Sterol Regulatory Element Binding Protein is a crucial regulator of Natural Killer cell metabolism and function

By

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other University. I hereby certify that this thesis is entirely my work. Any other assistance or contribution is mentioned under the Acknowledgements section.

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Raymond Donnelly

October 2015

Dedication

I dedicate this thesis to my parents Patsy and Grace and to my girlfriend Oana. Without their patience, understanding, support and most of all love, the completion of this work would not have been possible.

Never forget

"Whether you think you can, or you think you can't- You're right"

- **Henry Ford**

Publications

DONNELLY, R. P., LOFTUS, R. M., KEATING, S. E., LIOU, K. T., BIRON, C. A., GARDINER, C. M. & FINLAY, D. K. 2014. mTORC1-dependent metabolic reprogramming is a prerequisite for NK cell effector function. ***J Immunol,*** **193, 4477-84.**

The mammalian target of rapamycin complex 1 (mTORC1) is a key regulator of cellular metabolism and also has fundamental roles in controlling immune responses. Emerging evidence suggests that these two functions of mTORC1 are integrally linked. However, little is known regardingmTORC1 function in controlling the metabolism and function of NK cells, lymphocytes that play key roles in antiviral and antitumor immunity. This study investigated the hypothesis that mTORC1-controlled metabolism underpins normal NK cell proinflammatory function. We demonstrate thatmTORC1 is robustly stimulated in NK cells activated in vivo and in vitro. This mTORC1 activity is required for the production of the key NK cell effector molecules IFN-γ, which is important in delivering antimicrobial and immunoregulatory functions, and granzyme B, a critical component of NK cell cytotoxic granules. The data reveal that NK cells undergo dramatic metabolic reprogramming upon activation, upregulating rates of glucose uptake and glycolysis, and that mTORC1 activity is essential for attaining this elevated glycolytic state. Directly limiting the rate of glycolysis is sufficient to inhibit IFN-γ production and granzyme B expression. This study provides the highly novel insight that mTORC1-mediated metabolic reprogramming of NK cells is a prerequisite for the acquisition of normal effector functions.

DONNELLY, R. P. & FINLAY, D. K. 2015. Glucose, glycolysis and lymphocyte responses. ***Mol Immunol*****.**

Activated lymphocytes engage in robust growth and rapid proliferation. To achieve this, they tend to adopt a form of glucose metabolism termed aerobic glycolysis. This type of metabolism allows for the use of large amounts of glucose to generate energy, but also to support biosynthetic processes. This review article will discuss how aerobic glycolysis supports the biosynthetic demands of activated T cells, B cells and Natural Killer cells, and the emerging concept that glycolysis is integrally linked to the differentiation and function of these lymphocyte populations.

Abstract

Sterol Regulatory Element Binding Protein is a crucial regulator of Natural Killer cell metabolism and function

By Mr. Raymond Donnelly

Natural Killer (NK) cells are a lymphocyte subset that has a key role in anti-viral and anti-tumour immunity. While the concept of immunometabolism has recently been recognised as critical in determining functions of immune cell subsets, nothing is known regarding the regulation of NK cell metabolism. This study demonstrates for the first time that glucose metabolism is dramatically up-regulated in NK cells activated *in vitro* or *in vivo*. Rates of glucose uptake, glycolysis and mitochondrial respiration increase following cytokine stimulation, a response involving increased expression of glucose transporters and various glycolytic enzymes. mTORC1 regulated SREBP activity is identified as essential for IL-2/12 stimulated glycolysis in NK cells. Our data suggests that SREBP activity maintains cytosolic NAD^+, required for elevated glycolysis, via the citrate-malate shuttle. In contrast, IL-2/12/18 stimulated NK cells maintain SREBP activity and elevated glycolysis through a mechanism independent of mTORC1 activity. Activation induced glycolysis is crucial for normal NK cell function and perturbations that limit the rate of glycolysis disrupt the production of the key NK cell effector molecules IFNγ and granzyme b. This study defines the metabolic signature associated with NK cell activation and demonstrates for the first time that NK cell metabolism directly impacts NK cell effector function. This study has identified ways in which virally infected and transformed cells can evade the immune response through targeting NK metabolism; targeting mTORC1 activity or disrupting SREBP signalling and NAD^+ levels. Therefore with a greater understanding of NK cell metabolism we can strive towards improved therapeutics to restore immune homeostasis.

