC1 activity is essential for attaining this elevated glycolytic state. Directly limiting the rate of glycolysis is sufficient to inhibit IFN γ production and granzyme B expression. This study provides the highly novel insight that mTORC1-mediated metabolic reprogramming of NK cells is a prerequisite for the acquisition of normal effector functions.

Introduction

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that forms two protein complexes, mTORC1 and mTORC2, with roles in regulating immunological systems. mTORC1 in particular has emerged as a central regulator of immune responses. mTORC1 activity has been ascribed important roles in the regulation of a wide range of immune cells both in the innate and adaptive arms of immunity (1). It is now becoming clear that mTORC1-regulated cellular metabolism is crucial to the immunoregulatory function of this kinase complex in immune cells. Indeed, new evidence is emerging that metabolism plays a fundamental role in dictating immune cell differentiation and function. In cytotoxic T lymphocytes (CTL), mTORC1 activity is required to maintain the high rates of glycolysis that are essential to sustain normal migratory patterns and effector functions (2). mTORC1-regulated glycolysis is also linked to normal differentiation of effector CD4 T cells (3). Additionally, mTORC1 activity is involved in the initiation of lipid synthesis in T cell receptor stimulated CD8 T cells, a process that is required for blastogenesis and effective T cell activation (4). Similarly in innate immune cells, the immunoregulatory functions of mTORC1 have been linked to metabolic regulation. By regulating the level of glycolysis in activated dendritic cells, mTORC1 controls dendritic cell (DC) survival and ultimately the T cell response (5). Therefore, immune cell metabolism is integrally linked to the important immunoregulatory functions of mTORC1.

Remarkably, the role played by mTORC1 in controlling Natural Killer (NK) cell metabolism and function has not yet been defined. NK cells are lymphocytes that bridge innate and adaptive immunity with an important role in anti-viral and anti-tumour immune responses. Although NK cells are critical in early immune responses, they also regulate the ensuing adaptive immune response through the release of cytokines that modulate the downstream immune response, notably interferon (IFN) γ and tumour necrosis factor (TNF) α and by modulating DC numbers (6). More recently, it has been appreciated that NK cells can undergo clonal expansion and thus function in parallel with the adaptive immune system. In particular, NK cells can be induced to express the high affinity IL2 receptor in response to IL12, thus allowing them to respond to low doses of T cell derived IL2 (7, 8). Indeed, IL2 produced by T cells is critical for modulating NK cell activation in response to various pathogenic infections (7-9). Given our previous work linking mTORC1-regulated metabolism to normal CTL functions and the observation that the mTORC1 inhibitor rapamycin can disrupt NK cell effector functions (10-12), we hypothesised that mTORC1-regulated metabolism might be fundamental in the control of NK cell responses.

Indeed, the present study demonstrates that mTORC1 activity is essential for glycolytic reprogramming of activated NK cells and that this metabolic shift is a prerequisite for

normal NK cell effector functions, such as the production of IFN γ and increased expression of granzyme B.

Materials and Methods

Mice

C57BL/6J mice were purchased from Harlan (Bicester, U.K.) or the Taconic Laboratories (Germantown, NY) and maintained in compliance with Irish Department of Health and Children regulations and with the approval of the University of Dublin's ethical review board or in accordance with institutional guidelines for animal care and use at Brown University (Providence, RI).

In vivo NK cell activation with poly(I:C)

Mice were injected IP with 200 μ g of poly(I:C) in PBS (Invivogen) +/- 0.6 mg/kg rapamycin, (blood levels 71-94 ng/ml) (Fisher Scientific) or 1 g/kg 2-deoxyglucose (Sigma). Mice were sacrificed after 12 or 24 hours as indicated. Spleens were harvested and NK cells analysed.

Cell culture

Splenocytes were isolated and cultured in IL-15 (25 ng/ml, Peprotech) at 37 °C for 5 days. On day 5, the cells were supplemented with IL-15 (25 ng/ml) and cultured for a further 2 days. On day 7, cultured NK cells were stimulated for 18 hours with IL-2 (20 ng/ml, NCI preclinical repository) and/or IL-12 (10 ng/ml, Miltenyi Biotech) cytokines. Low dose IL15 (5 ng/ml) was added as a survival factor to unstimulated cultures or those stimulated with IL12 alone. Experiments were carried out in the presence or absence of 2-deoxyglucose (2DG, Sigma), rapamycin (20 nM, Fisher) and/or oligomycin (2 μ M, Sigma) inhibitors. NK cells were MACS purified using a NK isolation kit (Miltenyi Biotech) from day 7 cultures for biochemical analyses. Where indicated, NK cells were cultured in glucose-free medium supplemented with 10% dialyzed FCS (Fisher), 2 mM Glutamine (Invitrogen/Biosciences), 1 mM Sodium Pyruvate (Gibco), 1x concentration of MEM Vitamin Cocktail (Invitrogen/Biosciences), 1x concentration of selenium/insulin/transferrin Cocktail (Invitrogen/Biosciences), 50 μ M β -mercaptoethanol (Sigma) and 1% Penicillin/Streptomycin (Invitrogen/Biosciences) and with either glucose (10 mM) or galactose (10 mM).

