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**Amino acid-dependent mTORC1 and
cMyc signaling is essential for
Natural Killer cell metabolic and
functional responses**

A dissertation submitted to Trinity College Dublin in
candidature for the degree of Doctor of Philosophy

School of Biochemistry and Immunology,
Trinity College, Dublin

May 2017

By Róisín Loftus

Declaration

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Publications

mTORC1-Dependent Metabolic Reprogramming Is a Prerequisite for NK Cell Effector Function.

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Immunometabolism: Cellular Metabolism Turns Immune Regulator

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Metabolic Reprogramming Supports IFN- γ Production by CD56^{bright} NK Cells.

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Publications

TGF- β inhibits the activation and functions of NK cells by repressing the mTOR pathway.

Sébastien Viel, Antoine Marçais, Fernando Souza-Fonseca Guimaraes, **Roisin Loftus**, Jessica Rabilloud, Morgan Grau, Sophie Degouve, Sophia Djebali, Amélien Sanlaville, Emily Charrier, Jacques Bienvenu, Julien C. Marie, Christophe Caux¹⁰, Jacqueline Marvel, Liam Town, Nicholas D. Huntington, Laurent Bartholin, David Finlay, Mark J. Smyth and Thierry Walzer.

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Sterol regulatory element binding proteins (Srebp) are essential for metabolic and functional responses in activated Natural Killer cells.

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Table of Contents

Publications.....	i
Summary	viii
Abbreviations	x
Acknowledgements.....	xiii
Chapter 1	15
1.1. Natural Killer cells.....	16
1.1.1. Anti-tumor NK cell responses.....	16
1.1.2. Anti-viral NK cell response	17
1.1.3. NK cell effector functions	18
1.1.4. NK cell receptors	22
1.1.5. Immune evasion by virally infected and transformed cells	23
1.1.6. Cytokine-induced NK cell activation.....	25
1.1.7. Cytokine control of NK cell differentiation	27
1.1.8. Cytokine maintained NK cell homeostasis	28
1.1.9. NK cell immunotherapeutic strategies.....	28
1.1.10. The tumor microenvironment is metabolically restrictive.....	30
1.2. Immune cell energy metabolism.....	31
1.2.1. Cellular metabolism matches immune cell demands.....	35
1.2.2. Glutamine metabolism fuels energy production and signalling.....	37
1.2.3. Cellular metabolism directly regulates immune cell functions	39
1.2.4. T cell energy metabolism.....	40
1.2.5. Factors controlling metabolism and function of effector T cells.....	42
1.3. Mammalian target of rapamycin (mTOR)	44
1.3.1. Regulation of the mTOR signalling pathway.....	44
1.3.2. mTORC1 regulation of cellular metabolism	46
Aims	49
Chapter 2	50
Materials and Methods	50
2.1. Materials	51
2.1.1. Chemicals.....	51
2.1.2. Equipment.....	52
2.1.3. Animal Husbandry.....	53
2.1.4. Antibodies.....	54
2.2. Methods	56
2.2.1. Cell culture	56
2.2.2. Flow cytometry analysis	60
2.2.3. Metabolic Analysis.....	62
2.2.4. RNA analysis.....	65
2.2.5. Protein manipulation.....	70
2.2.6. ATP assay	72
Chapter 3	73
mTORC1-dependent metabolic reprogramming is a prerequisite for	
Natural Killer cell effector function	73
Introduction	74
3.1. Changes in NK cell metabolism correlate with the acquisition of effector functions	
.....	75

3.2. IL-2/12 cytokine activated NK cells undergo metabolic reprogramming to permit elevated levels of glucose metabolism	80
3.3. Metabolic alterations correlate with the acquisition of Natural Killer cell effector functions.....	88
3.4. Glucose metabolism regulates NK cell effector function.....	91
3.5. mTORC1 controls NK cell metabolism	100
3.6 mTORC1 controls NK cell functional responses	110
Chapter 3 discussion	114
Chapter 4	121
The transcription factor cMyc, not HIF1 α , controls NK cell metabolic and functional responses	121
Introduction	122
4.1 HIF1 α is dispensable for IL-2/12 induced metabolic responses in NK cells.....	123
4.2 HIF1 α is dispensable for IL-2/12 induced NK cell effector function.....	129
4.3 cMyc is required for IL-2/12 induced NK cell metabolic responses.....	133
4.4 cMyc is required for IL-2/12-induce NK cell effector functions	143
Chapter 4 discussion	151
Amino acids are indispensable for cytokine-induced Natural Killer cell metabolic and functional responses	151
Introduction	152
5.1 PI3K/Akt are not required for IL-2/12-induced cMyc expression.....	155
5.2 Amino acid transport through Slc7a5 controls cMyc, glycolysis and OxPhos in NK cells	159
5.3 Glutamine, but not glutamine metabolism, sustains NK cell metabolism and effector functions	175
Chapter 5 discussion	183
Chapter 6	193
Overall discussion	193
Bibliography.....	199

Table of Figures

CHAPTER 1

FIGURE 1.1.3 NK CELL CYTOTOXICITY	19
FIGURE 1.1.5. NK CELL ACTIVATING AND INHIBITORY RECEPTORS	24
FIGURE 1.2.1 CONFIGURING METABOLISM TO MATCH IMMUNE CELL FUNCTION	34
FIGURE 1.2.2. CELLULAR METABOLISM IS INTEGRALLY LINKED TO FUNCTION	38
FIGURE 1.3. MTORC1 INTEGRATES MICRO-ENVIRONMENTAL SIGNALS TO CONTROL CELLULAR PROCESSES	47

CHAPTER 2

FIGURE 2.2.4.1. DETERMINING GLYCOLYTIC RATES USING THE SEAHORSE METABOLIC FLUX ANALYSER.....	63
FIGURE 2.2.4.1 DETERMINING OXPHOS RATES USING THE SEAHORSE METABOLIC FLUX ANALYSER.....	64
TABLE 2.1.4.1 FLOW CYTOMETRY ANTIBODIES	55
TABLE 2.1.4.2 WESTERN BLOT ANTIBODIES	56
TABLE 2.2.1. CELL CULTURE STIMULANTS.....	58
TABLE 2.2.2 BUFFERS/MEDIA	59
TABLE 2.2.3 SEAHORSE INHIBITOR CONCENTRATIONS	65
TABLE 2.2.4.1 CDNA SYNTHESIS PROGRAM.....	66
TABLE 2.2.4.2 REAL TIME PCR RUN SETTINGS.....	68
TABLE 2.2.4.3: GENOTYPING MASTER MIX COMPONENTS.....	69
TABLE 2.2.4.4: MYC PCR GENOTYPING PROTOCOL.....	70
TABLE 2.2.4.5: TAMOXIFEN CRE PCR GENOTYPING PROTOCOL	70
TABLE 2.2.6 10% GEL COMPOSITION	71

CHAPTER 3

FIGURE 3.1. POLY (I:C) ACTIVATED NK CELLS INCREASE IN SIZE.....	76
FIGURE 3.2. LARGER NK CELLS FROM POLY(I:C) TREATED MICE ARE MORE METABOLICALLY ACTIVE.....	77
FIGURE 3.3. POLY(I:C) ENHANCES NK CELL IFN γ PRODUCTION <i>IN VIVO</i>	79
FIGURE 3.4. IL-15 INDUCES SPLENIC NK CELL EXPANSION	81
FIGURE 3.5. IL-2/12 CYTOKINE STIMULATION INDUCES A ROBUST INCREASE IN NK CELL SIZE, ACTIVATION MARKER EXPRESSION AND EFFECTOR FUNCTION	82
FIGURE 3.6. THE CYTOKINE COMBINATION IL-2/12 INDUCES A SYNERGISTIC INCREASE IN THE RATE OF NK CELL GLYCOLYSIS.....	83
FIGURE 3.7. IL-2/12 STIMULATED NK CELLS ARE MORE METABOLICALLY ACTIVE	84
FIGURE 3.8. IL-2/12 STIMULATED NK CELLS USE GLYCOLYSIS AS THE PRIMARY FORM OF GLUCOSE METABOLISM	86
FIGURE 3.9. IL-2/12 STIMULATED NK CELLS HAVE AN INCREASED GLYCOLYTIC CAPACITY DUE TO METABOLIC REPROGRAMMING	87
FIGURE 3.10. IL-2/12 STIMULATED NK CELLS DISPLAY ENHANCED IL2 RECEPTOR EXPRESSION THAT FACILITATES INCREASED IL2 SIGNALING	89
FIGURE 3.11. IL-2/12 STIMULATED NK CELLS DISPLAY SYNERGISTIC INCREASES IN SIZE AND EFFECTOR MOLECULE PRODUCTION	90
FIGURE 3.12. LIMITING GLYCOLYSIS <i>IN VIVO</i> USING 2DG PREVENTS POLY(I:C) INDUCED NK CELL BLASTOGENESIS AND INCREASED GLUCOSE UPTAKE.....	92
FIGURE 3.13. ELEVATED LEVELS OF GLYCOLYSIS ARE REQUIRED FOR IFN γ PRODUCTION IN POLY(I:C) ACTIVATED NK CELLS <i>IN VIVO</i>	93
FIGURE 3.14. LOW DOSES OF 2DG DO NOT CAUSE NK CELL ENERGY CRISIS.....	94
FIGURE 3.15. ELEVATED RATES OF GLYCOLYSIS ARE REQUIRED FOR IL-2/12 INDUCED NK CELL EFFECTOR FUNCTION	96
FIGURE 3.16. GALACTOSE DOES NOT SUPPORT ELEVATED RATES OF GLYCOLYSIS IN IL-2/12 ACTIVATED NK CELLS	97
FIGURE 3.17. GALACTOSE CANNOT SUPPORT INCREASED CELL GROWTH IN CYTOKINE ACTIVATED NK CELLS	98

FIGURE 3.18. GLUCOSE IS REQUIRED FOR THE ACQUISITION OF NK CELL EFFECTOR FUNCTIONS.....	99
FIGURE 3.19. MTORC1 ACTIVITY INCREASES UPON NK CELL ACTIVATION <i>IN VIVO</i>	101
FIGURE 3.20. CYTOKINE-STIMULATED NK CELLS DISPLAY INCREASED MTORC1 ACTIVITY	102
FIGURE 3.21. NK CELLS INCREASE GLYCOLYTIC GENE EXPRESSION FOLLOWING IL-2/12 CYTOKINE STIMULATION.....	103
FIGURE 3.22. MTORC1 ACTIVITY IS REQUIRED FOR NK CELL ACTIVATION-INDUCED BLASTOGENESIS <i>IN VIVO</i>	105
FIGURE 3.23. MTORC1 ACTIVITY IS REQUIRED FOR INCREASED GLUCOSE UPTAKE AND METABOLIC RECEPTOR EXPRESSION IN POLY(I:C) ACTIVATED NK CELLS <i>IN VIVO</i>	106
FIGURE 3.24. MTORC1 ACTIVITY IS REQUIRED FOR INCREASED METABOLIC RECEPTOR AND GLUCOSE UPTAKE IN IL-2/12 ACTIVATED NK CELLS.....	107
FIGURE 3.25. MTORC1 CONTROLS THE INCREASED LEVEL OF GLYCOLYSIS SEEN IN IL-2/12 STIMULATED NK CELLS	108
FIGURE 3.26. MTORC1 IS REQUIRED FOR INCREASED NK CELL GLYCOLYTIC CAPACITY AND METABOLIC REPROGRAMMING FOLLOWING CYTOKINE STIMULATION	109
FIGURE 3.27. MTORC1 ACTIVITY IS REQUIRED FOR NK CELL IFN γ PRODUCTION <i>IN VIVO</i>	111
FIGURE 3.28. INHIBITION OF MTORC1 DOES NOT HAVE A GLOBAL EFFECT ON NK CELL CYTOKINE PRODUCTION	112
FIGURE 3.29. MTORC1 ACTIVITY IS REQUIRED FOR THE ACQUISITION OF NK CELL EFFECTOR FUNCTION	113

CHAPTER 4

FIGURE 4.1. HIF1A EXPRESSION IS INCREASED IN CYTOKINE STIMULATED NK CELLS IN AN MTORC1-DEPENDENT MANNER.....	124
FIGURE 4.2. HIF1A ^{FLOX/FLOX} VAV CRE-RECOMBINASE SPLENOCYTES DISPLAY COMPARABLE EXPANSION OF NK CELLS	125
FIGURE 4.3. CYTOKINE STIMULATED HIF1A NULL NK CELLS ARE METABOLICALLY ACTIVE..	126
FIGURE 4.4. HIF1A IS NOT REQUIRED FOR CYTOKINE-INDUCED INCREASES IN NK CELL GLYCOLYSIS.....	127
FIGURE 4.5. HIF1A IS NOT REQUIRED FOR CYTOKINE-INDUCED INCREASES IN NK CELL OXPHOS	128
FIGURE 4.6. HIF1A DOES NOT CONTROL NK CELL METABOLIC REPROGRAMMING	130
FIGURE 4.7. HIF1A DOES NOT CONTROL THE EXPRESSION OF IFN γ IN CYTOKINE STIMULATED NK CELLS	131
FIGURE 4.8. HIF1A DOES NOT CONTROL THE EXPRESSION OF GRANZYME B IN CYTOKINE STIMULATED NK CELLS	132
FIGURE 4.9. NK CELLS INDUCE AN EARLY INCREASE IN CMYC EXPRESSION THAT IS MTORC1-DEPENDENT, BUT IS MTORC1 INDEPENDENT OVER 18HOURS.....	134
FIGURE 4.10. IL-2/12 STIMULATED NK CELLS MAINTAIN ELEVATED LEVELS OF CMYC PROTEIN AND MRNA EXPRESSION.....	135
FIGURE 4.11. INDUCIBLE CMYC KNOCKOUT MOUSE	136
FIGURE 4.12. CMYC ^{KO} NK CELLS DISPLAY DECREASED SIZE AND METABOLIC MARKER EXPRESSION FOLLOWING IL-2/12 STIMULATION.....	137
FIGURE 4.13. CMYC ^{KO} NK CELLS DISPLAY DECREASED LEVELS OF GLYCOLYSIS COMPARED TO CMYC ^{WT} NK CELLS FOLLOWING CYTOKINE STIMULATION.....	138
FIGURE 4.14. MYC ^{KO} NK CELLS HAVE REDUCED EXPRESSION OF GLYCOLYTIC GENES FOLLOWING IL-2/12 STIMULATION	140
FIGURE 4.15. CMYC ^{KO} NK CELLS DISPLAY DECREASED RATES OF OXPHOS FOLLOWING CYTOKINE STIMULATION.....	141
FIGURE 4.16. CMYC CONTROLS NK CELL MITOCHONDRIAL BIOGENESIS	142
FIGURE 4.17. IL-2/12 STIMULATED CMYC ^{KO} NK CELLS DISPLAY DECREASED IFN γ EXPRESSION	144
FIGURE 4.18 CMYC IS REQUIRED FOR NK CELL GRANZYME B PRODUCTION.....	145

CHAPTER 5

FIGURE 5. IL-2 SIGNALING DRIVE NK CELL METABOLIC AND FUNCTIONAL RESPONSES.....	154
FIGURE 5.1. AKT IS NOT REQUIRED FOR IL-2/12 INDUCED INCREASES IN NK CELL SIZE OR CD71 EXPRESSION	156

FIGURE 5.2. AKT IS NOT REQUIRED FOR IL-2/12 INDUCED CMYC EXPRESSION OR MTORC1 ACTIVITY	157
FIGURE 5.3. AKT IS NOT REQUIRED FOR NK CELL INTERFERON- γ PRODUCTION OR GRANZYME B EXPRESSION.....	158
FIGURE 5.4. NK CELL SLC7A5 EXPRESSION INCREASES IN CYTOKINE ACTIVATED NK CELLS	160
FIGURE 5.5. INHIBITION OF SLC7A5 REDUCES ACTIVATION-INDUCED INCREASES IN NK CELL SIZE AND CD71 EXPRESSION	161
FIGURE 5.6. SLC7A5 CONTROLS CYTOKINE-INDUCED CMYC EXPRESSION AND MTORC1 SIGNALING IN IL-2/12 ACTIVATED NK CELLS.....	162
FIGURE 5.7. LEUCINE IS REQUIRED FOR MTORC1 ACTIVITY, BUT NOT CMYC EXPRESSION IN CYTOKINE ACTIVATED NK CELLS	164
FIGURE 5.8. GLUTAMINE IS REQUIRED FOR CMYC EXPRESSION AND MTORC1 ACTIVITY IN IL-2/12 ACTIVATED NK CELLS	165
FIGURE 5.9. GLUTAMINE FUELS O-GLCNACYLATION	166
FIGURE 5.10. GLUTAMINE DEPRIVED NK CELLS FAIL TO INCREASE IN SIZE AND DISPLAY REDUCED CD71 EXPRESSION	168
FIGURE 5.11. IL-2/12 ACTIVATED NK CELLS TREATED WITH BCH EXHIBIT DECREASED LEVELS OF GLYCOLYSIS.....	169
FIGURE 5.12. IL-2/12 ACTIVATED NK CELLS TREATED WITH BCH EXHIBIT DECREASED LEVELS OF OXPHOS	170
FIGURE 5.13. NK CELLS ACTIVATED IN THE ABSENCE OF GLUTAMINE DISPLAY DECREASED LEVELS OF GLYCOLYSIS	171
FIGURE 5.14. NK CELLS ACTIVATED IN THE ABSENCE OF GLUTAMINE DISPLAY DECREASED LEVELS OF OXPHOS.....	172
FIGURE 5.15. SLC7A5 ACTIVITY IS REQUIRED FOR CYTOKINE-INDUCED INCREASES IN NK CELL PRO-INFLAMMATORY MOLECULE PRODUCTION.....	173
FIGURE 5.16. GLUTAMINE IS REQUIRED FOR CYTOKINE-INDUCED INCREASES IN NK CELL PRO-INFLAMMATORY MOLECULE PRODUCTION.....	174
FIGURE 5.17. GLUTAMINE IS NOT AN IMPORTANT FUEL FOR SUSTAINING ELEVATED LEVELS OF NK CELL OXPHOS.....	176
FIGURE 5.18. GLUTAMINE METABOLISM IS NOT REQUIRED FOR SUSTAINING ELEVATED LEVELS OF NK CELL OXPHOS.....	177
FIGURE 5.19. DON INHIBITS CTL PROLIFERATION AND REDUCES NK CELL O-GLCNACYLATION	178
FIGURE 5.20. GLUTAMINE, BUT NOT GLUTAMINE METABOLISM, IS REQUIRED FOR SUSTAINING INCREASED CELL SIZE IN IL-2/12 ACTIVATED NK CELLS	180
FIGURE 5.21. GLUTAMINE, BUT NOT GLUTAMINE METABOLISM, IS REQUIRED FOR SUSTAINING EFFECTOR MOLECULE PRODUCTION IN IL-2/12 ACTIVATED NK CELLS.....	181
FIGURE 5.22. GLUTAMINE IS REQUIRED TO SUSTAIN CMYC EXPRESSION AND MTORC1 ACTIVITY IN IL-2/12 ACTIVATED NK CELLS	182
 CHAPTER 6	
FIGURE 6.1 PI3K/AKT AND MTORC1 SIGNAL INDEPENDENTLY CONTROL T CELL FUNCTION IN RESPONSE TO IL-2	185
FIGURE 6.1 GLUTAMINE FACILITATES AMINO ACID UPTAKE THROUGH SLC7A5	188
FIGURE 6.2 CMYC EXPRESSION IS DYNAMICALLY REGULATED BY THE BALANCE OF SYNTHESIS AND DEGRADATION	192

Summary

Natural killer (NK) cells are innate cytotoxic lymphocytes that are essential to the immune response against virally infected and transformed cells. NK cells directly kill target cells through the release cytotoxic granules. In addition, they can orchestrate an adaptive immune response through the release of proinflammatory cytokines.

While the concept of immunometabolism has been recognized as critical in dictating the response of several immune cell subsets, nothing is currently known regarding the regulation of NK cell metabolism. In this study, we examined the changes that occur in NK cell metabolism following activation and determined the importance of cellular metabolism in controlling NK cell effector functions. Our findings reveal the dramatic metabolic changes that occur following NK cell activation; cytokine-activated NK cells significantly increase their rates of glycolysis and OxPhos. This increase in metabolism corresponds with complete metabolic reprogramming, where the mRNA expression of key glycolytic genes was significantly increased in NK cells following cytokine stimulation. Furthermore, this metabolic response was shown to be important in controlling the acquisition of NK cell effector functions; direct perturbations to the rate of glycolysis decreased NK cell effector molecule production.

Here, we show the metabolic regulator mammalian target of rapamycin complex 1 (mTORC1) is robustly stimulated in NK cells activated *in vivo* and *in vitro*. Furthermore, we found mTORC1 activity was important in regulating NK cell metabolism and thereby, NK cell effector functions *in vivo* and *in vitro*.

Although mTORC1 was identified as a key regulator of NK cell metabolism, the transcription factors that control NK cell metabolic reprogramming remained unknown. Using transgenic mouse models, we show that the transcription factor cMyc, but not HIF1 α , is crucial for NK cell cytokine-induced increases in glycolysis and OxPhos. The PI3K/Akt signaling pathway is required for mTORC1

activity and cMyc expression in many cell types. In contrast, this signaling axis was found to be dispensable for NK cell mTORC1 activity and cMyc expression. Thus, inhibiting the PI3K/Akt signaling pathway had no effect on NK cell effector molecule production. Another key regulator of cMyc expression in T cells is the System L amino acid transporter Slc7a5. Here, we find blocking amino acid transport through Slc7a5 inhibits cMyc expression in NK cells, and prevents the cytokine-induced metabolic response. Depriving activated NK cells of glutamine, which is important for the activity of Slc7a5, also resulted in a rapid decrease in cMyc protein expression. Interestingly, while glutamine is essential for maintaining elevated NK cell metabolism and NK cell cytotoxicity, this is due to its role in sustaining cMyc signaling and not due to the importance of glutamine as a cellular fuel. In fact, unlike other effector lymphocyte subsets, glutaminolysis is not required to sustain elevated levels of OxPhos in activated NK cells. These findings identify an amino acid-dependent cMyc signaling axis that is crucial for robust NK cell responses.

In conclusion, this study has widened our knowledge of the NK cell response during activation, and outlines the importance of cellular metabolism in the acquisition of NK cell effector functions. In doing so, our data may provide reason for why NK cell responses are often found defective at the site of human solid tumors or in the chronic-viral infection setting. Virally infected and transformed cells may limit the NK cell metabolic response by directly competing for nutrients in the microenvironment. Nutrient deprivation can negatively impact on mTORC1 activity and cMyc signaling, which we have shown are indispensable for the metabolic and functional response of NK cells. With a greater understanding of the importance of cellular metabolism, and having identified the key metabolic regulators of NK cells, the findings from this study help may help in the development of improved NK cell based immunotherapeutic strategies.

Abbreviations

2DG	2-deoxy-D-glucose
4E-BP1	Eukaryotic translation initiation factor binding protein
ADCC	Antibody dependent cell cytotoxicity
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ALL	Acute lymphoblastic leukemia
AMPK	AMP-activated protein kinase
APC	Antigen presenting cell
CAR	Chimeric antigen receptor
CTL	Cytotoxic T lymphocyte
CPT1A	Carnitine palmitoyltransferase 1A
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DEPTOR	DEP-containing mTOR interacting protein
ECAR	Extracellular acidification rate
eIF4A/B	Eukaryotic initiation factor 4 A/B
ERK	Extracellular signal-regulated kinase
FACS	Fluorescent-activated cell sorting
FADH ₂	Flavin adenine dinucleotide
FAO	Fatty acid oxidation
FOXO	Forkhead box protein O
FKBP12	FK506 binding protein 12
GAPDH	Glyceraldehyde 3-phosphatate dehydrogenase
Glut1	Glucose transporter 1
GSK3	Glycogen synthase kinase 3
HBP	Hexosamine biosynthetic pathway
HIF1 α	Hypoxic inducible factor 1 alpha
Hex2	Hexokinase 2
HLA	Human leukocyte antigen
HRE	Hypoxia response element

IFN γ	Interferon gamma
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
JAK	Janus kinase
KIR	Killer immunoglobulin-like receptor
LAK	Lymphokine activated killer cell
LAT1	L-type amino acid transporter 1
Ldha	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
mTORC1	Mammalian target of rapamycin complex 1
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF- κ B	Nuclear factor kappa enhancer of activated B cells#
NFAT	Nuclear factor of activated T cells
NK cell	Natural Killer cell
OCR	Oxygen consumption rate
OxPhos	Oxidative phosphorylation
PAMP	Pathogen associated molecular pattern
PDK-1	Phosphoinositide-dependent kinase 1
PEP	Phosphoenolpyruvate
PHD	Prolyl hydroxylase
PI3K	Phosphoinositide 3-kinase
PIM	Proviral Integrations of Moloney virus
PKB/AKT	Protein kinase B
PMA	Phorbol 12-myristate 13-acetate
Poly I:C	Polyinosinic:polycytidylic acid
PPAR γ	Peroxisome proliferation activated receptor gamma
RAPTOR	Regulatory associated protein of mTOR
RHEB	Ras homolog enriched in brain
RICTOR	Rapamycin insensitive companion of mTOR
Rplp0	Ribosomal protein lateral stalk subunit P0

S6	Ribosomal protein S6
S6K	P70-S6 kinase
Slc7a5	Solute carrier family 7 member 5
SMC1	Structural maintenance of chromosomes 1
SREBP	Sterol regulatory element-binding protein
STAT	Signal transducer and activator of transcription
T-Bet	T-cell-specific T-box transcription factor
TCA	Tricarboxylic acid cycle
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Teff	Effector T cell
T _H 1	T helper cell 1
T _H 17	T helper cell 17
T _H 2	T helper cell 2
TIL	Tumor infiltrating lymphocyte
Tmem	Memory T cell
Treg	Regulatory T cell
TLR	Toll like receptor
TRAIL	TNF-related apoptosis inducing ligand
TRAMP	TNF receptor-related apoptosis mediating protein
TSC	Tuberous sclerosis
VHL	Von Hippel-Lindau

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Chapter 1

Introduction

1.1. Natural Killer cells

Natural killer (NK) cells are innate lymphoid cells (ILC) that rapidly respond in the absence of prior sensitization (Ward et al., 2007). NK cells comprise one of the three major lymphocyte populations. They can be identified from the other two major populations, B and T cells, by their expression of unique cell-surface receptors (see section 1.1.4). NK cell surface receptors receive information from the microenvironment and direct the NK cell response accordingly. This may involve the direct killing of target cells through death receptor engagement or cytotoxic molecule release (see section 1.1.3). In addition, NK cells can orchestrate an adaptive immune response through the release of pro-inflammatory cytokines, which drive immune cell activation. Due to their ability to both rapidly respond to immunological challenges and initiate the production of adaptive cytokines such as IL-2, NK cells are intermediary between innate and adaptive immunity.

1.1.1. Anti-tumor NK cell responses

NK cells have long been regarded as the first line of defence in the anti-cancer immune response (Barlozzari et al., 1983). NK cells survey the body for transformed cells and directly eliminate them through the release of cytotoxic molecules (see section 1.1.3). In addition, NK cells can initiate an anti-cancer adaptive immune response through the release of pro-inflammatory cytokines such as IFN γ (Vivier et al., 2008). NKp46 is an activating receptor expressed specifically on the surface of both human and mouse NK cells. Walzer et al performed a NK cell depletion study using a transgenic mouse where the NKp46 promoter was under the control of the

diphtheria toxin receptor. In this mouse model, injection of diphtheria lead to a complete and selective ablation of NK cells *in vivo* within 24hours (Walzer et al., 2007). Importantly, NK cell-deficient mice displayed profoundly impaired tumor cell killing. In a separate study, mice in which the NK cell receptor NK1.1 was depleted genetically also displayed greatly advanced tumor progression and decreased survival (Kim et al., 2000). However, one caveat of targeting NK1.1 is the non-specific effects on other immune cell types; invariant NKT cells also express NK1.1 and are important in the anti-tumor immune response (Emoto et al., 2007).

The number of successful adoptive transfer studies involving NK cells highlights the protective role of NK cells in the anti-cancer immune response. For example, in a mouse lymphoma model, the adoptive transfer of cytokine-activated NK cells increased mouse survival and decreased the volume of established tumors (Ni et al., 2012). The success of such experimental models has led to the development of human NK cell-based immunotherapeutic strategies (further discussed in section 1.1.9).

1.1.2. Anti-viral NK cell response

NK cells also have an important role in the anti-viral immune response. Depletion of NK cells in the murine cytomegalovirus (MCMV) model of viral infection results in increased viral load and progression (Bukowski et al., 1984). Concurrently, adoptive transfer of NK cells confirms their protective role in MCMV viral clearance *in vivo* (Bukowski et al., 1985). Although NK cells have a direct role in MCMV clearance, they also influence the adaptive T cell anti-viral response (Robbins et al., 2007). NK cells reduce MCMV viral load through the suppression of viral replication and the direct killing of virally infected target cells. Reduced viral load decreases the production of immunosuppressive type 1 IFNs by Dendritic Cells (DC). In the

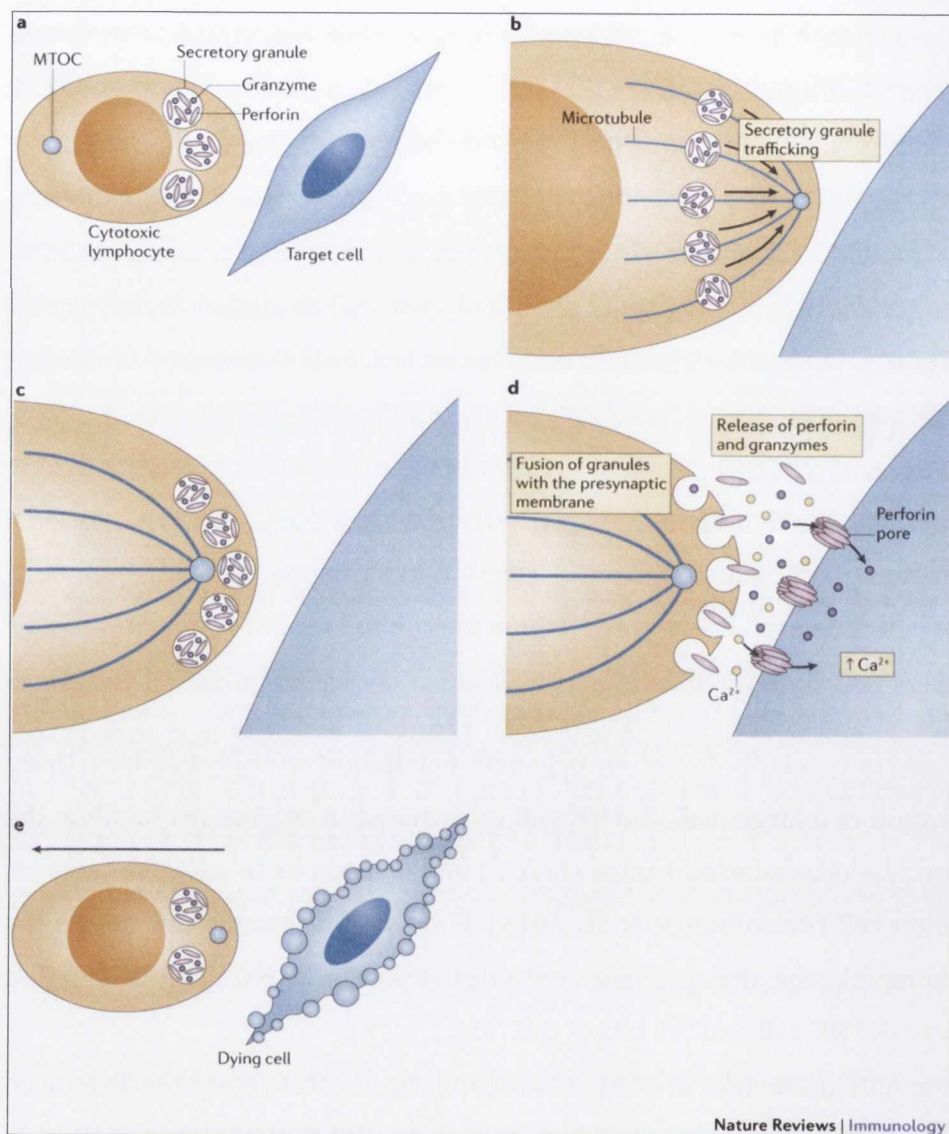
absence of type 1 IFN, CD8⁺ T cell proliferation and anti-viral functions are enhanced (Robbins et al., 2007).

Depletion of NK cells in pox-virus resistant mouse models further supports the indispensable role for NK cells in the anti-viral immune response. In a mouse model of ectromelia virus (mouse pox), depletion of NK cells resulted in viremia and death (Jacoby et al., 1989). NK cell depletion in mice with encephalitis induced by Theiler's virus or myocarditis induced by coxsackie B3 virus also displayed viremia and accelerated pathology (Paya et al., 1989, Fairweather et al., 2001).

1.1.3. NK cell effector functions

How do NK cells kill? NK cells express cytolytic effector molecules that are contained in specialised exocytic organelles called secretory lysosomes. Upon recognition of a target cell, the NK cell actin skeleton rearranges to allow the microtubule organisation centre (MTOC) and lysosomes to polarise towards the target cell (Voskoboinik et al., 2015). Following lysosomal docking to the plasma membrane, the cytotoxic contents are released into the lytic synapse between the NK cell and the target cell (Fig. 1.1.3).

Perforin and granzymes are important cytolytic effector molecules produced by NK cells. Perforin is a membrane disrupting protein that binds to target cells in a calcium-dependent manner (Voskoboinik et al., 2005). Once bound to the target cell membrane, perforin polymerises and forms a transmembrane channel 5-22nm in diameter (Law et al., 2010) (Kurschus et al., 2008). The transmembrane channel created by perforin allows for the free efflux of target cell ions and polypeptides. This disrupts target cell homeostasis, resulting in the activation of pro-apoptotic pathways (Osinska et al., 2014). In addition, the transmembrane created by perforin enhances granzyme-mediated cell death.



(Voskoboinik et al., 2015)

Figure 1.1.3 NK cell cytotoxicity

An NK cell encounters a target cell (a). The microtubule-organizing centre (MTOC) polarizes and lysosomal granules migrate towards the synapse between the NK cell and target cell (b). Cytotoxic granules fuse with the presynaptic membrane and release perforin and granzymes into the synaptic cleft (c, d). Perforins polymerise, forming transmembrane pores in the target cell. Granzymes enter the target cell by receptor-mediated endocytosis and initiate apoptosis of the target cell (d). The NK detaches from the dying target cell and moves on and searches for its next target cell (e).

The molecule granzyme is 4nM in diameter, hence the transmembrane created by perforin (5-22nM in diameter) assists in granzyme entry into the target cell. However, perforin-independent mechanisms of granzyme-induced cell death also exist. Studies using purified granzymes showed the importance of receptor-mediated endocytosis for granzyme-induced target cell killing (Baran et al., 2009, Thiery et al., 2011). Granzymes are serine proteases that initiate caspase-driven cell death pathways (Voskoboinik et al., 2015). Granzyme B is the most efficient at initiating target cell apoptosis by cleaving proteins after aspartate residues. This mechanism of action mimics and initiates caspase-mediated cell death (Voskoboinik et al., 2015). Granzyme A is another important granzyme that cleaves proteins after basic amino acids to induce caspase-mediated cell death. It is important to note that perforin and granzyme induced-cytotoxicity trigger apoptotic, not necrotic, cell death pathways. A calcium flux in the target cell is initiated by perforin binding, which causes endosomal fusion with the damaged target cell membrane, thereby sealing the cytotoxic contents inside. Pro-apoptotic pathways are then engaged to minimise host damage (Keefe et al., 2005), while accommodating the cross-presentation of antigen from apoptotic target cells to DCs subsets (Krebs et al., 2009). This permits a secondary, potent, antigen-specific adaptive immune response involving B and T cells. The low pH of cytolytic lysosomes (pH5) safeguards the NK cell from death by its own cargo. Perforins are active at neutral pH7, hence they are only activated upon release into the lytic synapse (Voskoboinik et al., 2015). How NK cells are resistant to these cytotoxic molecules once they are released into the lytic synapse surprisingly remains unclear. The fact that NK cells are serial killers, with the ability to kill on average four target cells each, makes them even more impressive effector immune cell. After reaching their killing capacity, NK cell effector molecule production is reduced and the cell adopts an 'exhausted' phenotype (Bhat and Watzl, 2007).

Another means of highly specific NK cell killing involves the death receptor pathway. Five death receptors have been characterised on the NK cell surface; TNF receptor-1, Fas/FasL (CD95), TNF-related apoptosis-inducing ligand (TRAIL) 1 and 2 and TNF-receptor-related apoptosis-mediated protein (TRAMP) (Warren and Smyth, 1999). Each of these death receptors shares a characteristic intracellular death receptor domain, which recruits adaptor proteins to induce caspase-dependent target cell apoptosis. The Fas/Fas-ligand pathway is important in the control of graft versus host disease (GvHD) (van Den Brink et al., 2000). In Fas-deficient mice, NK cells proved protective through the suppression of auto reactive B-cells. The depletion of NK cells in Fas deficient mice dramatically increased autoimmune severity (Takeda and Dennert, 1993).

In addition to the direct cytotoxic function of NK cells, the production of the pro-inflammatory cytokine IFN γ and chemokines CCR7 and CCR5 enhances the bystander immune response. These NK cell derived pro-inflammatory and chemotaxis molecules have been shown to activate, recruit and prime T cells, dendritic cells, macrophages as well as other NK cells in the microenvironment (Walzer et al., 2005, Martin-Fontecha et al., 2004).

Although NK cells are traditionally considered part of the innate immune system, strong evidence has emerged to suggest their adaptive-like features. Immunological memory is a hallmark of the adaptive immune response. The ability of a subgroup of NK cells to respond more robustly to MCMV infection suggested a capacity for memory-like properties. Indeed, the expression of the activating receptor Ly49H on NK cell surface is now used as a marker for 'memory' NK cells. Ly49H⁺ NK cells display a unique transcriptional signature that confers exquisite sensitivity for MCMV recognition (Daniels et al., 2001) (Sun et al., 2011).

1.1.4. NK cell receptors

NK cells express both activating and inhibitory receptors on their cell surface, which enable them to identify target cells and coordinate a rapid immune response. NK cell activating receptors contain immunoreceptor tyrosine based activating motifs (ITAMs) that trigger NK cell activation when a virally infected or transformed cell ligand is encountered. NK cell inhibitory receptors contain immunoreceptor tyrosine based inhibitory motifs (ITIMs), which regulate NK cell activation and prevent auto-reactivity through the recognition of self-molecules expressed on healthy cells.

NK cell activating receptors recognise pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). PAMPs are pathogen-associated molecules that are not normally expressed by host cells. For example, the influenza hemagglutinin is a glycoprotein expressed on the viral cell surface that is recognised by the natural cytotoxicity receptor NKp44 on NK cells (Arnon et al., 2001).

DAMPs relate to the aberrant expression of 'self-molecules', mainly stress ligands, which are present at low levels or absent on healthy cells but become upregulated in harmful situations. Examples of stress markers that are upregulated upon viral infection are the major histocompatibility class I homologues MICA and MICB, detected by the NK cell activating receptor NKG2D (Steinle et al., 2001) (Figure 1.1.5). Activating receptors transduce signal through the phosphorylation of a tyrosine residue in their cytoplasmic tail, allowing for the recruitment of the Src homology 2 domain containing kinases Syk or ZAP70 (Colucci et al., 1999).

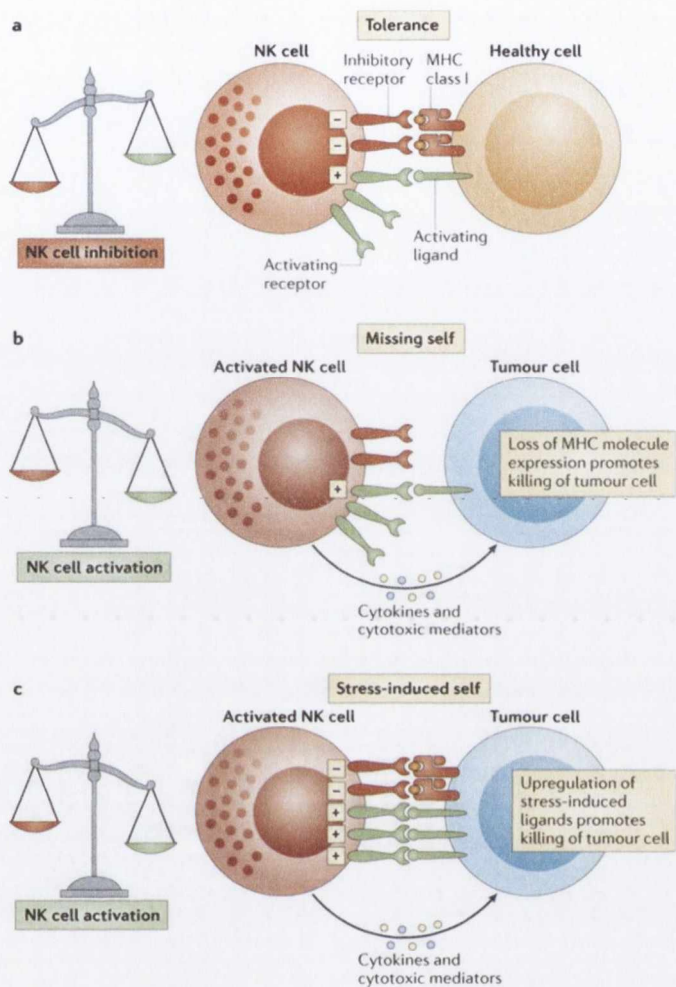
The engagement of inhibitory surface receptors, such as killer cell immunoglobulin-like receptors (KIR) or LY49 receptors in humans and mice respectively, provide a means for 'self-recognition' (Lanier, 2008). Self-recognition is crucial to prevent auto-reactivity and autoimmunity. Human leukocyte antigen (HLA) class I molecules are the human homolog of the

major histocompatibility complex (MHC). They are constitutively expressed on all cells of the body and function to present antigen-associated peptides, or indeed stress molecules, to surveying immune cells (Hewitt, 2003).

Another function of HLA molecules is to provide a 'self' signal to immune cells under homeostatic conditions. When an NK cell encounters HLA molecules on the surface of a cell, clustering of inhibitory receptors is initiated to induce NK cell tolerance to 'self' (Long, 2008) (Figure 1.1.5). As previously mentioned, NK cell inhibitory receptors contain immunoreceptor tyrosine based inhibitory motifs (ITIMs) in their cytoplasmic tail. These allow for the recruitment of SH2 domain containing protein tyrosine phosphatase 1 (SHP1) and SH2 domain-containing inositol 5-phosphatase (SHIP) to the NK cell-target cell interface, which blocks Ca^{2+} influx, thereby inhibiting NK cell activation (Lanier, 2008). It is the balance of NK cell activating and inhibitory receptors that decide whether an NK cell response is initiated or inhibited.

1.1.5. Immune evasion by virally infected and transformed cells

NK cells and CD8^+ T lymphocytes work together in the identification of transformed cells in the body. CD8^+ T cells raised specifically against tumor antigen patrol the body in search of HLA molecules exhibiting transformed cell antigen. Consequently, a common CD8^+ T cell immune evasion strategy employed by tumor cells involves the down regulation of HLA surface molecules. NK cells exploit this immune evasion strategy and use it to their advantage in the identification of transformed cells. A direct repercussion of diminished HLA expression is a decrease in NK cell inhibitory receptor engagement. Consequently, there is an increase in NK cell activation based on the 'missing self' principal (figure 1.1.5). Upon engagement with low MHC I expressing cells, NK cell $\text{IFN}\gamma$ production is induced. The release of $\text{IFN}\gamma$ in the tumor microenvironment initiates the transformation of quiescent dendritic cells into potent IL-12 producing cells, resulting in the priming of a CD8^+ T cell anti-tumor response (Mocikat et al., 2003).



Nature Reviews | Immunology

(Vivier et al., 2012)

Figure 1.1.5. NK cell activating and inhibitory receptors

The NK cell surface expresses activating and inhibitory receptors. NK cell inhibitory receptors induce NK cell tolerance and prevent auto-reactivity through the recognition of self-molecules expressed on healthy cells (a). Virally infected and transformed cells down regulate the cell surface expression of MHC to avoid detection by T cells. NK cells can recognise this immune evasion strategy and are activated by the 'missing-self' marker expression (b). NK cell activating receptors trigger NK cell activation when a virally infected, transformed or stressed cell ligand is encountered (c).

In addition to transformed cells, virally infected cells down-regulate HLA expression to avoid CD8⁺ T cell recognition. The missing self-principal states that NK cells recognize down-regulated HLA expression and use it as a marker for target cell killing. However, in contrast to cancer cells, viruses have employed a cunning mechanism to avoid NK cell recognition. The human cytomegalovirus (CMV) selectively up regulates HLA-E expression in infected cells (Tomasec et al., 2000). HLA-E is not recognized by CD8⁺ T cells but is a ligand for the NK cell inhibitory receptor NKG2A (Lee et al., 1998). This allows CMV infected cells to evade CD8⁺ T cell recognition, whilst preventing NK cell activation. Likewise, the herpes simplex virus (HSV) expresses decoy HLA I molecules, which bind to NK cell inhibitory receptors and prevent NK cell activation (Carrillo-Bustamante et al., 2013).