Summary

Natural killer (NK) cells are a type of cytotoxic lymphocyte essential to the innate immune system. The role NK cells play is analogous to that of cytotoxic T cells in the adaptive immune response in Humans. NK cells provide a swift response to viral-infected and tumour cells, with the ability to directly kill a target cell through the release cytotoxic granules, perforin and granzymes, as well as the ability to orchestrate an immune response through the release of IFNγ. NK cells have the ability to interact with cells from the innate and adaptive immune systems. NK cells have recognized stressed cells in the absence of antibodies and MHC, allowing for a much quicker immune reaction. Their function is regulated by a cohort of activating and inhibitory receptors. While the concept of immunometabolism has recently been recognised as critical in determining functions of immune cell subsets such as T cells, nothing is known regarding the regulation of NK cell metabolism. In this study we examined the changes that occur in NK cell metabolism following cytokine stimulation and the role that metabolism has in controlling NK cell function.

Our data identifies the changes that occur in NK cell glucose metabolism upon *in vitro* and *in vivo* stimulation. NK cell metabolism is dynamically regulated by multiple cytokines *in vitro*. In response to IL-2/12 cytokine stimulation, activated NK cells dramatically increase glucose uptake, and the rates of both glycolysis and OxPhos. Overall, there is a shift towards preferentially metabolising glucose to lactate, a metabolic process called aerobic glycolysis. This metabolic phenotype is also observed in NK cells stimulated with IL-2/12/18. In addition our data shows that elevated glycolysis is a prerequisite to IFNγ and granzyme b production in activated NK cells.

Our data shows that while different cytokine combinations can induce metabolic reprogramming in murine NK cells the mechanism controlling this

metabolic shift is dependent on the stimulus an NK cell receives. mTORC1 is a serine/threonine kinase with roles in regulating immunological systems. Indeed, it is becoming clear that mTORC1-regulated cellular metabolism is crucial to the immunoregulatory function of this kinase complex in immune cells. Our data also demonstrates that mTORC1 is required for the observed metabolic reprogramming in NK cells stimulated with IL-2/12 but not with IL-2/12/18.

The data shows that IL-2/12 and IL-2/12/18 stimulated NK cells increase *de novo* lipid synthesis in a SREBP dependent manner. Moreover SREBP activity is required to maintain elevated glycolysis in NK cells independently of the stimulation received. SREBP regulated glycolysis occurs independently of lipid synthesis. Our data suggests that SREBP activity is essential for maintaining the citrate-malate shuttle, required for increasing cytosolic NAD^+ levels needed to support elevated glycolytic flux. Moreover, we show that mTORC1 signalling is required for SREBP activity in IL-2/12 but not IL-2/12/18 stimulated NK cells, resulting in mTORC1 independent glycolysis in NK cells stimulated with IL-2/12/18.

In conclusion this study has widened our knowledge of NK cell glucose and lipid metabolism. Our data shows that elevated glycolysis is a prerequisite for discrete NK cell effector molecules; IFNγ and granzyme b. Furthermore, the data shows that SREBP activity is required for elevated glycolysis in cytokine stimulated NK cells. However, the mechanism controlling SREBP activity is dependent on the stimulation an NK cell receives. This study has identified ways in which virally infected and transformed cells can evade the immune response through targeting NK metabolism; targeting mTORC1 activity or disrupting SREBP signalling and NAD^+ levels. Therefore with a greater understanding of NK cell metabolism we can strive towards improved therapeutics to restore immune homeostasis.