Flow cytometric analysis

Cells (between 1×10^6 and 3×10^6 cells) were stained for 30 min at 4°C with saturating concentrations of antibody. Antibodies used were as follows: eFluor 450 NK1.1 (PK136), eFluor 660 NKp46, PerCP-eFluor 710 NKp46 (29A1.4), PE NKp46 (29A1.4), FITC CD3 (145-2C11), FITC TCR β , APC TCR β (H57-597), PE-Cy7 CD69 (H1.2F3), PerCP-Cy5.5 CD69 (H1.2F3), APC-Cy7 CD25 (PC61), APC CD71 (R17217) PE CD98 (RL388), APC IFN γ (XMG1.2), PE-Cy7 IFN- γ (XMG1.2), PE-Cy7 Granzyme B (NGZB), purchased from eBioscience and BD Pharmingen. Live cells were gated according to their forward scatter (FSC-A) and side scatter (SSC-A), single cells selected based on FSC-W and FSC-A and NK cells identified as NKp46⁺, NK1.1⁺, CD3⁻ cells. For intracellular cytokine staining, endocytosis was blocked using golgi plug (BD Pharmingen) for four hours. Cells were then

fixed and permeabilised using Cytofix/Cytoperm reagent (BD Pharmingen) as per manufacturer's instructions. Data were acquired on either a FACSCanto, a LSR Fortessa, or a FACSCalibur (Becton Dickinson) and analyzed using FlowJo software (TreeStar).

Phospho-S6 ribosomal protein intracellular staining

In vivo experiments: cells were fixed and stained as described previously (41) using PE anti-phospho-S6 ribosomal protein Ser 235/236 (eBiosciences). *In vitro* experiments: cells were fixed and stained as described previously (42) using anti-phospho-S6 ribosomal protein Ser 235/236 (Cell Signaling Technologies) and PE-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch).

Western blot analysis

Cells were lysed (2x10⁷/ml) in Tris lysis Buffer containing 10 mM Tris pH 7.05, 50mM NaCl, 30mM Na pyrophosphate, 50mM NaF, 5μM ZnCl₂, 10% Glycerol, 0.5% Triton, 1μM DTT and protease inhibitors. Lysates were centrifuged (4°C, 16,000g for 10 min) and separated by SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with antibodies recognizing phospho-Akt^{S473} phospho-S6 ribosomal protein^{S235/236}, phospho-S6K^{T389}, phospho-GSK3α/β^{S21/9} and Total Akt (Cell Signaling Technologies).

Quantitative real time PCR

Cultured NK cells were purified by magnetic bead sorting using a NK cell isolation kit (Milyenyi Biotech) prior to stimulations. RNA was extracted using the RNeasy RNA purification mini kit (QIAGEN) according to manufacturer's protocol. Purified RNA was reverse-transcribed using the qScript cDNA synthesis kit (Quanta Biosciences). Real time PCR was performed in triplicates in 96 well plate using iQ SYBR Green-based detection on a ABI 7900HT fast qPCR machine. For the analysis of mRNA levels the derived values were normalized to Rplp0 mRNA levels.

Primers:

Rplp0 forward: 5'-CATGTCGCTCCGAGGGAAG-3',

Rplp0 reverse: 5'-CAGCAGCTGGCACCTTATTG-3',

Ldha forward: 5'-CTGGGAGAACATGGCGACTC-3',

Ldha reverse: 5'-ATGGCCCAGGATGTGTAACC-3',

Glut1 forward: 5'-GGAATCGTCGTTGGCATCCT-3',

Glut1 reverse: 5'-CGAAGCTTCTTCAGCACACTC-3',

Hex2 forward: 5'-TCGCCTGCTTATTCACGGAG-3',

Hex2 reverse: 5'-TCGCCTGCTTATTCACGGAG-3'

Ifng forward: 5' ACGCTACACACTGCATCTTG 3'

Ifng reverse: 5' GTCACCATCCTTTTGCCAGTT C 3'

OCR and ECAR measurement

A XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) was used for real-time analysis of the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of NK cells cultured under various conditions. In brief, purified NK cells were adhered to CellTaq (BD Pharmingen) coated XF 24-well microplate (Seahorse Bioscience) at 750,000 cells per well, 10^7 cells/ml. Sequential measurements of ECAR and OCR following addition of the inhibitors (Sigma-Aldrich) oligomycin (2 μ M), rotenone (100 nM) plus antimycin (4 μ M) and 2-deoxyglucose (2DG) (30 mM) allowed for the accurate calculation of oxygen consumption due to OxPhos and acidification due to glycolysis.

Glucose uptake

3×10^6 splenocytes or 0.5×10^6 cultured NK cells were washed and incubated at 37 °C for 15 min in glucose-free media supplemented with 10% dialyzed FCS (Fisher), 2 mM Glutamine (Life technologies), 1 mM Sodium Pyruvate (Gibco), 1x concentration of MEM Vitamin Cocktail (Life technologies), 1 \times concentration of Selenium/Insulin Cocktail (Life technologies), 50 μ M β -mercaptoethanol (Sigma) and 1% Penicillin/Streptomycin (Life technologies). Splenocytes and cultured NK cells were then incubated at 37°C for a further 2 hours or 1 hour, respectively, in supplemented glucose-free media containing the fluorescently labelled glucose analogue 2-NBDG (Life technologies) at a final concentration of 50 μ M before analysis using flow cytometry.

Statistical analysis

GraphPad Prism 6.00 for Macintosh (GraphPad Software) was used for statistical analysis. A one-way ANOVA test was used throughout with the Tukey test for multiple comparisons. A students t-test was used when there were only 2 data sets for comparison. For comparison of relative IFN γ MFI values a one-sample t-test was used to calculate P values with the theoretical mean set to 1.00.