1.1.6. Cytokine-induced NK cell activation

Cytokines are indispensable for mounting a successful immune response. Cytokines which enhance NK cell function include IL-15, IL-12, IL-2 and IL-18 (Marcais et al., 2013). High concentrations of IL-15, alone or in combination with IL-12, synergistically facilitates NK cell activation and the acquisition of effector functions (Koka et al., 2004) (Fehniger et al., 1999). In the NK cell microenvironment, dendritic cells and monocytes are the primary physiological sources of IL-15 and IL-12, hence the activity of these cells influences NK cell function (Koka et al., 2004, Fehniger et al., 1999).

IL-2 is an adaptive cytokine that is primarily produced by CD4⁺ and CD8⁺ T cells (Boyman and Sprent, 2012). IL-2 signals through the JAK-STAT, PI3K and MAPK/ERK signalling pathways to enhance NK cell function. IL-2 induces NK cell differentiation into lymphocyte activated-killer (LAK) cells, which display increased effector molecule production and cytotoxicity (see section 1.1.10). IL-2 signals through JAK3 to enhance STAT5 activity. In

response to IL-2, STAT5 binds directly to the perforin promoter in human NK cells, thereby increasing NK cell cytotoxicity (Yu et al., 1999). In addition, IL-2 signalling enhances NK cell pro-inflammatory functions; inhibition of the MAPK/ERK pathway resulted in a dose-dependent decrease in IFN γ expression in human NK cells stimulated with IL-2 (Yu et al., 2000). One mechanism by which regulatory T cells limit NK cell responses is through their ability to tolerise Teff cells, thus limiting the amount of IL-2 available for NK cell activation (Gasteiger et al., 2013b).

IL-18 is one of eleven members of the IL-1 cytokine family (Marcais et al., 2013). True to its original name, IFN γ inducible factor (IGIF), IL-18 greatly enhances NK cell pro-inflammatory function by enhancing IFN γ expression. Macrophages and dendritic cells are the major cellular sources of active IL-18, however epithelial cells constitutively express the IL-18 inactive precursor (Shi et al., 2011). IL-18 signals through the MAPK p38 pathway to stabilize NK cell IFN γ mRNA transcripts (Mavropoulos et al., 2005). The expression of CD25, the high affinity IL-2 receptor subunit, is greatly enhanced on NK cells by IL-18 signalling (Leong et al., 2014). IL-18 has been shown to synergize with IL-12 and IL-15, resulting in enhanced and sustained NK cell effector function. In a mouse tumor model, adoptive transfer of IL-12/15/18 pre-activated NK cells, but not IL-15 or IL-2 stimulated NK cells, enhanced tumor cell clearance and mouse survival (Ni et al., 2012).

IL-10 is an anti-inflammatory cytokine produced by T helper 2 cells, macrophages, B cells and also NK cells (Couper et al., 2008). The direct effect of IL-10 signalling on NK cell activity remains somewhat controversial. Several studies show IL-10 signalling through JAK1/STAT3 enhances NK cell pro-inflammatory cytokine production and activating receptor expression (Zhu et al., 2014, Mocellin et al., 2004). Where others describe how IL-10 can negatively regulate NK cell responses, by decreasing the expression of NK cell activating cytokines IL-12 and IL-18 in macrophages and dendritic cells

respectively (Scott et al., 2006). In a sepsis study, the administration of IL-10 neutralizing antibodies was shown to enhance NK cell cytotoxicity and exacerbate pathology in mice (Scott et al., 2006).

1.1.7. Cytokine control of NK cell differentiation

In addition to the role of cytokines in regulating NK cell responses, cytokines control NK cell differentiation. Cytokines of the common gamma chain (γ_c) family including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 are implicated in the differentiation of murine NK cells from bone marrow hematopoietic progenitor cells (Vosshenrich et al., 2005). X-linked severe combined immunodeficiency patients have a mutation leading to the loss of γ_c function. As a result, NK cell numbers are diminished, emphasizing the importance of this family of cytokines in human NK cell development (Horino et al., 2013). Similarly, genetic ablation of the murine γ_c gene results in a complete absence of NK cell development (Vosshenrich et al., 2005).

The IL-2 and IL-15 hetero-trimeric receptors share common β and γ subunits. Importantly, genetic ablation of the β subunit results in a complete absence of NK cells in mice (Suzuki et al., 1997). However, this is not due to the role of IL-2 signalling in NK cell development; mice deficient for IL-2 or its α -receptor subunit do not show impaired NK cell numbers (Kundig et al., 1993, Vosshenrich et al., 2005). In contrast, the deletion of IL-15 (Vosshenrich et al., 2005, Cooper et al., 2002) or the IL-15 α -receptor resulted in severely impaired NK cell differentiation (Kawamura et al., 2003, Koka et al., 2003).

1.1.8. Cytokine maintained NK cell homeostasis

Beyond NK cell activation and development, cytokines are of critical importance for the maintenance of NK cells in the periphery and residing organs. Considering IL-2^{-/-}, IL-2Rα^{-/-}, IL-4^{-/-} and IL-7Rα^{-/-} mice display normal levels of peripheral NK cells, it is unlikely that these cytokines play a crucial role in supporting NK homeostasis. However, γc^{-/-}, IL-15^{-/-} and IL-15Rα^{-/-} mice show significant decreases in mature NK cell numbers. This is not solely due to an inability of NK cells in these mice to mature (as previously mentioned IL-15 is required for NK cell development). In addition, IL-15 has been shown to act as a critical survival factor for NK cells. This is exemplified by adoptive transfer studies in which the survival of transferred wild type mature NK cells into IL-15^{-/-} and IL-15Rα^{-/-} mice decrease dramatically (Cooper et al., 2002, Koka et al., 2003). Furthermore, IL-15 has been shown to selectively expand and proliferate NK cells *in vitro* (Zhao and French, 2012).

1.1.9. NK cell immunotherapeutic strategies

NK cells isolated directly from cancer patients are known to display reduced rates of proliferation, decreased response to cytokine stimuli, reduced effector molecule production and impaired maturation marker expression (Whiteside and Herberman, 1994, Mamessier et al., 2013, Critchley-Thorne et al., 2009). One early strategy to enhance the NK cell anti-cancer immune response involved cytokine-induced activation of endogenous host NK cells *in vivo*. The systemic administration of IL-2 was shown to induce NK cell differentiation into lymphocyte activated-killer (LAK) cells *in vivo*, which display increased effector molecule production and enhanced proliferation (Burns et al., 2003). However, the anti-tumor killing capacity of LAK cells in response to IL-2 had no significant impact on tumor

growth or progression *in vivo*. In addition, this treatment was accompanied by many negative side effects, with some patients experiencing vascular leak syndrome and others increased Treg expansion, which is associated with a poor prognosis (Rosenberg, 1984).

A more successful NK cell based anti-cancer immunotherapy involves the adoptive transfer of *ex vivo* activated NK cells. NK cell adoptive transfer therapies are showing great promise in the treatment of breast cancer and malignant glioma (deMagalhaes-Silverman et al., 2000, Ishikawa et al., 2004). NK cells used in adoptive transfer therapies may be autologous (from the host) allogenic (from another individual) or genetically engineered chimeric antigen receptor (CAR) NK cells. CARs are engineered receptors that have the extracellular specificity of an antibody combined with potent intracellular signaling adaptors (Carlsten and Childs, 2015). CD19 is a cell surface marker of B cells. CD19-CAR engineered NK cells display enhanced tumor cell cytotoxicity in a previously resistance B cell leukemia line (Romanski et al., 2016).

In addition, the use of monoclonal antibodies, which target tumor cell receptors and assist in NK cell antibody dependent cellular cytotoxicity (ADCC), have already been approved by the FDA and are in use in the clinic (Weiner et al., 2010). Rituximab is an anti-CD20 antibody, which binds to the CD20 receptor expressed on B cells. In combination with chemotherapy, the use of rituximab in B cell lineage-specific acute lymphoblastic leukaemia (ALL) has significantly improved patient outcome (Maury et al., 2016).

These studies highlight the importance of NK cells in the immune response against virally infected and transformed cells. However, a recent publication by the Vivier group outlines a rare cohort of patients that have a mutation in the γ -chain cytokine receptor subunit IL-2R γ . This mutation renders these patients deficient in all ILC subsets, including NK cells (Vely et al., 2016). Surprisingly, although these patients lack NK cells entirely, most patients remained healthy following continued allogenic hematopoietic stem cell

transplantation treatment. Furthermore, they displayed no increased susceptibility against viral or tumor infection. This study highlights the fact that although NK cells are important in the anti-tumor and anti-viral immune response, they are not essential for survival.

1.1.10. The tumor microenvironment is metabolically restrictive

Immunotherapies are an exciting prospect for the field of cancer therapy and have shown great efficacy in the treatment of haematological disorders such as ALL as previously described (1.1.9). However, the use of immunotherapies in the treatment of solid tumors is proving more challenging (Assmann and Finlay, 2016). One reason why immunotherapeutic strategies are often unsuccessful in treating solid tumors is due the metabolically restrictive tumor microenvironment, which limits infiltrating immune cell function. Tumor cells display a 10-fold greater rate of glucose uptake than activated lymphocytes, meaning they can efficiently sequester glucose in the tumor microenvironment, away from infiltrating immune cells (Cham and Gajewski, 2005). Indeed, glucose levels in the tumor microenvironment were measured and found to be 15 times less than that found in the circulation (0.6mM versus 9mM) (Ho et al., 2015). Therefore, within the solid tumor, infiltrating immune cells can encounter unfavorable conditions that may directly limit the rate of glycolysis.

In addition to glucose, tumor cells are known for being 'addicted' to glutamine (Wise and Thompson, 2010). This is largely due to the importance of glutamine in polyamine and nucleotide synthesis, which facilitates high rates of cancer cell proliferation (Cory and Cory, 2006). In the tumor microenvironment, it is therefore likely that glutamine levels will also become limiting. Glutamine is an important metabolic fuel for many immune cells (Wang et al., 2011b, Glick et al., 2014). Hence glutamine deprivation in the tumor microenvironment will limit the metabolism of tumor infiltrating

lymphocytes (TIL), which can negatively impact their anti-tumor responses. Due to the poor vascularisation found in solid tumors (West et al., 2001), heterogeneous regions of hypoxia containing oxygen levels as low as 0.07% have been measured (Brizel et al., 1999). Oxygen concentrations this low inhibit oxidative forms of infiltrating immune cell metabolism, which can further impact TIL anti-tumor responses. The next section of this chapter will discuss the importance of cellular metabolism for the acquisition of immune cell function.

1.2. Immune cell energy metabolism

In the past, cellular metabolism was considered a basic process required for the supply of energy to a cell. However in recent years, strong evidence has emerged that shows metabolic processes play a fundamental role in dictating the immune response. All cells of the body require adenosine triphosphate (ATP) to fuel cellular processes. Glucose is a simple sugar that is the basic building block of polysaccharides. Importantly, it acts as a carbon fuel source for the generation of cellular ATP. Glucose may be metabolized via two integrated pathways: Glycolysis and Oxidative Phosphorylation (OxPhos) (Zheng, 2012). In the cytoplasm, glycolysis converts glucose into pyruvate via multiple enzymatic steps (Figure 1.2.1). A small amount of ATP is generated in the process; a net of 2 ATP molecules per glucose molecule broken down (Zheng, 2012). However, a second means of generating ATP from pyruvate can occur in the mitochondria. This involves the conversion of pyruvate to acetyl Co A by the enzyme pyruvate dehydrogenase, which can then fuel the tricarboxylic acid (TCA) cycle. The TCA cycle generates the reducing equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) from NAD⁺ and FAD respectively (Zheng, 2012). NADH and FADH donate electrons into the electron transport chain (ETC) and fuel Oxidative Phosphorylation

(OxPhos). The components of the ETC are found in the inner mitochondrial membrane. Together with the TCA cycle, the ETC generates a net of 34 molecules of ATP per glucose molecule broken down, making this form of glucose metabolism extremely resourceful in terms of energy production (Zheng, 2012).

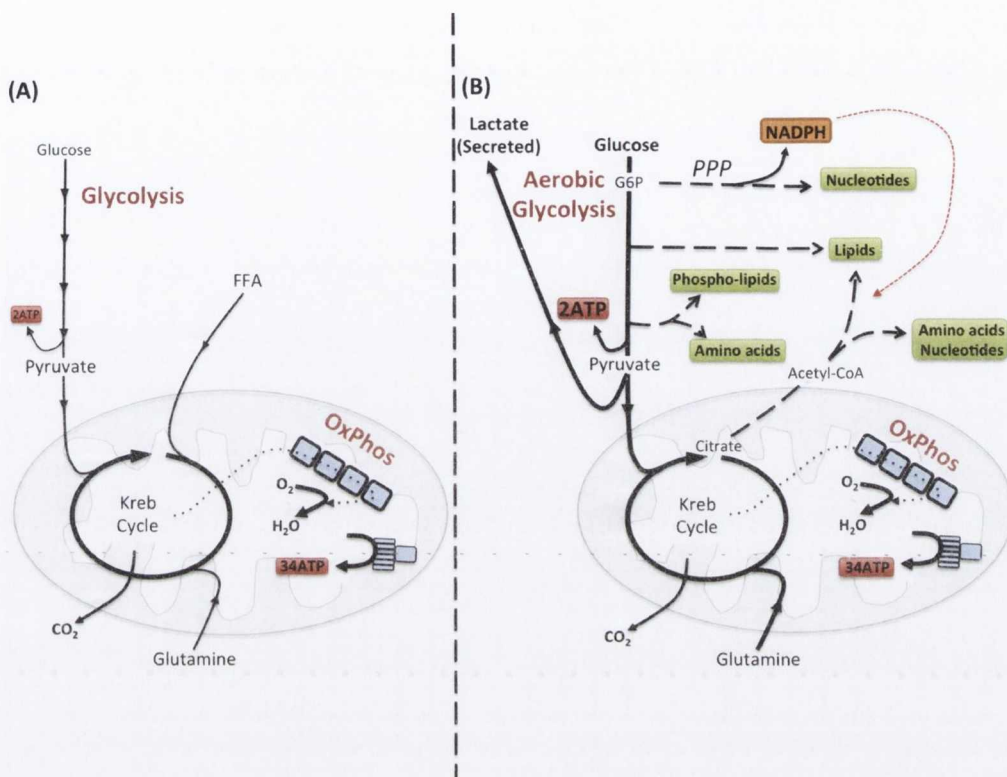
Glycolysis and OxPhos are integrally linked by the conversion of the end product of glycolysis, pyruvate, into acetyl-CoA, which can enter the TCA cycle to fuel OxPhos. Under hypoxic conditions, OxPhos is inhibited and pyruvate is preferentially converted into lactate, which is excreted from the cell. Under certain conditions, the conversion of pyruvate into lactate may also occur in normoxia (Chang et al., 2013b). Pro-inflammatory immune cells and transformed cells are known to often engage in this form of glucose metabolism termed 'Aerobic Glycolysis'.

Why do such cells adopt aerobic glycolysis when it produces less ATP per molecule of glucose broken down than OxPhos? Glycolytic intermediates provide an important source of carbon for biosynthetic processes, facilitating increased cell growth, the production of effector molecules and increased rates of proliferation. 3-phosphoglycerate can be converted into the amino acid serine, glucose-6-phosphate can be shunted into the pentose phosphate pathway (PPP) to make ribose-5-phosphate (the backbone of nucleotide synthesis) and NADPH (a reducing agent that facilitates lipid synthesis), and dihydroxyacetone phosphate can be converted into glycerol, which is also used for lipid synthesis (Fig. 1.2.1). Not only does aerobic glycolysis provide the building blocks for such anabolic processes, this metabolic profile prepares an immune cell for entering metabolically restrictive microenvironments, such as hypoxic tissue where OxPhos is not possible.

Pro-inflammatory immune cells also have the ability to dramatically increase flux through glycolysis, thereby supporting high levels of ATP production (Koppenol et al., 2011). This increased flux is facilitated by the conversion of pyruvate into lactate, generating the electron carrier NAD^+ . In the absence of

NAD⁺, glyceraldehyde-3-phosphate cannot be oxidized and flux through the glycolytic pathway is inhibited. Hence the preferential conversion of pyruvate to lactate assists in NAD⁺ regeneration and is essential for maintaining increased rates of glycolysis (Tan et al., 2013). Secondly, increased flux through glycolysis results in increased production of pyruvate. Mitochondria cannot upregulate the rate of OxPhos as quick as glycolytic machinery can make it. Hence, pyruvate is preferentially converted into lactate and excreted from the cell to avoid product inhibition when rates of glycolysis are increased.

Fatty acids are another a rich source of ATP that can be broken down via β -oxidation. For example, the oxidation of one palmitic acid molecule yields a net of 129 ATP molecules. M2 macrophages and regulatory T cells rely on fatty acid oxidation (FAO) as their primary source of energy production (Huang et al., 2014, Michalek et al., 2011).



(Loftus and Finlay, 2016)

Figure 1.2.1 Configuring metabolism to match immune cell function

(A) Oxidative forms of glucose metabolism support efficient energy production. The product of glycolysis, which yields a net of 2 molecules of ATP per glucose molecule broken down, can be converted into acetyl CoA. Acetyl CoA can then enter the TCA cycle and fuel Oxidative phosphorylation (OxPhos) in mitochondria. This yields 34 molecules of ATP per glucose molecule broken down. Other fuels that can support OxPhos are free fatty acids (FFA) and glutamine. (B) Aerobic glycolysis supports biosynthetic processes of the cell by providing the carbon source for lipids, amino acids and nucleotide synthesis. During aerobic glycolysis, a significant proportion of pyruvate is converted to lactate and secreted from the cell. This generates the NAD^+ required by GAPDH to support high flux through this pathway. Increased flux through glycolysis allows for large amounts of ATP to be produced.

1.2.1. Cellular metabolism matches immune cell demands

Different immune cells adopt distinct metabolic configurations that allow the cell to balance its requirements for energy, molecular biosynthesis and longevity (Loftus and Finlay, 2016). Pro-inflammatory immune cells acquire elevated levels of glucose uptake and increased rates of glycolysis, metabolizing glucose primarily into lactate (Chang et al., 2013a). In contrast, quiescent and anti-inflammatory immune cells mostly display low rates of glucose uptake, but metabolize it efficiently via OxPhos to meet their energy demands (Pearce and Pearce, 2013) (Figure 1.2.2). For example, the differentiation of a naïve macrophage into an activated pro-inflammatory M1 or anti-inflammatory M2 bears distinct metabolic traits: M1 macrophages adopt increased rates of aerobic glycolysis but M2 macrophages preferentially increase fatty acid β -oxidation (Lacy-Hulbert and Moore, 2006). Similarly, the differentiation of a naïve T cell into an activated effector T cell is accompanied by a shift in metabolism towards aerobic glycolysis (Chang et al., 2013b). In contrast, regulatory T cells (T_{reg}) mostly rely on oxidative forms of glucose metabolism to meet their energetic demands (Newton et al., 2016). These metabolic adjustments serve an important function during immune cell activation, where perturbations to cellular metabolism, or the factors controlling cellular metabolism, prevent immune cell activation, differentiation and function (Finlay et al., 2012b, Chang et al., 2013a).

Why do the metabolic configurations of an immune cell reflect its function? The biosynthetic demands of pro-inflammatory immune cells are often high, due to their requirement for increased clonal expansion and effector molecule production. As previously discussed, aerobic glycolysis supports efficient biosynthesis, hence why it is a characteristic metabolic profile of many effector immune cells.

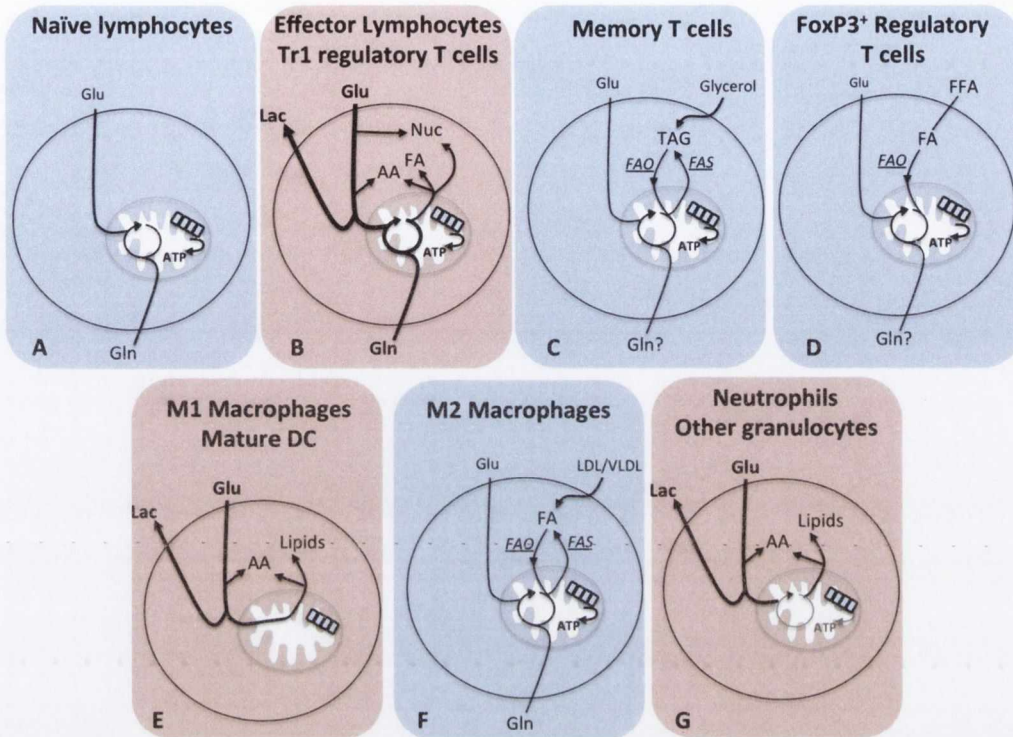
Oxidative forms of cellular metabolism are efficient at generating ATP. Regulatory cells tend to have less biosynthetic demands so ATP alone is sufficient to support cellular homeostasis. One exception to this is Foxp3-regulatory T cells (Tr1), which adopt elevated levels of glycolysis. Although these cells are regulatory, they have significantly more biosynthetic demands than classical Treg due to their direct killing of target cells through granzyme/perforin mediated cell lysis (Mascanfroni et al., 2015).

Long-lived immune cells tend to favour oxidative forms of metabolism over glycolytic. For example, the differentiation of an effector T cell into a memory T cell is accompanied by a shift in cellular metabolism from aerobic glycolysis to OxPhos. Mitochondrial membrane potential is integrally linked to the induction of cellular apoptosis (Gottlieb et al., 2003), hence it is hypothesised that oxidative forms of cellular metabolism promote enhanced mitochondrial integrity and therefore, longevity. Increased persistence is desirable for regulatory cells, such as memory T cells, which can recognise specific antigen from immunological insults that occurred years before. However, increased persistence would not be desirable for pro-inflammatory cells such as T_{eff}, as prolonged inflammation can lead to tissue damage and destruction. Therefore, the fact that oxidative but not glycolytic forms of cellular metabolism are linked to longevity is an elegant self-regulating mechanism by which the immune system keeps itself in check (Loftus and Finlay, 2016).

1.2.2. Glutamine metabolism fuels energy production and signalling

Amino acids are an important fuel source for many cell types. The amino acid glutamine can be broken down in the cell via glutaminolysis to form the TCA cycle intermediate alpha-ketoglutarate (α KG). Glutamine-derived α KG can replenish the TCA cycle, thereby fuelling OxPhos in a process termed anapleurosis. This form of cellular metabolism is important for the acquisition of some immune cell effector functions; inhibition of glutaminolysis in CD4⁺ T cells results in impaired IFN γ production and expansion (Lee et al., 2015).

Tracing studies revealed glutamine supports fatty acid synthesis under hypoxic conditions, through the generation of the TCA cycle intermediate citrate (Brose et al., 2014). Glutamine is also an indispensable nitrogen donor for purine and pyrimidine synthesis, implicating it in the controlling the rate of cellular proliferation (Cory and Cory, 2006). In addition, glutamine itself, being one of twenty amino acids, is generally required for protein synthesis. These studies highlight the importance of glutamine in fuelling various metabolic and biosynthetic cellular pathways. However, glutamine also has an important role in supporting immune cell signal transduction. Glutamine controls the expression and stability of signalling molecules due to its role in post-translational protein modifications. Glutamine, along with glucose, acetyl CoA, uridine and ATP, feeds the hexosamine biosynthetic pathway (HBP) for the synthesis of UDP-GlcNAc. UDP-GlcNAc is an uncharged, acetylated hexosamine sugar that can be attached to site-specific serine or threonine amino acid residues on proteins, thereby regulating protein stability. O-GlcNAcylation is now said to rival phosphorylation in terms of its importance in controlling protein expression/stability. Inhibition of GlcNAcylation in cytotoxic T lymphocytes results in defective T cell responses that lead to impaired T cell progenitor renewal and decreased peripheral T cell clonal expansion (Swamy et al., 2016).



(Loftus and Finlay, 2016)

Figure 1.2.2. Cellular metabolism is integrally linked to function

Blue panels represent cells with oxidative metabolism, red panels those with glycolytic metabolism. (A) Naïve T cells use glucose and glutamine and Oxphos. (B) Effector lymphocytes and Tr1 regulatory T cells have high rates of both glycolysis and OxPhos, metabolise glucose to lactate and use metabolic intermediates to support biosynthetic processes. (C) Memory T cells use glucose to generate mitochondrial citrate, which is exported into the cytosol to support lipid synthesis. These de novo synthesised fatty acids are used with imported glycerol to generate and store triacylglycerides (TAG). Acetyl-CoA generated following β -oxidation of these TAGs fuels OxPhos. (D) FoxP3⁺ regulatory T cells use exogenously derived fatty acids metabolised by β -oxidation to support OxPhos. (E) M1 macrophages and mature DC engage aerobic glycolysis for ATP synthesis and to support biosynthesis while also inactivating OxPhos. (F) M2 Macrophage metabolism is characterised by fatty acid β -oxidation and OxPhos. β -oxidation is fuelled by lipids that are scavenged from the external microenvironment and by lipids generated by de novo fatty acid synthesis. (G) Neutrophils are highly glycolytic with few functional mitochondrial and very low rates of OxPhos.

1.2.3. Cellular metabolism directly regulates immune cell functions

Intriguingly, it has been shown that key metabolic regulators are also immunomodulatory. A secondary role for the glycolytic enzyme GAPDH in controlling CD4⁺ T cell IFN γ production was recently described (Chang et al., 2013b). In a quiescent T cell, where rates of glycolysis are low, GAPDH binds to free IFN γ mRNA. This decreases the association of IFN γ with the polysome, hence reducing the rate of IFN γ translation. Upon activation, CD4⁺ T cells switch their metabolic balance towards aerobic glycolysis, displaying dramatically enhanced rates of glycolysis. This results in GAPDH sequestration into the glycolytic pathway. In this instance, IFN γ mRNA transcripts were shown to have increased association with polysomes, indicating increased rates of translation (Chang et al., 2013b). The Hentze group has since identified mRNA binding capacity of other glycolytic enzymes, such as enolase (Castello et al., 2015). These findings further extrapolate the 'moonlighting' ability of metabolic enzymes in the direct regulation of immune cell function.

The glycolytic intermediate phosphoenolpyruvate (PEP) is important for effector T cell responses. Susan Kaech's group elegantly showed that PEP accumulation inhibits Ca²⁺ uptake into the endoplasmic reticulum by suppressing the activity of the calcium uptake transporter SERCA. Increased calcium flux stabilises NFAT signalling following TCR engagement, which is required for IFN γ and CD40L expression (Ho et al., 2015). Therefore, in glucose restrictive microenvironments such as the tumor microenvironment where glycolysis and thereby PEP accumulation is limited, T cell effector responses will be compromised. In accordance with this, T cell functional responses in a mouse model of B16 melanoma were enhanced by the over-expression of phosphoenolpyruvate carboxykinase 1, which is the enzyme that converts OAA to PEP.

The TCA cycle intermediates succinate, citrate, fumarate and acetyl Co-A have each been shown to directly influence immune cell functions. The TCA cycle in M1 macrophages is broken in two places. The first break occurs after citrate (Jha et al., 2015). This allows for the repurposing of citrate for pro-inflammatory (prostaglandin) and anti-microbial (itaconic acid) molecule production. The second break in the M1 TCA cycle occurs after succinate. This results in succinate accumulation, which has been shown to enhance IL-1 β production through stabilisation of HIF1 α (Tannahill et al., 2013). In monocytes, fumarate induces epigenetic modifications that confer innate immune memory (Arts et al., 2016). In all cell types, acetyl Co A can directly influence protein and gene expression through the the acetylation of lysine residues in histones and other proteins (Pietrocola et al., 2015).

The methyltransferase EZH2 enhances Notch activity and controls the polyfunctionality of T cells i.e the ability to simultaneously express multiple effector molecules such as IFN γ , granzyme b, perforin and IL-2. EZH2 expression in T cells is negatively regulated by the microRNA miR101 and miR26a. It was recently shown that in a metabolically restrictive microenvironment, such as that found in ovarian cancer, the expression of miR101 and miR26a increases. This resulted in decreased EZH2 expression, leading to impaired T cell functional responses (Zhao et al., 2016). Although the mechanism by which glucose restriction enhances miR101 and miR26a expression remains to be elucidated, this study highlights the fact that cellular metabolism can influence immune cell function through the regulation of microRNA expression.

1.2.4. T cell energy metabolism

When this study began, nothing was known about NK cell metabolism. The only information regarding lymphocyte metabolism was with respect to T cells. Following TCR activation, T cells dramatically increase their rates of glucose uptake and glycolysis, producing large amounts of lactate via aerobic

glycolysis (Chang et al., 2013b). This metabolic switch, which favours efficient-biosynthesis over efficient-ATP production, facilitates T cell clonal expansion and pro-inflammatory molecule production.

Activated T cells can differentiate into long lasting, antigen-specific memory T cells. Unsurprisingly, the functions, and thereby metabolic demands, of memory T cell are distinct from that of an activated effector T cell. Unlike effector T cells, memory T cells (T_{mem}) do not have high biosynthetic demands. Therefore, T_{mem} rely primarily on OxPhos for the generation of ATP to maintain cellular homeostasis (Vander Heiden et al., 2009). Cellular metabolism has also been shown to influence the differentiation of multiple $CD4^+$ T helper subsets; T regulatory cells predominantly use mitochondrial OxPhos for development and survival (Michalek et al., 2011), whereas the development of T helper 17 (T_H17) cells requires elevated rates of glycolysis (Shi et al., 2011) (Fig 1.2.2). Experiments in which the rate of glucose metabolism was directly limited highlight the importance of glycolysis in controlling T cell differentiation and function. $CD4^+$ T cells cultured in galactose instead of glucose showed decreased effector molecule production and increased memory like properties (Chang et al., 2013b). Similarly, when the rate of glycolysis was inhibited in $CD8^+$ T cells using low doses of the glycolytic inhibitor 2-deoxyglucose, memory T cell differentiation was enhanced at the expense of effector T cell development (Sukumar et al., 2013). These experiments demonstrate the direct role for aerobic glycolysis in controlling effector lymphocyte responses.

More than just an increase in flux through glycolysis, activated $CD8^+$ T cells undergo complete metabolic reprogramming upon activation. This is supported by an increase in the expression of i) nutrient receptors, such as CD71 the transferrin receptor, ii) glucose transporters, such as GLUT 1, iii) amino acid transporters, such as Slc7a5 and iv) an increase in the transcription of key rate limiting glycolytic enzymes Hexokinase 2 (Hex2) and Lactate Dehydrogenase (LDHa). (Jacobs et al., 2008, Kelly et al., 2007, Fox

et al., 2005). (Marko et al., 2010, Vander Heiden et al., 2009). The next section will describe the key factors that control T cell metabolic reprogramming and thereby, T cell function.

1.2.5. Factors controlling metabolism and function of effector T cells

Two transcription factors that control T cell metabolic reprogramming are myelocytomatosis oncogene (cMYC) and hypoxia inducible factor 1 alpha (HIF1 α). cMyc expression is robustly induced early on following TCR triggering (Wang et al., 2011b, Sinclair et al., 2013). As previously discussed, T cells undergo a dramatic shift in cellular metabolism upon activation, significantly upregulating rates of glucose uptake and glycolysis (section 1.2.4). cMyc is indispensable for this process; in the absence of cMyc, T cell metabolic reprogramming is profoundly impaired (Wang et al., 2011b, Sinclair et al., 2013). Ldha and PKM2 are among the key glycolytic genes that are decreased in cMyc null T cells following TCR activation (Wang et al., 2011b). In addition to its role in glycolytic T cell metabolic reprogramming, cMyc is also required for activation-induced increases in T cell glutaminolysis (Wang et al., 2011a). cMyc activity is required for glutaminase 2 expression in T cells, which converts glutamine to alpha-ketoglutarate (Wang et al., 2011a, Colombo et al., 2010, Carr et al., 2010). Alpha-ketoglutarate is an important precursor of polyamine synthesis; hence it plays an important role in DNA synthesis and cellular replication. Indeed, cMyc null T cells display profound defects in their ability to proliferate following TCR activation (Wang et al., 2011a). Although cMyc expression is robustly increased following T cell activation, its expression is transient (Wang et al., 2011b). However, cMyc drives the stable expression of the transcription factor AP4, which promotes T cell growth and entry into the cell cycle (Chou et al., 2014). Mice deficient for AP4 show a significant decrease in the expression of Myc target genes such as Ldha (Chou et al., 2014). Furthermore, mice lacking AP4 display a decreased clearance of the

West Nile Virus (WNV) due to compromised T cell activation (Chou et al., 2014).

HIF1 α controls the metabolic changes that occur during the differentiation of an activated T cells into a CTL (Finlay et al., 2012a). IL-2 is an important cytokine in this process. CD8⁺ T cells deficient for HIF fail to sustain increased levels of glycolysis, even in the presence of IL-2. The metabolic defect of HIF null T cells is due to reduced mRNA expression of key glycolytic genes such as Hex2, Ldha, pfk1 and Glut1 (Finlay et al., 2012a). A HIF1 α dependent metabolic checkpoint governs the developmental fate of T_H17 and Treg cells. Due to the importance of HIF1 α in promoting glycolytic metabolism in T cells, the progression of Tregs is enhanced in the absence of HIF (OxPhos dependent) at the expense of T_H17 cell development (glycolysis dependent) (Shi et al., 2011).

Other determining factors that control T cell metabolism include Notch, a signaling receptor expressed on the surface of T cells. In activated T cells, IL-2 sustains notch signaling and enhances PI3K/Akt activity (Backer et al., 2014). The PI3K/AKT signaling pathway promotes increased glucose metabolism by enhancing glut1 expression on the T cell surface (Gerriets and Rathmell, 2012). Following receptor ligation, the notch intracellular domain (NICD) of its receptor is cleaved. This permits translocation into the nucleus where it acts as a transcriptional promoter of glycolysis-associated genes (Schulz et al., 2012). Notch signaling events are dependent upon PDK1, mTOR and t-bet activity in T cells (Kelly et al., 2007). Furthermore, Notch controls O-GlcNACylation during T cell development; through its regulation of glucose and glutamine transport (Swamy et al., 2016).

One kinase that dictates the fate of many immune cells, including T cells, is the mammalian target of rapamycin (mTOR). The role of mTOR in controlling the immune response will be discussed in the next section.

1.3. Mammalian target of rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine-threonine kinase that acts as a molecular sensor of the cellular microenvironment, incorporating environmental signals to alter cell responses accordingly (Shimobayashi and Hall, 2016). mTOR associates with various proteins to form one of two complexes, mTORC1 or mTORC2. Broadly speaking, mTORC1 controls cell growth in response to nutrient availability and growth factors, whereas mTORC2 mediates cell proliferation and survival (Sparks and Guertin, 2010). Most mTOR-based research has predominantly focused on mTORC1. Rapamycin is a potent, selective inhibitor of mTORC1. Upon entry into a cell, rapamycin forms a complex with the protein FKBP12 (FK506 binding protein of 12KDa) (Shimobayashi and Hall, 2014). The rapamycin-FKBP12 complex binds and impedes the activity of the mTORC1 complex by preventing substrate recruitment (Shimobayashi and Hall, 2014). The use of rapamycin has allowed many of the signalling pathways associated with mTORC1 to be elucidated. mTORC1 signal transduction plays a vital role in coordinating cell metabolism, growth, differentiation, immune cell activation and protein synthesis (Shimobayashi and Hall, 2014). Given its importance in the maintenance of homeostatic processes, deregulation of mTORC1 activity is implicated in pathological settings such as cancer progression and insulin resistance (Doe et al., 2012).

1.3.1. Regulation of the mTOR signalling pathway

mTOR is activated by increased levels of growth factors (e.g. the cytokine IL-2), nutrient availability (e.g. amino acids), oxygen availability and the overall energy status of the cell (ATP:AMP ratio) (Laplante and Sabatini, 2012) (Figure 1.3.). Many immune-modulating cytokines, such as IL-1, IL-2, IL-4, IL-12 and IFN γ activate mTOR through the PI3K/Akt signaling axis (Powell et al., 2012). When a growth factor, such as insulin, binds to its extracellular surface receptor, insulin receptor (IR), it activates PI3K and Ras

signaling pathways. The downstream effector kinases of these pathways; protein kinase B (Akt/PKB), extracellular-signal-regulated kinase 1/2 (ERK1/2), and ribosomal S6 kinase (RSK1), directly phosphorylate and inactivate the tuberous sclerosis complex (TSC1/2) (Laplane and Sabatini, 2012) (Figure 1.3.1). TSC1/2 are GTPases, which negatively regulate mTOR activity through the hydrolysis and inactivation of Ras homolog enriched in the brain (Rheb). Rheb is located on the lysosomal surface. Only in its active state, bound to GTP, may Rheb interact with the catalytic domain of mTOR to induce mTOR activation.

Amino acid availability is a critical regulator of mTORC1 activity. Amino acids activate Rags (Ras-related GTPases), which are tethered to the lysosomal surface and facilitate mTOR docking (Shimobayashi and Hall, 2016). Once at the lysosomal surface, mTORC1 can sense amino acid availability and the nutrient status of the cell through lysosomal sampling. Increased mTOR docking during nutrient replete conditions facilitates prolonged mTOR-Rheb interactions, thereby enhancing mTOR activation in response to amino acid availability (Ogmundsdottir et al., 2012, Cang et al., 2013). Another mechanism by which amino acids enhance mTORC1 activity is by inducing TSC1/2 dissociation from the lysosomal surface (Menon et al., 2014). Sestrins are a set of stress-responsive proteins that are activated when amino acid availability is low. Sestrins signal through TSC1/2 dependent and independent mechanisms to turn off mTORC1 activity (Ye et al., 2015).

If a cell becomes energy deprived, AMP-activated protein kinase (AMPK) is switched on due to its ability to sense cellular ATP: AMP levels. This kinase functions to suppress ATP-consuming anabolic processes whilst turning on ATP-generating catabolic ones, in an effort to restore energy balance (Hardie, 2008). mTOR activity is inhibited by AMPK phosphorylation and activation of TSC2 (Inoki et al., 2006).

Whilst PI3-kinase/Akt signalling is often upstream of mTORC1 activity in most cell types, a PI3K-Akt independent mechanism of mTORC1 activation in

IL-2 activated CD8⁺ T cells has been identified. Here, phosphoinositide-dependent kinase 1 (PDK1), and not PI3K-Akt signalling, was shown to be essential for mTOR activity in CD8⁺ T cells (Finlay et al., 2012a).

1.3.2. mTORC1 regulation of cellular metabolism

mTORC1 activity enhances cell growth and proliferation by promoting anabolic processes such as lipid and protein synthesis. mTORC1 phosphorylation of the transcriptional regulator eukaryotic initiation factor 4E binding protein 1 (4E-BP1), results in the initiation of cap-dependent translation (Richter and Sonenberg, 2005). Another downstream target of mTORC1 is the p70 ribosomal S6 Kinase 1 (S6K1). Phosphorylated S6K1 increases mRNA biogenesis, cap-dependent translation and elongation, and the translation of ribosomal proteins (Ma and Blenis, 2009) (Figure 1.3.). mTORC1 activity also enhances the transcription of ribosomal protein phosphatase 2A and the transcription initiation factor IA, both of which are involved in ribosomal biogenesis (Mayer et al., 2004). mTORC1 enhances glucose metabolism in activated T cells through the stabilization of important transcription factors such as HIF1 α and cMyc (figure 1.3). As previously mentioned, HIF1 α and cMyc are two important transcriptional regulators that directly control the expression of key glycolytic genes and glucose transporters in T cells (Finlay et al., 2012b, Wang et al., 2011b) (see section 1.2.5.).

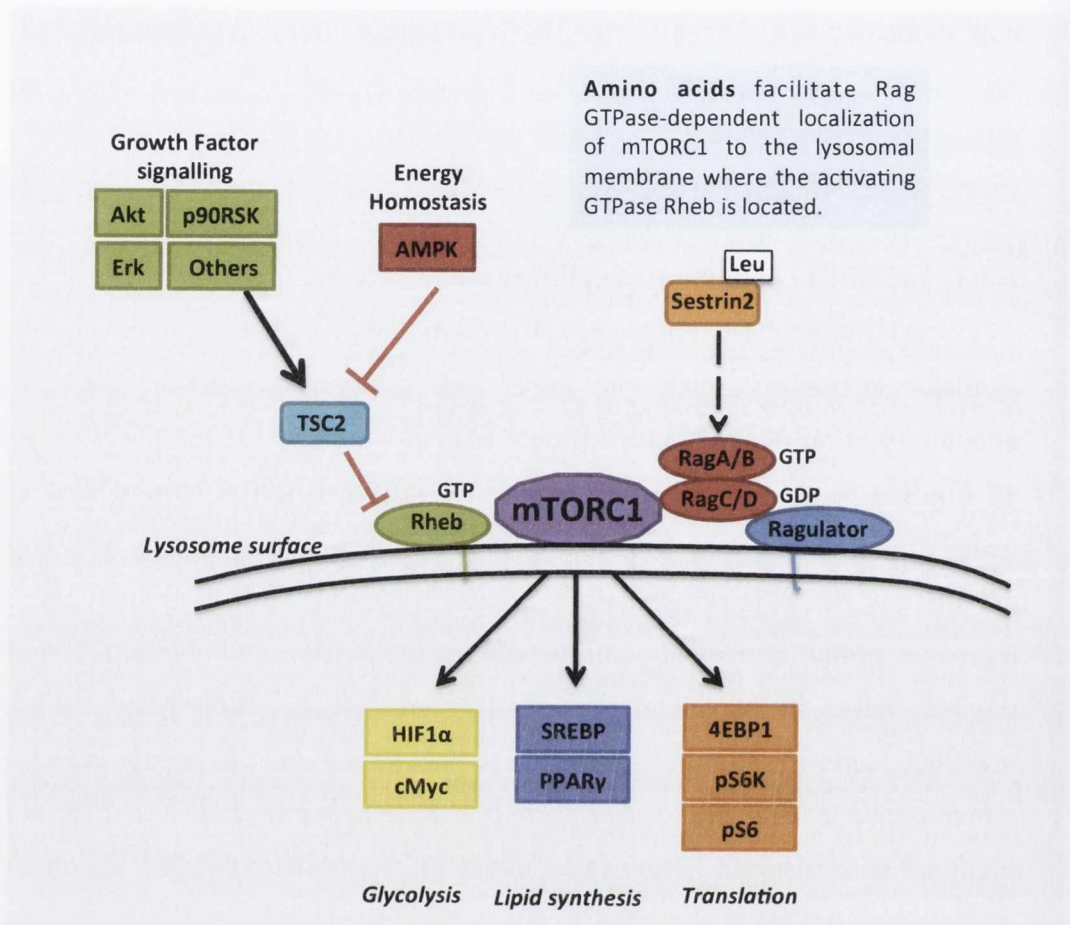


Figure 1.3. mTORC1 integrates micro-environmental signals to control cellular processes

The activity of mTORC1 is governed by myriad microenvironmental cues. The binding of growth factors, amino acids or cytokines to their respective cell surface receptors promotes mTORC1 activation through the inhibition of the TSC1/2 complex. In a similar manner, the energy status of the cell also influences mTORC1 activity. The TSC1/2 complex negatively regulates mTORC1 activity through hydrolysis and inhibition of Rheb. Rheb activity and amino acid availability are the two principal factors that definitively control mTORC1 activation. mTORC1 signalling promotes anabolic cellular processes such as protein synthesis, lipid synthesis, and increased glucose metabolism.