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Abbreviations

ACAT2	acetyl-CoA acetyltransferase 2
ACC1	Acetyl-CoA Carboxylase 1
ACLY	ATP-citrate lyase
ADCC	Antibody-dependent cell cytotoxicity
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CiC	Citrate Carrier
CPT1a	carnitine palmitoyltransferase 1A
CTLs	Cytotoxic T lymphocytes
DC	Dendritic Cell
DG	Dioxyglucose
DHAP	Dihydroxyacetone phosphate
EAE	experimental autoimmune encephalomyelitis
ECAR	Extra Cellular Acidification Rate
Eef2k	Eukaryotic elongation factor 2 kinase
eIf4E	Eukaryotic initiation factor 4E
ELF	E_{74}-like factor
ERRα	Estrogen-related receptor alpha
FAO	Fatty Acid Oxidation
$FADH_2$	Flavin adenine dinucleotide
FASN	Fatty acid synthase
FOXO	Forkhead box protein O
GAP	GTPase-activating domain
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Glut 1	Glucose transporter 1
GSK	Glycogen synthase kinase
HIF1α	Hypoxia-inducible factor 1α
HMCV	Human cytomegalovirus
HMGCoS	3-Hydroxy-3-Methylglutaryl-Coenzyme A Synthase

HLH	Hemophagocytosis lymphhistiocytosis
IFNγ	Interferon gamma
INSIG	Insulin induced gene
ITAMs	Immunoreceptor tyrosine-based activation motifs
ITIMs	Immunoreceptor tyrosine-based inhibition motifs
KIR	Killer immunoglobulin-like receptor
LAT	Linker of activation
LCFAs	Long Chain Fatty Acids
Ldha	Lactate dehtdrogenase
LXR	Liver X receptor
MCMV	Murine cytomegalovirus
MHC	Major Histocompatibility Complex
mTOR	Mammalian target of rapamycin
NADH	Nictotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural Killer
NO	Nitric Oxide
OCR	Oxygen consumption rate
OxPhos	Oxidative Phosphorylation
PDCD4	Programmed cell death 4
PI3K	Phosphoinositol 3-kinase
PIM	Proviral Integrations of Moloney virus
PP2A	Protein phosphatase 2A
PPARγ	Peroxisome proliferation activated receptor γ
ROS	Reactive oxygen species
S1P	Site 1 Protease
SCAP	SREBP cleavage-activating protein
SCD1	Stearoyl-CoA desaturase-1
Slfn2	Shlafen2
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein

TCA	Tricarboxylic acid
T_{effs}	Effector T cells
TGFβ	Transforming growth factor β
T_H1	T helper cell 1
T_H17	T helper cell 17
T_H2	T helper cell 2
TIF-IA	Transcription initiation factor IA
T_{regs}	Regulatory T cells
TSC	Tuberous Sclerosis complex

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Chapter 1-Introduction

1.1. NK cells

Natural killer (NK) cells are a major component of the innate immune system, as they respond rapidly to virally infected or transformed cells in the absence of prior priming (Herberman, 1982). NK cells comprise one of the 3 major lymphocyte populations (Trinchieri, 1989) and can be identified from the other two major populations; namely B and T cells, by the absence of B-and T-cell antigen receptors (surface immunoglobulin and T cell receptor, respectively). NK cells are critical in early immune surveillance against virally infected or transformed cells and help orchestrate the resulting immune response through direct killing but also through the release of cytokines such as interferon γ (IFNγ)(Sun and Lanier, 2011).

1.1.1. NK cell regulation

The magnitude of the NK cell effector response is highly dependent on the cytokine microenvironment and interactions with other immune cells and target cells, such as T cells, macrophages and Dendritic cells (DCs) (Long, 2007).

1.1.1.1. Cytokine Microenvironment

The cytokine environment of the NK cell is of critical importance at numerous stages of its life cycle in order for the NK cell to achieve its maximum capabilities in defense against virally infected and transformed cells. The stages can be broken down into three distinct phases; NK cell development, homeostasis and activation.