Results

mTORC1 activity is required for NK cell IFN γ production *in vivo*

NK cells are activated *in vivo* in response to viral infections. Polyinosinic:polycytidylic acid (poly(I:C)), a synthetic dsRNA analogue, is often used as a mimic of viral infection (13). To investigate whether NK cell activation *in vivo* is associated with increases in mTORC1 activity, mice were injected with poly(I:C) and splenocytes were isolated for analysis. There was a dramatic increase in phosphorylated S6 ribosomal protein (pS6), a readout of mTORC1 signalling, in NK cells analysed 12 hours and 24 hours after poly(I:C) injection (Fig. 1A-B). In contrast, poly(I:C) did not increase pS6 in T cells (Fig. 1A). To assess the importance of mTORC1 activity in poly(I:C)-activated NK cells, rapamycin was co-administered with poly(I:C) in mice. Rapamycin did not affect NK cell activation as measured by CD69 expression (Fig. 1C) but it had a significant effect on IFN γ production in splenic NK cells (Fig. 1D-E). Inhibition of mTORC1 did not have a global effect on cytokine production as poly(I:C)-stimulated increases in TNF α expression were not affected

by rapamycin treatment (Fig. 1F-G). When poly(I:C) was administered to splenocytes *in vitro* IFN γ production was similarly increased in NK cells in a mTORC1-dependent manner (Fig. 1H).

It was apparent that poly(I:C) does not result in T cell activation *in vivo* or *in vitro* as measured by pS6 and CD69 levels (Fig. 1A and data not shown). Given the evidence that NK cell activation can be closely linked to T cell responses and the observations that T cell-derived IL2 and the innate cytokine IL12 are important for optimal NK cell activation in response to various infections (7, 8), the effect of supplementing poly(I:C)-stimulated splenocytes with IL2 and IL12 was investigated. Poly(I:C) plus IL2/12-stimulated NK cells had significantly enhanced levels of IFN γ production compared to poly(I:C) alone both in terms of the frequency of IFN γ + NK cells and the amount of IFN γ protein expressed per NK cell, as measured by the mean fluorescence intensity (MFI) of IFN γ staining (Fig. 1I-J). Importantly, these elevated levels of IFN γ were inhibited by rapamycin treatment (frequency and MFI) (Fig. 1J). Thus, mTORC1 activity is important for IFN γ production in NK cells activated in response to innate stimuli and to the T cell-derived cytokine IL2.

The role for mTORC1-controlled cellular metabolism in dictating NK cell function was supported by the observation that NK cells producing IFN γ had increased metabolic activity. A comparison of poly(I:C)-activated cells, revealed that IFN γ -producing NK cells were larger, based on forward scatter, than those not producing IFN γ (Fig. 2A). Additionally, in the presence of rapamycin, the induced increased cell size of poly(I:C)-activated NK cells was impaired (Fig. 2B-C). A requirement for the increased cellular growth associated with blastogenesis is the provision of essential nutrients including the key fuel source glucose. Consistent with this, the larger NK cells in poly(I:C)-treated mice had elevated rates of glucose uptake, as measured using the fluorescent 2-deoxyglucose analogue 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (NBDG). They also had increased expression of important nutrient receptors including CD71 (transferrin receptor) and CD98 (a component of the L-amino acid transporter) (Fig. 2D). Rapamycin treatment inhibited the induction of elevated glucose uptake and the increased expression of CD71 and CD98 in NK cells (Fig. 2E). Thus, mTORC1 signalling in activated NK cells is required to allow NK cells up-regulate nutrient uptake for normal blastogenesis and also for the induction of IFN γ expression.

mTORC1 controls glycolytic metabolism in activated NK cells

The data suggested that mTORC1-dependent production of the pro-inflammatory cytokine IFN γ in activated NK cells is linked to the metabolic activity of NK cells. Pro-inflammatory immune cells tend to be characterised by elevated levels of glucose uptake and increased rates of glycolysis, metabolising glucose primarily to lactate rather than through oxidative phosphorylation (OxPhos) in the mitochondria (14). To investigate whether changes in cellular metabolism were associated with the pro-inflammatory functions of activated NK cells, we performed detailed metabolic analysis of purified NK cells following cytokine stimulation, measuring rates of glycolysis and OxPhos. Due to the numbers of cells required for these analyses, NK cells were first expanded for 7 days from isolated splenocytes (15). This was achieved using low dose IL15, a cytokine required for DC-mediated NK cell

priming *in vivo* (16-18). Indeed, this culture process provided large numbers of ‘primed’ NK cells that responded robustly to subsequent stimulation with distinct increases in cell size, CD69 expression and IFN γ production as expected (Supplementary Fig. 1).

Purified IL15-cultured NK cells were stimulated with IL2 and/or IL12 and rates of glycolysis and oxidative phosphorylation measured. IL2 stimulation increased the rate of glycolysis (measured as the extracellular acidification rate - ECAR) two-fold. Alone, IL12 had no effect on the rate of glycolysis, but the combination of IL2 and IL12 resulted in a synergistic increase in NK cell glycolysis (Fig. 3A). The synergistic action of IL2 and IL12 reflected IL12-induced expression of the high affinity IL2 receptor subunit CD25 (Fig. 3B) (7, 8), which facilitates enhanced IL2 signalling and substantially elevated mTORC1 activity (Fig. 3C). Consistent with increased glycolysis, IL2/12-stimulated NK cells had increased levels of glucose uptake (Fig. 3D). IL2/12 stimulation also increased the rate of OxPhos (measured as the oxygen consumption rate – OCR) in NK cells (Fig. 3E). Although increases in both glycolysis and OxPhos were observed in NK cells following cytokine stimulation, overall there was a shift in the balance of NK cell metabolism from OxPhos to glycolysis (Fig. 3F). These data clearly demonstrate that NK cells undergo distinct metabolic reprogramming following cellular activation and highlight a key role for the adaptive cytokine IL2 in promoting elevated levels of NK cell glucose uptake and glycolysis.