1.3.2. mTORC1 regulation of cellular metabolism (continued)

Regarding lipid synthesis, mTORC1 positively regulates the activity of Sterol regulatory element binding protein (SREBP) (Porstmann et al., 2008) and peroxisome proliferation-activated receptor- γ (PPAR γ) (Kim and Chen, 2004), both of which control the expression of proteins involved in lipid homeostasis. mTORC1 control of SREBP activity influences glucose-6-phosphate dehydrogenase (G6PDH) activity (Duvel et al., 2010), which catalyzes the oxidation of G6P to 6-phosphogluconolactone. This generates ribose for RNA and DNA synthesis, along with NADPH for fatty acid synthesis (Figure 1.3).

mTORC1 promotes glutamine anapleurosis by inhibiting SIRT4, a transcriptional repressor of glutamate dehydrogenase (GDH) (Csibi et al., 2013). mTORC1 activity increases the expression of transcription factors that enhance oxidative metabolism and mitochondrial biogenesis such as PPAR γ co-activator 1 α (Cunningham et al., 2007, Zhang et al., 2009).

Aims

Despite the emerging role for cellular metabolism in controlling immune cell function, nothing is currently known regarding the regulation of NK cell metabolism and so it is unclear if metabolic reprogramming plays a role in the acquisition of NK cell effector functions. This study aims to characterize the metabolic changes that accompany NK cell activation and the molecular mechanisms involved.

Key objectives:

- 1) Investigate the changes in NK cell metabolism that occur upon cytokine stimulation.
- 2) Explore the mechanisms responsible for changes in cytokine stimulated NK cell metabolism.
- 3) Determine if NK cell metabolism is linked to NK cell effector functions.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Chemicals

Glucose, Galactose, 2-Deoxyglucose, β -Mercaptoethanol, Albumin from bovine serum, research grade Ethanol, Phosphate buffered saline tablets, Oligomycin, Fluoro-Carbonyl Cyanide Phenylhydrazine (FCCP), dNTPs, 6-Diazo-5-oxo-L-norleucine (DON), Leucine, 2-Amino-2-norbornanecarboxylic acid (BCH), Rotenone, Antimycin, Ammonium Persulphate, 40% Acrylamide A3699, TEMED, 4-Hydroxytamoxifen, Triton X, Agar, RedTaq, Deoxynucleotide set, Sodium dodecyl-sulphate, Tris(hydroxymethyl)aminomethane 99%+, Nancy-520, Dimethyl sulfoxide (DMSO), 1,4-Dithiothreitol (DTT), proteinase K and Tween-20 were purchased from Sigma-Aldrich. Trypan Blue solution (0.4% in phosphate buffered saline), rapamycin, sterile DNase RNase and protease free DEPC treated and Nuclease free water for RNA work and Glycine were purchased from Fisher Scientific. RPMI medium 1640 (1X) [+]L-Glutamine, RPMI medium 1640 (1X) [-]L-Glutamine, RPMI medium 1640 (1X) [+]L-Glutamine [-]Glucose, Hanks Buffered Saline solution, Dulbecco's phosphate buffered saline, Penicillin-Streptomycin, Mem-vitamin solution (100X), L-Glutamine solution (100X), Insulin-Transferrin-Selenium-G Supplement (100X) were purchased from Biosciences. Fetal Bovine Serum (FBS) and dialysed FBS was purchased from Labtech International. Sodium pyruvate was purchased from Gibco. IL-12 and NK cell isolation kit II were purchased from Miltenyi Biotech. Seahorse XF media, base media and calibration buffer were purchased from Agilent technologies. IL-15 was purchased from Peprotech. IL-2 was purchased from NCI preclinical repository. Golgi plug, Cytofix/Cytoperm, Perm/Wash reagent, Celltak and Calcein-AM were purchased from BD Pharmingen. Dimethyloxaloyglycine (DMOG) was purchased from VWR. Poly (I:C) was purchased from Invivogen. RNeasy RNA purification mini kit was purchased from Qiagen. X-Ray films were purchased from Fujifilm. qScript cDNA supermix kit was from Quanta Biosciences. iQ

SYBR Green was purchased from VWR. ECL blotting reagent was from Merck Millipore. ATP assay determination kit was purchased from Perkin Elmer. Methanol and Absolute Alcohol were purchased from school stores.

2.1.2. Equipment

Pipettes, pipette tips, filtered tips, cell culture strainers (70mM), 2ml disposable syringes, 20mM filters, tissue culture dishes (6, 12, 24, 48 and 96 well), tissue culture flasks (25cm, 75cm and 175cm) and (15ml, 50ml) falcons were obtained from Fisher. AB-1900 High sided low profile PCR plates, Absolute qPCR seal, Scilogex MX-S Vortex and Scilogex D1008 Mini Centrifuge were from MSC. BD polystyrene 5ml FACS tubes were obtained from Unitech. Graphpad-prism software was obtained from Graphpad. FACS Canto and LSR Fortessa were from Becton Dickinson. FlowJo software was obtained from TreeStar. 7500 fast real time PCR system was from Thermo Fisher. XF 24-well microplate was from Seahorse Bioscience. 96 well flat black polystyrene plates were from Costar. LS Macs purification columns, Quadro Macs cell separator and AutoMACS pro separator were from Miltenyi Biotec. Prime Thermal Cycler and Dri-Block DB-2D were purchased from Techne. Grant JB Series Water Bath was obtained from Amlab Services. Bright-Line Hemocytometer from Hausser Scientific. Power Source 300 V was obtained from VWR International. Steri-Cycle CO₂ Incubator was from Thermo Forma. Heraeus Pico 17 centrifuge was from Thermo Scientific. 5810R centrifuge was from Eppendorf. Legend RT Centrifuge from Sorvall. 1500 Standard Fumehood from Phoenix Controls Corporation. Bioair Topsafe Fumehood from Crowthorne HiTec Services. GelDoc XR+ imaging system was purchased from BioRad. Nanodrop Spectrophotometer ND-1000 from Labtech International. FLUROstar OPTIMA luminescence reader from BMG labtech. Electrophoresis running system from Atto and XCELL II Blot Module transfer system from Invitrogen. Microplate reader was Fluostar optima from BMG Labtech. SpectraMax M3 was from Molecular Devices.

2.1.3. Animal Husbandry

C57BL/6J mice were purchased from Harlan (Bicester, U.K.) 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. For *in vivo* NK cell activation experiments, 200µg of poly(I:C) in PBS (Invivogen) +/- 0.6mg/kg rapamycin (Fisher Scientific) or 1g/kg 2-deoxyglucose (Sigma) was injected intra-peritoneal. Mice were sacrificed after 12 or 24 hours, spleens harvested and NK cells analyzed.

HIF1alpha^{flox/flox} vav cre, Slc7a5^{flox/flox} vav cre, cMyc^{flox/flox} and tamoxifen cre mice were obtained from Dundee University and maintained in compliance with UK Home Office Animals (Scientific Procedures) Act 1986 guidelines. In the vav cre mouse lines, cre recombinase expression is under the control of the vav promoter. Hematopoietic stem cells are the earliest known prenatal cells to express the vav gene, meaning all progenitor immune cells express the cre recombinase enzyme. This enzyme cleaves DNA at site directed sequences that have LoxP sites flanking a gene of interest, in this instance *HIF1α* or *Slc7a5*, resulting in its excision.

Tamoxifen cre mice were crossed with cMyc^{flox/flox} mice, both of which were on a C57BL/6J background. Tamoxifen cre mice express a cre-recombinase-estrogen receptor fusion protein that is maintained in the cytoplasm through interactions with HSP90. When 4-OH tamoxifen is added this binds to the ER and displaces HSP90, resulting in the nuclear translocation of the Cre-ER fusion protein. The Cre recombinase then catalyzes the recombination of LoxP sites in the DNA resulting in the deletion of the cMyc gene.

2.1.4. Antibodies

2.1.4.1. Flow cytometry antibodies

Antibodies used for flow cytometry: eFluor 450 NK1.1 (PK136), PerCP-eFluor 710 CD335 (29A1.4), PE CD335 (29A1.4), FITC CD3 (145-2C11), APC CD71 (R17217) PE CD98 (RL388), PE-Cy7 Granzyme B (NGZB) were purchased from eBioscience. PE-Cy7 CD69 (H1.2F3), PerCP-Cy5.5 CD69 (H1.2F3), APC-Cy7 CD25 (PC61), APC IFN- γ (XMG1.2), PE-Cy7 IFN- γ (XMG1.2) and Fc block anti-CD16/CD32 (2.4G2) were purchased from BD pharmingen. Alexa Fluor 647 anti O-GlcNAc was purchased from Novus. 2-NBDG was purchased from Invitrogen. Western blot antibody S6 ribosomal protein (Ser235) was incubated along with a secondary antibody PE-conjugated donkey anti-rabbit immunoglobulin G purchased from Jackson ImmunoResearch for pS6 Flow cytometry analysis. MitoTracker Red CMXRos and LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit were obtained from Thermo Fisher. See table 2.1.4.1.

2.1.4.2. Western blot antibodies

Western Blot antibodies raised against S6 ribosomal protein, phospho-S6 S235/236, phospho-S6K Thr 389, ribosomal S6K, phospho-4E-BP1, 4E-BP1, cMYC and total PKB were purchased from Cell signaling. HIF-1 α was from Novus Biologicals. SMC1 was purchased from Bethyl Laboratories Inc. β -actin was obtained from Sigma. See table 2.1.4.2.

Antibody (clone)	Source	Dilution	Location
Fc block (2.4G2)	BD Pharmingen	1/100	Extracellular
eFluor 450 NK1.1 (PK136)	eBioscience	1/200	Extracellular
PerCP-eFluor 710 CD335 (29A1.4)	eBioscience	1/200	Extracellular
PE CD335 (29A1.4)	eBioscience	1/200	Extracellular
FITC CD3 (145-2C11)	eBioscience	1/400	Extracellular
APC TCRb (H57-597)	BD Pharmingen	1/100	Extracellular
Alexa Fluor 488 Anti GFP	Life technologies	1/100	Intracellular
PE-Cy7 CD69 (H1.2F3)	BD Pharmingen	1/200	Extracellular
PerCP-Cy5.5 CD69 (H1.2F3)	BD Pharmingen	1/200	Extracellular
APC-Cy7 CD25 (PC61)	BD Pharmingen	1/400	Extracellular
APC CD71 (R17217)	eBioscience	1/200	Extracellular
PE CD98 (RL388),	eBioscience	1/200	Extracellular
Mitotracker	Thermo Fisher	1/10,000	Extracellular
Bv510 CD71 (C2F2)	BD horizon	1/200	Extracellular
PE TCRβ (H57-597)	BD Pharmingen	1/200	Extracellular
Live dead aqua	Thermo Fisher	1/1,000	Extracellular
APC IFN-g (XMG1.2),	BD Pharmingen	1/200	Intracellular
PE-Cy7 IFN-γ (XMG1.2)	BD Pharmingen	1/200	Intracellular
PE-Cy7 Granzyme B (NGZB)	eBioscience	1/200	Intracellular
Phospho S6 ribosomal protein (Ser 235)/ PE-conjugated donkey anti-rabbit immunoglobulin G	Cell Signalling/ Jackson ImmunoResearch	1/100	Intracellular

Table 2.1.4.1 Flow cytometry antibodies

Antibody	Source	Dilution	2° Ab
S6 ribosomal protein	Cell Signaling	1/500 5% BSA/PBST	Mouse
phospho-S6 S235/236	Cell Signaling	1/2,500 5% BSA/PBST	Rabbit
phospho-p70 S6K Thr 389	Cell Signaling	1/2,500 5% BSA/PBST	Rabbit
S6K	Cell Signaling	1/500 5% BSA/PBST	Rabbit
4E-BP1	Cell Signaling	1/2,500 5% milk/PBST	Rabbit
phospho-4E-BP1	Cell Signaling	1/5,000 5% BSA/PBST	Rabbit
MYC	Cell Signaling	1/2,500 5% BSA/PBST	Rabbit
HIF-1α	Novus Biologicals	1/1,000 5% milk/PBST	Rabbit
SMC1	Bethyl Laboratories	1/2,500 5% milk/PBST	Rabbit
PKB	Cell Signaling	1/2,000 5% BSA/PBST	Rabbit
β-actin	Sigma	1/10,000 5% BSA/PBST	Mouse

Table 2.1.4.2 Western blot antibodies

2.2. Methods

2.2.1. Cell culture

2.2.1.1 Natural Killer cell culture

Under sterile conditions, murine spleens were extracted and gently minced with a plunger from a 2ml syringe through a 70uM strainer into 4ml cold media in a 6 well plate. The cell suspension was filtered through the strainer until a homogeneous cell solution, free from any clumps, was obtained. The cell solution was spun at 1400rpm for 4 minutes at room temperature. Supernatant was decanted and the cell pellet was resuspended. Erythrocytes were lysed with 3ml sterile ddH₂O for 3 seconds before the addition of 10ml RPMI media to discontinue the lysis process. Cells were spun down at 1400rpm for 4 minutes.

Splenocytes were resuspended at 2×10^6 cells/ml in RPMI media and cultured in 6 well dishes: 5ml per well at 37°C for 6 days. Splenocytes were fed with IL-15 (Peprotech) on day 0 and day 4. The biological activity of IL-15 varied between batches. For this reason, each batch of IL-15 was titrated for the optimal concentration to expand primed NK cells that were not overtly activated compared to ex vivo splenocytes (between 5-25ng/ml).

On day 6, fresh media was warmed to 37 °C and cultured NK cells were washed out of IL15 and stimulated for 18 hours with the cytokines: IL-15 (2-10ng/ml, Peprotech), IL-2 (20ng/ml, NCI preclinical repository), IL-12 (10ng/ml, Miltenyi Biotech) and inhibitors; 2-deoxy-D-glucose (Sigma), DMOG (VWR, 200uM), DON (2uM Sigma), rapamycin (20nM, Fisher) or BCH (25mM, Sigma). BCH is a competitive inhibitor of Slc7a5. For this reason, the concentration of amino acids in RPMI 1640 media was diluted 1 in 2 with Hanks Buffer Saline Solution (Biosciences) for all BCH experiments. Where indicated, NK cells were MACS purified using the NK Cell Isolation Kit II (Miltenyi Biotech) according to the manufacturer's guidelines from day 6 cultures for biochemical analyses. Where indicated, cultured NK cells were stimulated in glucose-free, glutamine-free or leucine-free medium containing dialysed FCS supplemented with or without the addition of glucose (10mM Sigma) or galactose (10mM), or L-glutamine (2mM) or leucine (50µg/ml). For experiments in which glutamine was deprived following 18hour activation, cells were washed three times with 15ml warmed glutamine free media to remove all extracellular glutamine.

2.2.1.2 Cytotoxic T lymphocyte (CTL) culture

For CTL cultures, spleens were processed in the same manner as NK cells until erythrocyte lysis stage (see 2.2.1.1). Following erythrocyte lysis, splenocytes were resuspended at 10^6 cells/ml in RPMI 1640 media supplemented with 2c11 (500ng/ml) and IL-2 (20ng/ml, NCI preclinical

repository). After 36hours activation, cells were spun at 1,300rpm for 4 minutes and resuspended in 50ml fresh RPMI media containing IL-2 (20ng/ml) in a T75 flask. After 24hours, cells were diluted to 0.3×10^6 cells/ml and supplemented with fresh IL-2 (20ng/ml). Cells were re-fed IL-2 (20ng/ml) and maintained at 0.3×10^6 cells/ml every 24hours for a further 3 days.

Compound	[Stock]	[Final]
IL-2	20µg/ml	20ng/ml
IL-15	20ug/ml	5ng/ml
IL-12	50µg/ml	10ng/ml
DMOG	1mM	200uM
Rapamycin	1mM	20nM
Oligomycin	2mM	2µM
2-DG	1M	1mM
Galactose	1M	10mM
Glucose	1M	10mM
DON	2uM	2uM
BCH	25mM	25mM

Table 2.2.1. Cell culture stimulants

Buffers/Media	Composition
Cell culture media	RPMI medium (+) L-Glutamine, FCS (10%), Penicillin/Streptomycin (1%), β -mercaptoethanol (50uM)
Supplemented glucose/glutamine free media	10% Dialayzed FCS, 1mM Sodium Pyruvate, 1x Vitamin Cocktail, 1x Selenium/Insulin Cocktail, 50 μ M β -mercaptoethanol and 1% Penicillin/Streptomycin, +/- 2mM Glutamine, +/- Glucose 10mM, +/- Galactose 10mM
RNA lysis Buffer	RLT buffer (Qiagen), 10% b-mercaptoethanol
FACS Buffer	Dulbecco's phosphate buffered saline, Cell culture media (10%)
MACS Buffer	To make up 1L: 800mls ddH ₂ O, 4 PBS tablets, 3.2mls EDTA (1M) 0.5% FCS
Cell-tak	For 20 wells: 13ul Cell-Tak, 4180.5ul Sodium Bicarbonate (0.1M), 6.5ul NaOH (1M)
Running Buffer	25mM Tris, 192mM Glycine, 0.1% SDS
Transfer Buffer	25mM Tris, 192mM Glycine, 10% methanol
Stripping Buffer	0.7% β -mercaptoethanol, 2% SDS, 62.5mM Tris
Protein lysis buffer	100mM Tris/HCl pH 6.7, 20% Glycerol, 4% SDS, 5% β -Mercaptoethanol, 0.1% bromophenol blue
Ear digest buffer	25mM NaCl, 50mM Tris/HCl, 0.1% SDS
50X TAE	2M Tris, 17.4M acetic acid, 64mM EDTA
2% agarose gel	2g Agarose, 100ml 1X TAE, 20ml Nancy520
PBST	1x PBS, 1ml Tween-20

Table 2.2.2 Buffers/Media

2.2.2. Flow cytometry analysis

2.2.2.1. Extracellular staining

Following stimulation, cells were transferred to labeled FACS tubes and washed with 1ml FACS buffer per tube before being centrifuged, at 1400 rpm for 3 minutes. Cell pellets were resuspended, followed by the addition of Fc block (50ul per sample of 1 in 50 Fc block:FACS buffer) to block non-specific antibody binding. After 5minutes Fc block at 4°C, extracellular staining antibodies for NK cell surface markers were added in a final volume of 100ul antibody master mix per sample (see 2.1.4.1). Cells were incubated in the dark for 20 min at 4°C. Cells were washed with 1ml FACS buffer and centrifuged at 1400rpm 3 minutes before acquisition by flow cytometry. Live cells were gated according to their forward scatter and side scatter. Data were acquired on a FACS Canto or FACS Fortessa (Becton Dickinson) analysed using FlowJo software (TreeStar).

2.2.2.2. Intracellular staining

For intracellular staining exocytosis was blocked using golgi plug (BD Pharmingen, contains Brefeldin A) four hours prior to the end of the period of stimulations. Where appropriate, Fc block and extracellular staining was carried out as outlined above. Subsequently, cells were washed using FACS buffer and fixed using fixation/permeabilization solution Cytofix/Cytoperm (BD Pharmingen) as per manufacturer's instructions. Cells were then allowed to incubate at 4°C for 20 min. Following fixation and permeabilization, cells were washed with 1ml 1X perm wash and centrifuged at 1400rpm for 3 minutes. Cell pellets were dislodged and intracellular antibodies added in a final volume of 100ul 1X perm wash/tube and incubated for 20min at 4°C. Cells were washed with 1ml 1X perm wash and centrifuged at 1400rpm 3 minutes before acquisition. Data were acquired on a FACS Canto or FACS Fortessa (Becton Dickinson) and analysed using FlowJo software (TreeStar).

2.2.2.3. Phospho-S6 ribosomal protein intracellular staining

Following the 18 hour incubation after the stimulations, cells were washed in 1ml FACS buffer and fixed and permeabilised using Cytofix/Cytoperm (BD Pharmingen) as per manufacturer's instructions. Cells were incubated with primary anti-phospho-S6 ribosomal protein Ser 235/236 (Cell Signalling Technologies) for 30 min at RT 1ul/sample. Cells were then washed in FACS buffer and incubated with PE-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch) for 30 min at RT in the dark. Samples were washed with FACS buffer and data acquired on a FACS Canto flow cytometer. As a negative control, rapamycin (20nM, 20 min, 37°C) was used to inhibit mTORC1 induced S6 phosphorylation.

2.2.2.4. 2-NBDG uptake assay

0.5x10⁶ cultured cells were washed with glucose free media and re-suspended in pre-warmed glucose-free media, supplemented with 10% dialyzed FCS (Fisher), 2mM Glutamine (Invitrogen/Biosciences), 1mM Sodium Pyruvate (Gibco), 1x concentration of MEM Vitamin Cocktail (Invitrogen/Biosciences), 1x concentration of selenium/insulin/transferrin Cocktail (Invitrogen/Biosciences), 50µM β-mercaptoethanol (Sigma) and 1% Penicillin/Streptomycin (Invitrogen/Biosciences), at a concentration of 6x10⁶cells/ml. After 15 minutes incubation at 37°C, cells were resuspended in 1 ml of supplemented glucose-free media with 50µM of the fluorescently labelled glucose analogue 2-NBDG (Life technologies), or in glucose-free media alone as a control. Cells were then incubated for a further 2 hours at 37°C after which they were washed twice in supplemented glucose-free media. Extracellular staining was carried out as outlined above before acquisition on a FACS Canto or FACS Fortessa (Becton Dickinson).

2.2.3. Metabolic Analysis

2.2.3.1. Metabolic Seahorse analysis

A XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) was used for real-time analysis of the extra cellular acidification rate (ECAR), as a measure of glycolysis; and oxygen consumption rate (OCR), as an indicator of Oxidative Phosphorylation rates, of NK cells stimulated under various conditions. One day prior to analysis, the seahorse cartridge plate was hydrated using 1ml calibration buffer per well in a non-CO₂ incubator at 37°C according to manufacturers instructions.

Wells of the 24well seahorse cell plate were coated with 200ul Cell-tak solution (BD Pharmingen) (See Table 2.2) and incubated at room temperature for 20 minutes before being washed twice with ddH₂O (500ul/well). Meanwhile, 3x10⁶ NK cells per condition were transferred to 15ml falcons and washed twice in 5ml pre-warmed seahorse media. Cells were resuspended at 7.5x10⁶ cells/ml for NK cells, or 5x10⁶ cells/ml for T cells, in seahorse media supplemented with relevant treatments. 100ul of cells were added to designate cell-tak coated wells in quadruplicate of the seahorse cell plate. The cell plate was gently centrifuged at 1200 rpm for 3 minutes with the brake off before incubation in a non-CO₂ incubator for 20 minutes. Meanwhile, 70ul of inhibitors were added to designate ports of the seahorse cartridge plate prior to calibration. Inhibitor final concentrations were as follows; oligomycin (2μM), fluoro-carbonyl cyanide phenylhdrazone (FCCP 0.5μM), rotenone (100nM) plus antimycin (4μM) and 2DG (30mM) see table 2.2.4.1. Following 20 minute incubation of cell plate, 500ul seahorse media containing relevant treatments was added to each well, and 600ul seahorse media was put in the blank wells. After calibration of the seahorse cartridge, the cell plate was put in the seahorse machine for analysis.

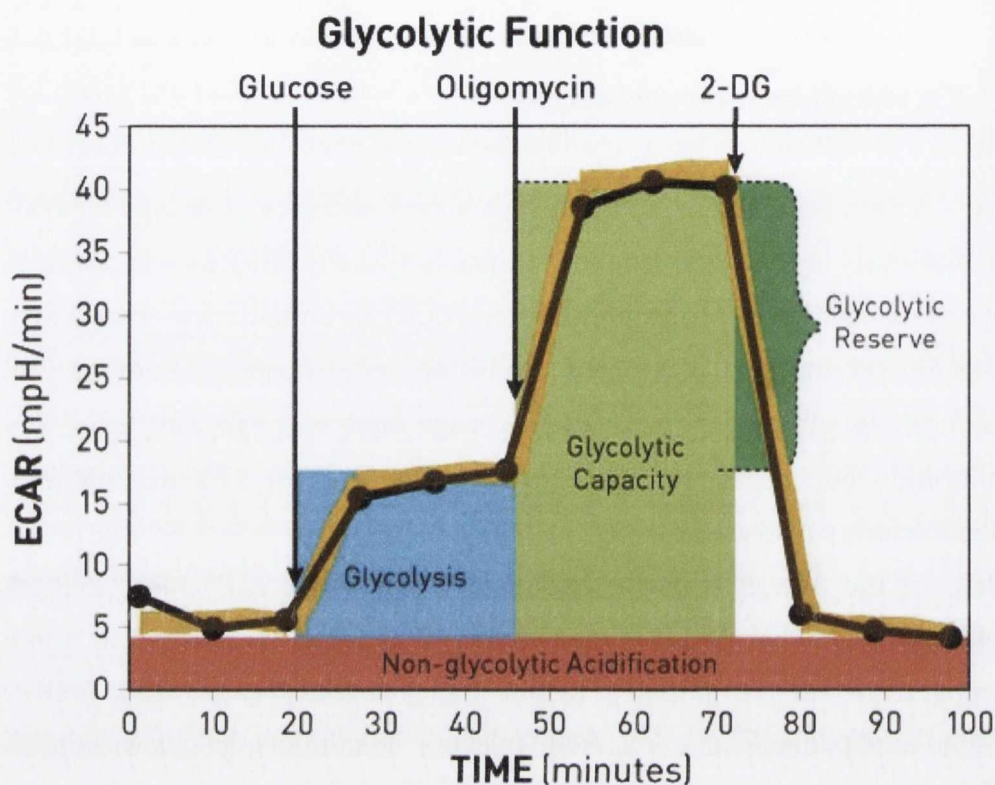


Figure 2.2.3.1. Determining Glycolytic rates using the Seahorse metabolic flux analyser

Basal rates of glycolysis are determined by the change in extracellular acidification in the media over time, resulting from the release of protons during glycolysis (ECAR). Oligomycin inhibits ATP synthase, the enzyme that generates ATP from ADP at the end of the electron transport chain (ETC). In order to avoid energy crisis, the rate of glycolysis is maximally increased to the cells '**glycolytic capacity**'. 2-DG is a direct glycolytic inhibitor; therefore in the presence of 2DG, any change in acidification of the media can be attributed to processes other than glycolysis. This allows us to calculate the actual **rate of glycolysis**.

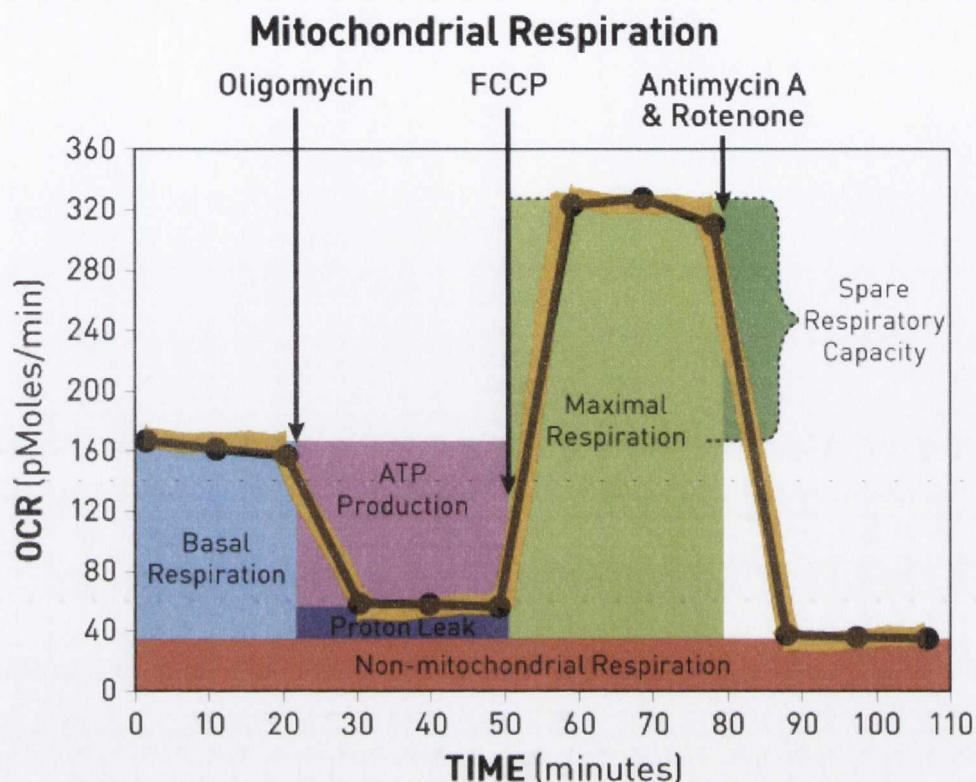


Figure 2.2.3.1 Determining OxPhos rates using the Seahorse metabolic flux analyser

Basal rates of OxPhos can be determined by the amount of oxygen being consumed over time (OCR). Oligomycin inhibits ATP synthase, the enzyme that generates ATP from ADP at the end of the ETC. FCCP disrupts the proton gradient across the inner mitochondrial membrane, which is coupled to electron transport, thus allowing a free flow of protons through the inner mitochondrial membrane. In the presence of FCCP, the rate of OxPhos is maximally increased allowing the **Maximum Respiratory Capacity (MRC)** to be measured. The **Spare Respiratory Capacity (SRC)** can be calculated by subtracting the basal rate of OxPhos from the MRC. Antimycin A and Rotenone inhibit complex III and I of the Electron transport chain respectively, therefore any residual oxygen consumption measured after the addition of these two compounds is not due to mitochondrial respiration. All these measurements together allow us to calculate the actual **rate of OxPhos**.

Compound	Stock	Final Conc	Working stock	Seahorse media (ul)	Inhibitor (ul)
Oligomycin	2mM	2uM	19.2uM	1980.8	19.2
FCCP	1mM	0.5uM	5.3uM	1989.4	10.6
Rot/Antimycin	1/5mM	100/4uM	1.16/46.4uM	1979.2	2.3/18.5
2DG	1M	30mM	378mM	1231	769

Table 2.2.3 Seahorse inhibitor concentrations

2.2.4. RNA analysis

2.2.4.1. RNA isolation

Cultured NK cells were purified by magnetic separation and 1×10^6 pure NK cells were stimulated for 18 hours. After 18 hours, stimulated cells were centrifuged at 1400rpm for 3 minutes. The supernatant was discarded and cells were washed with 1ml cold PBS. Cells were re-centrifuged at 1400rpm for 3 minutes and the cell pellet was resuspended in 600µl RLT buffer containing 1% β-Mercaptoethanol. RNA lyates were stored at -80°C. The RNeasy mini kit (Qiagen) was used to isolate the RNA according to the manufacturer’s protocol. RNA quality and quantity were assessed using a Nanodrop ND-1000 (NanoDrop® Technologies).

2.2.4.2. cDNA synthesis

1µg of isolated RNA was made up to 16 µl with ddH2O and added to 4 µl of qScript cDNA Supermix (5x) (Quanta BioSciences), containing the reverse transcriptase enzyme required to carry out cDNA synthesis. The reverse transcription was carried out in Techne’s 3Prime Thermal Cycler at the following incubation times:

Time	Temperature
5 minutes	25°C
30 minutes	42°C
5 minutes	85°C
Hold	4°C

Table 2.2.4.1 cDNA synthesis program

2.2.4.3. Primer design

Primers for quantitative real time PCR (qRT-PCR) were designed using the Primer BLAST tool located on the NCBI website (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primers designed have a GC clamp at the 3' end, they span an exon-exon junction, have less than 1degree between melting temperatures, GC content is between 40-60% and have at least 3 mismatches with any other sequence in the gene of interest. Sequences were verified in Ensembl (<http://www.ensembl.org/index.html>).

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'- CCATCCGGAGTTGACCTCAC-3'
cMYC forward: 5'- GCGTTGGAAACCCCGACAG -3'
cMYC reverse: 5'- CTTCCAGATATCCTCACTGGGC-3'
Slc7a5 forward: 5'- CTGGATCGAGCTGCTCATC-3'

Slc7a5 reverse: 5'- GTTCACAGCTGTGAGGAGC-3'

Glutaminase forward: 5' TTGCGAACATCTGATCCCAGG-3'

Glutaminase reverse: 5' TCATCGATGTGTGAGGTAAAAGAC-3'

Glutaminase 2 forward: 5'- CTTCCAAAAGTGTGTGAGCAGC-3'

Glutaminase 2 reverse: 5'- GCCCCAAAGGTCTGGGTTTG-3'

CD71 forward: 5'- TAGAGGCGCTTCCTAGTACTCC-3'

CD71 reverse: 5'- CACTAGCCTTCATGTTATTGTCCG-3'

PHD3 forward: 5'- CTGGTCCTGTACTGCGGGAG-3'

PHD3 reverse: 5'- GACCCCTCCGTGTAACCTGG-3'

PKM1 forward: 5'- AAACAGCCAAGGGGGACTAC-3'

PKM1 reverse: 5'- TTATAAGAGGCCTCCACGCTG-3'

PKM2 forward: 5'- GCTATTCGAGGAACCTCCGCC-3'

PKM2 reverse: 5'- AAGGTACAGGCACTACACGC-3'

TNFa forward: 5'-AGGCACTCCCCCAAAGATG-3'

TNFa reverse: 5'-TGGTTTGTGAGTGTGAGGGTC-3'

IFNg forward: 5'- ACGCTACACACTGCATCTTG -3'

IFNg reverse: 5'-GTCACCATCCTTTTGCCAGTTC -3'

GzymB forward: 5'- TGCTACTGCTGACCTTGTCTCTG-3'

GzymB reverse: 5'-TCTGGGCCCCCAAAGTGAC-3'

2.2.4.4. Quantitative Real Time PCR

Quantitative Real time PCR was performed in a 96 well plate format using iQ SYBR Green based detection (VWR) in a 7900HT Fast Real-Time PCR System (Applied Biosciences) to quantify the relative amounts of steady-state mRNA expression for genes of interest against RpLp0 as an internal control. Each 10ul reaction contained: 0.5ul cDNA, 0.4ul (10pmol/ul) sense and antisense primer, 5ul SYBR Green supermix and 3.7ul nuclease free water. The plate was sealed with ABsolute qPCR seal (MSC) and run under the fast cycle conditions;

Stage	Temperature (°C)	Time	Cycles
1	95°C	1min	1
2	95°C	1sec	35
2	57°C	20sec	35
3	95°C	15sec	1
3	55°C	30sec	1
3	95°C	15sec	1

Table 2.2.4.2 Real time PCR run settings

Each reaction was performed in duplicate. Rplp0 was used for normalization and the relative mRNA levels calculated using the following equation:

$$\text{Rel. mRNA expression} = 2^{(ct_{uc} - ct_{us})} / 2^{(ct_{rc} - ct_{rs})}$$

Where ct is the threshold cycle, u is the mRNA of interest, r is the reference gene, uc is the sample every other sample is made relative to and s is every other sample.

2.2.4.5 Genotyping

Ear digestion

45ul of ear lysis buffer and 5ul of 10mg/ml proteinase K was added to each ear punch in 1.5ml eppendorfs. Eppendorfs were placed in a heating block set to 55°C overnight, to allow the ears to digest. 100ul DEPC DNase nuclease free water was added to each sample before heating to 100°C for 7 minutes in order to inactivate proteinase K. DNA samples were stored at -20°C until use.

Primer sequences

CRE-forward: 5'-CGGTCGATGCAACGAGTGATGAGG-3'

CRE-Reverse: 5'-CCAGAGACGGAAATCCATCGCTCG-3'

MYC- forward: 5'- CCGCGGCACTAGGACTTGATG-3'

MYC-Reverse: 5'-GCTCAGATCACGACTCACCGCAG-3'

For MYC PCR, ear templates were diluted 1 in 2 in DEPC water. For tamoxifen Cre PCR, templates were diluted 1 in 3 in DEPC water. 2ul of template was added to 23ul of MYC master mix, while 1ul of template was added to 24uls of master mix (See table below for appropriate master mixes).

Component	Tamoxifen cre (ul)	MYC (ul)
Buffer	5ul (of 5X buffer)	2.5 (of 10X buffer)
MgCl ₂	2.5ul	2ul
Forward primer	0.5ul (of 10pmol/ul)	0.1 (of 100pmol/ul)
Reverse primer	0.5ul (of 10pmol/ul)	0.1 (of 100pmol/ul)
Enzyme	0.1ul of GoTaq	0.5ul of RED taq
DEPC water	14.4ul	14.8ul
1% Igepal	-	2.5ul
dNTP	1ul (of 10mM)	0.5ul (of 10mM)

Table 2.2.4.3 Genotyping master mix components

Stage	Temperature (°C)	Time	Cycles
0	95°C	Hold (hot start)	N/A
1	95°C	3min	1
2	95°C	30sec	35
2	60°C	40sec	35
2	72°C	90sec	35
3	72°C	10min	1
3	4°C	Hold	1

Table 2.2.4.4 MYC PCR genotyping protocol

Stage	Temperature (°C)	Time	Cycles
0	94°C	Hold (hot start)	N/A
1	94°C	5min	1
2	94°C	1min	35
2	63°C	2min	35
2	72°C	3min	35
3	72°C	5min	1
3	4°C	Hold	1

Table 2.2.4.5 Tamoxifen Cre PCR genotyping protocol

2.2.5. Protein manipulation

2.2.5.1 Protein Extraction

Cultured NK cells were purified by magnetic separation and 2 x 10⁶/condition pure NK cells were stimulated for 18 hours. After 18 hours, stimulated cells were centrifuged at 1400rpm for 4 minutes. Cell pellets were washed with 1ml cold PBS and recentrifuged at 1400rpm for 4 minutes. Cell pellets were resuspended and lysed in 1X protein lysis sample buffer + DTT 25uM (see table 2.2.1) at a concentration of 10x10⁶cells/ml. Samples were denatured at 95°C for 15 minutes before use and stored at -20°C.

2.2.5.2 Sodium Dodecyl-Sulphate Poly-Acrylamide Gel Electrophoresis

Cell lysates are separated based on their molecular weight. Gels were run using the AE6450 system from Atto Corporation. The recipe for one 10% acrylamide gel is shown in table 2.2.6. 8% and 14% gels were also used depending on protein size.

Reagent	Stacking gel	Resolving gel
1M Tris	625ul (pH 6.8)	2.25ml (pH 8.8)
1% SDS	500ul	600ul
40% acrylamide	500ul	1.5ml
1.5% APS	250ul	300ul
TEMED	5ul	6ul
ddH2O	3.12ml	1.344ml

Table 2.2.5 10% gel composition

2.2.5.3 Western Blot

For the transfer of protein onto nitrocellulose membrane, SDS-PAGE gels were removed from the running plates and assembled into a gel-membrane sandwich as dictated in the manufacturer's protocol of the transfer apparatus. Nylon sponge pads, Whatmann 3-MM chromatography paper and Hybond-C extra Nitrocellulose membrane (Amersham Biosciences) were soaked in transfer buffer containing 10% methanol. Nitrocellulose membrane was activated in absolute methanol before use. The sandwich was loaded into the transfer apparatus with the nitrocellulose membrane closest to the anode. Proteins were transferred at 45V for 2 hours or 15V overnight on ice. To determine quality of transfer, membranes were stained with Ponceau S solution for 30 seconds, before gently rinsing with water. Membranes were blocked in a 5% milk/PBST solution for 40 minutes at

room temperature. Membranes were washed three times in PBST for 5 minutes before incubation in primary antibody (see table 2.1.4.2.) for 4 hours at room temperature. After primary antibody incubation, membranes were washed three times for 5 minutes in PBST before incubation with secondary antibody (1/5000 in 5% milk/PBST) for 1 hour at room temperature. After secondary antibody probing, membranes were washed three times for 5 minutes in PBST. ECL (Merk Millipore) was applied to the membrane and luminescence assessed by GelDoc XR⁺ system.

2.2.6. ATP assay

For ATP analysis, 100ul of MACS purified NK cells were stimulated in a 96 well plate at 1×10^7 cells/ml stimulated for 18 hours in triplicate. Cells were lysed and relative ATP levels in mM per 10^6 cells were measured using an ATP assay determination kit (Perkin Elmer) as per the manufacturer's instructions on a FLUORostar OPTIMA luminescence reader. ATP is a marker for cell viability because it is present in all metabolically active cells and the concentration declines very rapidly when the cells undergo necrosis or apoptosis. The ATPlite assay system is based on the production of light caused by the reaction of ATP with added luciferase and d-luciferin. This is illustrated in the following reaction scheme:

ATP + d-Luciferin + O₂ (in the presence of luciferase and Mg²⁺) gives you Oxyluciferin + AMP + PPi + CO₂ + Light.

The emitted light is proportional to the ATP concentration within certain limits.

Chapter 3

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

Introduction

mTORC1 is a serine/threonine kinase that is indispensable for the activation of many immune cells (Finlay et al., 2012b, Powell et al., 2012). It is becoming clear that mTORC1-regulated cellular metabolism is crucial to the immune-modulatory function of this kinase. Indeed, evidence is emerging which demonstrates cellular metabolism dictates immune cell differentiation and function (see section 1.2.3). In CTLs, mTORC1 activity is required to maintain the high rates of glycolysis that are essential to sustain normal migratory patterns and effector functions (Finlay et al., 2012a). mTORC1 regulated glycolysis is also linked to normal differentiation of effector CD4⁺ T cells (Shi et al., 2011). However, the importance of mTORC1 in controlling NK cell metabolism and function has not yet been defined. NK cells are lymphocytes that bridge innate and adaptive immunity, and they have an important role in anti-viral and anti-tumor immune responses. Although NK cells are critical in early immune responses, they also regulate the adaptive immune response through the release of pro-inflammatory cytokines such as IFN γ and TNF α (Vivier et al., 2011). More recently, it was appreciated that NK cells can undergo clonal expansion and function in parallel with the adaptive immune system. NK cells can be induced to express the high affinity IL-2R in response to IL-12, thus allowing them to respond to low doses of T cell derived IL-2 (Lee et al., 2012, Bihl et al., 2010). Indeed, IL-2 is an important cytokine for the NK cell response against viral infection (Gasteiger et al., 2013a, Bihl et al., 2010, Lee et al., 2012). Given that mTORC1-regulated metabolism is linked to normal CTL functions we hypothesized that mTORC1 regulated metabolism might be fundamental in the control of NK cell responses.

All experiments from chapter 3 were carried out in collaboration with Dr Raymond Donnelly.

3.1. Changes in NK cell metabolism correlate with the acquisition of effector functions

To investigate whether NK cell activation *in vivo* is associated with changes in NK cell metabolism, mice were injected with poly(I:C) and splenocytes isolated after 24 hours for analysis. Polyinosinic:polycytidylic acid (poly(I:C)) is a synthetic dsRNA analogue that is used as a mimic of dsRNA pathogen-associated molecular patterns (PAMPs) produced during viral infection (Caskey et al., 2011). Following poly(I:C) injection, CD3⁺NK1.1⁺NKp46⁺ NK cells increased the expression of CD69, an early activation-associated glycoprotein, validating this method of NK cell activation *in vivo* (Fig.3.1A) (Enric Esplugues et al Blood 2005). Interestingly, poly(I:C) activated NK cells also displayed substantial increases in cell size (FSC), as compared to the PBS control mice (Fig.3.1B). Increased cell size, which occurs during lymphocyte blastogenesis (Preston et al., 2015), is synonymous with increased cell growth. Moreover, changes in cellular metabolism are required for increased cellular growth, to allow for the provision of essential biosynthetic precursors and nutrients. Consistent with this, larger NK cells in poly(I:C)-treated mice displayed elevated rates of nutrient uptake, as measured by uptake of the fluorescent 2-deoxyglucose analogue 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (NBDG) (Fig.3.2A). Many forms of nutrient uptake, such as glucose uptake, are passive. Hence increased NBDG uptake implies increased expression of key nutrient transporters. Indeed, poly(I:C) treated NK cells displayed increased expression of the important nutrient receptors CD71 (transferrin receptor) and CD98 (a component of the L-amino acid transporter) (Fig.3.2B-C). These data provide insight into the metabolic changes NK cells acquire upon activation *in vivo*. We hypothesized that these activation-induced changes in cellular metabolism were required for NK cell functional responses. To investigate this, the production of the pro-inflammatory cytokine IFN γ was assessed in poly(I:C) activated NK cells *in vivo*.

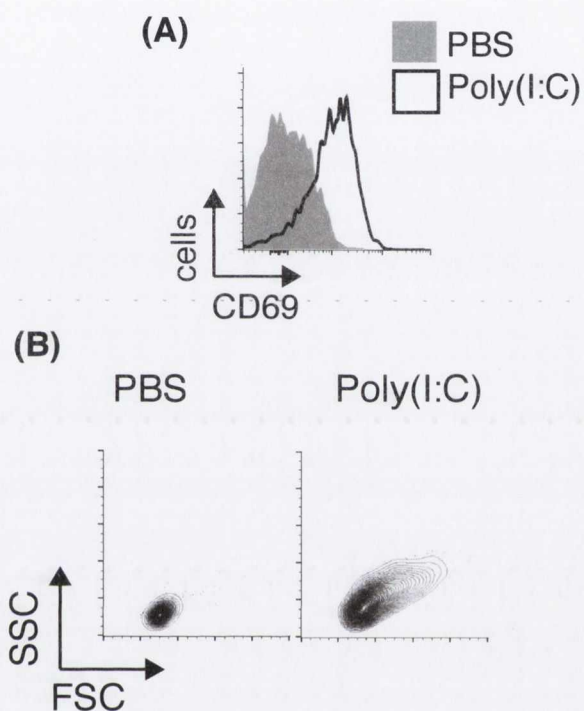


Figure 3.1. Poly (I:C) activated NK cells increase in size

Mice were administered PBS or 200mg poly(I:C) by intra peritoneal injection and spleens were harvested after 24hrs. Splenocytes were isolated, and NK1.1⁺ NKp46⁺ CD3⁻ cells analyzed by flow cytometry for changes in CD69 expression (A) and size (FSC) (B). Data are representative of 8–10 mice for each condition from two separate experiments.