Metabolic analysis of IL2/12-stimulated NK cells in the presence of rapamycin revealed that mTORC1 activity was required for elevated levels of glycolysis but not OxPhos (Fig. 3G-H). This reflects that mTORC1 signalling is required to promote the expression of the glucose transporter, Glut1, and key rate limiting glycolytic enzymes, Hexokinase 2 (Hex2) and Lactate dehydrogenase a (Ldha) (Fig. 3I). Given that the alternative mTOR complex, mTORC2, has also been described to control cellular glycolysis through regulating the activity of the Akt serine/threonine kinase (19) and the reports that prolonged rapamycin treatment can destabilise the mTORC2 signalling complex (20), it was important to demonstrate that the observed metabolic effects of rapamycin in NK cells were not due to altered mTORC2 activity. Therefore the activity of mTORC2 was investigated by immunoblot analysis of IL2/12-stimulated NK cells in the presence or absence of rapamycin. The data show that in cytokine-activated NK cells rapamycin treatment had no effect on the phosphorylation of the mTORC2 substrate Akt on serine 473, or on Akt kinase activity, as measured by the phosphorylation of the Akt substrate GSK3 (Fig. 3J). In contrast, the ATP competitive mTOR inhibitor AZD-8055 that targets both mTORC1 and mTORC2, prevents the phosphorylation of Akt on serine 473 and inhibits Akt-mediated phosphorylation of GSK3. Therefore, mTORC1, and not mTORC2, activity in NK cells is critical for the glycolytic reprogramming that occurs following cytokine stimulation.

mTORC1-maintained glycolysis in NK cells is required for IFN γ production and granzyme B expression

To address if mTORC1-controlled metabolism underpins the immunoregulatory functions of this kinase in NK cells, we investigated the levels of IFN γ production in cultured NK cells treated with IL2/12 +/- rapamycin (Fig. 4A-B) and found them to directly correlate with

measured rates of glycolysis (Fig. 3G). Additionally, IL2/12-mediated up-regulation of granzyme B expression in NK cells was inhibited by rapamycin (Fig. 4A-B) demonstrating that the mTORC1 pathway was also required for granzyme B expression. To determine whether mTORC1-controlled glycolysis would account for the effect of rapamycin on the expression of IFN γ and granzyme B, experiments were designed to directly limit the rate of glycolysis in cytokine-activated NK cells. As a first approach, glycolysis was limited using suboptimal doses of the glycolytic inhibitor 2-deoxyglucose (2DG) as described previously (21). Cultured NK cells were activated in the presence of low doses of 2DG (0.5-1mM). Although NK cells up-regulated the expression of CD69 normally, IFN γ production and granzyme B expression was greatly diminished (Fig. 4C-D).

Galactose is a carbon fuel source that is metabolised by glycolysis following conversion to glucose-6-phosphate (G6P) through the Leloir pathway (22). However, the rate at which galactose is converted to G6P is slow and acts to limit the overall rate of glycolysis. Therefore, a second complementary approach to limit the rate of glycolysis in activated NK cells involved culturing NK cells in galactose rather than glucose. Metabolic analysis confirmed that galactose did not support elevated rates of glycolysis; however, the cells had normal rates of OxPhos (Fig. 4E). Galactose-cultured NK cells up-regulated CD69 normally in response to IL2/12 but IFN γ production and granzyme B expression were substantially reduced (Fig. 4F-G). Importantly, IL2/12-stimulated NK cells cultured in glucose or galactose had equivalent levels of mTORC1 activity as measured by pS6 levels (Fig. 4H), demonstrating glycolysis to be acting downstream of mTORC1 in the regulation of NK cell function. Therefore, the data demonstrate that increasing glycolytic metabolism in activated NK cells is a prerequisite for normal pro-inflammatory NK cell function.

Elevated levels of glycolysis correlate with the frequency of activated NK cells producing IFN γ (Fig. 4) but also with the amount of IFN γ protein expressed per IFN γ + NK cell, as determined by the MFI of IFN γ staining (Fig. 5A). In CD4 T cells, a mechanism has been described where the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (Gapdh) has an additional role outside glycolysis and can destabilise IFN γ mRNA to control the levels of IFN γ protein produced per T cell (23). As these dual functions for Gapdh appear to be competitive, this model predicts that increasing the rate of glycolysis in NK cells would result in IFN γ mRNA stabilisation and increased IFN γ protein levels. Indeed, rapamycin treatment and the resultant decrease in glycolysis levels correlates with decreased IFN γ mRNA in activated NK cells (Fig. 5B). To test whether glycolytic enzymes such as Gapdh can modulate IFN γ expression in NK cells we used the pharmacologic ATP synthase inhibitor, oligomycin, to increase the rate of NK cell glycolysis. NK cells were activated with IL2/12 in the presence of rapamycin for 14 hours before the addition of oligomycin for a further 5 hours and the levels of IFN γ protein (MFI) measured. The addition of oligomycin to NK cells activated in the presence of rapamycin resulted in a 2.5-fold increase in the rate of glycolysis (Fig. 5C) to an equivalent level to IL2/12 stimulated NK cells in the absence of rapamycin (Fig. 3A). These elevated rates of glycolysis had no effect on the MFI of IFN γ expression in IFN γ + NK cells (Fig. 5D). Therefore, acutely restoring elevated rates of glycolysis does not restore the levels of IFN γ protein expression in NK cells. This argues that glycolytic regulation of IFN γ mRNA stability, akin to that described by Chang et al (23), does not have a substantial impact upon IFN γ protein expression levels in activated

NK cells. Therefore, it is likely that there are alternative mechanisms linking the rate of glycolysis to the amount of IFN γ protein expression in activated NK cells.

To determine whether elevated levels of glycolysis are required for NK cells to produce IFN γ *in vivo*, mice were injected with poly(I:C) in the presence or absence of the glycolytic inhibitor 2DG. This inhibitor will be preferentially taken up by cells with high levels of Glut1 expression and elevated levels of glucose uptake such as activated NK cells, but also other activated immune cell subsets. Administration of 2DG did not affect poly(I:C)-mediated NK cell activation as measured by CD69 expression (Fig. 6A) but activated NK cells were smaller in size and had lower rates of glucose uptake in the presence of 2DG, consistent with decreased glycolysis (Fig. 6B-D). Crucially, *in vivo* 2DG administration significantly reduced IFN γ production by activated NK cells (Fig. 6E), which is consistent with the observed effect of glycolytic inhibition in *ex vivo* NK cells (Fig. 4D-G). It is clear that *in vivo* inhibition of glycolytic cells did not globally disrupt NK cell function as poly(I:C) induced expression of TNF α to equivalent levels independent of the presence or absence of 2DG (Fig. 6F).

The data presented here demonstrate that mTORC1 activity is required for glycolytic reprogramming in activated NK cells and that this is a prerequisite for the acquisition of normal effector functions such as IFN γ production and increased granzyme B expression.

Discussion

This study reveals mTORC1-regulated metabolic reprogramming to be central to its immunoregulatory functions in NK cells. As mTORC1 activity has been previously demonstrated to be required for elevated levels of glycolysis in activated CD4 Th17 and CD8 cytotoxic T cells (2, 3), our current data extends the characterization of mTORC1 as a key regulator of glycolytic metabolism in an innate lymphocyte subset. Indeed, the metabolic changes described in this study to accompany NK cell activation are similar to those that occur in pro-inflammatory effector T cell subsets (2, 3, 24, 25). In activated T cells, elevated glycolysis has been linked to normal effector functions including granzyme B expression by activated CD8 T cells, and inhibition of glycolysis disrupts the expression of IFN γ in activated CD4 T cells (21, 23). Both activated NK cells and effector T cells, that cannot engage in elevated glycolysis, can maintain energy homeostasis through OxPhos but it is clear that oxidative metabolism alone is not sufficient to allow for cell growth or to sustain normal effector functions of activated lymphocytes. Therefore, a common theme is emerging that suggests that glycolytic metabolic reprogramming is an essential step for lymphocytes to successfully acquire pro-inflammatory effector functions.

The exact mechanism linking glycolysis to the expression of these effector molecules is not clear. In CD4 T cells, Gapdh can bind to the AU-rich 3' UTR region of IFN γ mRNA resulting in mRNA destabilisation (23). Consistent with a role for glycolytic enzymes such as Gapdh in regulating IFN γ mRNA in NK cells, limiting the rate of glycolysis decreased the levels of IFN γ protein expressed by IFN γ -producing cells. One prediction of this model is that acutely increasing the rate of glycolysis would result in stabilisation of IFN γ mRNA and an increase in IFN γ protein expression. However, in our experiments, the increased rates

of glycolysis that resulted from acute oligomycin treatment had no effect on IFN γ protein levels, arguing that glycolytic enzymes such as Gapdh do not have a significant impact upon IFN γ expression in IL2/12-activated NK cells. While our data demonstrate that the levels of granzyme B protein expression are also linked to the rate of glycolysis in NK cells, unlike IFN γ mRNA the 3'UTR of granzyme B mRNA does not contain an AU-rich region. This further suggests that mechanisms independent of the regulation of mRNA stability are likely to be involved in controlling effector protein expression levels. Together these data show that mTORC1-regulated glycolysis is critical for promoting IFN γ expression in activated NK cells and also regulates the protein levels of the key NK cell effector molecules IFN γ and granzyme B, and thus will have a significant impact upon NK functional outputs.

This study highlights a crucial role for signalling through the high affinity IL2 receptor, CD25, in promoting elevated rates of NK cell glycolysis and concomitant enhanced NK cell effector functions. It is becoming clear that NK cells act in parallel with T cells of the adaptive immune system during immune responses to diverse immunological challenges (6-8). While it is clear that T cells are not essential for NK cell responses (26-29), our research suggests that the adaptive response may enhance NK cell effector function through the actions of the T cell-derived cytokine IL2 in promoting elevated NK cell glycolysis. Additionally, given that a key mechanism through which Tregs control NK cell responses is through sequestering IL2 (9, 30), it seems likely that Treg-mediated suppression of NK cell effector functions will involve decreased IL2 stimulated levels of NK cell glycolysis. Therefore, the data presented here promote the idea that T cell-controlled IL2 availability may be a factor that impacts upon the regulation of NK cell metabolism and function.