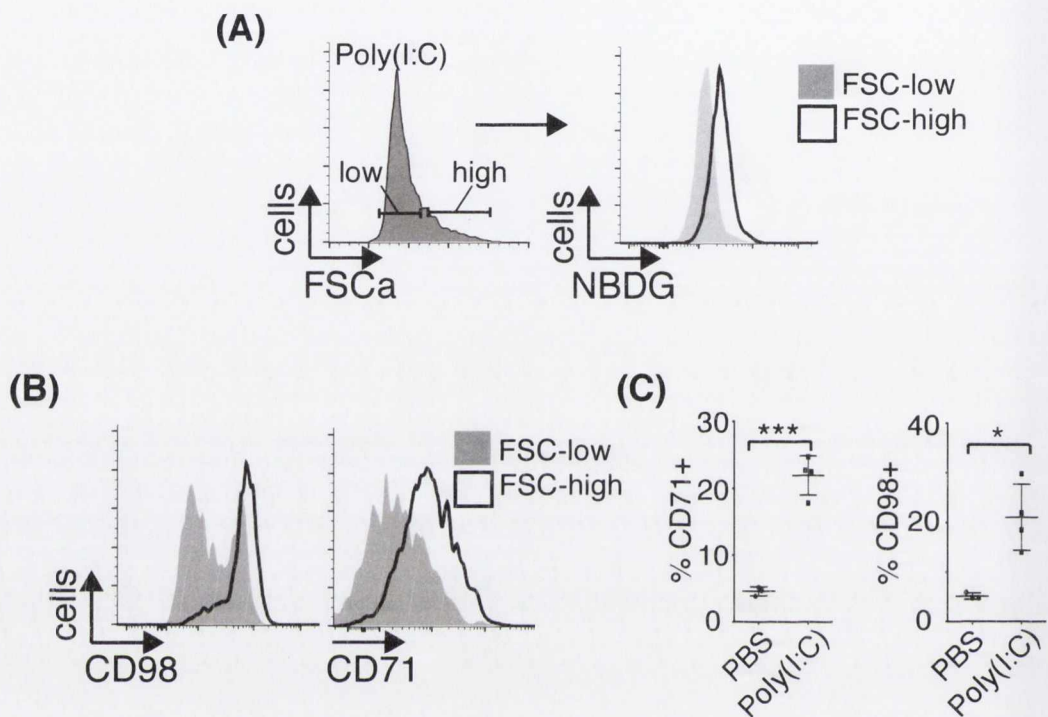


Figure 3.2. Larger NK cells from poly(I:C) treated mice are more metabolically active

Mice were administered PBS or 200mg poly(I:C) by peritoneal injection and spleens were harvested after 24hrs. Splenocytes were isolated, and NK1.1⁺ NKp46⁺ CD3⁻ cells (or NK1.1⁺ NKp46⁺ TCRβ⁻ NK cells for NBDG experiments) from either stimulation were segregated based on cell size (left panel A). Small versus large NK cells were analyzed for levels of nutrient uptake (NBDG)(right panel A), CD98 and CD71 expression (B, C). Data are mean +/- SEM or are representative of 4 mice for each condition from two separate experiments. * $p < 0.05$, *** $p < 0.001$.

Poly(I:C)-activated NK cells increase the expression of IFN γ as compared to the PBS control treated mice (Fig.3.3A,B). To investigate the link between IFN γ production and NK cell growth, poly(I:C) activated NK cells were stratified based on size and IFN γ production was assessed. Interestingly, there was a distinct difference in the amount of IFN γ being produced by poly(I:C) activated NK cells based on cell size; NK cells with increased size (FSC) produced more IFN γ -than smaller NK cells (Fig.3.3C). Together, these data show for the first time that NK cells activated *in vivo* display increased metabolic marker expression and nutrient uptake, which correlate with increased cell growth effector molecule production.

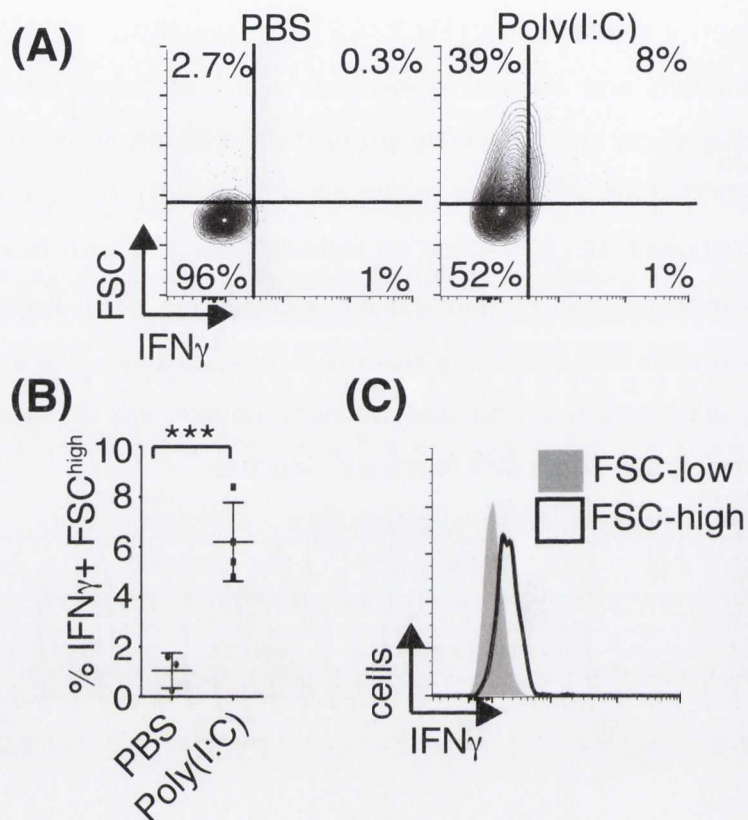


Figure 3.3. Poly(I:C) enhances NK cell IFN γ production *in vivo*

Mice were administered PBS or 200 mg poly(I:C) by peritoneal injection. Spleens were harvested after 24hrs and NK1.1⁺ NKp46⁺ CD3⁻ NK cells were analyzed by flow cytometry for levels of IFN γ (A, B). FSC of NK cells was compared for IFN γ ⁺ and IFN γ ⁻ NK cells from poly(I:C)-treated mice (C). Data are mean $\pm$ SEM or are representative of 4 mice for each condition from two separate experiments. *** p < 0.001

3.2. IL-2/12 cytokine activated NK cells undergo metabolic reprogramming to permit elevated levels of glucose metabolism

Pro-inflammatory immune cells tend to be characterized by elevated levels of glucose uptake and increased rates of glycolysis, metabolizing glucose primarily to lactate rather than oxidative phosphorylation (OxPhos) in the mitochondria (Chang et al., 2013a). For detailed metabolic analysis large numbers of purified NK cells were required, which could not be achieved using NK cells isolated directly *ex vivo*. For this reason, NK cells were first expanded for 6 days from isolated splenocytes (Zhao and French, 2012). This culture system was achieved using low dose IL-15 (5-25ng/ml), a cytokine required for DC-mediated NK cell priming *in vivo* (Lucas et al., 2007, Koka et al., 2004, Dubois et al., 2002). Indeed, this culture process provided large numbers of primed NK cells (Fig.3.4A-B) that responded robustly to subsequent cytokine stimulation with increases in CD69 expression, cell size and IFN γ production (Fig.3.5).

Given the evidence that NK cell activation can be closely linked to T cell responses, and the observation that T cell-derived IL-2 and the innate cytokine IL-12 are important for optimal NK cell activation in response to various infections (Bihl et al., 2010, Lee et al., 2012), the effect of stimulating IL-15 cultured NK cells *ex vivo* with IL-2 and IL-12 was investigated. Purified IL-15-cultured NK cells were stimulated with IL-2 and/or IL-12 and rates of glycolysis and Oxidative Phosphorylation (OxPhos) measured using the seahorse metabolic flux analyser. IL-2 stimulation increased the rate of glycolysis (measured as the extracellular acidification rate - ECAR) two-fold. Alone, IL-12 had no effect on the rate of glycolysis; however, the combination of IL-2 and IL-12 resulted in a synergistic increase in NK cell glycolysis (Fig.3.6). Consistent with increased metabolism, IL-2/12 stimulated NK cells displayed increased levels of NBDG uptake, cell size and an increase in the expression of metabolic receptors CD71 and CD98 (Fig.3.7). These data suggest IL-2/12 stimulated NK cells are highly metabolically active.

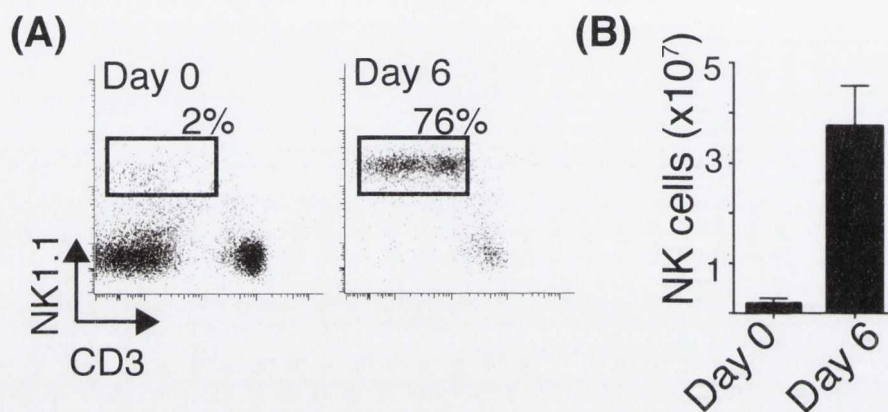


Figure 3.4. IL-15 induces splenic NK cell expansion

Splenocytes were isolated from murine spleens, counted and subjected to flow cytometry analysis. The remainder were cultured in IL-15 (5-25ng/ml) for 6 days at 2×10^6 cells/ml. Cells were harvested on day 6, counted and FACS analysis was performed to assess NK cell numbers and purity. Data shown is representative of the frequency (A) and numbers (B) of NK1.1⁺ TCR β ⁻ NK cells in splenocytes analyzed directly ex vivo (day 0) and following 6 days culture in IL-15.

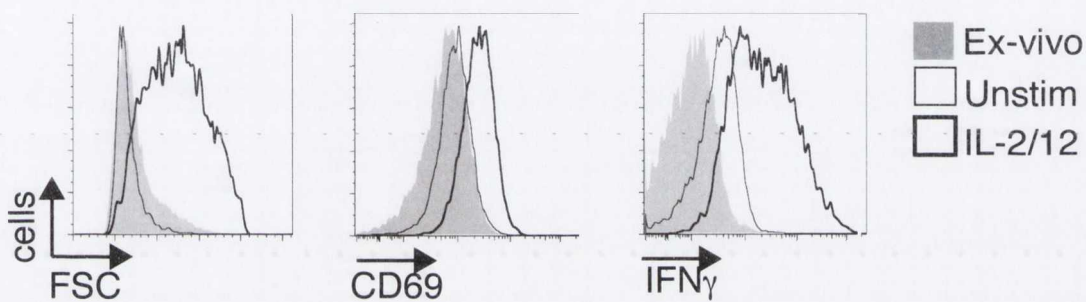


Figure 3.5. IL-2/12 cytokine stimulation induces a robust increase in NK cell size, activation marker expression and effector function

Flow cytometry analysis of cell size (FSC), activation marker expression (CD69) and effector molecule production (IFN γ) in NK1.1⁺ NKp46⁺ CD3⁻ ex vivo splenocytes and 6 day IL-15 cultured cells stimulated for 18hrs with IL-2/12 or left unstimulated.

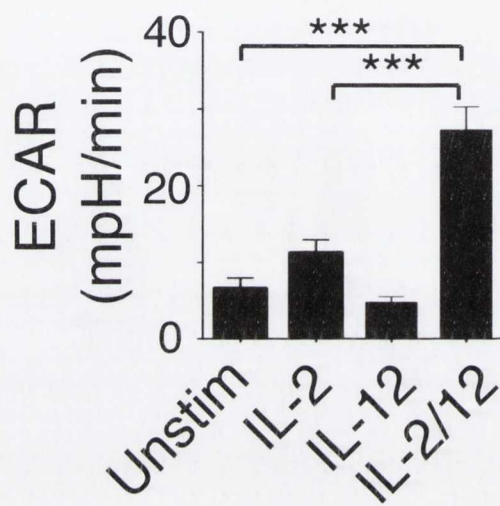


Figure 3.6. The cytokine combination IL-2/12 induces a synergistic increase in the rate of NK cell glycolysis

Seahorse analysis of the rate of glycolysis (ECAR) in purified, 6 day cultured NK cells stimulated for 18 hrs with IL-2, IL-12, or IL-2 plus IL-12 or left unstimulated. Data are mean +/- SEM of three separate experiments. *** $p < 0.001$.

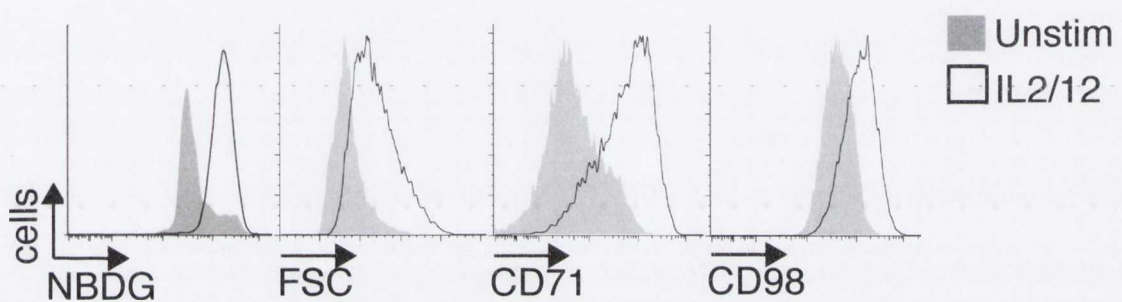


Figure 3.7. IL-2/12 stimulated NK cells are more metabolically active

Flow cytometry analysis of the rate of glucose uptake (NBDG), changes in cell size (FSC), CD71 and CD98 expression in NK1.1⁺ NKp46⁺ CD3⁻ (or NK1.1⁺ NKp46⁺ TCR β ⁻ for glucose uptake) cultured NK cells stimulated for 18hr with IL-2/12 or left unstimulated. Data are representative of five experiments.

Indeed, the rate of OxPhos (measured as the oxygen consumption rate – OCR) in IL-2/12 stimulated NK cells also significantly increased in response to cytokine stimulation (Fig.3.8A). Although increases in both glycolysis and OxPhos were observed in cytokine activated NK cells, overall there was a shift in the balance of NK cell metabolism from OxPhos to glycolysis (Fig.3.8B).

Increased aerobic glycolysis in cytokine stimulated NK cells may reflect elevated flux through glycolysis and the preferential conversion of pyruvate to lactate, instead of it entering the mitochondria to fuel OxPhos. Alternatively, it may be due to intrinsic rewiring of metabolic machinery, termed metabolic reprogramming. Metabolic reprogramming increases the expression of glycolysis-associated genes, to support elevated rates of glucose uptake and metabolism. To explore these two possibilities, the 'glycolytic capacity' of IL-2/12 stimulated NK cells was measured. The glycolytic capacity is the ECAR rate following the addition of oligomycin in the seahorse analyser. Oligomycin inhibits ATP synthase in the mitochondria, which prevents ATP production via OxPhos. Consequentially, the rate of glycolysis increases to the maximal rate possible in order for energy homeostasis to be maintained. As a result, the glycolytic capacity rate reflects the glycolytic machinery available to the cell. IL-2/12 stimulated NK cells display an increased glycolytic capacity as compared to unstimulated NK cells, suggesting that this cytokine combination induces an increase in NK cell metabolic machinery (Fig.3.9A). Indeed, real time PCR analysis confirmed IL-2/12 stimulated NK cells have increased mRNA expression of key glycolytic genes and enzymes; the expression of the glucose transporter Glut1, and crucial rate limiting glycolytic enzymes Hexokinase 2 (Hex2) and Lactate dehydrogenase a (Ldha) were significantly increased in IL-2/12 stimulated NK cells (Fig.3.9B).

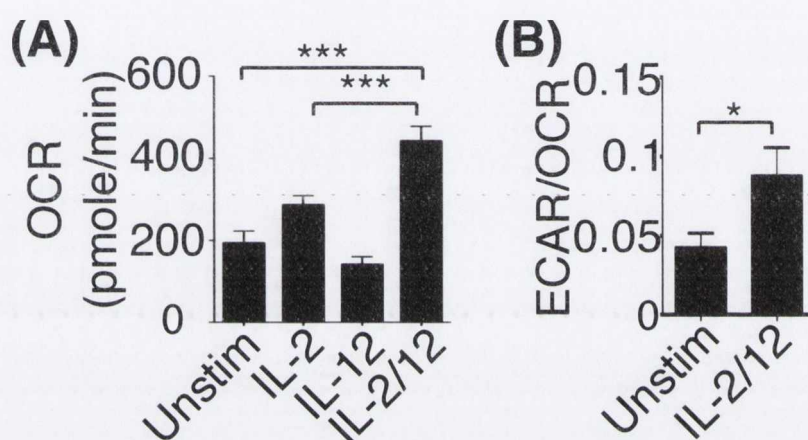


Figure 3.8. IL-2/12 stimulated NK cells use glycolysis as the primary form of glucose metabolism

Seahorse analysis of the rate of OxPhos (OCR) in MACS purified, cultured NK cells stimulated for 18hrs with IL-2, IL-12, or IL-2 plus IL-12 or left unstimulated (A) and the ratio of glucose utilization for glycolysis versus OxPhos in NK cells stimulated with IL-2/IL-12 or left unstimulated for 18hrs (B). Data are mean +/- SEM of three separate experiments. *p<0.05, ***p < 0.001.

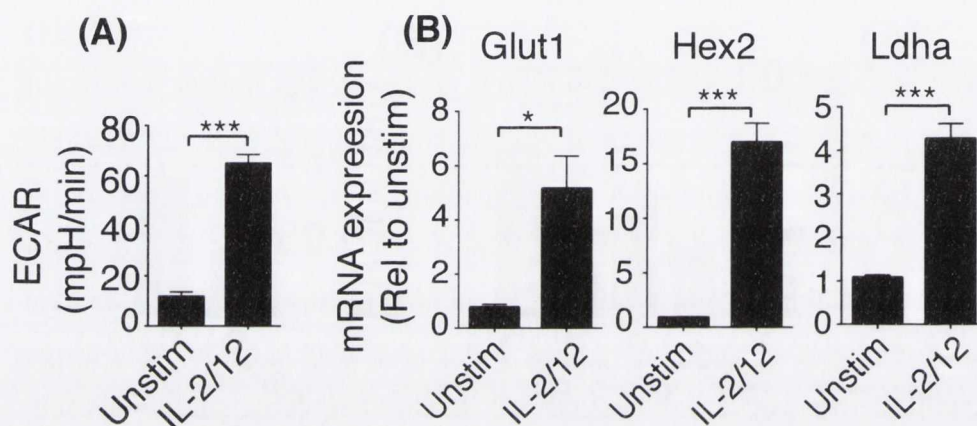


Figure 3.9. IL-2/12 stimulated NK cells have an increased glycolytic capacity due to metabolic reprogramming

MACS purified, cultured NK cells were stimulated for 18hrs with IL-2/12, or were left unstimulated and analyzed using a seahorse flux assay for their glycolytic capacity rate following the addition of oligomycin (A) or lysed for mRNA analysis looking at the expression of Glut1, Hex2, and Ldha genes (B). Data are mean +/- SEM of three to five experiments. *p < 0.05, ***p < 0.001.

3.3. Metabolic alterations correlate with the acquisition of Natural Killer cell effector functions

These data clearly demonstrate NK cells undergo metabolic reprogramming upon cytokine stimulation, which led us to ask whether increased levels of glucose metabolism correlated with the acquisition of NK cell effector functions. Interestingly, the effector function of cytokine activated NK cells *ex vivo* paralleled the observed metabolic changes. Alone, IL-2 does change the expression of the high affinity IL-2 receptor CD25. However, IL-12 alone or in combination with IL-2, induced a robust increase in CD25 expression (Fig.3.10A). This increased expression of CD25 was hypothesized to permit enhanced IL-2 signaling. Indeed, there was a synergistic increase in pS6 levels, as an indicator of mTORC1 activity, observed in IL2/12 activated NK cells compared to either cytokine alone (Fig.3.10B). Furthermore, the enhanced IL2 signaling in IL-2/12 activated NK cells resulted in a synergistic increase in cell size (FSC) and IFN γ production (Fig.3.11A,B). Combined, these data clearly demonstrate that NK cells undergo metabolic reprogramming following activation, involving elevated levels of glycolysis that parallel with increased effector molecule production.

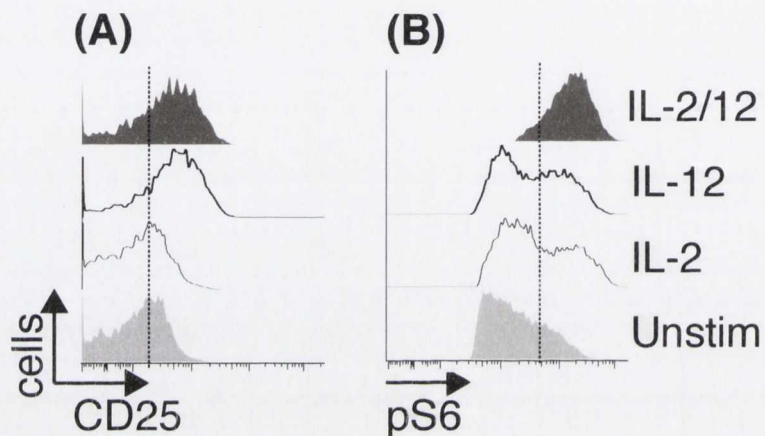


Figure 3.10. IL-2/12 stimulated NK cells display enhanced IL2 receptor expression that facilitates increased IL2 signaling

Flow cytometry analysis of NK1.1⁺ NKp46⁺ CD3⁻ NK cells stimulated for 18hrs with IL-2, IL-12, or IL-2 plus IL-12 or left unstimulated looking at CD25 expression (A) and pS6 levels (B). Data are representative of three to five experiments.

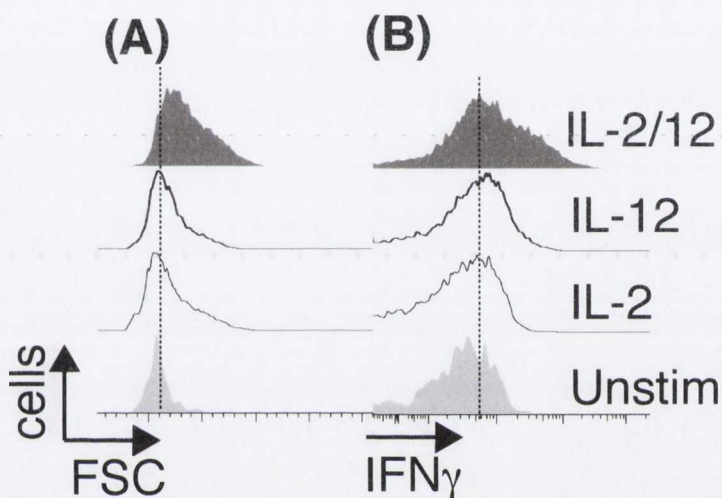


Figure 3.11. IL-2/12 stimulated NK cells display synergistic increases in size and effector molecule production

Flow cytometry analysis of NK1.1⁺ NKp46⁺ CD3⁻ NK cells stimulated for 18hrs with IL-2, IL-12, or IL-2 plus IL-12 or left unstimulated looking at cell size (FSC) (A) and IFN γ expression (B). Data are representative of five experiments.

3.4. Glucose metabolism regulates NK cell effector function

The effector functions of CD4⁺ T cells rely on their ability to increase the rate of glycolysis upon activation (Chang et al., 2013b). To determine whether the elevated levels of glycolysis observed in activated NK cells are required for NK cell effector functions, experiments were designed to directly limit the rate of glycolysis *in vivo* and *in vitro*. Firstly, the glycolytic inhibitor 2-deoxyglucose (2DG), as previously described (Sukumar et al., 2013), was used. 2DG is a glucose analogue, with just one hydroxyl group replaced by a hydrogen residue. It is metabolized by the first step of glycolysis, involving its phosphorylation by Hexokinase 2 to yield 2DG-6-phosphate. However, it cannot be further metabolised by phosphoglucose isomerase leading to an accumulation of 2-DG-6P in the cell and subsequent inhibition of glycolysis.

To determine whether elevated levels of glycolysis are required for NK cell IFN γ production *in vivo*, mice were injected with poly(I:C) in the presence or absence of 2DG. Administration of 2DG did not affect poly(I:C)-mediated NK cell activation as measured by CD69 expression (Fig.3.12A). However, NK cells activated in the presence of 2DG were smaller in size (Fig.3.12B, C) and had lower rates of 2-NBDG uptake (Fig.3.12D), consistent with decreased glycolysis. Crucially, *in vivo* 2DG administration significantly reduced IFN γ production by activated NK cells (Fig.3.13A). It is clear that *in vivo* inhibition of glycolysis did not globally disrupt NK cell function, as poly(I:C) induced expression of TNF α increased to equivalent levels, independent of the presence or absence of 2DG (Fig.3.13B). Parallel experiments were performed using IL-15 cultured NK cells. In these experiments, low doses of 2DG were used to limit glycolytic flux, but not block glycolysis completely, therefore avoiding a cellular energy crisis. We confirmed that low doses of 2DG (0.5-1mM) did not result in decreased ATP levels, which were apparent with higher doses of 2DG (10mM) (Fig.3.14).

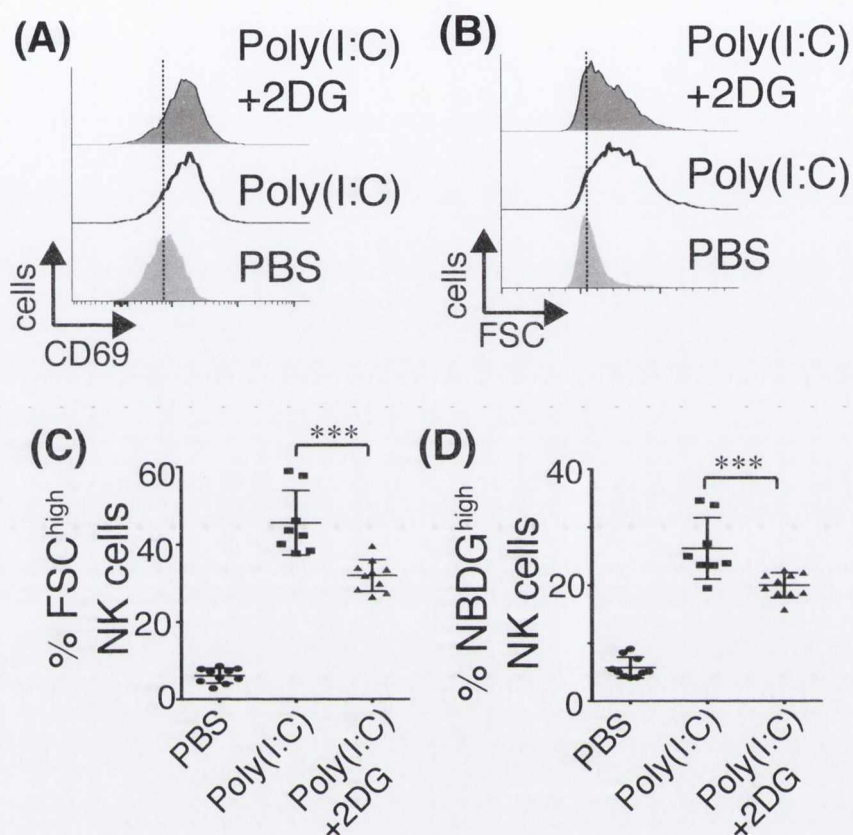


Figure 3.12. Limiting glycolysis in vivo using 2DG prevents poly(I:C) induced NK cell blastogenesis and increased glucose uptake

Mice were administered PBS or 200mg poly(I:C) alone or in combination with 2DG (1g/kg) by peritoneal injection, and spleens were harvested after 24hrs. Splenocytes were isolated, and NK1.1⁺ NKp46⁺ CD3⁻ NK cells (or NK1.1⁺ NKp46⁺ TCR β ⁻ NK cells for glucose uptake) were analyzed for CD69 expression (A), cell size (B-C) and rates of glucose uptake (NBDG) (D). Data are $\pm$ SEM or are representative of 8–10 mice for each condition from two separate experiments. ***p < 0.001.

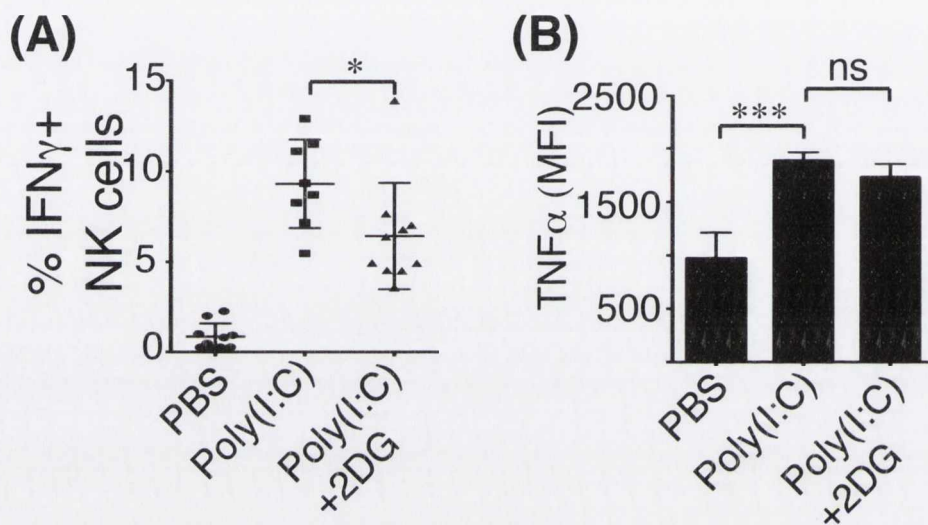


Figure 3.13. Elevated levels of glycolysis are required for IFN γ production in poly(I:C) activated NK cells *in vivo*

Mice were administered PBS or 200mg poly(I:C) alone or in combination with 2DG (1g/kg) by peritoneal injection, and spleens were harvested after 24hrs. Splenocytes were isolated, and NK1.1⁺ NKp46⁺ CD3⁻ NK cells were analyzed for IFN γ (A) and TNF α expression (B). Data are representative of 8–10 mice for each condition from two separate experiments. *p < 0.05, ***p < 0.001.

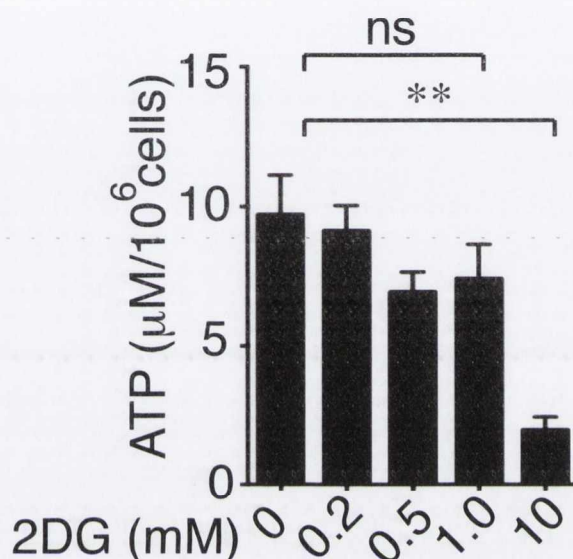


Figure 3.14. Low doses of 2DG do not cause NK cell energy crisis

Cultured NK cells were stimulated with IL-2/12, in the presence or absence of 2DG at the stated concentrations for 18hrs. Cellular ATP levels were determined using the Perkin Elmer ATP assay kit according to manufacturers instructions and read on a FLUOstar OPTIMA luminescence reader. Data are mean +/- SEM of three separate experiments **p < 0.01

Consistent with the *in vivo* findings, IL-15 cultured NK cells activated in the presence of low doses of 2DG (0.5-1mM) activated normally as measured by CD69 expression (Fig.3.15A), yet their effector functions were compromised; in the presence of 2DG, NK cell IFN γ (Fig.3.15B,D) and granzyme B (Fig.3.15C,E) expression were reduced in a dose dependent manner.

These data demonstrate the importance of glycolysis in controlling Natural Killer cell growth and effector responses *in vivo* and *in vitro*. In addition to the use of the glycolytic inhibitor 2DG, a second, complimentary approach was used to assess the importance of elevated rates of glycolysis in controlling NK cell effector responses. Galactose is a carbon fuel source that is metabolised by glycolysis following its conversion to glucose-6-phosphate (G6P) through the Leloir pathway (Frey, 1996). However the rate at which galactose is converted to G6P is slow, and therefore using galactose as an alternative fuel source to glucose acts to limit the overall rate of glycolysis. Metabolic analysis confirmed that rates of glycolysis were reduced in NK cells cultured in galactose containing media to compared to those cultured in glucose containing media (Fig3.16A); however, NK cells had normal rates of OxPhos as predicted (Fig.3.16B). NK cells cultured in galactose containing media up-regulated CD69 equivalently to glucose cultured NK cells in response to IL-2/12 (Fig.3.17A), however NK cell growth was substantially impaired (Fig.3.17B). Crucially, IFN γ production and granzyme B expression were also substantially reduced (Fig.3.18A,B). These data further demonstrate that there is an integral link between the rate of NK cell glucose metabolism, NK cell growth and the production of NK cell effector molecules.

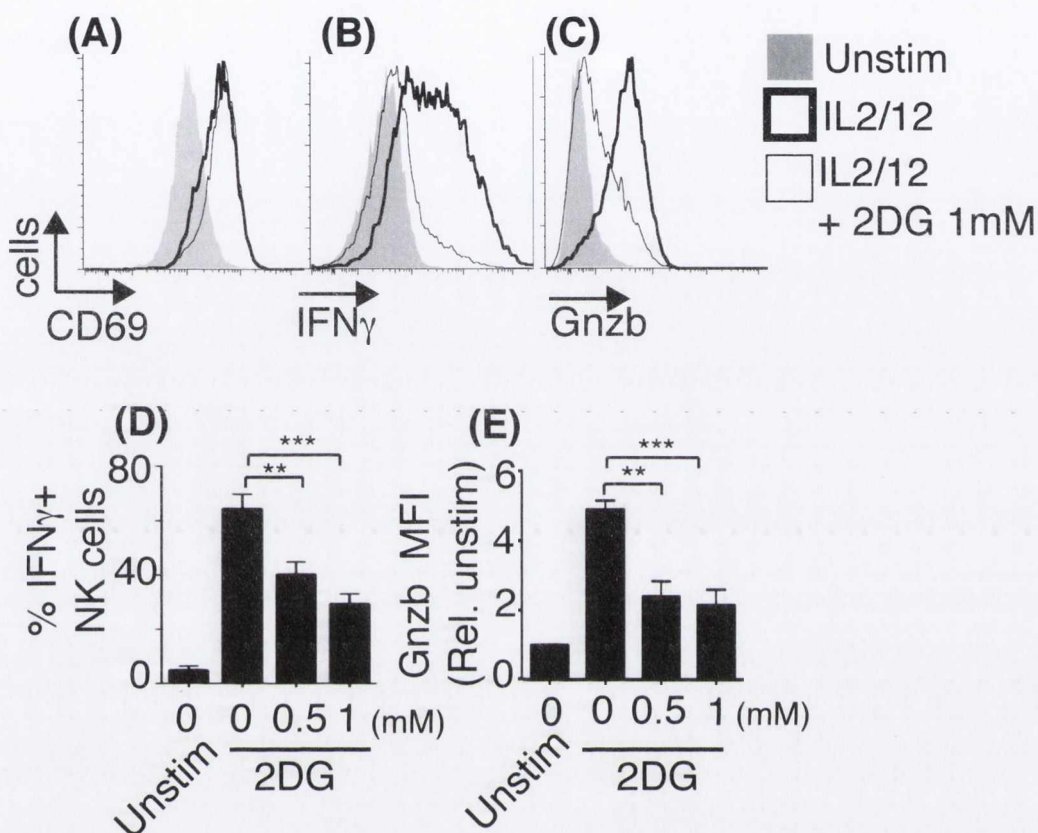


Figure 3.15. Elevated rates of glycolysis are required for IL-2/12 induced NK cell effector function

Cultured NK cells were left unstimulated or treated with IL-2/12, with or without 2DG at the stated concentrations, for 18hrs and were analyzed by flow cytometry for CD69 (A), IFN γ (B,D) and granzyme b (C,E) expression in NK1.1⁺ NKp46⁺ CD3⁻ NK cells. Data are mean $\pm$ SEM or are representative of three to five experiments. **p < 0.01, ***p < 0.001.

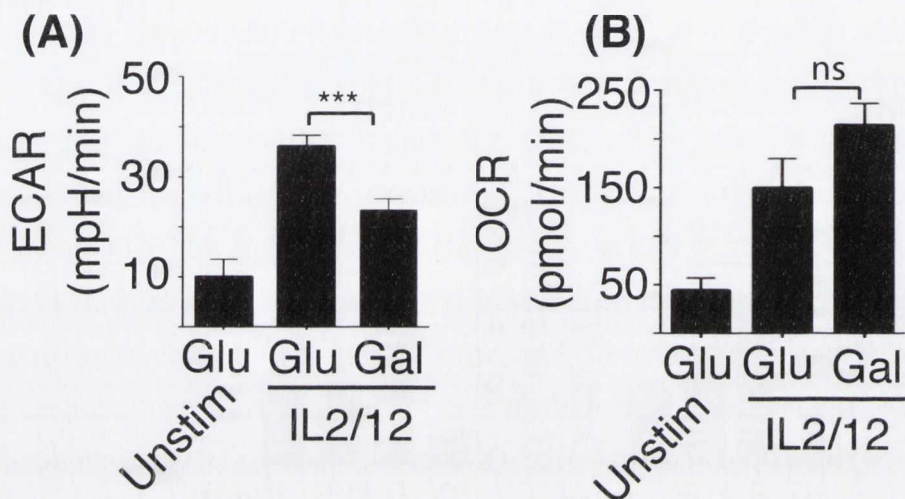


Figure 3.16. Galactose does not support elevated rates of glycolysis in IL-2/12 activated NK cells

Cultured NK cells were left unstimulated in media containing glucose (10 mM) or treated with IL-2/12 in media containing glucose (10 mM) or galactose (10 mM) for 18hrs and analyzed for rates of glycolysis (A) and OxPhos (B). Data are mean +/- SEM of three separate experiments. ***p < 0.001.

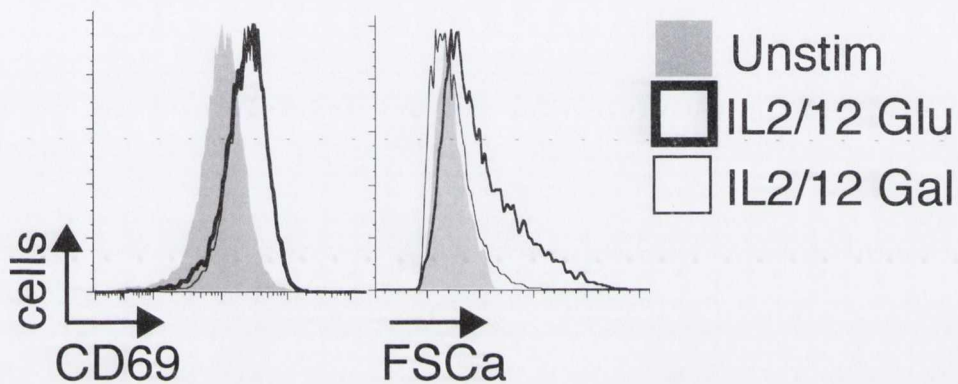


Figure 3.17. Galactose cannot support increased cell growth in cytokine activated NK cells

Cultured NK cells were either left unstimulated in media containing glucose (10mM) or treated with IL-2/12 in media containing glucose (10mM) or galactose (10mM) for 18hr and analyzed by flow cytometry for levels of CD69, and cell size (FSCa) in NK1.1⁺ NKp46⁺ CD3⁻ NK cells. Data are representative of five experiments.

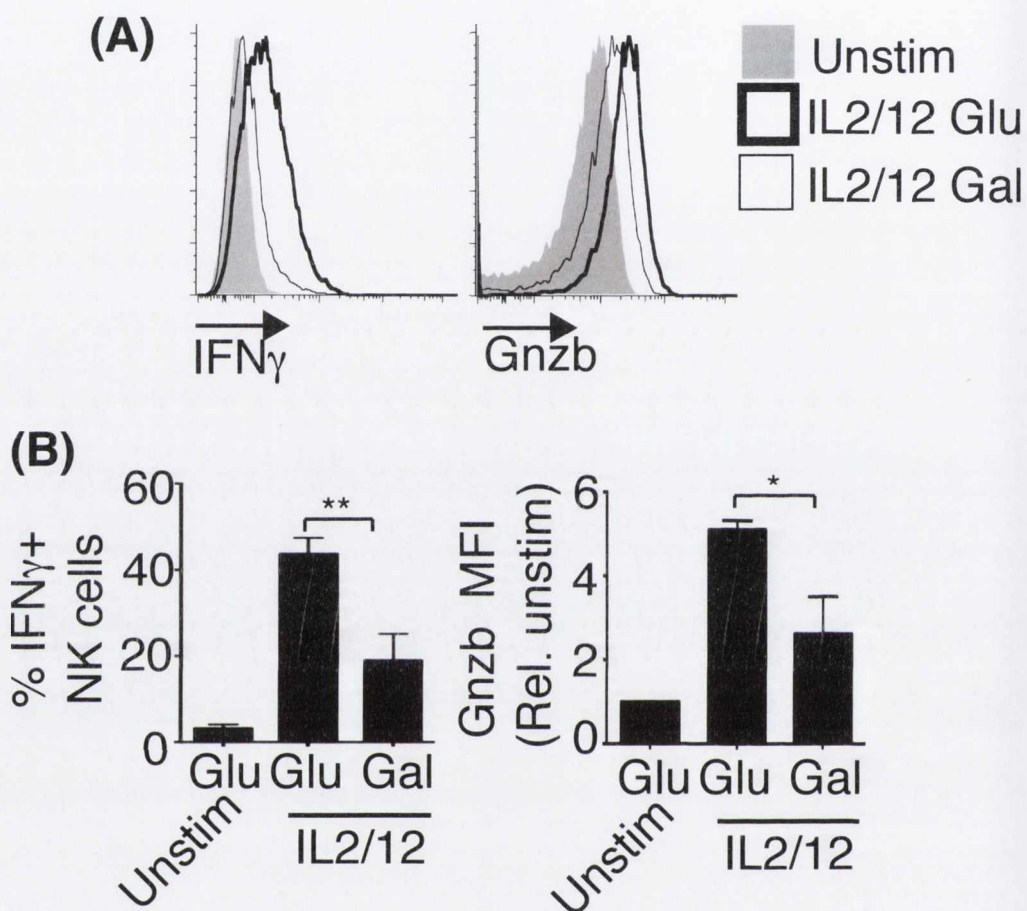


Figure 3.18. Glucose is required for the acquisition of NK cell effector functions

Cultured NK cells were either left unstimulated in media containing glucose (10mM) or treated with IL-2/12 in media containing glucose (10mM) or galactose (10mM) for 18hr and analyzed by flow cytometry for the production of IFN γ and granzyme B (A, B) in NK1.1⁺ NKp46⁺ CD3⁻ NK cells. Data are mean $\pm$ SEM or are representative of five experiments.

3.5. mTORC1 controls NK cell metabolism

mTORC1 is a serine threonine kinase that governs the metabolic and functional responses of many lymphocytes, including activated CD8⁺ T cells (Finlay et al., 2012b). Given the functional similarities between NK cells and CD8⁺ T cells, the role of mTORC1 in NK cell activation was investigated.