We have described a new regulatory axis that is required for the acquisition of normal NK cell effector functions. This has widespread implications for our understanding of NK cell immune responses to viral infection, tumours and other inflammatory situations. In lymphocytes, mTORC1 is an acute sensor of the immune microenvironment. Amongst other things, it monitors the availability of nutrients such as glucose and amino acids (31, 32). Sites of inflammation can become nutrient-depleted due to competition for nutrients between the large numbers of infiltrating immune cells. Indeed, virally infected cells increase rates of glucose uptake and glycolysis, thereby limiting the levels of glucose available for immune cells in the local microenvironment (33-35). Equally, malignant cells within solid tumours have hugely elevated rates of nutrient uptake and promote nutrient deprivation within the tumour microenvironment (36, 37). Therefore, these environments that are important to the immunological functions of NK cells can limit mTORC1 activity resulting in reduced glucose uptake and glycolysis in activated NK cells that would ultimately disrupt pro-inflammatory NK cell functions. Indeed, NK cells isolated from human solid tumours have been found to be defective in their pro-inflammatory functions including IFN γ production and cytotoxicity (38-40).

In summary, the present study demonstrates that normal effector functions of activated NK cells are reliant on successful mTORC1-dependent metabolic reprogramming that increase rates of glucose uptake and glycolysis. This regulatory axis has important implications for NK cell responses within infection and tumour microenvironments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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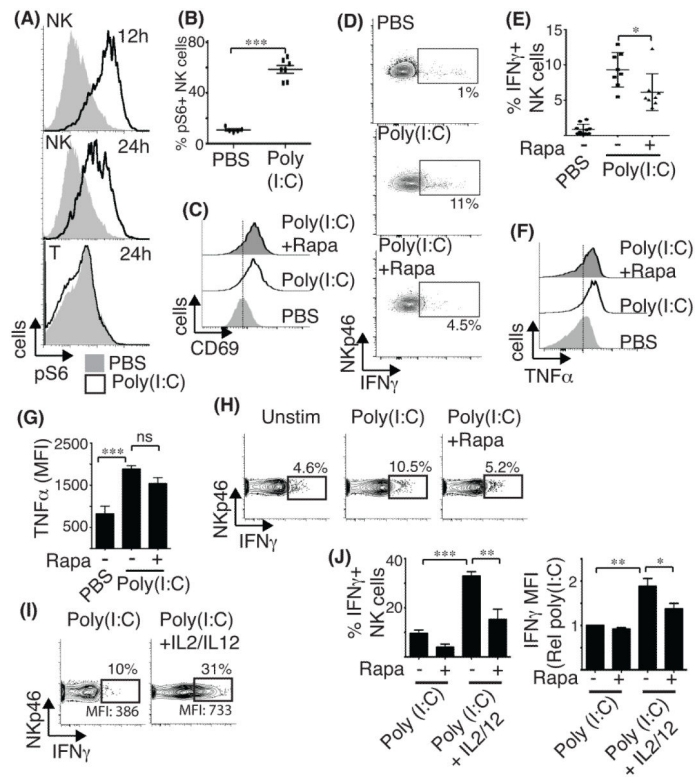


Figure 1. mTORC1 activity is required for IFN γ production in NK cells *in vivo*

(A-E) Mice were administered PBS, 200 μ g poly(I:C) alone or in combination with rapamycin (0.6 mg/kg) by peritoneal injection. (A-B) Spleens were harvested after 12 hours or 24 hours and NKp46+ TCR β - NK cells (A-B) or TCR β + T cells (A) analysed by flow cytometry for levels of phospho-S6 ribosomal protein (pS6). (C-G) Spleens were harvested after 24 hours for analysis. NK1.1+ NKp46+ CD3- NK cells were analysed by flow cytometry for CD69 expression (C), IFN γ production (D-E), and TNF α expression (F-G). (H-J) Splenocytes were stimulated *ex vivo* with poly(I:C) +/- IL2/12 +/- rapamycin or left untreated for 18 hours and IFN γ production, frequency and MFI, was analysed in NK1.1+ NKp46+ CD3- cells. Data is mean +/- S.E.M or representative of 5-10 mice for each condition from 2 separate experiments (A-E), 4 mice for each condition (F-G), 3 separate experiments (H-J) (ns, non significant, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$).

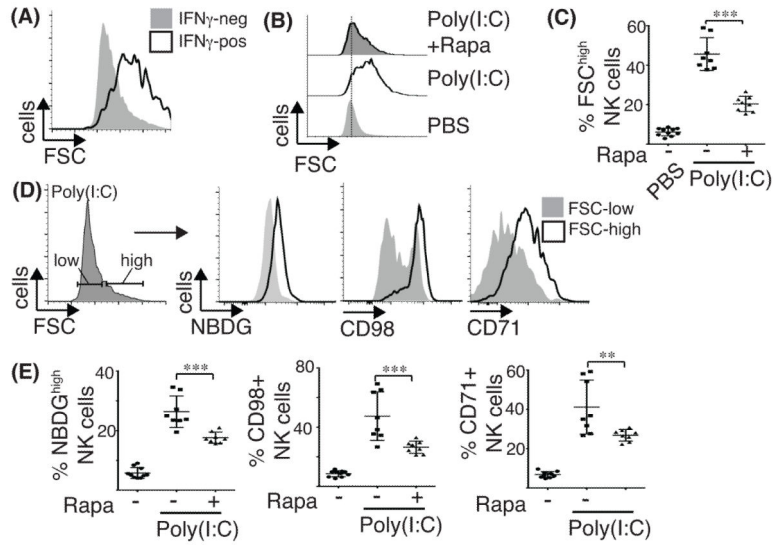


Figure 2. Rapamycin treatment prevents normal blastogenesis of NK cells activated *in vivo* by poly(I:C)