Ribosomal S6 protein is a downstream target in the mTORC1 signaling pathway, and the degree of phosphorylation on ser235/236 residue is a useful tool to assess mTORC1 activity. To investigate whether mTORC1 activity was elevated during NK cell activation *in vivo*, pS6 was measured by flow cytometry in NK cells isolated 12 or 24 hours after poly(I:C) injection. Strikingly, poly(I:C) treated NK cells displayed a dramatic increase in pS6 levels, indicative of increased mTORC1 activity (Fig.3.19). In contrast, poly(I:C) did not increase pS6 levels in T cells, highlighting the specificity of this model for activating NK cells *in vivo* (Fig.3.19).

Cell numbers from the poly(I:C) *in vivo* model for NK cell activation limited our analysis of mTORC1 signaling to FACS based assays. However, cell numbers attained using IL15 cultured NK cells allowed for in depth analysis of mTORC1 signaling by western blotting. In addition to measuring pS6 in these experiments, the level of phosphorylated p70 S6 kinase, a direct mTORC1 substrate, was also assessed. Interestingly, IL-2/12 cytokine stimulated NK cells displayed increased mTORC1 activity after just 4hours that was maintained over a 20hour period (Fig.3.20). Importantly, this increase in mTORC1 activity correlated with NK cell metabolic reprogramming events; mTORC1 is active during the period NK cells increase the mRNA expression of the glycolysis-associated genes Glut1, Hex2 and LDHa (Fig.3.21).

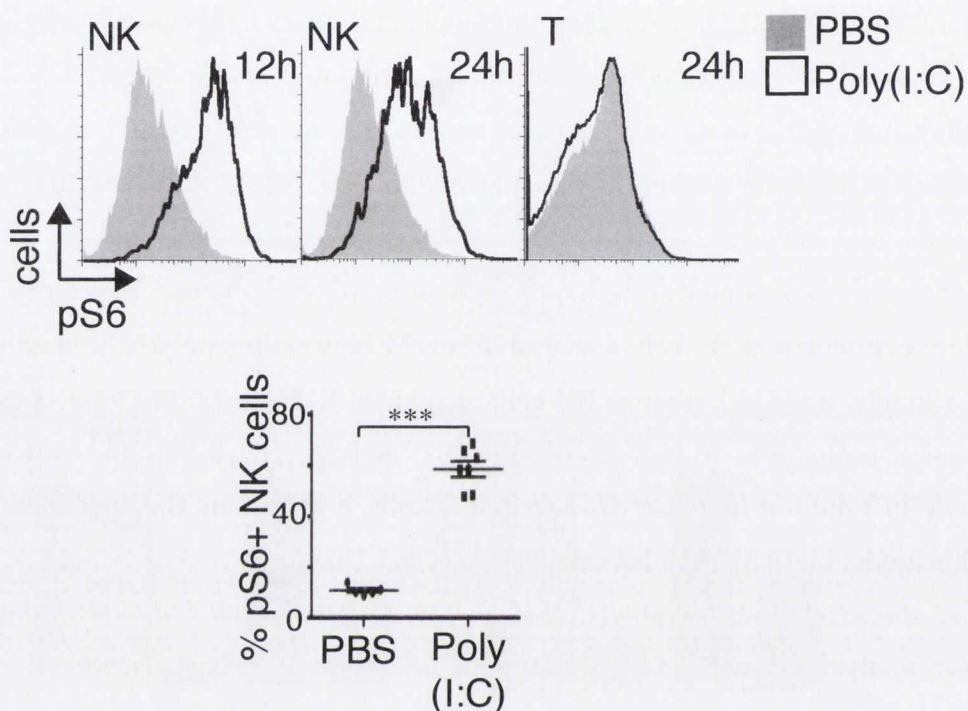


Figure 3.19. mTORC1 activity increases upon NK cell activation *in vivo*

Mice were administered PBS or 200mg poly(I:C) by peritoneal injection. Spleens were harvested after 12 or 24hrs, and NKp46⁺ TCRβ⁺ NK cells (A-B) or TCRβ⁺ T cells were analyzed by flow cytometry for levels of pS6. Data are mean +/- SEM or are representative of 5-10 mice for each condition from two separate experiments. ***p < 0.001.

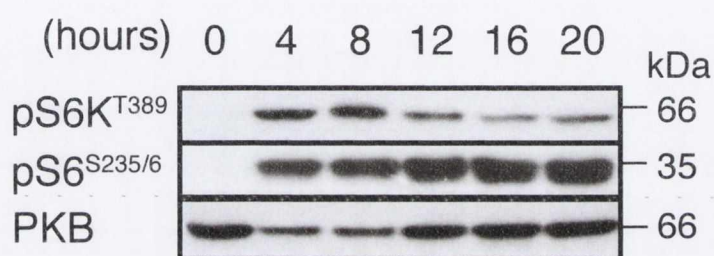


Figure 3.20. Cytokine-stimulated NK cells display increased mTORC1 activity

MACS purified 6 day cultured NK cells were activated with IL-2/12 and lysed at 10^6 cell/ml at the time points indicated (0-20hrs). Samples were analysed by immunoblot for pS6K_{t389}, pS6_{s235/236} and total PKB. Data is representative of 3 independent experiments.

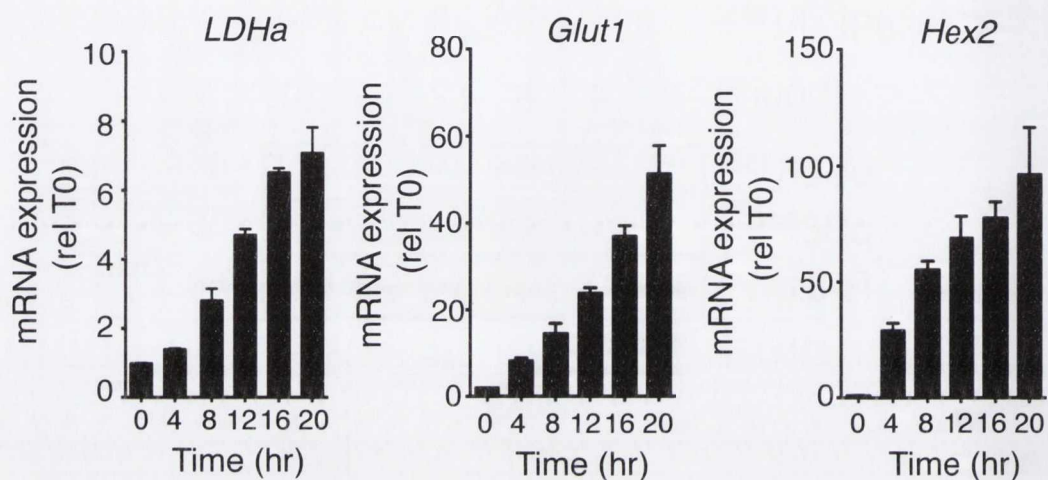


Figure 3.21. NK cells increase glycolytic gene expression following IL-2/12 cytokine stimulation

MACS purified, cultured NK cells were stimulated with IL-2/12 at the timepoints indicated (0-20hrs) and analyzed for the expression of LDHa, Glut1, and Hex2 mRNA. Data are mean $\pm$ SEM of three experiments.

The data show that mTORC1 activity increases during NK cell activation. To define the importance of mTORC1 signaling for NK cell responses *in vivo*, the mTORC1 inhibitor rapamycin was co-administered with poly(I:C) in mice. Importantly, rapamycin did not affect NK cell activation as measured by CD69 expression (Fig.3.22A), however it had a profound effect on NK cell size; NK cells from rapamycin treated mice had reduced FSC (Fig.3.22B-C). Furthermore, rapamycin treatment inhibited NBDG uptake (Fig.3.23A) and the expression of CD71 and CD98 remained low in NK cells from poly(I:C)/rapamycin treated mice (Fig.3.23B,C). These data suggest an important role for mTORC1 in controlling NK cell metabolism *in vivo*.

The role of mTORC1 in controlling activation-induced changes in NK cell metabolism was confirmed *ex vivo*; IL-2/12 stimulated NK cells in the presence of rapamycin failed to engage in cell growth and remained small in size (Fig.3.24A). They also had reduced expression of CD71 or CD98 (Fig.3.24B,C) and reduced NBDG uptake (Fig.3.24D). Rapamycin treated IL-2/12 stimulated NK cells also displayed a significant decrease in the rate of glycolysis (Fig.3.25A), while the rate of OxPhos was not unaffected (Fig.3.25B). Furthermore, the glycolytic capacity of rapamycin treated NK cells was also significantly reduced (Fig.3.26). This finding suggests that mTORC1 is important in controlling NK cell metabolic reprogramming. Indeed, RT-PCR analysis revealed a decrease in the expression of the glucose transporter glut1 and key glycolytic enzymes Hex2 and Ldha in the presence of rapamycin (Fig.3.27A-C). Together, these data show a crucial role for mTORC1 in controlling the metabolic reprogramming in response to IL2/12 stimulation.

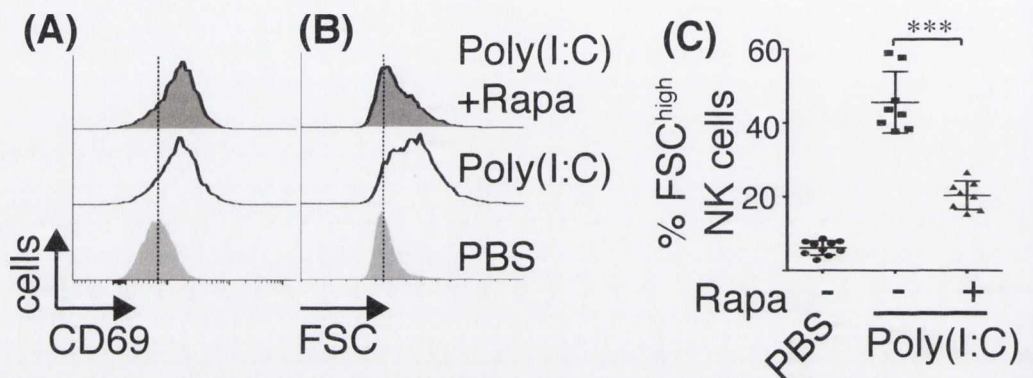


Figure 3.22. mTORC1 activity is required for NK cell activation-induced blastogenesis *in vivo*

Mice were administered PBS or 200mg poly(I:C) alone or in combination with rapamycin (0.6 mg/kg) by peritoneal injection. Spleens were harvested after 24hrs, and NK1.1⁺ NKp46⁺ CD3⁻ NK cells were analyzed by flow cytometry for levels of CD69 (A) and cell size (B-C). Data are mean $\pm$ SEM or are representative of 5–10 mice for each condition from two separate experiments. *** $p < 0.001$.

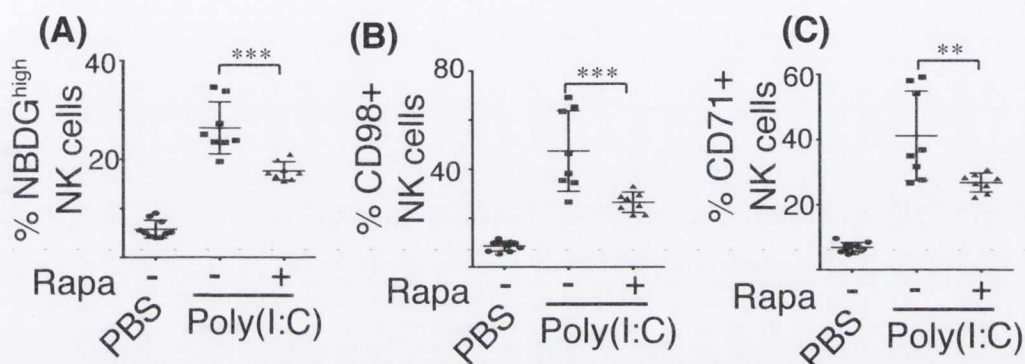


Figure 3.23. mTORC1 activity is required for increased glucose uptake and metabolic receptor expression in poly(I:C) activated NK cells *in vivo*

Mice were administered PBS or 200mg poly(I:C) alone or in combination with rapamycin (0.6 mg/kg) by peritoneal injection, and spleens were harvested after 24 h. Splenocytes were isolated, and NK1.1⁺ NKp46⁺ CD3⁻ NK cells (or NK1.1⁺ NKp46⁺ TCRb⁻ NK cells for NBDG) were analyzed by flow cytometry. The frequency of NK cells with high levels of NBDG (A) and expression of CD98 (B) and CD71 (C) were analysed. Data are mean $\pm$ SEM of 8–10 mice for each condition from two separate experiments. ** $p < 0.01$, *** $p < 0.001$.

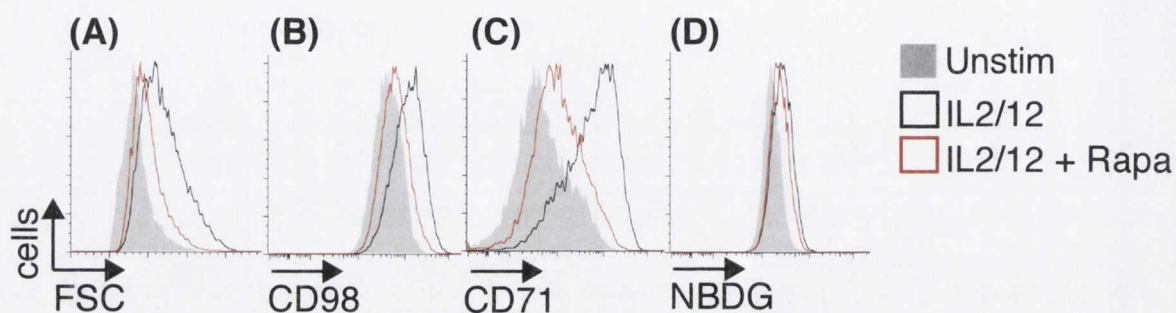


Figure 3.24. mTORC1 activity is required for increased metabolic receptor and glucose uptake in IL-2/12 activated NK cells

Cultured NK cells were left unstimulated or stimulated with IL-2/12, with or without rapamycin (20nM), for 18hrs. NK1.1⁺ NKp46⁺ CD3⁻ cells (or NK1.1⁺ NKp46⁺ TCR β ⁻ NK cells for NBDG) were analyzed by flow cytometry for changes in size (A), CD71 (B), CD98 (C) and glucose uptake (D) fluorescence. Data are representative of five experiments.

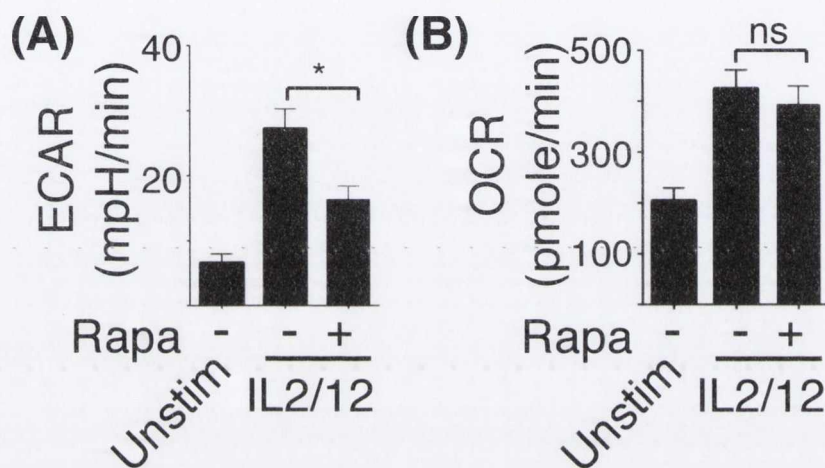


Figure 3.25. mTORC1 controls the increased level of glycolysis seen in IL-2/12 stimulated NK cells

MACS purified, cultured NK cells were stimulated for 18hrs with IL-2/12, with or without rapamycin (20 nM), or were left unstimulated and analyzed for the rates of glycolysis (A) and OxPhos (B). Data are mean +/- SEM of three experiments. *p < 0.05

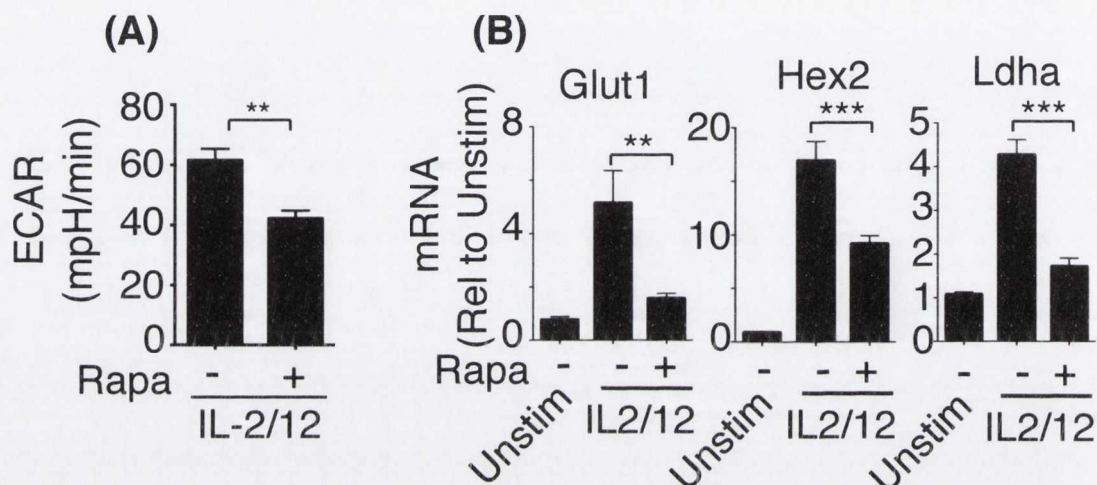


Figure 3.26. mTORC1 is required for increased NK cell glycolytic capacity and metabolic reprogramming following cytokine stimulation

MACS purified, cultured NK cells were stimulated for 18hrs with IL-2/12, with or without rapamycin (20nM) and were analyzed for the maximal glycolytic capacity following the addition of oligomycin (A) or the mRNA expression of Glut1, Hex2 and Ldha (B). Data are mean $\pm$ SEM of three experiments. ** $p < 0.01$, *** $p < 0.001$.

3.6 mTORC1 controls NK cell functional responses

The data shown thus far highlight the integral link between NK cell glucose metabolism and NK cell effector responses. mTORC1 has been identified as a key regulator of NK cell metabolism, hence the role for mTORC1 in controlling NK cell functional responses was next explored.

To assess the importance of mTORC1 in controlling NK cell effector functions *in vivo*, the expression of the pro-inflammatory cytokine IFN γ was assessed in NK cells isolated from poly(I:C) and rapamycin co-administered mice. Strikingly, the production of IFN γ in rapamycin treated NK cells was significantly decreased (Fig.3.27A,B). Importantly, 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.3.28A-B). The importance of mTORC1 in controlling NK cell effector responses was also evident in IL-2/12 stimulated, cultured NK cells *ex vivo*; rapamycin treatment significantly decreased both IFN γ (Fig.3.29A) and granzyme B (Fig.3.29B) expression.

In summary, the data in this results chapter paved a new way for the metabolic regulation of Natural killer cells, showing that NK cells undergo metabolic reprogramming upon activation, the importance of this metabolic shift in the acquisition of NK cell effector responses and finally, highlighting the imperative role of mTORC1 signaling in controlling the metabolic and functional responses of Natural Killer cells.

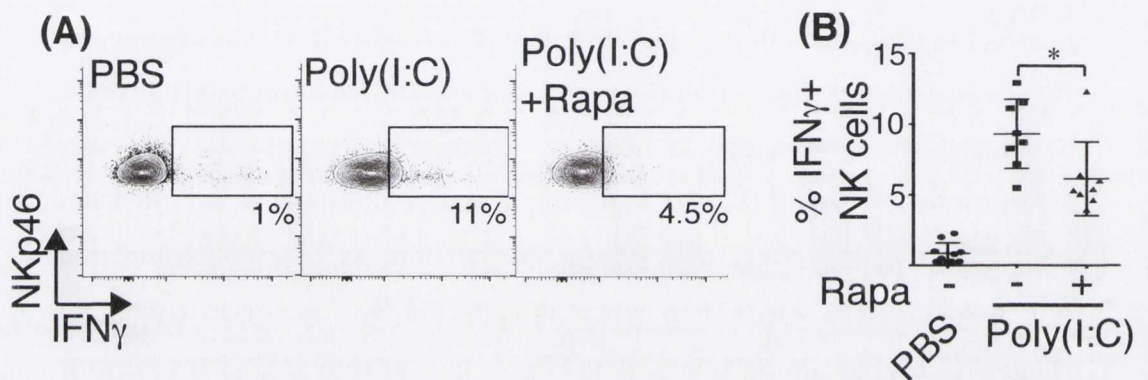


Figure 3.27. mTORC1 activity is required for NK cell IFN γ production *in vivo*

Mice were administered PBS or 200mg poly(I:C) alone or in combination with rapamycin (0.6 mg/kg) by peritoneal injection. Spleens were harvested after 24hra, and NK1.1⁺ NKp46⁺ CD3⁻ NK cells were analyzed by flow cytometry for levels of IFN γ (A-B). Data are mean $\pm$ SEM or are representative of 5–10 mice for each condition from two separate experiments. * $p < 0.05$.

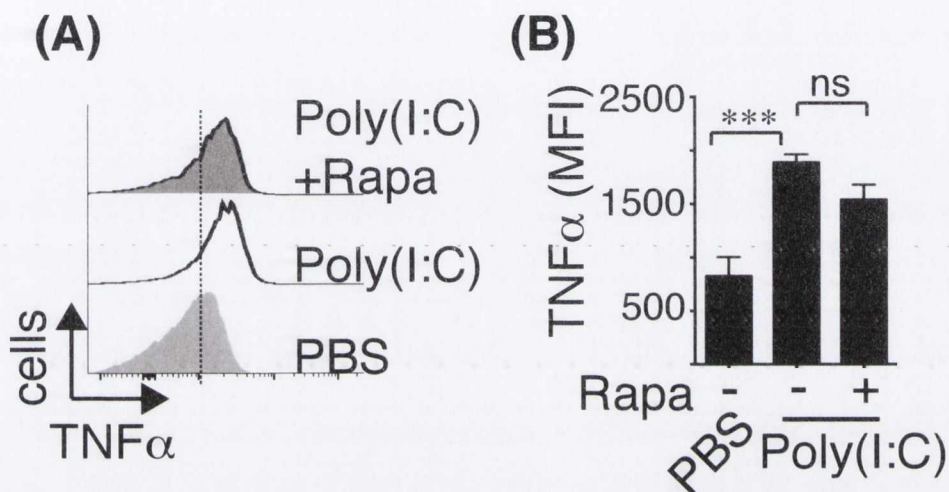


Figure 3.28. Inhibition of mTORC1 does not have a global effect on NK cell cytokine production

Mice were administered PBS or 200mg poly(I:C) alone or in combination with rapamycin (0.6 mg/kg) by peritoneal injection. Spleens were harvested after 24hrs, and NK1.1⁺ NKp46⁺ CD3⁻ NK cells were analyzed by flow cytometry for levels of TNF α (A-B). Data are mean $\pm$ SEM or are representative of four mice for each condition. *** $p < 0.001$.

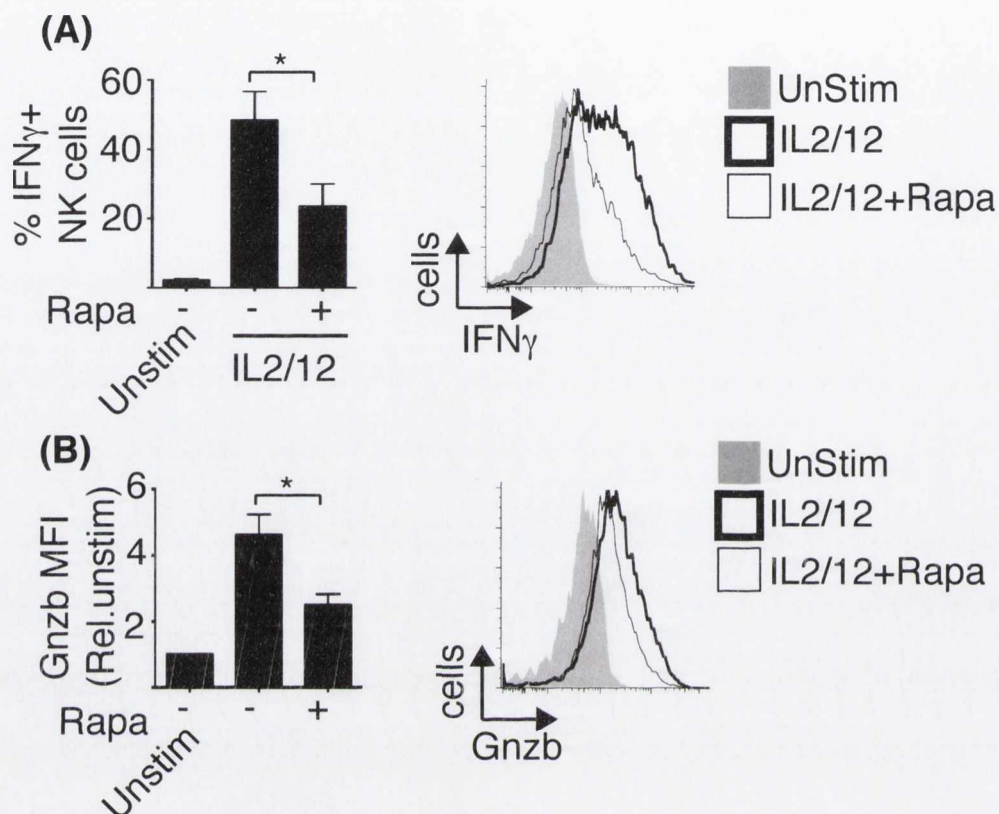


Figure 3.29. mTORC1 activity is required for the acquisition of NK cell effector function

Cultured NK cells were left unstimulated or treated with IL-2/12, with or without rapamycin (20nM), for 18hrs. NK1.1⁺ NKp46⁺ CD3⁻ cells were analyzed by flow cytometry for IFN γ (A) and granzyme B (B) expression. Data are mean $\pm$ SEM or are representative of five experiments. *p < 0.05.

Chapter 3 discussion

NK cells are critical effector cells in the immune response against virally infected or transformed cells. Significant progress has been made in terms of identifying how NK cells recognise target cells and their regulation at a cellular level by cytokines. Here we demonstrate the fundamental contribution that cellular metabolism makes to NK cell function. We have successfully measured and defined changes in NK cell metabolism following activation *in vitro* and *in vivo*. We have shown that in response to IL-2/12 cytokine stimulation, activated NK cells dramatically increase in size and display increased rates of glycolysis and OxPhos. Overall, there is a shift towards preferentially metabolising glucose to lactate, a metabolic process termed aerobic glycolysis.

This increase in NK cell metabolism coincided with complete metabolic reprogramming, with an observed increase in the mRNA expression of key glycolytic genes. The metabolic profile of activated NK cells resembles that of pro-inflammatory effector T cell subsets (Finlay et al., 2012, Michalek et al., 2011a, Shi et al., 2011). The initial activation of a T cell, through the T cell receptor (TCR) induces elevated rates of glucose uptake and glycolysis (Wang et al., 2011a). This metabolic shift is maintained by various cytokines, including IL-2, as the T cell differentiates into a CD4+ or CD8+ T effector cell. In contrast, regulatory or memory T cells display low levels of glycolysis and utilize oxidative glucose and lipid metabolism to maintain energy homeostasis (Michalek et al., 2011a, Shi et al., 2011, van der Windt et al., 2012). These distinct, characteristic metabolic profiles of effector and regulatory immune cells demonstrate how immune cells adapt their metabolism to meet functional demands.

This led us to the hypothesis that cellular metabolism has an important role in dictating NK cell responses. In order to test this hypothesis, we developed strategies to limit the increase in glycolysis observed in activated NK cells.

Firstly, we stimulated NK cells in an alternative carbon source (galactose) that cannot support elevated levels of glycolysis. Secondly, we directly inhibited glycolysis by using low doses of 2DG, an inhibitor of hexokinase 2. In support of our hypothesis, a key finding from this study is that NK cell glycolysis is integrally linked to NK cell effector functions, including the production of IFN γ and granzyme B. It is interesting that in CTLs, cells with many analogous functional roles to NK cells, glycolytic metabolism is also essential for normal effector functions. Thus, IL-2 maintained CTLs that cannot maintain elevated glycolysis do not sustain the expression of key CTL effector molecules including components of cytolytic granules, namely perforin and granzymes (Finlay et al., 2012, Sukumar et al., 2013). As in the case of NK cells, 2DG administered to differentiating CTLs blocks the expression of granzyme B (Sukumar et al., 2013) and it also blocks production of IFN γ in CD4 $^{+}$ T cells (Chang et al., 2013). Our data shows that the rate of glucose uptake segregates NK cells based on their production of IFN γ , that is, IFN γ producing cells are increased in size with elevated rates of glucose uptake. Similarly, glucose uptake has been shown to segregate CD8 $^{+}$ T cells based on the expression of granzyme B; high levels of glucose uptake in cells with high granzyme B expression (Sukumar et al., 2013). B cells treated with 2DG displayed reductions in antibody production (Caro-Maldonado et al., 2014). Activated CD8 $^{+}$ T cells exhibiting lower levels of glucose uptake are the precursors for long-lived memory T cells while those with elevated glucose uptake become short lived effector T cells (Sukumar et al., 2013). Indeed, increasing glucose uptake in TCR stimulated CD4 $^{+}$ T cells through transgenic expression of the Glut1 glucose transporter is sufficient to promote increased numbers of IFN γ^{+} effector T cells (Michalek et al., 2011a). In an analogous fashion, this report demonstrates that elevated glucose uptake and glycolysis is required for robust IFN γ production in IL-2/12 stimulated NK cells. Together, these studies define a new paradigm of metabolic regulation of effector lymphocyte function in which achieving

elevated rates of glucose uptake and glycolysis is a prerequisite for the generation of functional proinflammatory effector T and NK cells subsets. Therefore, the emerging theory is that rates of glucose uptake and glycolysis directly impact upon lymphocyte differentiation and activation.

While mTORC1 is emerging as a key molecule for the control of T cell metabolism and function, the role played by mTORC1 in other lymphocyte subsets, in particular NK cells, has not been well defined. Therefore, very little is known about the role mTORC1 plays in NK cell activation and function. Recent studies have shown that NK cell progenitors have high metabolic activity and this mTORC1-dependent glycolytic metabolism decreases as NK cell differentiation progresses (Marcais et al., 2014). Indeed, deletion of mTORC1 in early NK cell progenitors disrupts NK cell maturation and peripheral homeostasis (Marcais et al., 2014). The data presented here demonstrates an essential role for mTORC1 for elevated rates of glucose uptake and glycolysis. In addition, mTORC1 activity is required for metabolic reprogramming in IL-2/12 stimulated NK cells, as demonstrated by decreased expression of glycolytic genes and glucose uptake upon rapamycin treatment. This data was mirrored *in vivo* with poly (I:C) activated NK cells treated with rapamycin demonstrating reduced glucose uptake. Moreover, our findings implicate mTORC1 regulated glycolysis in normal NK cell effector functions in *in vitro* IL-2/12 and *in vivo* poly (I:C) activated NK cells. mTORC1 has an emerging role as a key regulator of lymphocyte metabolism and lymphocyte differentiation and function (Powell and Delgoffe, 2010). Thus, disruption of mTORC1 activity through either pharmacological or genetic based strategies prevents the differentiation of TH1 and TH17 effector T cells, while promoting the generation of Tregs (Delgoffe et al., 2009, Delgoffe et al., 2011a, Gao et al., 2007, Kang et al., 2008). Equally, in CD8⁺ T cells, mTORC1 is essential to allow for elevated glucose uptake and glycolysis in CTL differentiation (Finlay et al., 2012). Indeed, concomitant with decreased glycolysis, CD8⁺ T cells lacking mTORC1 activity acquire

memory T cell like characteristics while rapamycin treated mice generate enhanced CD8+ T cell memory (Araki et al., 2009, Finlay et al., 2012, Sinclair et al., 2008). In this study we show that IL-2/12 stimulated NK cells treated with rapamycin (thus lacking mTORC1 activity) are unable to up-regulate glycolysis and cell growth, which are essential for the acquisition of NK cell effector functions; IFN γ and granzyme B production. Interestingly, TNF α expression is not inhibited by rapamycin *in vivo* or *in vitro* suggesting that elevated glycolysis is not required for all NK cell effector functions and confirming that rapamycin or 2DG did not globally block NK cell function. In more recent studies by other groups published after this, inhibition of mTORC1 (induced with high dose of IL-15) in NK cells was also found to inhibit the expression of effector molecules key for NK cell function, namely IFN γ and granzyme B (Marcais et al., 2014, Nandagopal et al., 2014). Furthermore, a recent collaborative human study showed that the mTORC1 inhibitor rapamycin can inhibit the production of IFN γ in IL-2 stimulated CD56bright NK cells (Keating et al., 2016). While mTORC1 appears to be required for glucose metabolism in T cells and NK cells, this is not the case for all lymphocytes. mTORC1 activity is not required for B cell glucose metabolism (Doughty et al., 2006). Together, these studies demonstrate that mTORC1 is a key regulator of glycolytic metabolism and also a key determinant of lymphocyte activation and differentiation.

Our data shows that IL-2/12 induced elevated glycolysis drives IFN γ and granzyme B production in NK cell stimulated *in vitro*. Our *in vivo* studies mirrored this result; while we could not directly measure glycolysis, poly(I:C) activated NK cells increased glucose uptake, which is reflective of increased glycolysis. Poly(I:C) is a synthetic analog of double stranded RNA (dsRNA), a molecular pattern associated with viral infection. While IL-2 and IL-12 are important proinflammatory cytokines involved in the clearance of viral infections natural and synthetic dsRNAs are known to induce type I interferons (IFN) and other cytokine production. Poly(I:C) is recognized by

Toll-like receptor 3 (TLR3) (Alexopoulou et al., 2001, Matsumoto et al., 2002). Upon poly(I:C) recognition, TLR3 activates the transcription factor interferon regulatory factor 3 (IRF3), through the adapter protein Toll-IL-1 receptor (TIR) domain-containing adapter inducing IFN- β (TRIF) (Yamamoto et al., 2003). Activation of IRF3 results in the production of type I IFNs, especially IFN- β . A second pathway involves the recruitment of TNF receptor-associated factor 6 (TRAF6) or receptor interacting protein 1 (RIP1), with the subsequent activation of the transcription factors NF- κ B and AP-1 (Kawai and Akira, 2008). Activation of this pathway triggers the production of inflammatory cytokines and chemokines such as TNF- α , IL-6 and CXCL10. Poly(I:C) is also recognized by the cytosolic RNA helicases retinoic acid-inducible protein 1 (RIG-I) and melanoma differentiation-associate gene 5 (MDA-5) (Kato et al., 2006). Therefore, our data suggests that NK cell glucose metabolism is dynamically regulated with varying cytokine stimuli.

It is clear that aerobic glycolysis is important to facilitate the biosynthetic demands of effector lymphocyte subsets, and limiting the rate of glycolysis results in decreased growth and proliferation in some effector lymphocytes (Doughty et al., 2006). However, glycolytic maintenance of cellular biosynthesis is not the only mechanism by which glycolysis regulates effector lymphocyte function. Indeed, limiting the rate of glycolysis can have relatively little effect on global cellular biosynthesis, while having profound effects on key lymphocyte effector functions. For example, activated CD4⁺ T cells cultured in galactose have decreased rates of glycolysis but are equal in size and proliferative capacity to those maintained in glucose with elevated levels of glycolysis. However, T cells cultured in galactose have dramatically reduced production of cytokines IFN γ and IL2 (Chang et al., 2013). Similarly, when mTORC1 activity, which promotes elevated levels of glycolysis in CTLs, was disrupted, CTLs had substantially reduced rates of glycolysis although they were comparable in size and the rate of proliferation to control

glycolytic CTLs (Finlay et al., 2012). However, despite relatively normal levels of molecular biosynthesis rapamycin treated CTLs are functionally very distinct (Finlay et al., 2012). Similar observations have been made for CD8+ T cells responding to LCMV infection *in vivo*. CD8+ T cells in rapamycin treated mice would be predicted to be non-glycolytic, yet they undergo normal clonal expansion, indicating sufficient biosynthetic capacity, but under these conditions T cell differentiation is altered (Araki et al., 2009). However, our data showed that not only did inhibiting glycolysis decrease NK cell function but we also observed reduced cell growth. Therefore, we cannot rule out the importance of glycolysis driven cellular biosynthesis in controlling NK cell function. Importantly, glycolysis is not universally required for all NK cell functions, as there was no observed decrease in TNF α production. This suggests that glycolysis has a more direct role in regulating the effector functions of activated lymphocytes.

A direct role for cellular metabolism in regulating the effector functions of activated lymphocytes is best described for GAPDH, the sixth enzyme in the glycolytic pathway, which is also described to have RNA binding activities. GAPDH was first shown to bind to AU rich regions of mRNA transcripts 20 years ago and has since been shown to also bind to viral encoded RNA of influenza and hepatitis virus (Nagy and Rigby, 1995, De et al., 1996, Schultz et al., 1996, Zang et al., 1998). Additionally, in myeloid cells GAPDH is a component of the IFN γ -activated inhibitor of translation (GAIT) complex that binds defined 3' untranslated region (UTR) elements within a family of inflammatory mRNAs and suppresses their translation (Mukhopadhyay et al., 2009). GAPDH binding to RNA involves the nucleotide-binding domain that is used to bind the cofactor NAD $^{+}$ when catalysing the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate within glycolysis (Nagy and Rigby, 1995). Therefore, GAPDH activities as an enzyme in glycolysis and as a RNA binding protein are mutually exclusive. In CD4+ T cells, GAPDH has been shown to have an important role in regulating the

translation of IFN γ and IL2 mRNA, thus providing a direct link between the rate of glycolysis and CD4 $^{+}$ T cell effector function (Chang et al., 2013). This study suggested that in highly glycolytic CD4 $^{+}$ T cells GAPDH is fully engaged in its function within the glycolytic pathway. However, when the rate of glycolysis is reduced, the requirement for GAPDH within glycolysis is decreased and some GAPDH molecules are now free to bind to the AU rich regions in the 3'UTR of IFN γ and IL2 mRNA and repress their translation (Chang et al., 2013). In 2DG treated NK cells, as well as NK cells cultured in galactose, the decreased IFN γ being produced is consistent with the model described by Chang et al. Indeed, this decrease in IFN γ can also be seen in NK cells treated with rapamycin. Future experiments to determine the role of GAPDH mRNA binding in NK cells would therefore be of great interest. However, the mRNA of other effector molecules regulated by glycolysis, such as granzyme B, does not contain AU-rich regions in their 3'UTR arguing that there are additional mechanisms linking glycolysis to lymphocyte function. Interestingly, there are potentially other metabolic enzymes that act in dual roles in this mRNA binding capacity. A recent study identified enzymes from glycolysis, the TCA cycle, amongst other cellular pathways as mRNA binding proteins (Castello et al., 2012). This research revealed the abundant potential for metabolic pathways to directly impact upon cellular functions through controlling mRNA and thus protein expression. Moreover the glycolytic enzyme hexokinase has been reported to be required for IL-1 β maturation via regulation of the NLRP3 inflammasome (Moon et al., 2015).

In conclusion this chapter demonstrates that the proinflammatory functions of IL-2/12 in vitro activated and Poly (I:C) in vivo activation of NK cells are reliant on successful mTORC1 dependent metabolic reprogramming that permits elevated rates of glucose uptake and glycolysis. This regulatory axis has important implications for NK cell responses within infection and tumor microenvironments, which will be further discussed in chapter 6.

Chapter 4

The transcription factor cMyc, not HIF1 α , controls NK cell metabolic and functional responses

Introduction

Data presented in chapter 3 demonstrates the importance of glucose metabolism for the acquisition of Natural Killer cell effector functions. In addition, the kinase mTORC1 was identified as a crucial mediator in coordinating NK cell activation-associated metabolic reprogramming (Donnelly et al., 2014). Two transcription factors that have been shown to be important for glycolytic reprogramming in immune cell subsets are hypoxia-inducible factor 1 alpha (HIF1 α) and myelocytomatosis oncogene (cMyc).

Both HIF1 α and cMyc coordinate the metabolic changes of T_{eff} cells during distinct stages of activation and differentiation. Where cMyc is indispensable for the early metabolic reprogramming events following TCR triggering (Wang et al., 2011b), HIF1 α coordinates the changes in cellular metabolism that accompany the differentiation of an activated T cell into a cytotoxic T lymphocyte (CTL) (Finlay et al., 2012b). HIF1 α and cMyc share many of the same metabolic target genes; both enhance the transcription of Glut1, Hex2 and Ldha (Finlay et al., 2012b, Wang et al., 2011b). However each transcription factor also has its own distinct target-gene profile; HIF1 α enhances Phd3 expression whereas cMyc enhances Cd71 expression (Finlay et al., 2012b) (Wang et al., 2011b).

IL-2 is an important cytokine for the HIF1 α -dependent transcriptional reprogramming events that occur during CTL differentiation (Finlay et al., 2012b). Given the use of IL-2, in combination with IL-12, in our NK cell activation system, the role of HIF1 α in controlling cytokine-activated NK cell metabolism was first explored.

4.1 HIF1 α is dispensable for IL-2/12 induced metabolic responses in NK cells

Western blot analysis revealed IL-2/12 cytokine stimulated NK cells increase HIF1 α protein expression in an mTORC1-dependent manner (Fig.4.1A). Measuring the mRNA expression of Phd3, a HIF1 α target gene, also correlated with HIF1 α protein expression. IL-2/12 stimulation induced a seven-fold increase in Phd3 mRNA that was blocked by rapamycin (Fig.4.1B). To investigate the importance of HIF1 α for NK cell activation, HIF1 α -null NK cells were cultured from the spleens of HIF1 $\alpha^{\text{lox/lox}}$ vav-cre mice (HIF1 α^{KO}) obtained from the University of Dundee. In this mouse line, cre recombinase expression is under the control of the vav promoter, which is active early on during haematopoiesis resulting in cre recombinase-mediated excision of floxed HIF1 α alleles in all immune cell subsets (see section 2.1.3). HIF1 $\alpha^{\text{lox/lox}}$ mice that did not contain the vav-cre transgene (HIF1 α^{WT}) were used as wild type controls in these experiments. Deletion of HIF1 α alleles in HIF1 α^{KO} NK cells was confirmed by real-time PCR analysis (Fig.4.2A). HIF1 α^{KO} mice displayed comparable frequencies of NK cells in splenocytes to HIF1 α^{WT} mice (Fig.4.2B). Moreover, HIF1 α^{KO} NK cells expanded equivalently to wild type cultures *in vitro* in low dose IL-15 over 6 days (Fig.4.2C). Together, these data suggest HIF1 α is not required for NK cell development. However, it was observed that the expression of NK1.1 was reduced in HIF1 α^{KO} NK cells compared to HIF1 α^{WT} NK cells (Fig.4.2B,C). IL-2/12 stimulated HIF1 α^{KO} cells were comparable in size (FSC) and had equivalent expression of the nutrient receptor CD71 compared to HIF1 α^{WT} NK cells (Fig.4.3A,B). In addition, HIF1 α^{KO} NK cells had comparable levels of 2-NBDG uptake to HIF1 α^{WT} NK cells (Fig.4.3C). For detailed metabolic analysis of HIF1 α^{KO} NK cells, a seahorse flux assay was performed. The rate of glycolysis in HIF1 α^{KO} NK cells was equivalent to that of HIF1 α^{WT} NK cells stimulated with IL-2/12 (Fig.4.4). In addition, the rate of OxPhos was also comparable between HIF1 α^{WT} and HIF1 α^{KO} NK cells (Fig.4.5).

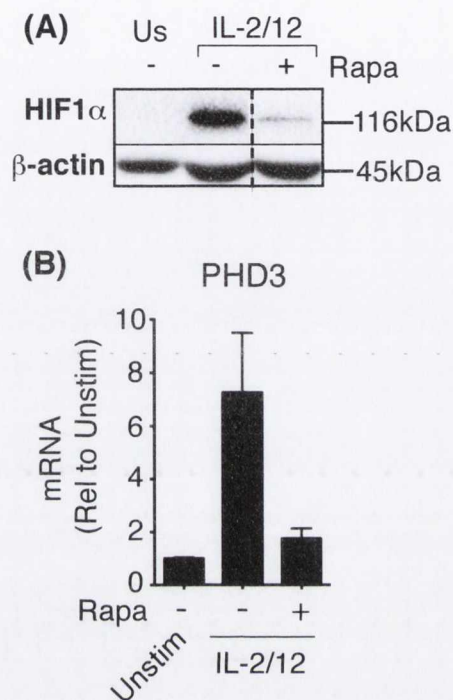


Figure 4.1. HIF1 α expression is increased in cytokine stimulated NK cells in an mTORC1-dependent manner

MACS purified 6 day cultured NK cells were left unstimulated or were activated with IL-2/12 in the presence or absence of rapamycin (20nM). Samples were lysed for protein or RNA after 18hrs. Protein samples were analysed by immunoblot for HIF1 α expression with β -actin as a loading control (A). HIF1 α activity was assessed by mRNA expression of Phd3 (B). Data are mean $\pm$ SEM or are representative of three to five independent experiments.

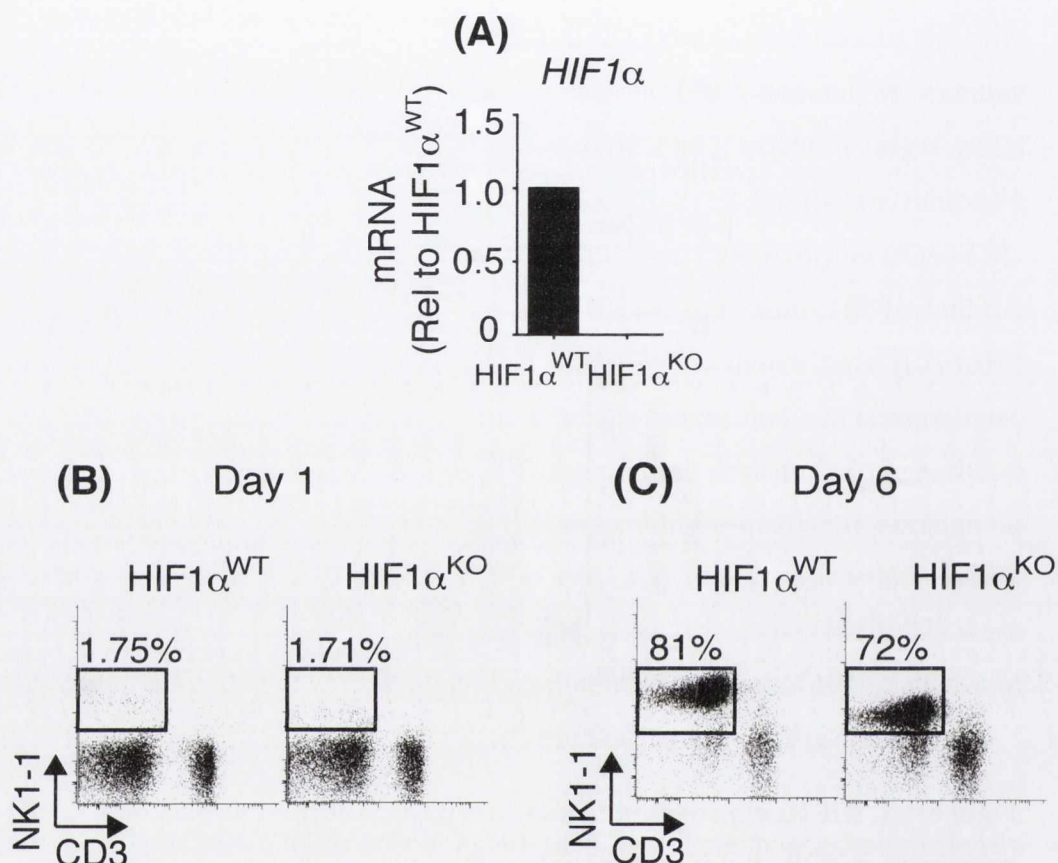


Figure 4.2. HIF1α^{fllox/fllox} vav cre-recombinase splenocytes display comparable expansion of NK cells

MACS purified 6 day cultured NK cells from HIF1α^{fllox/fllox} vav cre positive (HIF1α^{KO}) and cre negative (HIF1α^{WT}) mice were activated with IL-2/12 and samples were lysed for RNA after 18hrs. HIF1α mRNA expression was assessed by RT-PCR analysis (A). Splenocytes were isolated from HIF1α^{KO} and HIF1α^{WT} mice and the expression of NK1.1⁺ CD3⁻ cells were assessed by flow cytometry (B). Splenocytes were cultured in low dose IL-15 for 6 days and NK cell expansion was assessed by flow cytometry looking at NK1.1⁺ CD3⁻ cells (C). Data are representative or mean +/- SEM of five independent experiments.

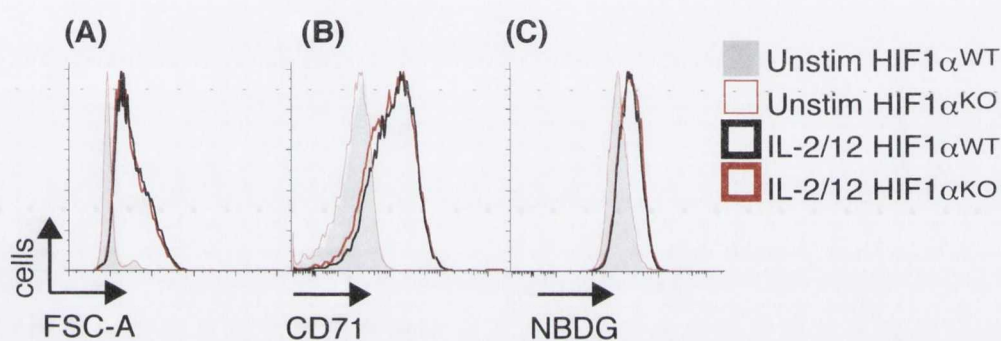


Figure 4.3. Cytokine stimulated HIF1 α null NK cells are metabolically active

6 day cultured NK cells from HIF1 α ^{KO} and HIF1 α ^{WT} mice were left unstimulated or stimulated with IL-2/12 for 18hr and NK1.1⁺ NKp46⁺ CD3⁻ cells (or NK1.1⁺ NKp46⁺ TCR β ⁻ cells) were analysed by flow cytometry for changes in cell size (A) CD71 (B) and NBDG uptake (C). Data are representative of five independent experiments.

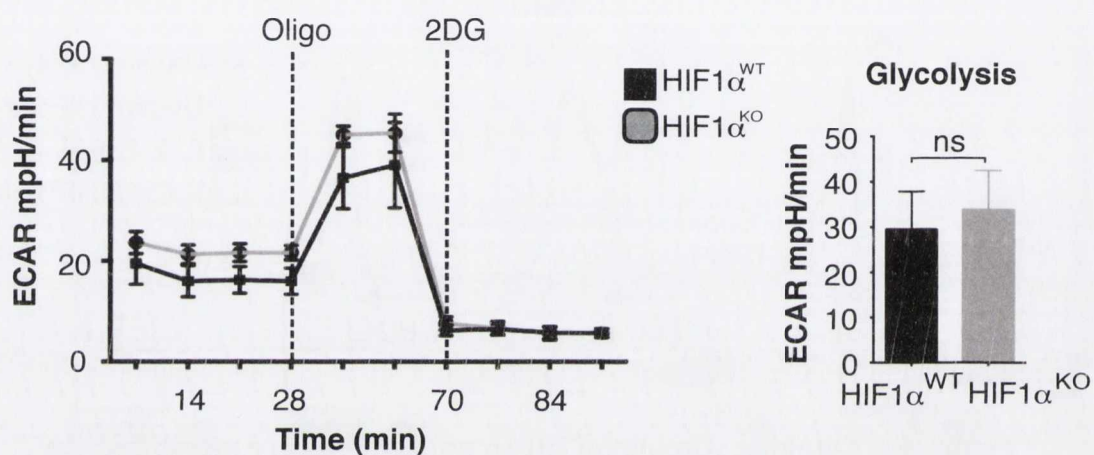


Figure 4.4. HIF1α is not required for cytokine-induced increases in NK cell glycolysis

MACS purified 6 day cultured NK cells from HIF1α^{KO} and HIF1α^{WT} mice were stimulated for 18hrs with IL-2/12 and the rate of glycolysis (ECAR) was assessed using a seahorse extracellular flux assay. Data are mean +/- SEM or are representative of four independent experiments. ns: non-significant

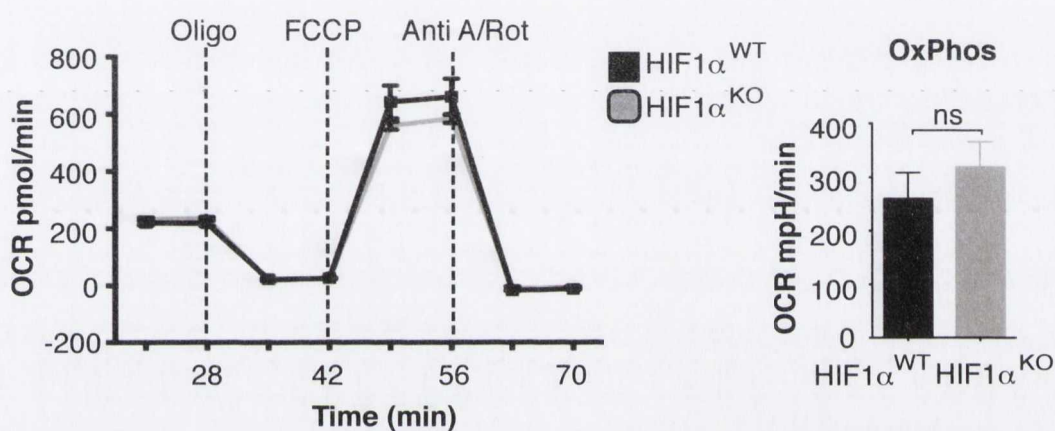


Figure 4.5. HIF1 α is not required for cytokine-induced increases in NK cell OxPhos

MACS purified 6 day cultured NK cells from HIF1 α^{KO} and HIF1 α^{WT} mice were stimulated for 18hrs with IL-2/12 and the rate of OxPhos (OCR) was assessed using a seahorse extracellular flux assay. Data are mean $\pm$ SEM or are representative of four independent experiments. ns: non-significant

The glycolytic capacity of HIF1 α ^{KO} NK cells was also the same as HIF1 α ^{WT} NK cells stimulated with IL-2/12 (Fig.4.6A). This argues HIF1 α is dispensable for cytokine stimulated NK cell metabolic reprogramming. Indeed, RT-PCR analysis confirmed IL-2/12 cytokine stimulation increased the expression of the glycolytic receptors and enzymes Glut1, Hex2 and Ldha to the same degree in HIF1 α ^{WT} NK cells and HIF1 α ^{KO} NK cells (Fig. 4.6B-D). Taken together, these experiments clearly show that HIF1 α is not required for NK cell metabolic reprogramming and HIF1 α ^{KO} NK cells are highly metabolically active following cytokine stimulation.

4.2 HIF1 α is dispensable for IL-2/12 induced NK cell effector function

Chapter 3 demonstrated that NK cell glucose metabolism is closely linked to NK cell effector function (Donnelly et al., 2014). The prediction was that HIF1 α ^{KO} NK cells would have normal effector function because HIF1 α is not required for NK cell metabolic responses. Cultured HIF1 α ^{KO} and HIF1 α ^{WT} NK cells were stimulated with IL-2/12 for 18 hours and the expression of NK cell effector molecules IFN γ and granzyme b were assessed by flow cytometry. As predicted, there was no difference in IFN γ production in HIF1 α ^{KO} NK cells compared to HIF1 α ^{WT} control cells. (Fig.4.7). Similarly, the expression of granzyme b in HIF1 α ^{KO} and HIF1 α ^{WT} NK cells was equivalent (Fig. 4.8). These data show that HIF1 α is not required for IL-2/12 induced NK cell metabolic or functional responses.

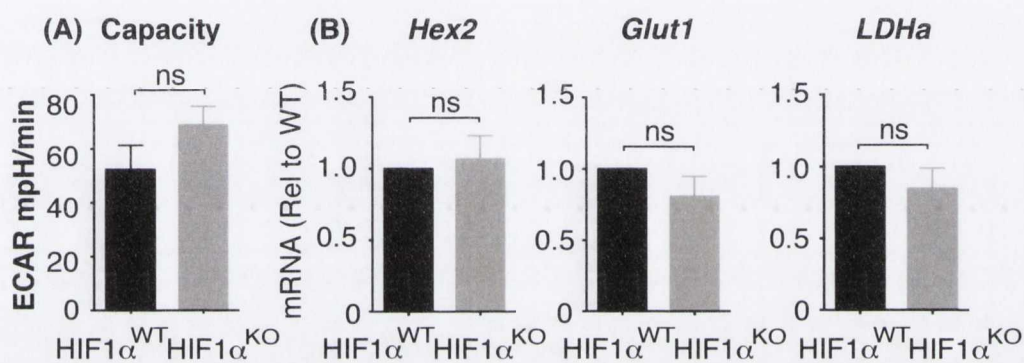


Figure 4.6. HIF1 α does not control NK cell metabolic reprogramming

MACS purified 6 day cultured NK cells from HIF1 α^{KO} and HIF1 α^{WT} mice were stimulated for 18hrs with IL-2/12 and either the glycolytic capacity was assessed using the seahorse extracellular flux analyser (A) or the mRNA expression of glycolytic genes *Hex2*, *Glut1* and *LDHa* were measured (B). Data are mean +/- SEM of four independent experiments. ns: non- significant

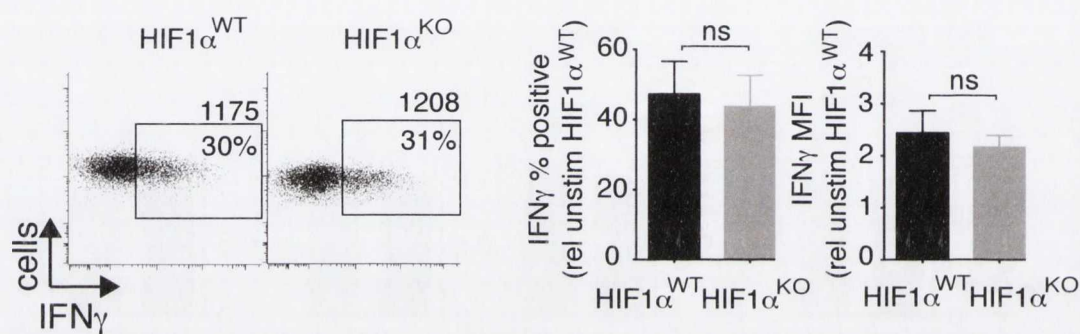


Figure 4.7. HIF1 α does not control the expression of IFN γ in cytokine stimulated NK cells

Cultured HIF1 α^{KO} and HIF1 α^{WT} NK cells were stimulated with IL-2/12 for 18hr and IFN γ expression was assessed by flow cytometry in NK1.1 $^{+}$ NKp46 $^{+}$ CD3 $^{-}$ cells. Data are mean $\pm$ SEM or are representative of five independent experiments. ns: non- significant



Figure 4.8. HIF1 α does not control the expression of granzyme B in cytokine stimulated NK cells

Cultured HIF1 α ^{KO} and HIF1 α ^{WT} NK cells were stimulated with IL-2/12 for 18hr and granzyme B expression was assessed by flow cytometry in NK1.1⁺ NKp46⁺ CD3⁻ cells. Data are mean $\pm$ SEM or are representative of five independent experiments. ns: non-significant

4.3 cMyc is required for IL-2/12 induced NK cell metabolic responses

Western blot analysis showed NK cells rapidly increase cMyc protein expression within 30 minutes following IL-2/12 cytokine stimulation (Fig. 4.9A). The early IL-2/12-mediated induction of cMyc expression was dependent on mTORC1 activity (Fig. 4.9A). However, over the course of 18 hours, rapamycin treated NK cells increased cMyc protein expression to a similar level as IL-2/12 activation alone (Fig. 4.9B). Elevated levels of cMyc protein were maintained over a 20 hour period in IL-2/12 stimulated NK cells (Fig. 4.10A). Furthermore, levels of *cMyc* mRNA transcripts were also increased following 20 hr cytokine stimulation (Fig. 4.10B). These data show cMYC expression is highly induced during NK cell activation.

To define the importance of cMyc in controlling NK cell metabolic responses, cMyc null NK cells were generated from cMyc *flox/flox* tamoxifen cre mice (cMyc^{KO}) (see 2.1.3). All control mice used in transgenic cMyc experiments were cMyc^{WT/WT} tamoxifen cre mice (cMyc^{WT}) and were administered tamoxifen to control for non-specific cre recombinases toxicity. mRNA and/or western blot analysis of IL-2/12 stimulated Myc^{WT} versus cMyc^{KO} NK cells was used to assess knockout efficiency in each experiment (Fig. 4.11A, B). A knockout efficiency of 80% or greater was consistently achieved.

Interestingly, NK cells from cMyc^{KO} mice remained small compared to Myc^{WT} cells following IL-2/12 stimulation (Fig. 4.12A). In addition, the expression of CD71, a known cMyc target gene, did not increase in cMyc^{KO} NK cells stimulated with IL-2/12 (Fig. 4.12B). cMyc^{KO} NK cells also displayed a marked decrease in the level of NBDG uptake following IL-2/12 stimulation, compared to cMyc^{WT} NK cells (Fig. 4.12C). For detailed metabolic analysis, a Seahorse extracellular flux assay was performed. Strikingly, the rate of glycolysis was significantly decreased in cMyc^{KO} NK cells compared to cMyc^{WT} following IL-2/12 stimulation (Fig. 4.13).

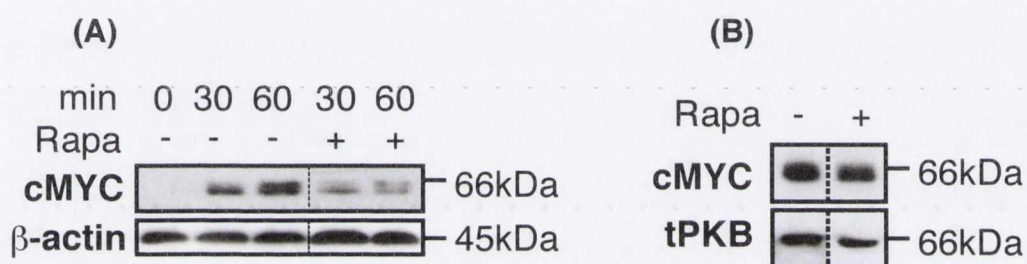


Figure 4.9. NK cells induce an early increase in cMyc expression that is mTORC1-dependent, but is mTORC1 independent over 18hours

Purified 6 day cultured NK cells were stimulated with IL-2/12 in the presence or absence of rapamycin (20nM) at the time points indicated (A) or for 18hours (B). Levels of cMyc, β-actin and total PKB protein were assessed by immunoblot analysis. Data are representative of three experiments.

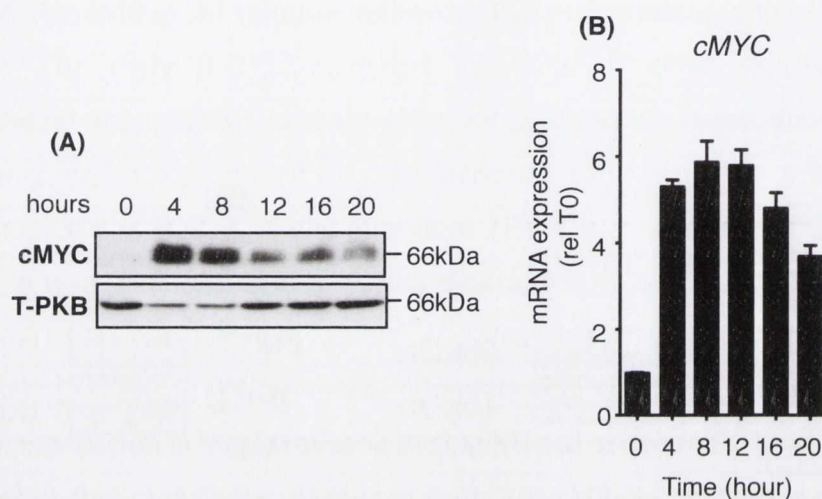


Figure 4.10. IL-2/12 stimulated NK cells maintain elevated levels of cMyc protein and mRNA expression

Purified 6 day cultured NK cells were left unstimulated or were stimulated for the time points indicated with IL-2/12 and levels of cMyc protein (A) and cMyc mRNA (B) were assessed by immunoblot analysis and RT-PCR respectively. Data are representative of three experiments.

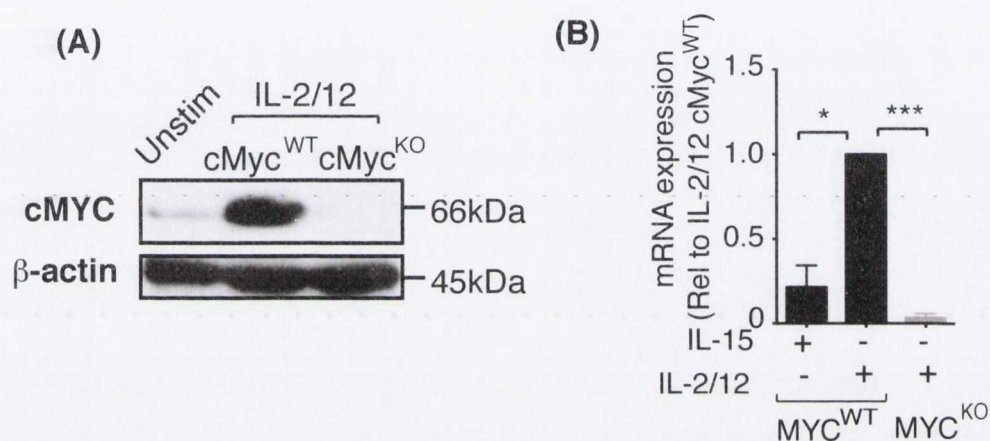


Figure 4.11. Inducible cMyc knockout mouse

Splenocytes isolated from cMyc^{WT} and cMyc^{KO} mice were cultured at 2×10^6 cell/ml in low dose IL-15 plus 4-OHT (0.6 μ M) for 6 days. NK cells were MACS purified and stimulated with IL-2/12 or left unstimulated for 18hrs before the expression of cMYC protein (A) and mRNA (B) was assessed. Data are representative of five experiments. * p < 0.05, *** p < 0.005

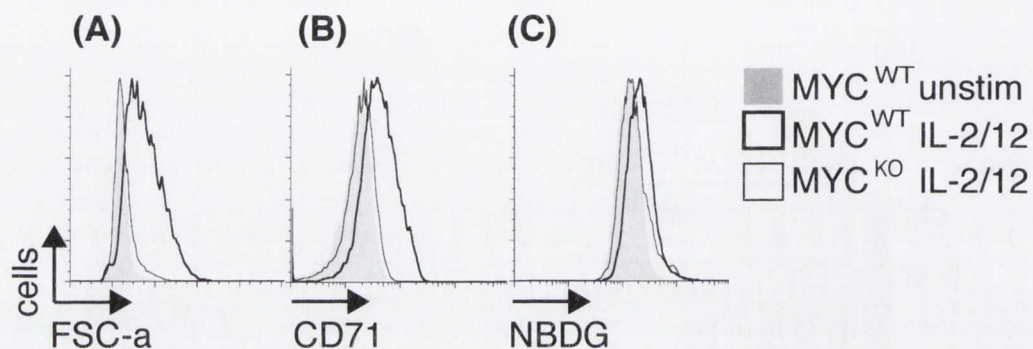


Figure 4.12. cMyc^{KO} NK cells display decreased size and metabolic marker expression following IL-2/12 stimulation

Cultured cMyc^{WT} and cMyc^{KO} NK cells were left unstimulated or were stimulated with IL-2/12 for 18hr and cell size (A), CD71 expression (B) and nutrient uptake (NBDG) (C) were measured by flow cytometry in NK1.1⁺ NKp46⁺ CD3⁻ cells (or NK1.1⁺ NKp46⁺ TCR β ⁻ cells for NBDG). Data are representative of five experiments.

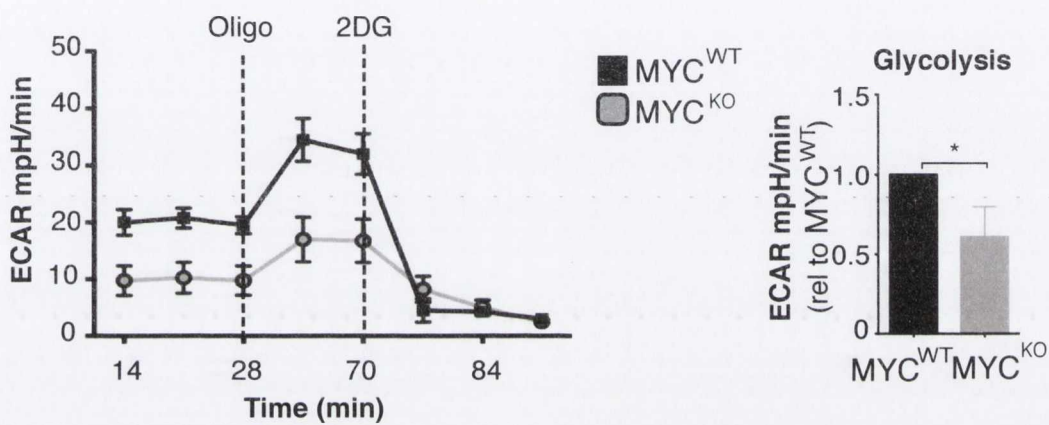


Figure 4.13. cMyc^{KO} NK cells display decreased levels of glycolysis compared to cMyc^{WT} NK cells following cytokine stimulation

MACS purified 6 day cultured NK cells from cMyc^{WT} and cMyc^{KO} mice were stimulated for 18hrs with IL-2/12. The rate of glycolysis was measured using the seahorse extracellular flux analyser. Data are mean +/- SEM or are representative of four independent experiments. * p 0.05

Furthermore, the glycolytic capacity of cMyc^{KO} NK cells was significantly reduced (Fig. 4.14A). To investigate whether the decreased glycolytic capacity observed was due to a failure in metabolic reprogramming in the cMyc^{KO}, the mRNA expression of important glycolysis-associated genes were measured. The expression of LDHa and PKM2 in cytokine stimulated cMyc^{KO} NK cells were decreased compared to cMyc^{WT} NK cells (Fig. 4.14B). These data implicate cMyc as the key transcription factor, which controls cytokine-induced NK cell metabolic reprogramming.

The rate of NK cell OxPhos was compared between cMyc^{WT} and cMyc^{KO} NK cells. Interestingly, the rate of OxPhos, the spare respiratory capacity and the maximal respiratory capacity were decreased in IL-2/12 stimulated cMyc^{KO} NK cells when compared to cMyc^{WT} NK cells (Fig. 4.15A-D). It is currently unknown whether the mitochondrial mass of NK cells changes during cytokine activation. However cMyc has been directly linked to mitochondrial biogenesis, through its regulation of the expression of mitochondrial associated genes such as TFAM (Li et al., 2005). To investigate whether the decreased rate of cMyc^{KO} OxPhos was associated with reduced mitochondrial mass, mitochondria of cMyc^{WT} and cMyc^{KO} NK cells were stained using mitotracker red CMX Ros. There was an increase in mitochondrial mass in MYC^{WT} NK cells following IL-2/12 cytokine stimulation (Fig. 4.16). In IL-2/12 stimulated cMyc^{KO} NK cells, there was a significant decrease in mitochondrial mass compared to cMyc^{WT} NK cells (Fig. 4.16).

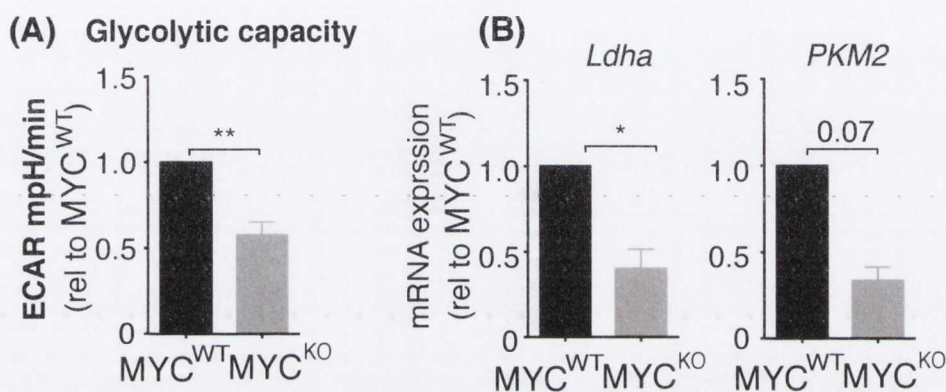


Figure 4.14. MYC^{KO} NK cells have reduced expression of glycolytic genes following IL-2/12 stimulation

MACS purified 6 day cultured NK cells from cMyc^{WT} and cMyc^{KO} mice were stimulated for 18hrs with IL-2/12. The glycolytic capacity was measured using the seahorse extracellular flux analyser (A) or cells were analyzed for the expression of LDHa and PKM2 mRNA. Data are mean +/- SEM of four (A) or three (B) experiments. * $p < 0.05$, ** $p < 0.01$

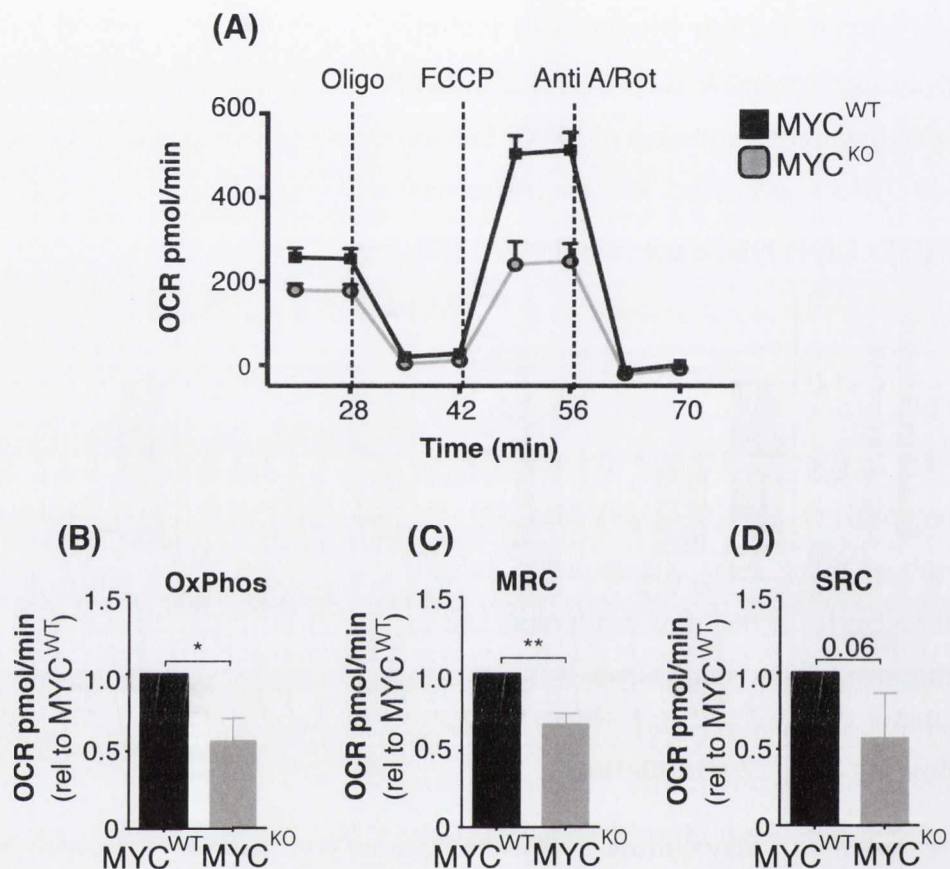


Figure 4.15. cMyc^{KO} NK cells display decreased rates of OxPhos following cytokine stimulation

MACS purified 6 day cultured NK cells from cMyc^{WT} and cMyc^{KO} mice were stimulated for 18hrs with IL-2/12. The rate of OxPhos (B), the maximal respiratory capacity (OCR following addition of FCCP) (C) and the spare respiratory capacity (D) were measured using the seahorse extracellular flux analyser. Data are mean +/- SEM or are representative of four independent experiments. * $p < 0.05$, ** $p < 0.01$

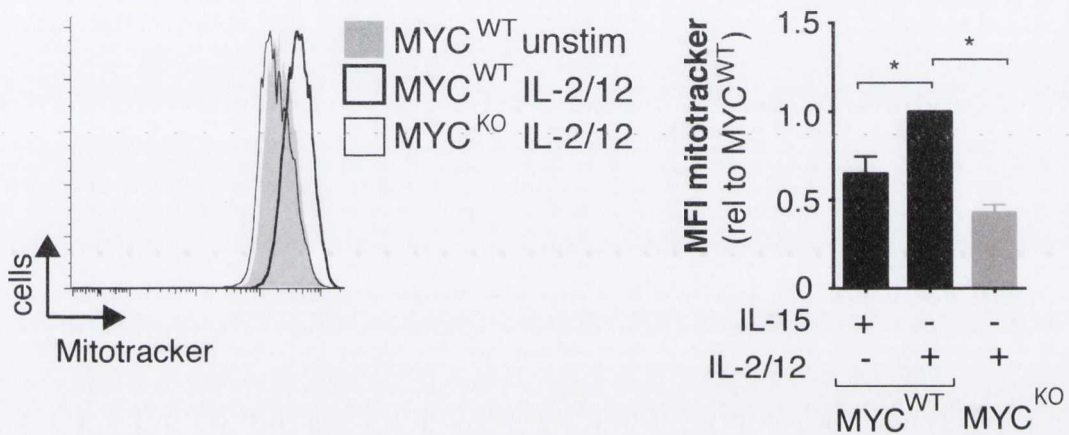


Figure 4.16. cMyc controls NK cell mitochondrial biogenesis

Cultured NK cells from cMyc^{WT} and cMyc^{KO} mice were left unstimulated or stimulated with IL-2/12 for 18hrs. NK1.1+ NKp46+ CD3- cells were analyzed by flow cytometry for changes in mitochondrial mass. Data are mean +/- SEM or are representative of three experiments. * p < 0.05

4.4 cMyc is required for IL-2/12-induce NK cell effector functions

The data thus far highlight the importance of cMyc for cytokine-induced NK cell metabolic responses. As previously mentioned, there is an integral link between NK cell metabolism and NK cell effector functions. Given the metabolic defect observed in cMyc^{KO} NK cells, we hypothesised this would negatively impact on the effector function of cytokine-activated cMyc^{KO} NK cells. Indeed, the expression of the pro-inflammatory cytokine IFN γ (Fig. 4.17A-C) and effector molecule granzyme b (Fig. 4.18A, B) were significantly reduced in cMyc^{KO} NK cells compared to MYC^{WT} NK cells.

These data identify a key role for cMyc in controlling cytokine induced NK cell metabolic and functional responses.

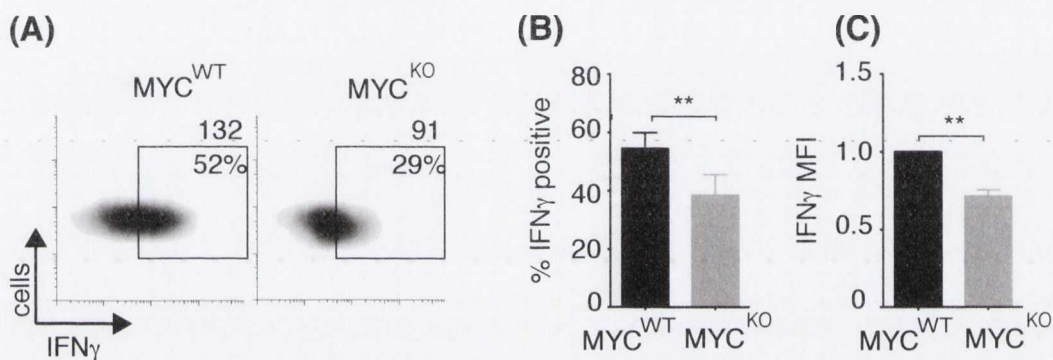


Figure 4.17. IL-2/12 stimulated cMyc^{KO} NK cells display decreased IFN γ expression

Cultured NK cells from cMyc^{WT} and cMyc^{KO} mice were stimulated with IL-2/12 for 18hrs and NK1.1+ NKp46+ CD3- cells were analyzed by flow cytometry for IFN γ production (A,-C). Data are mean $\pm$ SEM or are representative of five experiments. ** $p < 0.01$.

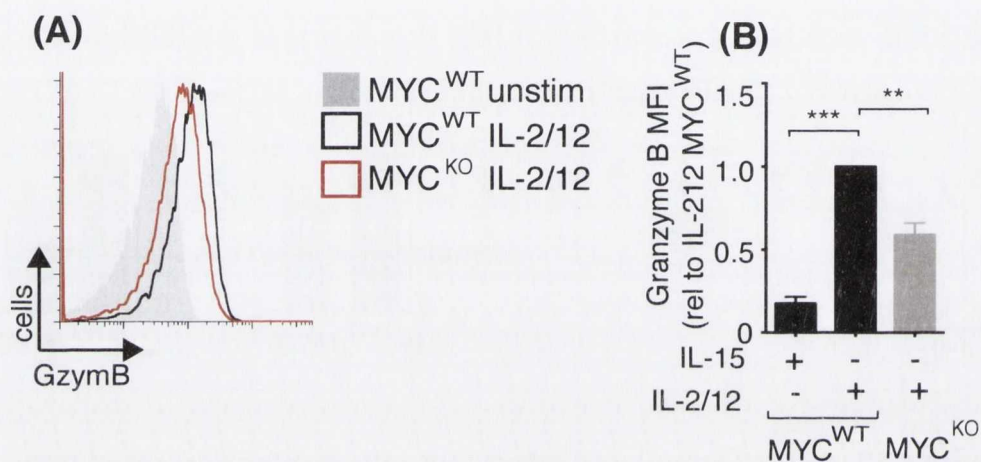


Figure 4.18 cMyc is required for NK cell granzyme b production

Cultured NK cells from cMyc^{WT} and cMyc^{KO} mice were stimulated with IL-2/12 for 18hrs and NK1.1+ NKp46+ CD3- cells were analyzed by flow cytometry for granzyme B production (A, B). Data are mean +/- SEM or are representative of five experiments. **p < 0.01, ***p < 0.005.

Chapter 4 discussion

The early findings from this study underpin a fundamental role for mTORC1 in controlling NK cell activation-induced metabolic and functional responses. However, the transcriptional regulators that control NK cell metabolic reprogramming remained unknown. Two of the most important transcription factors that control immune cell metabolism were investigated in cytokine activated NK cells; HIF1 α and cMyc.

HIF1 α is an important transcription factor that sustains activation-induced increases in T cell metabolism by enhancing the expression of key glycolysis-associated genes (Finlay et al., 2012a, Cramer et al., 2003). HIF1 α is highly expressed in Th1, Th17 and CD8⁺ T cells, and enhances the expression of Glut1 (glucose transporter 1), Hex2 (Hexokinase 2), Ldha (Lactate dehydrogenase) and PKM (pyruvate kinase muscle) (Shi et al., 2011, Finlay et al., 2012a). In this respect, HIF1 α enhances the glycolytic capacity of activated T cells, facilitating the metabolic shift required for the acquisition of T cell effector functions. Interestingly, T cells which do not favour this glycolytic shift, such as Tregs, maintain a low level of HIF1 α expression upon activation (Shi et al., 2011). HIF1 α control of immune cell metabolism is not limited to cells of the adaptive immune system. HIF1 α expression is also enhanced in activated M1 macrophages and neutrophils, and is required for the metabolic shift observed in these innate effector immune cells (Cramer et al., 2003). In the absence of HIF1 α , M1 macrophages, monocytes and neutrophils display defective glucose metabolism and fail to achieve highly glycolytic metabolic profiles upon activation (Cheng et al., 2014, Cramer et al., 2003).

The expression of HIF1 α was increased in cytokine activated NK cells in an mTORC1-dependent manner. However, in contrast to the indispensable role of HIF1 α in controlling the metabolic adaptations of the immune cells mentioned above, HIF1 α was not required for cytokine-induced increases in

NK cell metabolism. Deletion of HIF1 α using a HIF1 $\alpha^{\text{lox/lox}}$ vav-cre mouse model obtained from the University of Dundee did not impair NK cell blastogenesis following cytokine stimulation. Increased cell size often correlates with increased rates of cellular metabolism (Fox et al., 2005). Indeed, detailed metabolic analysis using the seahorse extracellular flux analyser revealed HIF1 α^{KO} NK cells had comparable rates of glycolysis and OxPhos to HIF1 α^{WT} NK cells following cytokine stimulation. These data reflect what is currently known regarding the dispensable role of HIF1 α in the early metabolic reprogramming events of T cells (Wang et al., 2011b). In this study, HIF1 α null T cells displayed comparable increases in their rate of glycolysis, glutaminolysis and FAO to wild type T cells 24hours after TCR activation (Wang et al., 2011b).

In accordance with our earlier finding that cellular metabolism is integrally linked to NK cell effector function, it was unsurprising that HIF1 α^{KO} NK cells displayed normal effector functions, due to their ability to upregulate glycolysis upon activation. IFN γ and granzyme b expression in cytokine stimulated HIF1 α^{KO} NK cells were comparable to HIF1 α^{WT} NK cells. In contrast, the importance of HIF1 α for CTL, macrophage and neutrophil glucose metabolism resulted in impaired effector functions of these cells in the absence of HIF1 α activity (Cramer et al., 2003, Finlay et al., 2012a, Shi et al., 2011). Early HIF1 α deletion in CD4 $^{+}$ T cells skews the dichotomy of Treg and Th17 differentiation. In the absence of HIF1 α , CD4 $^{+}$ T cells preferentially differentiate into Tregs, which do not rely on HIF1 α -induced increased glucose metabolism, at the expense of Th17 effector cell differentiation (Shi et al., 2011). CD8 $^{+}$ T cells lacking HIF display defects in maturation and mobility (Finlay et al., 2012b). Furthermore, deletion of von Hippel-Lindau (VHL), a negative regulator of HIF activity, results in CD8 $^{+}$ T cells with increased granzyme B and TNF α production in response to LCMV infection (Doedens et al., 2013).

HIF is a heterodimeric transcription factor consisting of an oxygen-regulated alpha subunit and a constitutively expressed beta subunit known as 'Aryl hydrocarbon Receptor Nuclear Translocator' (ARNT). There are three isoforms of the HIF alpha subunit; HIF1 α , HIF2 α and HIF3 α . All isoforms are similar in their binding sites and function (Pawlus and Hu, 2013). Interestingly, several reports have observed that deletion of one HIF subunit provokes a compensatory increase of the other (Carroll and Ashcroft, 2006). HIF2 α knockdown in HepG2 cells, a liver carcinoma cell line, displayed a compensatory increase in HIF1 α expression, which enhanced cell survival (Schulz et al., 2012). Future experiments exploring the role of the HIF2 α subunit may provide insight into any contribution of HIF isoform compensation.

cMyc is an important transcription factor that controls the metabolic response of T cells upon activation (Chou et al., 2014, Wang et al., 2011b). Interestingly, we found that cMyc expression was low in unstimulated NK cells, but could be rapidly and robustly induced following cytokine stimulation (30minutes). The early induction of cMyc in cytokine activated NK cells was mTORC1 dependent, however at later time points (18 hours post stimulation) rapamycin treatment had no effect on the levels of cMyc protein. Indeed, mTORC1-independent cMyc expression has been described in CD8 $^{+}$ T lymphocytes, both in response to TCR-stimulation and in IL2 maintained cytotoxic T cells (Sinclair et al., 2013, Finlay et al., 2012a). In another study, in a combination of CD4 $^{+}$ and CD8 $^{+}$ TCR activated T cells, there was a partial reduction in cMyc expression following rapamycin treatment (Wang et al., 2011b). Interestingly, the switch in mTORC1-dependence, from early cMyc induction being dependent on mTORC1 to late stability being independent, corresponds with NK cell metabolic activity and reprogramming. The prediction is that cMyc expression during the initial activation of NK cells may be dependent on mTORC1 mediated translation whereas at later time points other factors such as amino acid levels dictate

cMyc levels (Figure 6.2). Indeed, amino acid availability has been shown to control cMyc expression in IL2 maintained CD8+ cytotoxic T cells, where inhibition of mTORC1 has no effect (Sinclair et al., 2013, Finlay et al., 2012a).

T cells undergo a dramatic shift in cellular metabolism upon activation, significantly upregulating glycolytic and glutaminolytic metabolic pathways (Wang et al., 2011b). cMyc is indispensable for this process; in the absence of cMyc, T cell metabolic reprogramming is profoundly impaired (Wang et al., 2011b, Sinclair et al., 2013). *Ldha* and *PKM2* are among the key glycolytic genes that are decreased in cMyc null T cells following TCR activation (Wang et al., 2011b). Interestingly, using a conditional knockout mouse line, we found that cMyc was indispensable for cytokine-induced increases in NK cell size; cytokine activated NK cells from cMyc^{KO} were significantly smaller than those from cMyc^{WT} mice. This defect in NK cell blastogenesis correlated with an impaired metabolic response. cMyc^{KO} NK cells displayed reduced rates of glycolysis and OxPhos following cytokine stimulation compared to cMyc^{WT} NK cells. In line with what is known regarding cMyc-dependent metabolic reprogramming in T cells (Wang et al., 2011b), we also found cMyc activity was required for NK cell metabolic reprogramming. The expression of key glycolytic genes *PKM2* and *Ldha* were reduced in cMyc^{KO} NK cells following cytokine stimulation. These data implicate cMyc as an indispensable metabolic regulator in cytokine activated NK cells. Interestingly, we also found that NK cell mitochondrial mass was reduced in cytokine stimulated cMyc^{KO} NK cells. A strong association between cMyc and mitochondrial biogenesis exists in the literature (Li et al., 2005, Morrish and Hockenbery, 2014). cMyc has been shown to enhance the expression of many mitochondrial associated genes such as cytochrome c (complex IV of the ETC), *CLIC4* (a mitochondrial chloride channel) and *TFAM* (mtDNA transcription factor) (Morrish et al., 2003, Shiao et al., 2006, Li et al., 2005). Future experiments comparing the transcriptome of cMyc^{WT} and cMyc^{KO} NK

cells would reveal the key cMyc-induced genes that control NK cell mitochondrial mass.