(A-E) Mice were administered PBS, 200 μ g poly(I:C) alone or in combination with rapamycin (0.6 mg/kg) by peritoneal injection and spleens harvested after 24 hours. Splenocytes were isolated and NK1.1+ NKp46+ CD3⁻ NK cells (or NK1.1+ NKp46+ TCR β - NK cells for NBDG experiments) analysed by flow cytometry. The forward scatter of NK cells was compared for IFN γ -positive versus IFN γ -negative NK cells from poly(I:C)-treated mice (A). Differences in NK cell size (B) and the frequency of FSC^{high} NK cells (C) in each treatment group were analysed. (D) Poly(I:C)-stimulated NK cells were segregated based on cell size (left) and small versus large NK cells analysed for levels of glucose uptake (NBDG), CD98 and CD71 expression (right). (E) Analysis of the frequency of NK cells with high levels of NBDG, and expression of CD71 and CD98. Data is mean \pm S.E.M or representative of 8-10 mice for each condition from 2 separate experiments. (** $p < 0.01$, *** $p < 0.001$).

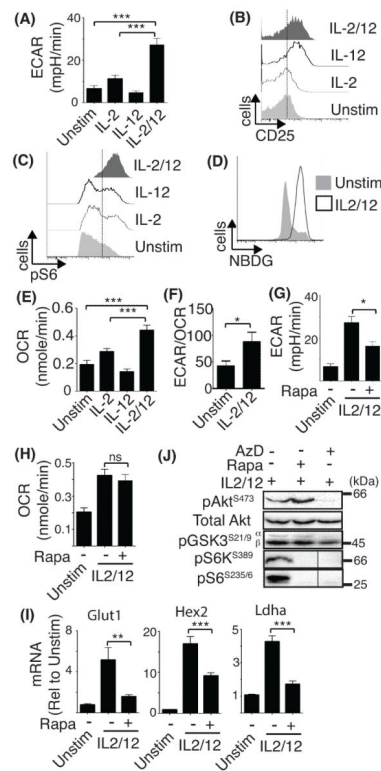


Figure 3. mTORC1 is required for metabolic reprogramming of activated NK cells

Analysis of the rate of glycolysis (ECAR) (A), CD25 expression (B), mTORC1 activity as measured by pS6 levels (C), glucose uptake (NBDG) (D) and OxPhos (OCR) (E) in cultured NK cells stimulated for 18 hours with IL2, IL12, IL2 plus IL12 or left unstimulated. (F) Ratio of glucose utilization for glycolysis to OxPhos in NK cells stimulated with IL2/12 or left unstimulated or 18 hours. (G-I) Cultured NK cells were stimulated for 18 hours with IL2/12 +/- rapamycin (20 nM) or left unstimulated and analysed for the rates of glycolysis (G) and OxPhos (H) and the expression of Glut1, Hex2 and Ldha mRNA (I). (J) Immunoblot analysis of cultured NK cells stimulated with IL2/12 for 18 hours +/- rapamycin (20 nM), +/- AZD-8055 (1 μM). Data is mean +/- S.E.M or representative of 3-5 experiments. (ns, non significant, * p<0.05, ** p<0.01, ***p<0.001).

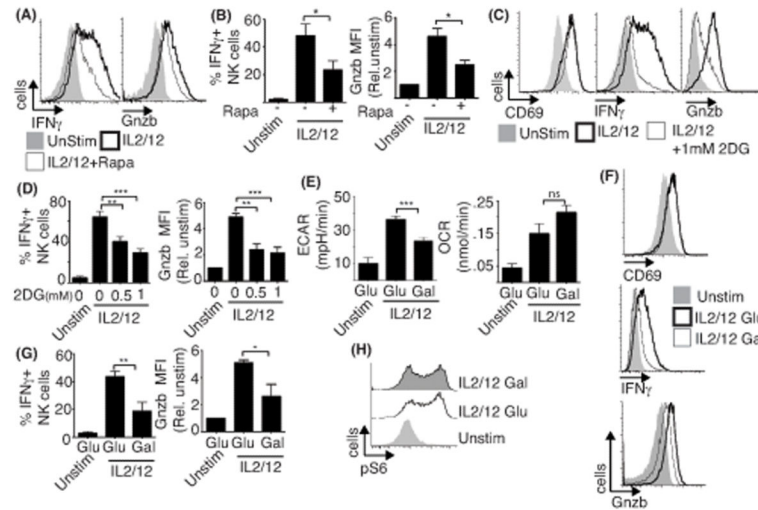


Figure 4. Elevated rates of glycolysis are required for normal NK cell effector functions (A-B) Cultured NK cells either unstimulated or treated with IL2/12 +/- rapamycin for 18 hours were analysed by flow cytometry for IFN γ and granzyme B (GnzB) expression in NK1.1+ NKp46+ CD3- NK cells. (C-D) Cultured NK cells either unstimulated or treated with IL2/12 +/- 2-deoxyglucose (2DG) at the stated concentrations for 18 hours were analysed by flow cytometry for CD69 (C), IFN γ and GnzB (C-D) expression in NK1.1+ NKp46+ CD3- NK cells. (E-G) Cultured NK cells either unstimulated in media containing 10 mM glucose or treated with IL2/12 in media containing glucose (10 mM) or galactose (10 mM) for 18 hours were analysed; for rates of glycolysis and OxPhos (E); and by flow cytometry for levels of CD69 (F), and IFN γ , GnzB (F-G) and pS6 (H), in NK1.1+ NKp46+ CD3- NK cells. Data is mean +/- S.E.M or representative of 3-5 experiments (A-D, F-G), 8 replicates from 2 separate experiments (E) or 3 separate experiments (H). (ns, non significant, * p<0.05, ** p<0.01, ***p<0.001).