These findings highlight the importance of cMyc for the metabolic response of cytokine stimulated NK cells. Chapter 3 identified an integral link between NK cell metabolism and the acquisition of NK cell effector functions (Donnelly et al., 2014). Due to the importance of cMyc in controlling NK cell metabolism, it was unsurprising that cMyc^{KO} NK cells displayed reduced IFN γ and granzyme B expression. Indeed, these data correspond with previous findings in Teff cells, where a loss of cMyc led to decreased effector molecule production (Wang et al., 2011b, Sinclair et al., 2013, Preston et al., 2015). What's particularly interesting is that the amount of cMyc a cell has, was recently shown to determine the cells functional fate. When a T cell interacts with an antigen presenting cell (APC), its cellular contents do not divide evenly between the two daughter progenitor cells (Verbist et al., 2016, Pollizzi et al., 2016). The expression of cMyc not only differs, but determines the fate of the daughter cells; where high levels of cMyc induce effector T cell differentiation and low levels of cMyc correlate with long-lived memory T differentiation (Verbist et al., 2016, Pollizzi et al., 2016). Hence, cMyc is a crucial determining factor which controls lymphocyte functional fate.

In conclusion, the data presented in chapter 4 highlight the importance of cMyc, but not HIF1 α , in cytokine activated NK cell responses. Cytokine stimulated cMyc^{KO} NK cells displayed significantly reduced rates of glycolysis and OxPhos, which correlated with impaired metabolic reprogramming. These findings reflect what is known regarding the transcriptional control of T cell metabolism following TCR activation (Wang et al., 2011b). In addition, because NK cell metabolism is integrally linked to NK cell effector function, we also show cMyc activity is indispensable for the functional responses of cytokine activated NK cells. These data further our knowledge and understanding regarding the metabolic regulation of NK cells and highlight the integral link between NK cell metabolism and NK cell effector function.

Chapter 5

**Amino acids are indispensable for
cytokine-induced Natural Killer cell
metabolic and functional responses**

Introduction

The data thus far outline the metabolic alterations that occur following NK cell activation, and shows there is an integral link between NK cell glucose metabolism and NK cell effector functions. In chapter 3, we identified IL-2 as the key cytokine that drives NK cell metabolic responses, due to its role in enhancing the activity of the serine threonine kinase mTORC1. In chapter 4, the transcription factor cMyc, but not HIF1 α , was implicated in controlling cytokine-induced NK cell metabolic reprogramming events. However, the data reveal a complex relationship between mTORC1 and cMyc. While mTORC1 activity is required for maximal cMyc expression following initial cytokine stimulation, it is not required for maintaining cMyc protein levels at later timepoints, once NK cells have undergone metabolic reprogramming. Chapter 5 will investigate the molecular mechanisms linking IL-2 to these two NK cell metabolic regulators; mTORC1 and cMyc.

IL-2 is a strong activator of the PI3K/Akt signaling pathway, which controls many cellular processes including proliferation, immune cell activation, cellular differentiation and cell survival (Otten and Germain, 1991, Rolf et al., 2013). The PI3K/Akt signaling pathway has an important role in regulating the metabolic adaptations of immune cells. PI3K/Akt signaling enhances the cell surface expression of Glut1 by inhibiting its internalization (Wieman et al., 2007). Consequently, inhibition of Akt in T cells led to decreased rates of glucose uptake, whereas overexpression of Akt increased T cell glucose uptake (Jacobs et al., 2008). In line with the role of cellular metabolism in controlling immune cell function, inhibition of PI3K/Akt signaling in T cells led to decreased IFN γ production (Jacobs et al., 2008). The PI3K/Akt signaling axis controls mTORC1 activity in many cell types (Dibble and Cantley, 2015). However, a PI3K/Akt independent mTORC1-signaling axis has recently been described in CD8 $^{+}$ T cells (Finlay et al., 2012a).

PI3K activity has also been shown to control the rate of cMyc transcription in B cells through a $\text{Nf}\kappa\beta$ -dependent signaling axis (Grumont et al., 2002). However, whether PI3K/Akt signaling is required for mTORC1 activity or cMyc expression in cytokine activated NK cells remained unknown.

IL-2 enhances Jak/STAT signaling, which has been linked to mTORC1 activity and cMyc expression in T cells (Preston et al., 2015). IL-2 stimulated CTL display elevated levels of cMyc protein expression, which are lost upon inhibition of JAK kinase activity (Preston et al., 2015). Following IL-2 stimulation, the amino acid transporter Slc7a5 is highly induced on CD8⁺ T cells (Sinclair et al., 2013). Importantly, Slc7a5 activity is fundamental to a successful T cell response, due to its role in regulating both cMyc expression and mTORC1 activity (Sinclair et al., 2013). Importantly, despite the high induction of many other amino acid transporters, loss of Slc7a5 alone profoundly impaired T cell activation; Slc7a5null T cells were smaller in size, had reduced rates of proliferation and decreased rates of glycolysis due to impaired metabolic reprogramming following TCR activation (Sinclair et al., 2013). Our data show that in response to IL-2, cMyc expression and mTORC1 activity control the metabolic and functional response of NK cells. Whether amino acids, or the amino acid transporter Slc7a5, are required for IL-2 induced NK cell signal transduction remains to be determined.

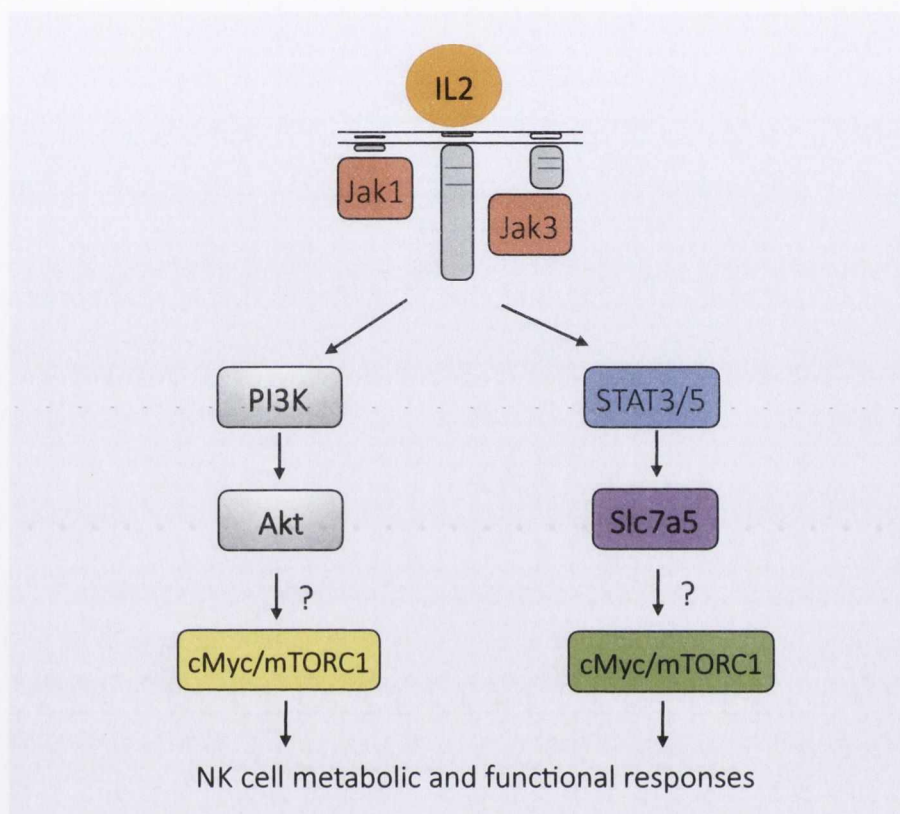


Figure 5. IL-2 signaling drives NK cell metabolic and functional responses

IL-2 enhances the JAK/STAT signaling pathway, which is important for PI3K/Akt activity and Slc7a5 expression in other immune cell types. In NK cells, IL-2 drives the metabolic shift that permits enhanced effector functions through the control of mTORC1 activity and cMyc expression. However, it is not yet known whether IL-2 driven PI3K/Akt signaling, or amino acid availability and Slc7a5 expression, are important for expression or activity of the NK cell metabolic regulators cMyc or mTORC1.

5.1 PI3K/Akt are not required for IL-2/12-induced cMyc expression

The data thus far identify a key role for the transcription factor cMyc in coordinating NK cell metabolic and functional responses. The Akt signaling pathway is one of the major pathways activated by IL-2, and has been described to regulate cMyc protein expression (Grumont et al., 2002). Therefore, we considered whether Akt signaling is important for NK cell responses. NK cells were stimulated with IL-2/12 for 18 hours in the presence or absence of an Akt inhibitor (Akti, 1 μ M) (Fig. 5.1A). Interestingly, NK cell size and CD71 expression were comparable in the presence or absence of Akti, arguing Akt signal transduction is not required for NK cell metabolic responses (Fig. 5.1B).

The fact that the expression of CD71, a known cMyc target gene, was unaffected by Akti suggested cMyc protein expression is not controlled by Akt signalling. Indeed, cMyc protein expression was not affected following the addition of Akti for 1 hour to 18 hour cytokine activated NK cells (Fig. 5.2A). It was also observed that mTORC1 activity in IL-2/12 activated NK cells is independent of Akt signalling; phosphorylation of the mTORC1 substrate p70-S6-kinase (S6K) or the downstream S6K substrate S6-ribosomal protein (S6) were unaffected by Akti, but were reduced by the mTORC1 inhibitor rapamycin (Fig. 5.2B). These data show that Akt signalling is dispensable for NK cell cMyc expression and mTORC1 activity.

The importance of Akt signalling for NK cell functional responses were next investigated. Considering cMyc expression remained unchanged in the presence of these inhibitors, it was hypothesised there would be no functional defects in Akti treated NK cells. Indeed, the effector functions of 18hr IL-2/12 activated NK cells in the presence of Akti remained unchanged; IFN γ (Fig. 5.3A) and granzyme B (Fig. 5.3B) expression were equivalent in the presence or absence of the Akt inhibitor. Therefore, the data clearly demonstrate that Akt is not required for cMyc-dependent NK cell metabolic and functional responses.

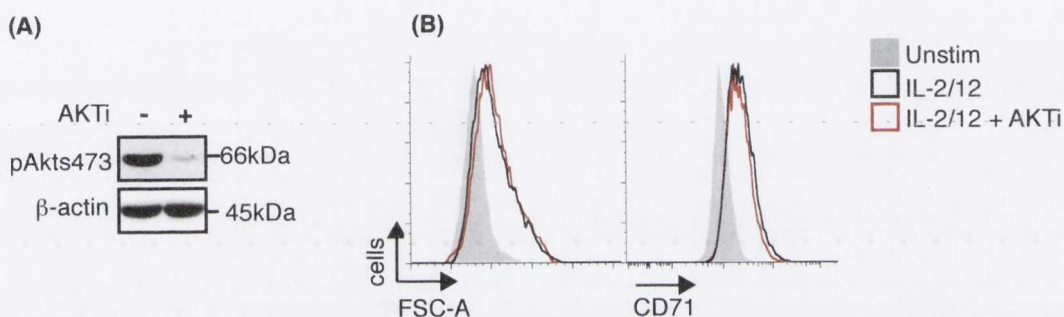


Figure 5.1. Akt is not required for IL-2/12 induced increases in NK cell size or CD71 expression

MACS purified 6 day cultured NK cells were activated with IL-2/12 for 18hours before the addition of Akt1/2i (1uM) for 1hour. Cells were lysed for protein at 10^6 cell/ml and analysed by immunoblot for levels of pAktS473 (A). β -actin was used as a loading control (A). Cultured NK cells were left unstimulated or activated with IL-2/12, with or without Akt1/2i (1uM) for 18hrs and were analyzed by flow cytometry for cell size (FSC) or CD71 expression in NK1.1⁺ NKp46⁺ CD3⁻ cells (B). Data are representative of five experiments.

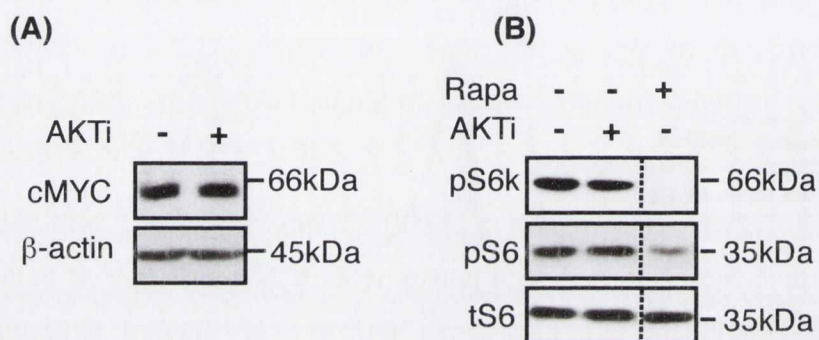


Figure 5.2. Akt is not required for IL-2/12 induced cMyc expression or mTORC1 activity

MACS purified 6 day cultured NK cells were activated with IL-2/12 for 18hours before the addition of Akt1/2i (1uM) or rapamycin (20nM) for 1hour. Cells were lysed for protein at 10^6 cell/ml and analysed by immunoblot for levels of cMyc (A) or pS6K_{t389}, pS6_{s235/236} (B). β -actin or total S6 were used as loading controls. Data is representative of three independent experiments.

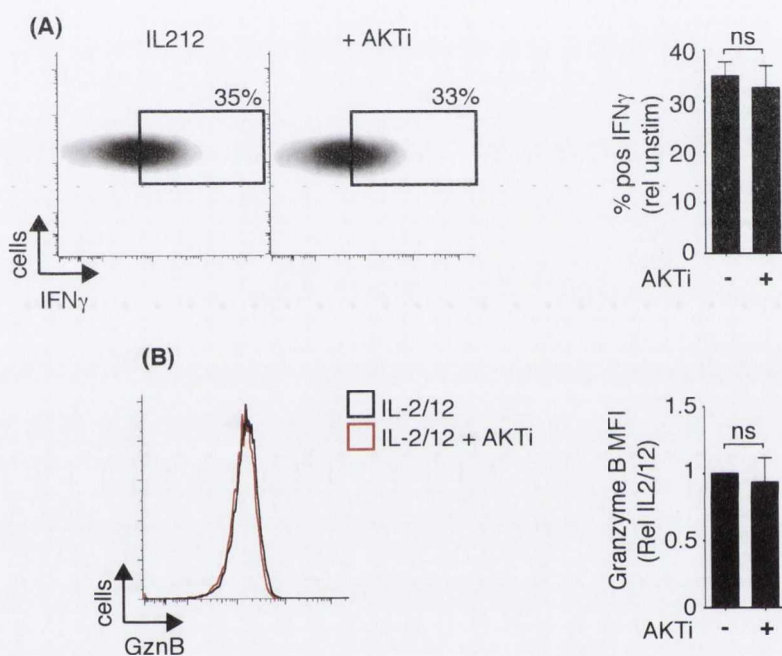


Figure 5.3. Akt is not required for NK cell interferon- γ production or granzyme B expression

Cultured NK cells were left unstimulated or activated with IL-2/12, with or without Akt1/2i (1uM) for 18hrs. IFN γ (A) and granzyme n (B) expression were measured in NK1.1 $^+$ NKp46 $^+$ CD3 $^-$ NK cells. Data are mean $\pm$ SEM or are representative of five experiments.

5.2 Amino acid transport through Slc7a5 controls cMyc, glycolysis and OxPhos in NK cells

Having demonstrated that Akt signalling is not important for NK cell metabolic or functional responses, we next considered other mechanisms that might link IL-2/12 stimulation to cMyc expression and therefore, NK cell metabolism. Both mTORC1 and cMyc have been described to be sensitive to amino acid levels (Averous et al., 2014, Sinclair et al., 2013). Therefore, we hypothesised that the regulation of amino acid transport in IL-2/12 stimulated NK cells might be important in the control of NK cell responses.

An inspection of the data available on Immgen showed Slc7a5 to be dramatically upregulated in NK cells 1day post MCMV infection (Fig. 5.4A). Slc7a5, also called LAT1, is a member of the system L amino acid transporter that transports large neutral amino acids into the cell. Slc7a5 has been shown to be important for metabolic and functional responses of CD8⁺ T cells (Sinclair et al., 2013). It was confirmed that Slc7a5 expression was induced in NK cells following IL-2/12 stimulation through RT-PCR analysis (Fig. 5.4B). Interestingly, inhibition of Slc7a5 activity in IL-2/12 stimulated NK cells using 2-Amino-2-norbornanecarboxylic acid (BCH), a specific inhibitor of the system L amino acid transporter, resulted in a significant decrease in NK cell size and CD71 expression, suggesting cMyc signalling may be decreased (Fig. 5.5). Strikingly, the addition of BCH (25mM) to IL-2/12 activated NK cells resulted in a rapid loss of cMyc expression within 30 minutes (Fig. 5.6A). In addition, a significant decrease in mTORC1 signalling was also observed; the addition of BCH substantially decreased pS6 levels within 30 minutes (Fig. 5.6B). To confirm the importance of Slc7a5-mediated amino acid transport for cMyc expression, NK cells were cultured from the spleens of Slc7a5^{flox/flox} Vav-Cre (Slc7a5^{KO}) and Slc7a5^{flox/flox} (Slc7a5^{WT}) mice. Indeed, cMyc protein expression was also markedly decreased in IL-2/12 stimulated NK cells lacking the expression of Slc7a5 (Fig.5.6C).

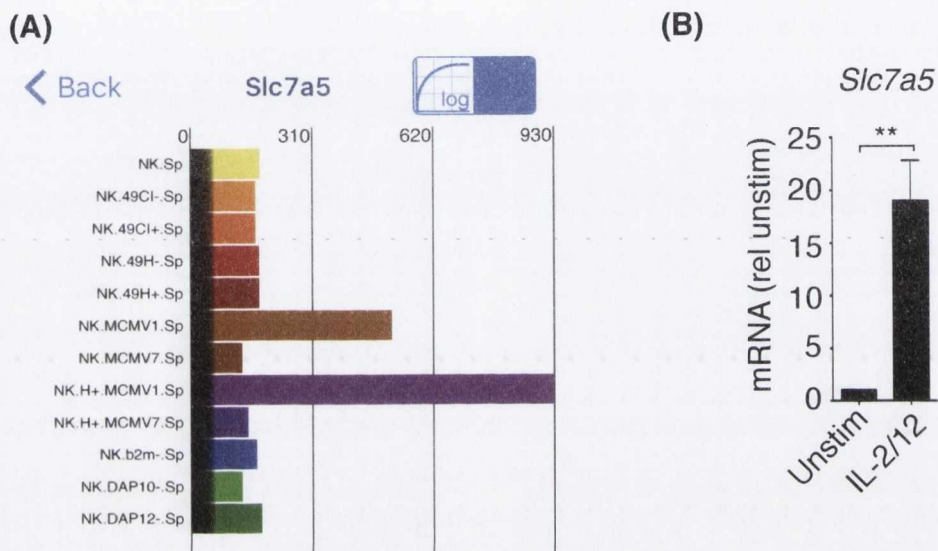


Figure 5.4. NK cell Slc7a5 expression increases in cytokine activated NK cells

Immgen data showing Slc7a5 to be dramatically upregulated in NK cells 1day post MCMV infection (A). MACS purified, cultured NK cells were left unstimulated or were activated with IL-2/12 and analyzed for the expression of Slc7a5 mRNA (B). Data are mean +/- SEM of five experiments. ** p<0.01

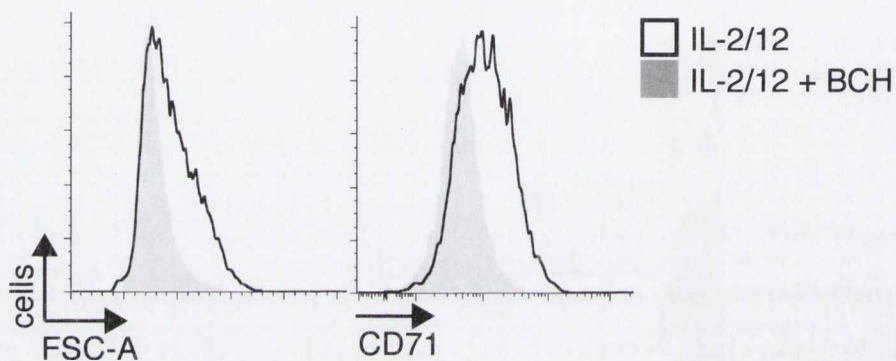


Figure 5.5. Inhibition of Slc7a5 reduces activation-induced increases in NK cell size and CD71 expression

Cultured NK cells activated with IL-2/12 in the presence or absence of BCH (25mM) for 18hrs were analyzed by flow cytometry for cell size (FSC) or CD71 expression in NK1.1⁺ NKp46⁺ CD3⁻ NK cells. Data are representative of five experiments.

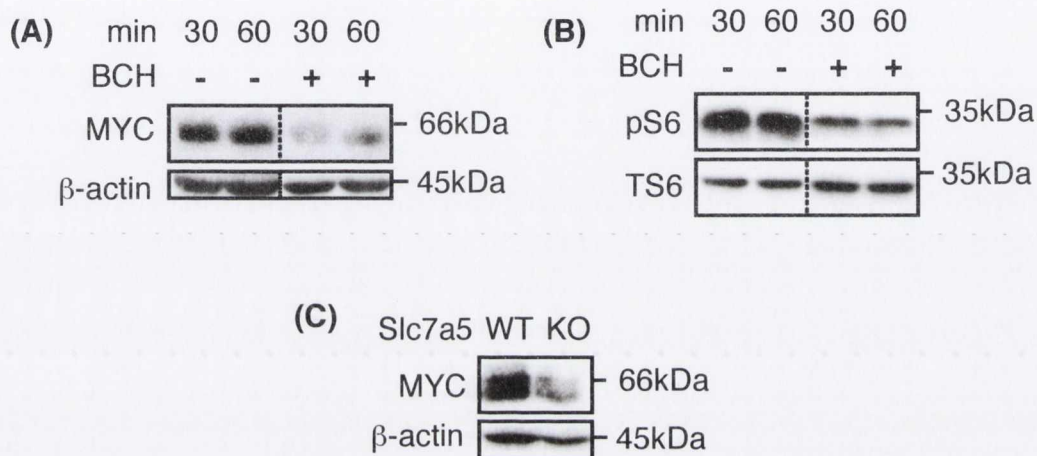


Figure 5.6. Slc7a5 controls cytokine-induced cMyc expression and mTORC1 signaling in IL-2/12 activated NK cells

MACS purified cultured NK cells were activated with IL-2/12 for 18hours before the addition of BCH for the time points indicated. cMyc expression (A) and levels of pS6_{s235/236} (B) were assessed. β-actin and total S6 were used as a loading controls, respectively. MACS purified 6 day cultured NK cells from Slc7a5 *flox/flox* vav cre positive (Slc7a5^{KO}) and cre negative (Slc7a5^{WT}) mice were activated with IL-2/12. cMyc expression was measured by immunoblot (C). β-actin was used as a loading control. Data is representative of two (C) or three (A, B) experiments.

Slc7a5 is important for the transport of multiple amino acids into the cell including leucine, methionine, histidine, phenylalanine, tryptophan and tyrosine (Nicklin et al., 2009). The transport of leucine through Slc7a5 has been shown to be important in the control of mTORC1 activity in CD8⁺ T cells. Indeed, NK cells activated in media lacking leucine lacked mTORC1 activity but, interestingly, these cells have normal expression of cMyc (Fig. 5.7.). This data further confirms the observations in chapter 4 that cMyc expression is not dependent on mTORC1 signalling in 18hour IL2/12 stimulated NK cells (Fig. 4.9A).

Slc7a5 is an obligate anti-porter, meaning it must transport an amino acid out of the cell to import other amino acids into the cell. Slc7a5 has a very low K_m for glutamine export (Nicklin et al., 2009) and so it is usually glutamine that facilitates amino acid import through Slc7a5. Given that glutamine is important for amino acid import through Slc7a5, we predicted that glutamine withdrawal would similarly affect cMyc expression in IL-2/12 stimulated NK cells as BCH treatment. Indeed, glutamine withdrawal resulted in a rapid decrease in cMyc expression within 30 minutes (Fig.5.8A). In addition, glutamine deprived NK cells displayed reduced mTORC1 signalling (Fig.5.8B). While this data argues that glutamine is required to sustain mTORC1 signalling and cMyc protein expression in IL2/12 activated NK cells, it should be noted that glutamine has also be linked to the regulation of cMyc levels because it fuels the hexosamine pathway. This pathway generates the substrate for O-GlcNAcylation reactions and cMyc is O-GlcNAcylated in a glutamine dependent manner leading to decreased cMyc degradation (Swamy et al., 2016). Therefore, we investigated whether the rapid loss (within 30 minutes) of cMyc protein expression observed following glutamine withdrawal could be due to reduced cellular O-GlcNAcylation. When 18 hour IL2/12 stimulated NK cells were switched into glutamine free media there was a global decrease in cellular O-GlcNAcylation after 24 hours but no observable decrease after 5 hours (Fig.5.9).

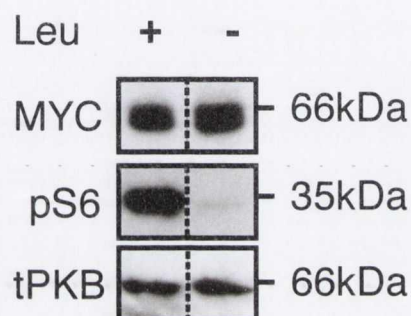


Figure 5.7. Leucine is required for mTORC1 activity, but not cMyc expression in cytokine activated NK cells

MACS purified cultured NK cells were activated with IL-2/12 for 18hours in the presence or absence of leucine (50µg/ml). cMyc expression and levels of pS6_{s235/236} were assessed. Total PKB was used as a loading control. Data is representative of four independent experiments.

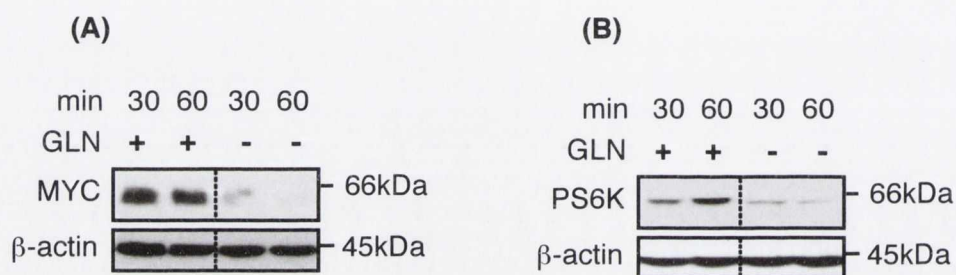


Figure 5.8. Glutamine is required for cMyc expression and mTORC1 activity in IL-2/12 activated NK cells

MACS purified 6 day cultured NK cells were activated with IL-2/12 before being switched into glutamine depleted or supplemented media (2mM). Samples were lysed for protein at 10×10^6 cells/ml at the time points indicated and analysed by immunoblot for cMyc expression (A) or pS6K_{t389} levels (B). β-actin was used as a loading control. Data is representative of 3 independent experiments.

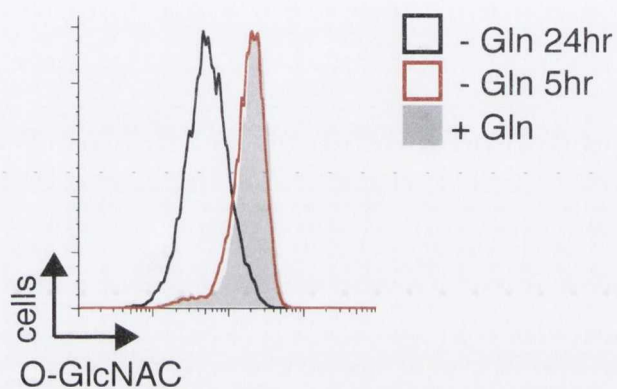


Figure 5.9. Glutamine fuels O-GlcNAcylation

NK cells were activated with IL-2/12 for 18hr before being switched into glutamine depleted or supplemented media (2mM) for the time points indicated. O-GlcNAc levels were analyzed by flow cytometry in NK1.1⁺ NKp46⁺ CD3⁻ cells. Data is representative of one experiment.

Therefore, while glutamine is important for cellular O-GlcNAcylation in NK cells, the decrease in cMyc expression observed following 30min glutamine deprivation is unlikely to be as a result of decreased O-GlcNAcylation.

Consistent with an important role for glutamine in controlling cMyc signalling, 18hour glutamine deprived NK cells stimulated with IL-2/12 failed to engage in blastogenesis and had low levels of CD71 expression (Fig. 5.10). Given the importance of Slc7a5 in controlling cytokine-induced cMyc expression, we next determined the impact of the Slc7a5 inhibitor BCH or glutamine deprivation on IL-2/12-induced metabolic responses. Strikingly, 18hr BCH treated NK cells had significantly reduced rates of glycolysis and reduced glycolytic capacity compared to control cells (Fig. 5.11). In addition, mitochondrial respiration (OxPhos, MRC, SRC) was also reduced in NK cell stimulated with IL-2/12 in the presence of BCH (Fig. 5.12). The metabolic profile of NK cells activated in the absence of glutamine was comparable to that of BCH treated cells; glutamine deprived NK cells had reduced levels of glycolysis (Fig 5.13) and OxPhos (Fig 5.14). Indeed, these data are consistent with our observations in Myc^{KO} NK cells (Fig. 4.13,15).

The effect of Slc7a5 inhibition on NK cell functional responses was next investigated. Consistent with the observed metabolic defects, NK cells stimulated with IL-2/12 in the presence of BCH displayed reduced IFN γ production (Fig 5.15A) and granzyme B expression (Fig 5.15B). Furthermore, NK cells activated in the absence of glutamine also displayed significantly compromised effector functions with a reduction in interferon (Fig. 5.16A) and granzyme b expression (Fig. 5.16B).

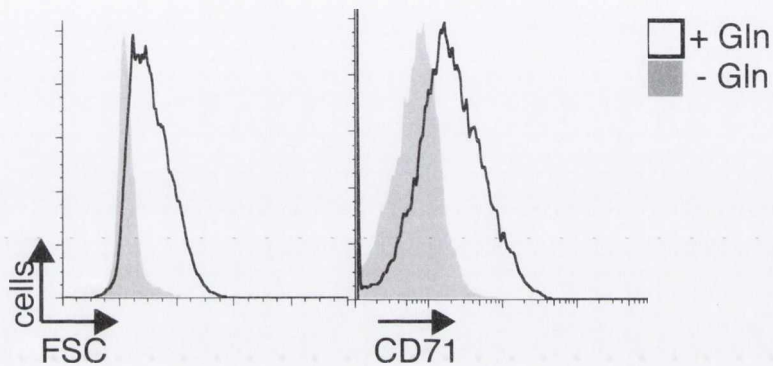


Figure 5.10. Glutamine deprived NK cells fail to increase in size and display reduced CD71 expression

Cultured NK cells activated with IL-2/12 in the presence or absence of glutamine (2mM) for 18hrs were analyzed by flow cytometry for cell size (FSC) or CD71 expression in NK1.1⁺ NKp46⁺ CD3⁻ NK cells. Data are representative of five experiments.

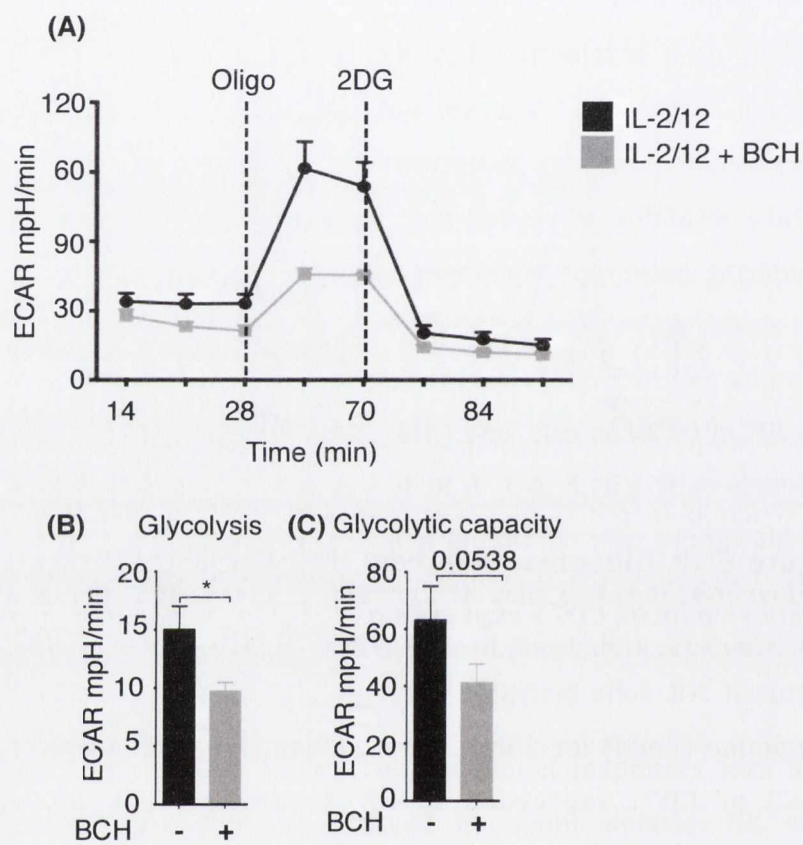


Figure 5.11. IL-2/12 activated NK cells treated with BCH exhibit decreased levels of glycolysis

Cultured NK cells were activated with IL-2/12 in the presence or absence of BCH (25mM) for 18hrs and levels of NK cell glycolysis were measured using the seahorse extracellular flux assay. Data are mean +/- SEM or are representative of four experiments. * $p < 0.05$

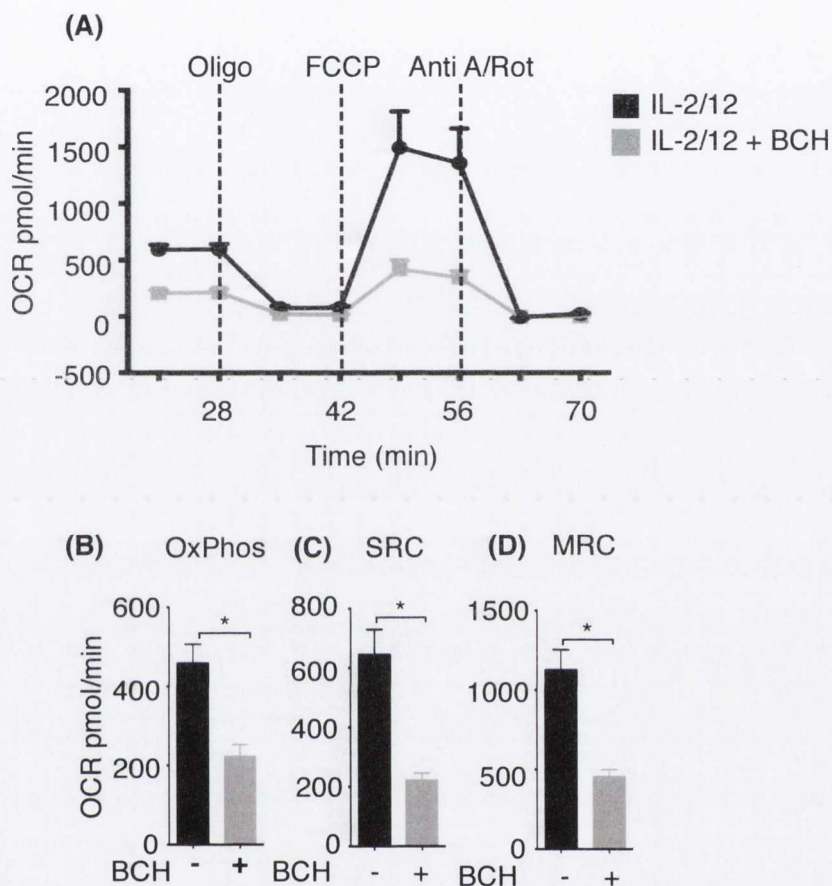


Figure 5.12. IL-2/12 activated NK cells treated with BCH exhibit decreased levels of OxPhos

Cultured NK cells were activated with IL-2/12 in the presence or absence of BCH (25mM) for 18hrs and levels of NK cell OxPhos were measured using the seahorse extracellular flux assay. Data are mean $\pm$ SEM or are representative of four experiments. * $p < 0.05$

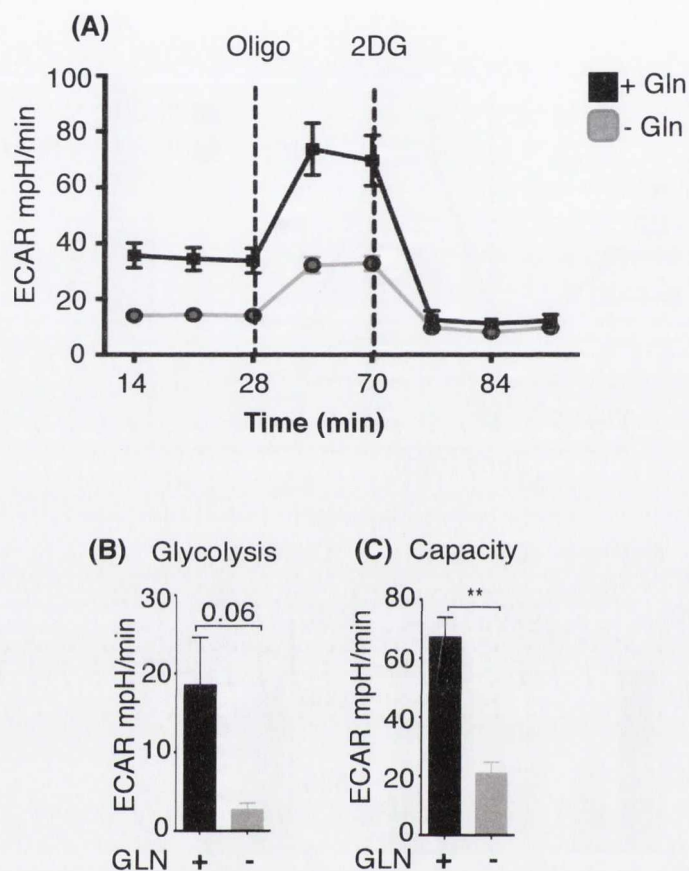


Figure 5.13. NK cells activated in the absence of glutamine display decreased levels of glycolysis

MACS purified 6 day cultured NK cells were activated with IL-2/12 in the presence (2mM) or absence of glutamine for 18hrs and levels of NK cell glycolysis were measured using the seahorse extracellular flux assay. Data are mean +/- SEM or are representative of four experiments. **p < 0.01

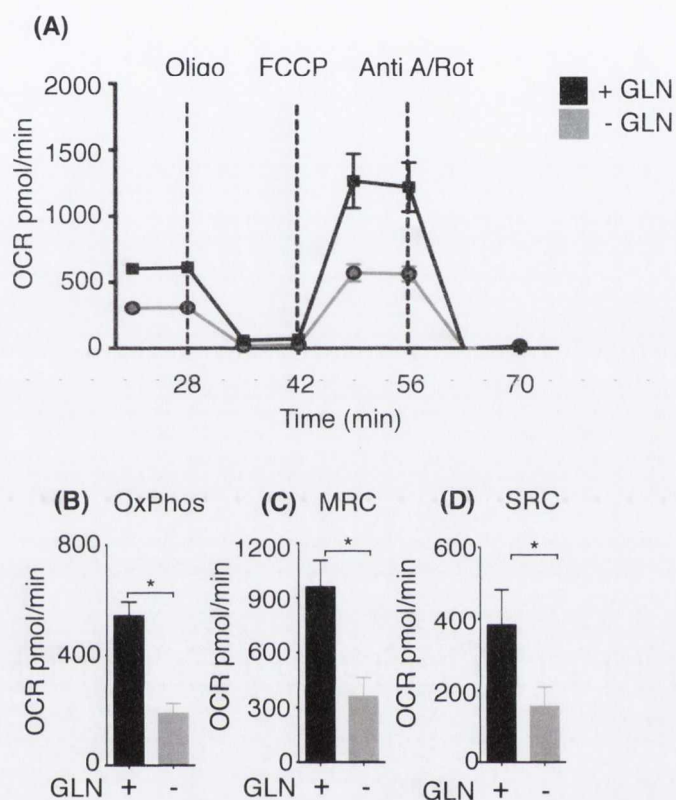


Figure 5.14. NK cells activated in the absence of glutamine display decreased levels of OxPhos

MACS purified 6 day cultured NK cells were activated with IL-2/12 in the presence (2mM) or absence of glutamine for 18hrs and levels of NK cell OxPhos were measured using the seahorse extracellular flux assay. Data are mean +/- SEM or are representative of four experiments. *p < 0.05

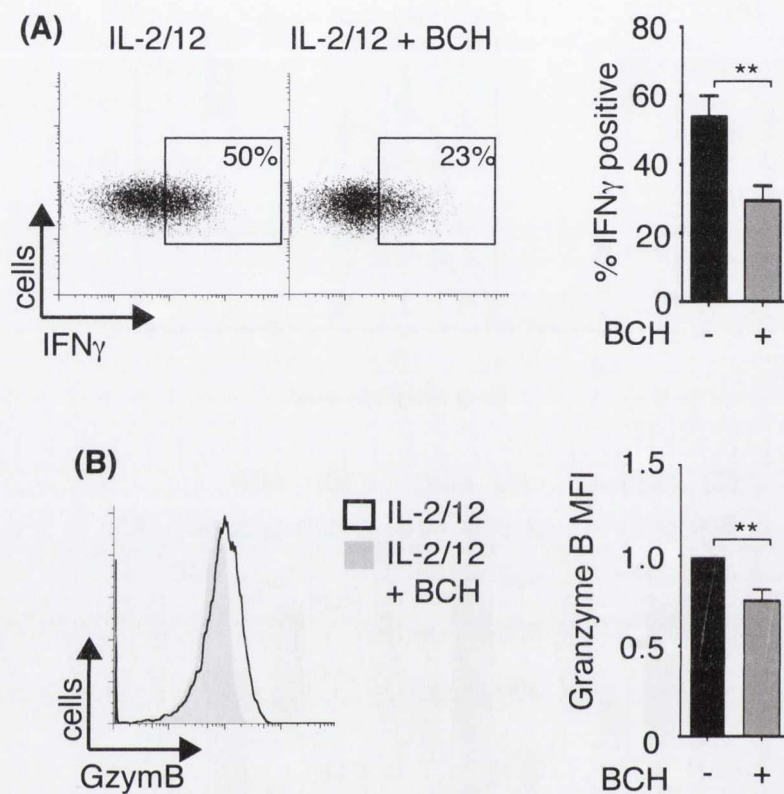


Figure 5.15. Slc7a5 activity is required for cytokine-induced increases in NK cell pro-inflammatory molecule production

Cultured NK cells were activated with IL-2/12 in the presence or absence of BCH (25mM) for 18hrs and were analyzed by flow cytometry for IFN γ expression (A) and granzyme b production (B) in NK1.1⁺ NKp46⁺ CD3⁻ cells. Data are mean +/- SEM of five separate experiments. **p < 0.01

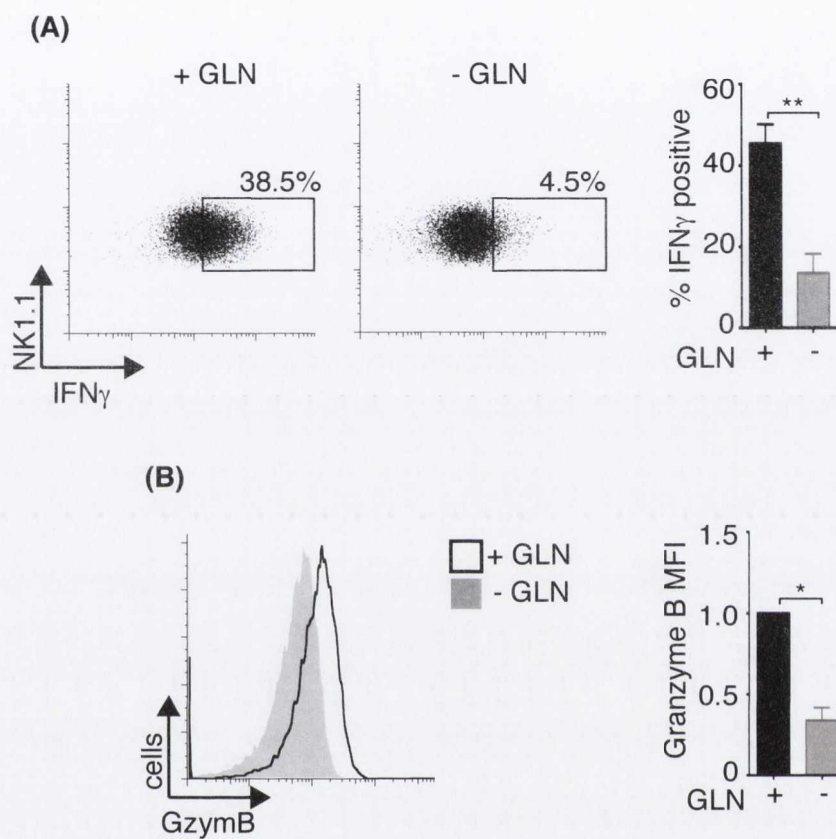


Figure 5.16. Glutamine is required for cytokine-induced increases in NK cell pro-inflammatory molecule production

Cultured NK cells were activated with IL-2/12 in the presence or absence of glutamine (2mM) for 18hrs and were analyzed by flow cytometry for IFN γ expression (A) and granzyme b production (B) in NK1.1⁺ NKp46⁺ CD3⁻ cells. Data are representative of five experiments. * $p < 0.05$, ** $p < 0.01$

5.3 Glutamine, but not glutamine metabolism, sustains NK cell metabolism and effector functions

These data outline a role for glutamine in controlling metabolic signalling pathways in IL-2/12 stimulated NK cells. However, glutamine is also a key metabolic fuel for many immune cells. Glutamine can be taken into the cell, broken down and converted into the TCA cycle intermediate alpha-ketoglutarate (α KG). Glutamine-derived α KG can directly feed into the TCA cycle in a process termed anapleurosis. This form of cellular metabolism is important for fuelling biosynthesis and energy production in many immune cell subsets (Wang et al., 2011b, Lee et al., 2015). Therefore, we considered whether glutamine is a fuel that supports the elevated levels of OxPhos observed in cytokine activated NK cells. NK cells stimulated with IL-2/12 for 18 hours were switched into glutamine-deficient media for 1 hour prior to metabolic analysis. Alternatively, NK cells were treated with an inhibitor of glutamine metabolism 6-diazo-5-oxo-L-norleucine (DON) that inhibits glutamine-utilizing enzymes including glutaminase, the first enzyme in glutaminolysis. Interestingly, the rate of NK cell OxPhos, the maximal respiratory capacity and the spare respiratory capacity of the NK cells were not affected by glutamine deprivation (Fig. 5.17) or by the inhibition of glutamine metabolism using DON (Fig. 5.18).