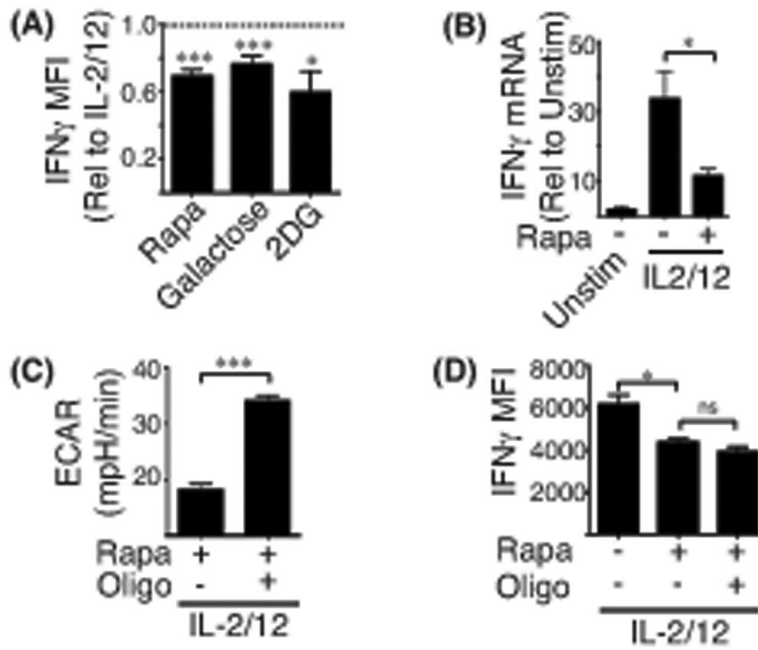


Figure 5. Elevated NK cell glycolysis is required for maximal IFN γ protein expression
 (A) IL2/12-stimulated cultured NK cells were treated +/- rapamycin, +/- 2DG, in the presence of glucose versus galactose as in figure 4. The data show the relative levels of IFN γ MFI in NK cells with decreased levels of glycolysis compared to the relevant IL2/12 control (dotted line). (B) IFN γ mRNA expression in cultured NK cells unstimulated or stimulated with IL2/12 +/- rapamycin for 18 hours. (C) Rate of glycolysis (ECAR) in cultured NK cells stimulated with IL2/12 plus rapamycin before and after the inhibition of ATP synthase with the addition of oligomycin (2 μ M). (D) Cultured NK cells were activated with IL2/12 for 18 hours +/- rapamycin or activated with IL2/12 for 14 hours before the addition of oligomycin added for 5 hours. Data is mean +/- S.E.M 4-12 experiments (A), 3 experiments (B-D) (ns, non significant, * $p < 0.05$, *** $p < 0.001$).

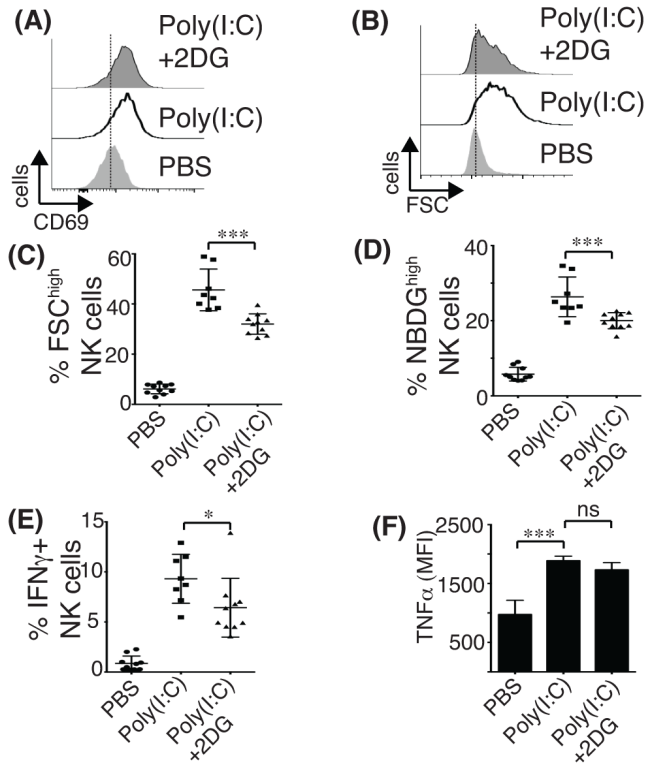


Figure 6. Disruption of NK cell glycolysis *in vivo* inhibits NK cell growth and effector function (A-F) Mice were administered PBS, 200 μ g poly(I:C) alone or in combination with 2DG (1 g/kg) by peritoneal injection and spleens harvested after 24 hours. Splenocytes were isolated and NK1.1+ NKp46+ CD3⁻ NK cells (or NK1.1+ NKp46+ TCR β ⁺ NK cells for NBDG experiments) were analysed for CD69 expression (A), cell size (B, C), glucose uptake (NBDG) (D), IFN γ (E) and TNF α production (F). All data mean \pm S.E.M or representative of 8-10 mice for each condition from 2 separate experiments. (ns, non significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).