Glutaminolysis is indispensable for T cell proliferation (Lee et al., 2015). To confirm the efficacy of DON as an inhibitor of glutamine metabolism, CTL proliferation was monitored in the presence or absence of DON (2 μ M) or in the presence or absence of glutamine (2mM) as a non-proliferative control. Indeed, DON treated CTL displayed a significant reduction in expansion compared to IL-2 maintained CTL (Fig. 19A). O-GlcNAcylation is an important post-translational modification which directly regulates protein stability (Swamy et al., 2016). Glutamine and glucose derived metabolites fuel O-GlcNAcylation.

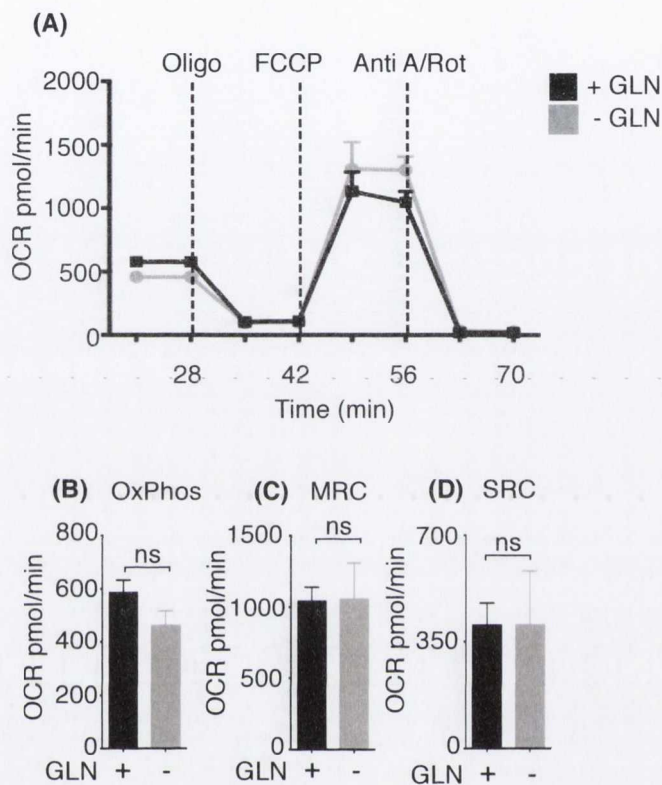


Figure 5.17. Glutamine is not an important fuel for sustaining elevated levels of NK cell OxPhos

MACS purified 6 day cultured NK cells were activated with IL-2/12 for 18hours. 1hour prior to seahorse analysis, cells were switched into glutamine depleted or supplemented seahorse media (2mM). Levels of NK cell OxPhos and the spare respiratory capacity following the addition of FCCP were measured using the seahorse extracellular flux assay. Data are mean +/- SEM or are representative of four experiments. ns: non-significant

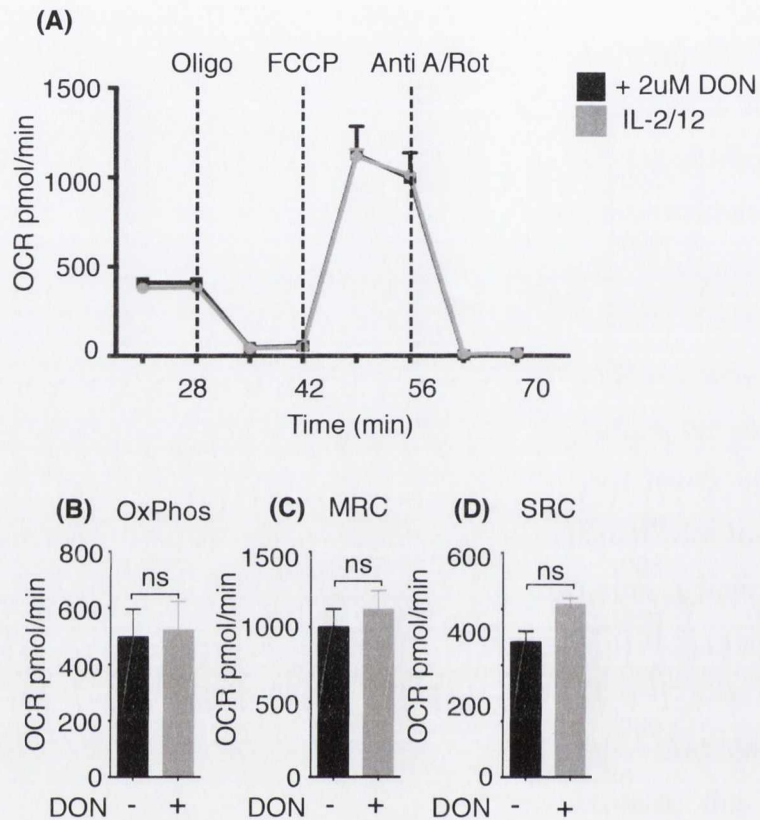


Figure 5.18. Glutamine metabolism is not required for sustaining elevated levels of NK cell OxPhos

MACS purified 6 day cultured NK cells were activated with IL-2/12 for 18hours. 1hour prior to seahorse analysis, cells were switched into seahorse media in the presence or absence of DON (2uM). Levels of NK cell OxPhos and the spare respiratory capacity following the addition of FCCP were measured using the seahorse extracellular flux assay. Data are mean +/- SEM or are representative of four experiments. ns: non-significant



Figure 5.19. DON inhibits CTL proliferation and reduces NK cell O-GlcNAcylation

IL2-induced proliferation of cytotoxic T lymphocytes (CTL) was measured as the increase in cell number after 24, 48 and 72 hours in the presence or absence of glutamine, with or without DON (2uM). The dashed line indicates the density at which the cells were seeded at 0.3×10^6 cells/ml (A). 18hour IL-2/12 activated NK cells were switched into glutamine depleted or supplemented (2mM) media in the presence or absence of DON (2uM) for 7rs. O-GlcNAc levels were analyzed by flow cytometry in NK1.1⁺ NKp46⁺ CD3⁻ cells. Data are mean $\pm$ SEM of six (A) or one (B) experiment. *p < 0.05, **p < 0.01

To further confirm the use of DON in our system, IL-2/12 activated NK cells were treated with DON (2 μ M) for 7 hours and levels of O-GlcNAc were measured. As a positive control, NK cells were switched into glutamine free media. Indeed, glutamine deprivation or DON treatment decreased O-GlcNAc levels in cytokine activated NK cells (Fig.5.19B).

These data show that glutamine is not a fuel for OxPhos in cytokine activated NK cells. However, when 18hr IL-2/12 activated NK cells were treated with DON or deprived of glutamine for a subsequent 18 hours, clear differences in NK cell phenotype emerged. Inhibition of glutamine metabolism in activated NK cells using DON for 18 hours had no impact on NK cell size, yet glutamine deprived cells were consistently smaller in size (Fig. 5.20). Furthermore, IFN γ production (Fig. 5.21A) and granzyme B expression (Fig. 5.21B) were unaffected in DON treated NK cells in contrast to the glutamine deprived group. It was hypothesised that the distinct differences observed between glutamine deprived NK cells versus DON treatment may be due to differences in signalling.

Western blot analysis was used to investigate the impact of short-term glutamine withdrawal on NK cell signal transduction. 18hr IL-2/12 activated NK cells were washed of glutamine and transferred into IL-2/12 supplemented media that was either depleted or supplemented with glutamine (2mM). The expression of cMyc was reduced after just 30minutes glutamine deprivation, with a further reduction in cMyc expression observed after 1hour (Fig. 22A). In contrast, 18hr IL-2/12 stimulated NK cells treated with DON displayed no differences in cMyc expression (Fig. 22B). Therefore, the differences in phenotype between glutamine deprived or DON treated NK cells reflects the fact that glutamine is essential to sustain cMyc expression.

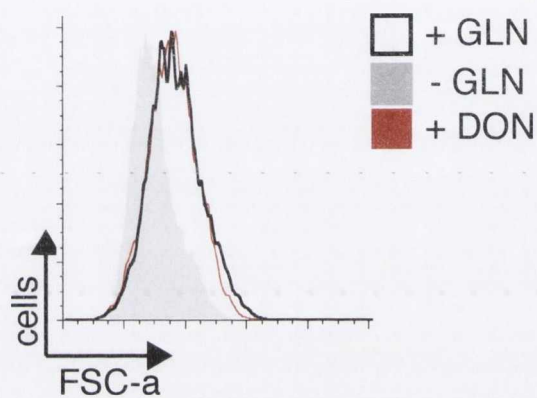


Figure 5.20. Glutamine, but not glutamine metabolism, is required for sustaining increased cell size in IL-2/12 activated NK cells

18hour IL-2/12 activated NK cells were switched into glutamine depleted or supplemented (2mM) media in the presence or absence of DON (2uM) for 18hrs. Changes in cell size (FSC) were analyzed by flow cytometry in NK1.1⁺ NKp46⁺ CD3⁻ cells. Data are representative of five experiments.

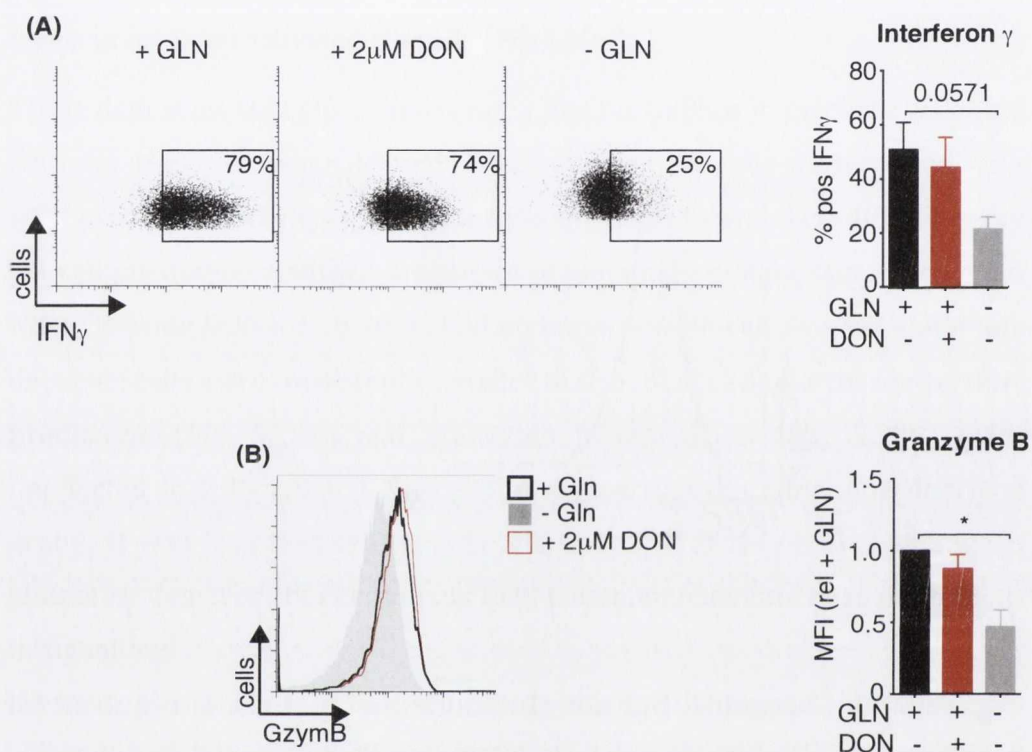


Figure 5.21. Glutamine, but not glutamine metabolism, is required for sustaining effector molecule production in IL-2/12 activated NK cells

18hour IL-2/12 activated NK cells were switched into glutamine depleted or supplemented (2mM) media in the presence or absence of DON (2uM) for 18hrs. IFN γ and granzyme b expression were analyzed by flow cytometry in NK1.1⁺ NKp46⁺ CD3⁻ cells. Data are mean $\pm$ SEM or are representative of four experiments. * $p < 0.05$

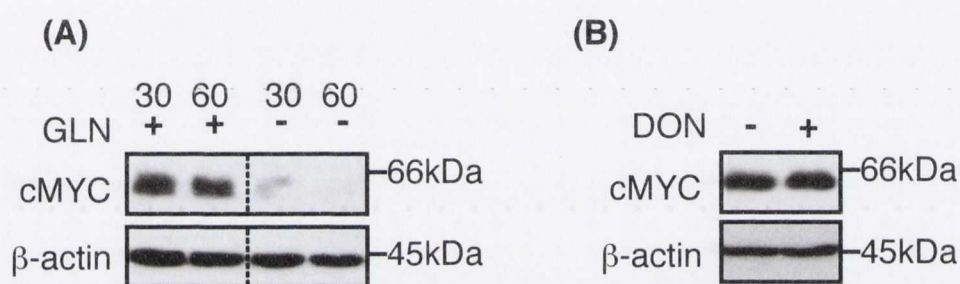


Figure 5.22. Glutamine, but not glutamine metabolism, is required to sustain cMyc expression IL-2/12 activated NK cells

MACS purified cultured NK cells were activated with IL-2/12 for 18hrs before being switched into glutamine depleted or supplemented media (2mM) in the presence or absence of DON (2uM). Samples were lysed for protein at 10×10^6 cells/ml at the timepoints indicated and analysed by immunoblot for cMyc levels. β -actin was used as a loading control. Data is representative of three experiments.

Chapter 5 discussion

The highly conserved PI3K/Akt signaling axis controls many aspects of cell biology including cellular metabolism, proliferation, immune cell activation, cellular differentiation and cell survival (Otten and Germain, 1991, Rolf et al., 2013). PI3K/Akt signaling enhances mTORC1 activity through the phosphorylation and inactivation of TSC2, a negative regulator of mTORC1 (Dibble and Cantley, 2015)(Figure 1.3). As previously discussed, mTORC1 is a serine threonine kinase that is indispensable for the metabolic and functional responses of many immune cells (Finlay et al., 2012b, Cheng et al., 2014, Donnelly et al., 2014). mTORC1 activity is dynamically regulated by a wide variety of signaling pathways (Figure 1.3). Although PI3K/Akt signaling is required for mTORC1 activity in many cell types, this is not always the case. IL-2 is a strong activator of both PI3K/Akt and mTORC1 signaling in CD8⁺ T cells (Finlay et al., 2012a). Indeed, PI3K/Akt and mTORC1 signaling pathways are similar in their ability to control T cell effector functions by enhancing pro-inflammatory (IFN γ) and cytotoxic (perforin, granzyme b) molecule expression (Macintyre et al., 2011). However, the key signaling components of these two pathways differ. For instance, mTORC1 signaling through HIF1 α enhances T cell metabolic and functional responses whereas PI3K control of foxo influences T cell IFN γ expression (Fig 5.1) (Zhao et al., 2016). Due to the converging roles of these two signaling pathways in controlling immune cell function, a common misconception is PI3K signaling is exclusively upstream of mTORC1 activity. Although PI3K/Akt signaling is required for mTORC1 activity in some cell types, PI3K/Akt activity is not required to maintain mTORC1 activity in IL-2 maintained CD8⁺ T cells (Finlay et al., 2012b), nor was it required to sustain the metabolism of IL-2 maintained CTL (Macintyre et al., 2011). Instead, in these cells it is IL-2 regulated amino acid uptake that is the critical determinant of mTORC1 activity (Preston et al., 2015, Sinclair et al., 2013). Interestingly, we also found that Akt activity was not required for cytokine induced NK cell metabolic signal transduction; inhibition of Akt had

no effect on cMyc expression or mTORC1 activity in cytokine-activated NK cells. In accordance with the role of mTORC1 and cMyc expression in NK cell metabolic responses, inhibition of Akt had no effect on NK cell size or effector molecule production. These data reveal that, similarly to CD8⁺ T cells, NK cell metabolic signal transduction is not dependent on the PI3K/Akt signaling pathway.

These findings do not preclude a role for PI3K/Akt signaling in controlling NK cell responses in the context of different stimuli. For instance, the Seung Hwan-Lee group also investigated the role of the PI3K-Akt-mTORC1 signaling pathway in 24hr IL-15 primed splenic NK cells, that were further stimulated for 5 hours with IL-12 or Ly49H (Nandagopal et al., 2014). We have previously shown that alone, IL-15 or IL-12 do not increase mTORC1 activity in NK cells (Fig. 3.10,11). Other data from our lab also suggests that engagement of NK cell activating receptors does not induce robust mTORC1 activity. Hence the Nandagopal study was essentially investigating the role of mTORC1 and PI3K signaling in IL-15 induced priming of NK cells. In this study, inhibition of Akt activity or inhibition of mTORC1 activity impaired IL-15 induced NK cell priming, leading to a decrease in NK cell IFN γ and granzyme b production in response to further cytokine stimulation or receptor activation (Zhou et al., 2006). Bear in mind, although both the PI3K/Akt and mTORC1 signaling pathways have converging roles in controlling the effector function of CTL, this does not mean they are in a linear pathway (Zhao et al., 2016). Indeed, 1hr inhibition of Akt in our system had no effect on mTORC1 activity or cMyc expression in IL-2/12 activated NK cells. Direct signal transduction analysis would be required to determine the role of PI3K/Akt signaling in controlling mTORC1 activity in IL-15 primed NK cells.

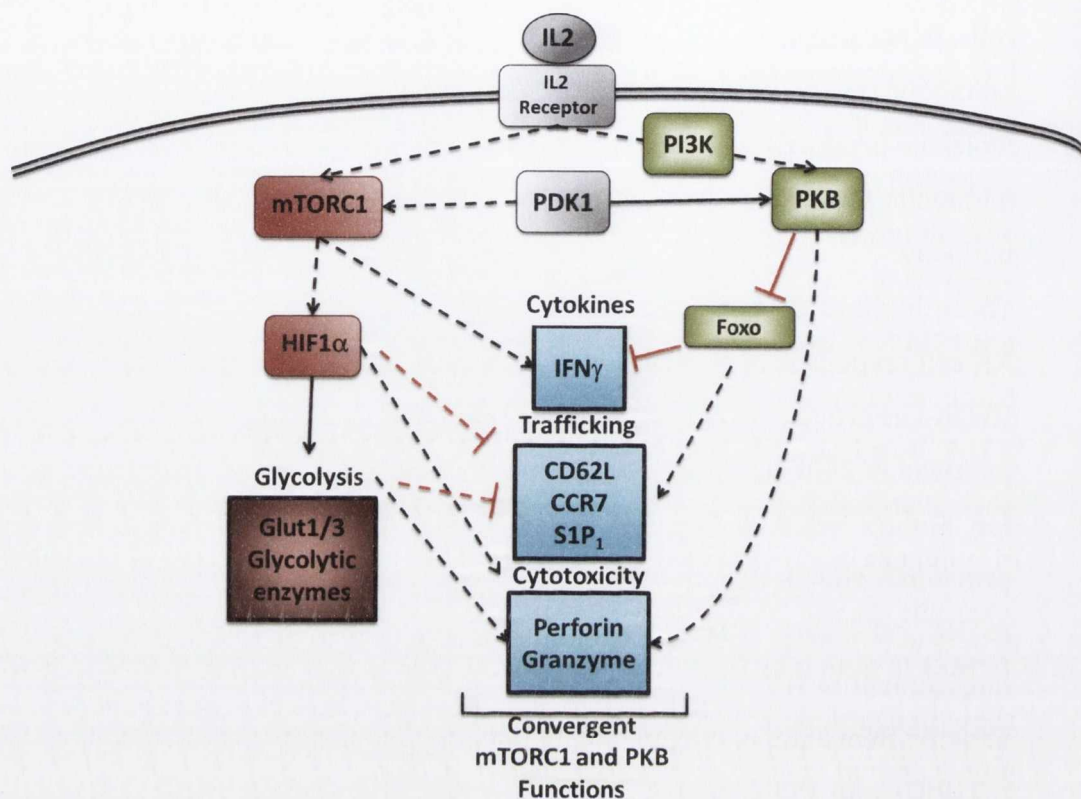


Figure 6.1 PI3K/Akt and mTORC1 signal independently control T cell function in response to IL-2

IL-2 is a strong activator of both PI3K/Akt and mTORC1 signaling in CD8⁺ T cells. These signaling pathways converge through distinct mechanisms to regulate key CTL effector molecules production (perforin and granzyme) and CD8⁺ T-cell trafficking. mTORC1 signaling through HIF1 α enhances T cell metabolic and functional responses whereas PI3K control of foxo influences T cell IFN γ expression.

Although the findings from this study differ from ours regarding the importance of Akt for NK cell functional responses, what was clear was the importance of mTORC1 in controlling NK cell effector functions. In an MCMV viral infection model, rapamycin treatment significantly impaired NK cell responses (Zhou et al., 2006). These data reflect our earlier findings, which show mTORC1 is required for NK cell effector function. In summary, our data shows Akt activity is not required for NK cell cMyc expression or mTORC1 activity.

The amino acid transporter Slc7a5 is fundamental to a successful T cell response due to its role in regulating cMyc expression (Sinclair et al., 2013). Despite the robust induction of many other amino acid transporters, loss of Slc7a5 alone profoundly impaired T cell activation; Slc7a5null T cells had decreased cMyc expression, they were smaller in size, had reduced rates of proliferation, reduced effector molecule production and decreased rates of glycolysis due to impaired metabolic reprogramming following TCR activation (Sinclair et al., 2013). Our data show cMyc is central to the metabolic and functional responses of cytokine activated NK cells. Strikingly, we found that Slc7a5 was required for cMyc expression in cytokine activated NK cells; pharmacological inhibition or genetic deletion of Slc7a5 resulted in reduced cMyc expression in cytokine activated NK cells. Due to the importance of cMyc for the metabolic response of NK cells, inhibition of Slc7a5 had a profound effect on the rate of NK cell glycolysis and OxPhos, which led to defective NK cell functional responses. These findings, along with data from the Cantrell group in the University of Dundee, define a signalling paradigm whereby Slc7a5 activity and amino acid availability are essential factors in controlling cMyc signal transduction, which is crucial for effector lymphocyte functional responses (Sinclair et al., 2013).

Slc7a5 is an obligate anti-porter, meaning it must transport an amino acid out of the cell to import other amino acids into the cell. Slc7a5 has a very low K_m for glutamine export and so it is usually glutamine that facilitates amino acid import through Slc7a5 (Vely et al., 2016). Interestingly, we found that glutamine deprivation mirrored Slc7a5 inhibition; withdrawal of glutamine or pharmacological inhibition of Slc7a5 activity resulted in a rapid loss of cMyc expression (30minutes). In addition, mTORC1 activity was also decreased following glutamine withdrawal or inhibition of Slc7a5 activity. Glutamine has previously been reported to control mTORC1 activity in T cells by regulating Slc7a5 activity (Sinclair et al., 2013). Slc7a5 is important for the transport of multiple amino acids into the cell, including leucine (Vely et al., 2016). While there is no intracellular glutamine sensor that controls mTORC1 activity, sestrins are a known set of stress-responsive proteins that are activated when leucine availability is low, and which turn off mTORC1 activity (Ye et al., 2015). Therefore, glutamine itself is not required for mTORC1 activity, however glutamine facilitates leucine uptake through Slc7a5 which is indispensable for mTORC1 activity (Vely et al., 2016, Sinclair et al., 2013). In support of this, Sinclair et al showed glutamine alone is not sufficient to sustain mTORC1 activity in amino acid depleted CD8⁺ T cells. However the addition of both glutamine and leucine restored mTORC1 activity to amino acid replete levels (Sinclair et al., 2013). Indeed, our data also supports the model that glutamine regulates amino acid import through Slc7a5. Glutamine withdrawal resulted in a rapid decrease in mTORC1 activity in cytokine stimulated NK cells, which mirrored that of leucine deprivation. However, glutamine control of intracellular leucine levels does not explain the importance of glutamine for sustaining cMyc expression; leucine deprivation did not affect cMyc expression in cytokine activated NK cells. In addition to leucine, Slc7a5 transports other amino acids including methionine, histidine, phenylalanine, tryptophan and tyrosine (Vely et al., 2016). Glutamine permits the uptake of all these amino acids through Slc7a5.

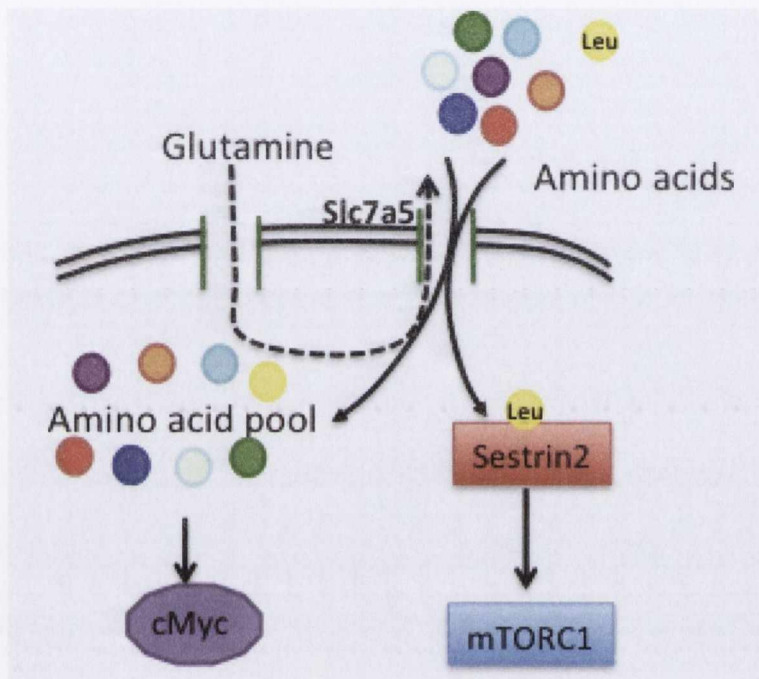


Figure 6.1 Glutamine facilitates amino acid uptake through Slc7a5

Slc7a5 controls the uptake of large neutral amino acids such as leucine, methionine, histidine, phenylalanine, tryptophan and tyrosine into the cell. Slc7a5 is an obligate anti-porter, meaning it must transport an amino acid out of the cell to import other amino acids into the cell. Slc7a5 has a very low K_m for glutamine export and so it is usually glutamine that facilitates amino acid import through Slc7a5. Glutamine controlled leucine uptake through Slc7a5 is required for mTORC1 activity. Glutamine controlled uptake of many other amino acids through is required for cMyc expression.

cMyc is a nutrient sensor that is acutely sensitive to intracellular amino acid levels (Sinclair et al., 2013). Although leucine is required for mTORC1 activity and not cMyc expression, perhaps glutamine control of other Slc7a5 amino acids regulates cMyc expression. If this were true, we would hypothesise that in the absence of glutamine, Slc7a5 activity is lost and the intracellular amino acid pool is depleted, resulting in a loss of cMyc translation.

The short timeframe in which glutamine withdrawal or BCH treatment reduced cMyc expression in cytokine activated NK cells reflects the fact that cMyc is actively degraded through ubiquitin-mediated targeting to the proteasome (Gregory and Hann, 2000). cMyc is a labile protein that can be rapidly expressed and degraded in lymphocytes (Preston et al., 2015, Swamy et al., 2016, Sinclair et al., 2013) (Fig. 6.2). Due to its role in controlling protein translation, mTORC1 activity has been shown to enhance the expression of cMyc in some cells (Shimobayashi and Hall, 2016). mTORC1 signalling through S6K enhances the association of eukaryotic initiation factor A (eIF4A) to eIF4B within the translation preinitiation complex (PIC). Importantly, knockdown of either eIF4A/B lead to a loss of cMyc protein expression in mouse embryonic fibroblasts (Shimobayashi and Hall, 2016). In addition to the translational regulation of cMyc, post-translational modifications have an important role in controlling cMyc expression. GSK3 mediated phosphorylation of cMyc on Thr-58 targets it for degradation via ubiquitin-mediated degradation. However, the addition of UDP-GlcNAC on the same residue, Thr58, competes with this phosphorylation, thereby enhancing cMyc stabilisation (Figure 6.1) (Swamy et al., 2016). In addition to facilitating amino acid transport through Slc7a5, another way glutamine could regulate cMyc expression is by fuelling O-GlcNACylation of Thr58, which prevents GSK3 mediated phosphorylation and degradation of cMyc. Indeed, 24hr glutamine deprivation lead to a significant reduction in global O-GlcNACylation levels in cytokine stimulated NK cells. However, 5hour glutamine deprived NK cells sustained high expression of O-GlcNACylation.

Given the temporal dynamics that glutamine withdrawal decreases cMyc expression (30 minutes), it is not likely that this is the primary mechanism behind glutamine control of cMyc expression. Furthermore, pharmacological inhibition of Slc7a5 would not decrease O-GlcNAcylation levels as glutamine import is not affected, however BCH treatment led to a rapid loss of cMyc expression in cytokine activated NK cells. These data further argue glutamine fuelled O-GlcNAcylation is not the primary mechanism in which glutamine controls cMyc expression in cytokine activated NK cells.

Another key finding of this study was that glutamine is not an important metabolic fuel for cytokine activated NK cells. Glutamine catabolism can generate the TCA cycle intermediate α -ketoglutarate (α KG), which can directly feed into the TCA cycle and fuel OxPhos in a process termed anapleurosis (Le et al., 2012). This form of cellular metabolism is important for fuelling biosynthesis and energy production in B and T cells, particularly when extracellular levels of glucose become limiting (Cham and Gajewski, 2005, Wang et al., 2011b). In contrast to other lymphocytes, our data shows glutamine is not an important fuel for cytokine activated NK cells. If glutamine was a metabolic fuel for the TCA cycle and OxPhos in cytokine activated NK cells, withdrawal of glutamine would lead to a rapid decrease in the rate of NK cell OxPhos. However, our data show that withdrawal of glutamine 1 hour prior to Seahorse analysis had no impact on the rate of NK cell OxPhos. In addition, inhibition of glutaminolysis had no effect on the rate of OxPhos in cytokine activated NK cells. These data show in contrast to other lymphocytes, glutamine is not an important fuel for maintaining elevated rates of cytokine induced NK cell OxPhos. Future work involving glutamine tracing studies to reveal the exact contribution of glutamine as a metabolic fuel would be of great interest. The data also shows that glutamine withdrawal, but not inhibition of glutamine metabolism, reduces cMyc expression in cytokine activated NK cells. As previously discussed, O-GlcNAcylation of Thr58 has been shown to regulate cMyc expression in IL-2

activated T cells (Swamy et al., 2016)(Figure 6.1). In our system, DON treated NK cells displayed a substantial decrease in global O-GlcNACylation levels, without cMyc protein expression being affected. Again, this data suggests O-GlcNACylation is not the primary mechanism in which glutamine controls cMyc expression in cytokine activated NK cells.

In conclusion, glutamine plays an indispensable role in controlling NK cell signal transduction, even though it is not an important fuel for NK cell metabolism. The importance of glutamine for maintaining elevated levels of cMyc expression in cytokine activated NK cells is most likely due to its role in facilitating Slc7a5 amino acid transport; withdrawal of glutamine or pharmacological inhibition of Slc7a5 activity in cytokine activated NK cells resulted in a rapid loss of cMyc expression (30minutes). These findings have important implications for the design of NK cell based immunotherapies, which will be further discussed in the final chapter.

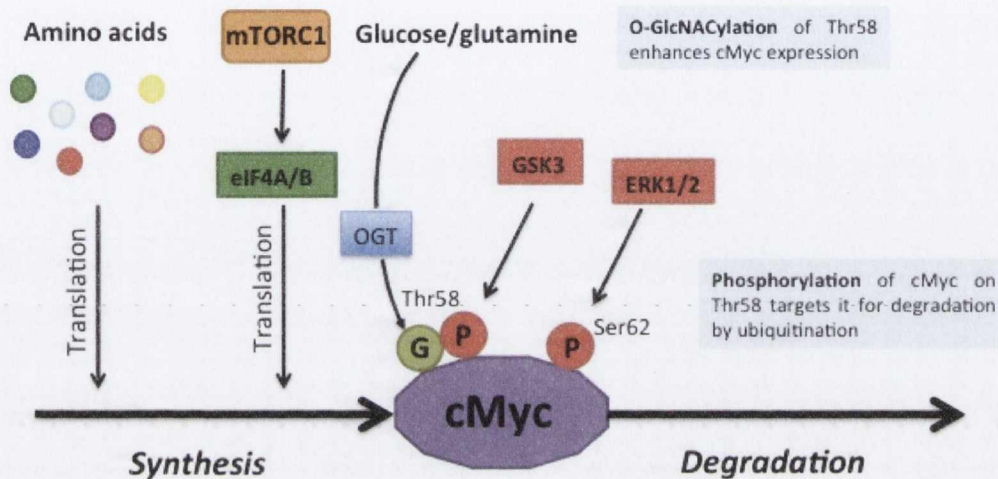


Figure 6.2 cMyc expression is dynamically regulated by the balance of synthesis and degradation

Amino acid availability is crucial for cMyc expression and facilitates cMyc translation. mTORC1 can regulate cMyc levels by enhancing its translation, although mTORC1-independent mechanisms of cMyc regulation also exist. Glutamine and glucose fuel the hexosamine biosynthetic pathway for the synthesis of UDP-GlcNAC. O-GlcNAcylation of cMyc on Thr58 enhances its stability by competing with phosphorylation of this same residue, Thr58. Phosphorylation of cMyc on Thr58 by GSK3 targets it for degradation via ubiquitination. ERK1/2 phosphorylation on ser62 is required to prime cMyc protein for phosphorylation on thr58. cMyc protein is a labile protein that is regulated by a balance of synthesis and degradation.

Chapter 6

Overall discussion

This study has characterised the changes in Natural Killer cell metabolism that occur upon activation. We have shown that elevated levels of glucose metabolism are imperative for the acquisition of NK cell effector functions. Experiments in which the rate of glycolysis was directly limited impaired NK cell IFN γ and granzyme b expression. Competition for glucose between the large numbers of infiltrating immune cells at sites of inflammation can result in nutrient depletion (Lawless et al 2017 in press). In addition, the amount of glucose available for immune cells during viral infections can become limiting; viruses can reprogram infected cells to up-regulate glucose uptake, which facilitates viral replication (Yu et al., 2011, Thai et al., 2014). However, the solid tumor microenvironment remains the best-characterised pathological setting whereby nutrient restriction can limit infiltrating immune cell responses (Hirayama et al., 2009, Ho et al., 2015). Tumor cells display a 10-fold greater rate of glucose uptake than activated lymphocytes, meaning they can efficiently sequester glucose in the tumor microenvironment, away from infiltrating immune cells (Cham and Gajewski, 2005). Indeed, glucose levels in the tumor microenvironment were measured and found to be 15 times less than that found in the circulation (0.6mM versus 9mM) (Ho et al., 2015). Therefore, at the sites of solid tumors, and during viral infection, infiltrating NK cells can encounter unfavorable conditions that may directly limit the rate of glycolysis. Due to our finding that NK cell metabolism is integrally linked to NK cell effector function, our data suggests that the NK cell anti-tumor and anti-viral functions will be compromised by the metabolically restrictive conditions of the tumor and inflammatory microenvironments. Indeed, NK cells infiltrating a range of human solid tumors have been shown to display decreased effector molecule production (Mamessier et al., 2011, Platonova et al., 2011, Rocca et al., 2013).

Immunotherapies, which harness a patient's own immune system to fight cancer, are revolutionizing the field of oncology and dramatically improving patient survival. One immunotherapeutic strategy involves monoclonal antibodies. Monoclonal antibodies bind antigen with high specificity and affinity, to assist the immune system in detecting and eliminating transformed cells (Scott et al., 2012). T cells express checkpoint receptors on their cell surface, which transduce inhibitory signals to downregulate T cell effector function and enhance tolerance. Programmed cell death receptor 1 (PD-1) is a T cell checkpoint receptor that interacts with programmed cell death receptor ligand 1 (PD-L1), found naturally expressed on macrophages and dendritic cells (Scott et al., 2012). Cancer cells often hijack T cell checkpoint receptor interactions and over-express their ligands to suppress the anti-cancer T cell response (Armand, 2015). Monoclonal antibodies which block PD-1/PD-L1 interactions 'take the breaks off' the anti-cancer T cell response and are proving extremely effective in the treatment of hematological malignancies such as Hodgkin's lymphoma, non-Hodgkin's lymphoma and multiple myeloma (Armand, 2015). In addition, although PD-1 is not normally expressed on NK cells, a recent study shows NK cells from multiple myeloma patients also express PD-1 on their cell surface. In this study, the use of anti-PD1 or anti-PD-L1 monoclonal antibodies enhanced NK cell detection and cytotoxicity against multiple myeloma transformed cells (Benson et al., 2010).

Although the use of checkpoint inhibitors are proving successful in the treatment of hematological malignancies, relatively low efficacy has been observed in the treatment of solid tumors (Assmann and Finlay, 2016). This may be in part due to the nutrient restrictive microenvironment of solid tumors, which can negatively affect immune cell signaling. In this study, we found that mTORC1 activity is essential for NK cell metabolic and functional responses. mTORC1 is an acute sensor of the immune microenvironment, and its activity reflects nutrient and growth factor availability. Sustained

glucose, amino acid or cytokine deprivation inhibits mTORC1 activity in immune cells (Sinclair et al., 2013, Rolf et al., 2013). Hence in the solid tumor microenvironment, where nutrient availability is low, mTORC1 activity will be turned off in infiltrating immune cells. Our data shows that in the absence of mTORC1 activity, NK cell metabolic and functional responses are impaired. These findings have important implications for the development of NK cell based immunotherapies. The use of chemo and radiotherapies to limit tumor size would be extremely beneficial alongside NK cell anti-cancer immunotherapeutic strategies. Although inherently toxic, the use of such agents could help tip the nutrient balance in favor of infiltrating lymphocytes, by limiting tumor cell metabolism, and enhancing glucose availability for immune cells.

The adoptive transfer of NK cells is showing great promise in the treatment of many forms of cancer, including breast cancer and malignant glioma (deMagalhaes-Silverman et al., 2000, Ishikawa et al., 2004). NK cells used in adoptive transfer therapies may be autologous (from the host) allogeneic (from another individual) or genetically engineered chimeric antigen receptor (CAR) NK cells. Early adoptive transfer studies used autologous NK cells that were activated with IL-2 *in vivo* (Burns et al., 2003). Although these studies successfully induced NK cell differentiation into lymphokine activated killer cells (LAK), the anti-tumor efficacy was unsatisfactory and often came with detrimental side effects (Rosenberg, 1984).

NK cells isolated from cancer patients are known to display inherent defects in their cytotoxicity, making this form of NK cell immunotherapy somewhat redundant (Rocca et al., 2013, Platonova et al., 2011, Mamessier et al., 2011). The use of allogeneic NK cells from healthy donors or NK cell lines has proved more successful and has the added benefit of increased immunogenicity (Sutlu and Alici, 2009). Furthermore, the development of a new class of genetically engineered NK cells holds great promise for the future of adoptive transfer-based immunotherapies. Chimeric antigen

receptor (CAR) cells are genetically modified immune cells that express specific tumor antigens. For example, T cells expressing CARs specific against CD19 antigen are currently in clinical trials for treating acute lymphoblastic leukemia (ALL) (Grupp et al., 2013). With the success of CAR engineered T cells, the future of NK CAR based immunotherapeutic strategies is bright. Our work highlights the importance of cellular metabolism in controlling NK cell effector functions. As previously discussed, a major challenge for tumor-infiltrating lymphocytes is overcoming the nutrient restrictive microenvironment of solid tumors. Genetically engineered NK CAR cells, designed to overexpress glucose transporters such as GLUT 1, could preferentially enhance NK cell glucose uptake in metabolically restrictive environments and enhance NK cell anti-tumor functions. Indeed, over expression of GLUT1 in T cells was shown to enhance effector T cell frequency and increased T cell pro-inflammatory functions (Jacobs et al., 2008).

Our study also highlights the importance of IL-2 signaling in promoting increased rates of NK cell glycolysis and enhanced NK cell effector functions. NK cells often work in parallel with T cells during an immunological challenge (Vivier et al., 2011, Lee et al., 2012). Although T cells are not essential for NK cell activation (Sun and Lanier, 2011), our research suggests that the adaptive immune response may enhance NK cell effector functions through the production of IL-2. Indeed, one mechanism by which regulatory T cells control NK cell responses is through sequestering IL-2 (Kerdiles et al., 2013). NK cell adoptive transfer studies often employ IL-2 to activate NK cells and induce their differentiation into LAK (Burns et al., 2003). However, the concentration of IL-2 in the tumor microenvironment is often limiting, particularly when Treg are enriched, which is associated with a poor prognosis (Curiel et al., 2004). By using a combination of IL-12 and IL-18, both of which enhance the expression of the high affinity IL-2 receptor CD25, NK cell responsiveness to low concentrations of IL-2 *in vivo* will be enhanced (Ni et al., 2012). Indeed, the combination of IL-12/15/18 has already shown

efficacy in a murine NK cell adoptive transfer study, due to its ability to enhance CD25 expression (Ni et al., 2012).

In addition to glucose, tumor cells are known for being 'addicted' to glutamine (Wise and Thompson, 2010). This is largely due to the importance of glutamine in polyamine and nucleotide synthesis, which facilitates high rates of proliferation (Cory and Cory, 2006). In the tumor microenvironment it is therefore likely that glutamine levels will become limiting. Our data shows that glutamine is required for sustaining cMyc expression in cytokine-activated NK cells. cMyc is a crucial transcription factor that controls NK cell metabolic and functional responses. Interestingly, we found that although glutamine is required for cMyc expression in NK cells, glutamine metabolism was dispensable. A recent study in CD4⁺ T cells shows targeting glutamine metabolism can limit T cell effector functions and improve allogeneic graft acceptance (Lee et al., 2015). However in contrast to CD4⁺ T cells, we have shown that the administration of glutaminase inhibitors such as DON, which inhibit tumor cell proliferation, do not affect NK cell effector responses. Furthermore, inhibition of tumor cell proliferation using glutamine metabolism inhibitors would increase glutamine availability in the tumor microenvironment, thereby allowing for sustained NK cell cMyc expression and improved anti-cancer NK cell responses. Indeed, glutamine metabolism is currently being targeted as an anti-cancer therapy (Lukey et al., 2013).

In conclusion, this study has broadened our understanding of NK cell metabolism and shows the importance of cellular metabolism in the acquisition of NK cell effector functions. Virally infected and transformed cells have adapted ways to evade the immune response, through targeting lymphocyte metabolism. With a greater understanding of NK cell metabolism and its importance for NK cell effector functions, we can now strive towards the development of improved immunotherapeutics that enhance the metabolic adaptations of NK cells and tip the balance in favor of the anti-cancer immune response.

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