1.1.1.2. NK cell Development

Cytokines of the common gamma chain (γ_c) family, which include IL-2, IL-4, IL-7, IL-9, IL-21 and IL-15, are critical for the development of lymphoid lineage cells. Mice that have lost the γ_c have impaired maturation in B, T and NK cell compartments (Sun and Lanier, 2011). It has been identified that IL-15 has an important role during NK cell development and continuous critical roles in the homeostasis and survival of peripheral NK cells (Ma et al., 2006). Receptors for IL-7, IL-2 and IL-15 are expressed at multiple stages of immature NK cell development and *in vitro* analysis has shown that IL-2, IL-7 and IL-15 can support NK cell differentiation (Loza et al., 2002, Miller et al., 1994, Mrozek et al., 1996, Williams et al., 1997). However other studies have shown that IL-2−/−, IL-2Rα−/−, IL-7−/−, and IL-7Rα−/− mice do not have significant impairments in the development of NK cells (von Freeden-Jeffry et al., 1995, Maki et al., 1996, Kundig et al., 1993, Vosshenrich et al., 2005). Therefore, while IL-7 and IL-2 are required to support NK cell differentiation *in vitro*, this role probably does not reflect their more non-essential contribution to NK cell differentiation *in vivo*. On the other hand, IL-15−/−, IL-15Rα−/− and γ_c −/− mice all have major impairments in NK cell development with significant reductions in the amount of mature NK cells. These studies suggest that *in vivo* signals from IL-15 are essential for NK cell maturation (Vosshenrich et al., 2005, Kawamura et al., 2003). Cytokine receptors that are connected to the adapter protein MyD88 are also essential for NK cell development, namely IL-1R in humans (Hughes et al., 2010) and IL-18R in the mouse (Chaix et al., 2008). Thus while IL-2, IL-7, IL-1 and IL-18 are all involved to some extent, the most important cytokine for NK cell development is IL-15.

1.1.1.3. NK cell Homeostasis

Peripheral mature NK cells can exist in the absence of immunogenic stimuli for approximately eight days (Koka et al., 2003). In addition to what is known about the development of NK cells, evidence is emerging that cytokines are also critically important in maintaining the life span of mature NK cells. Analysis of IL-2−/−, IL-2Rα−/−, IL-7 and IL-7Rα−/− mice showed normal levels of mature NK cells and thus it is unlikely that these cytokines play a role in maintaining mature NK cells. However analysis of IL-15−/− and IL-15Rα−/− mice showed a significant decrease in mature NK cell frequencies. This could be due to the inability of NK cells to mature as previously described as IL-15 is required for their development. Strikingly adoptive transfer of mature NK cells into IL-15−/− and IL-15Rα−/− mice also showed significant decreases in mature NK cell numbers (Cooper et al., 2002, Koka et al., 2003). Kota *et al.* observed that the life span of mature NK cells was dramatically reduced from 8 days to 5-6 hours. NK cells do not proliferate in the absence of IL-15 and in addition the reported loss in NK cell number also indicates that IL-15 is a critical survival cytokine for mature NK cells (Koka et al., 2003). Moreover, a recent study showed that IL-15 can also expand and cause NK cells to proliferate *in vitro* (Zhao and French, 2012).

1.1.1.4. Cytokine induced activation and function

NK cells have a range of receptors (see Fig 1.1) that can either stimulate or inhibit NK cell effector functions (Vivier et al., 2004, Bryceson et al., 2006). NK cell receptors can be activated by interactions with soluble ligands such as cytokines or by interacting with cell surface receptors. Some of the cytokines shown to be strong activators of NK cell effector functions include: type 1 IFN, IL-12, IL-18, IL-2 and IL-15 (Walzer et al., 2005, Trinchieri, 1989). IL-18 signaling is remarkably similar and as for IL-1 and IL-33 signaling in general (Weber et al., 2010) that involves MyD88- and TRAF6-dependent pathways to activate NF-κB, as well as JNK and p38 MAPK cascades (Arend et

al., 2008). Alternatively in the family of cytokines sharing the common cytokine-receptor γ_c in their receptor complexes each cytokine binds to a specific α chain, which forms a receptor complex with the γ_c. In case of IL-2 and IL-15, trimeric high affinity complexes, which include common IL-2Rβ and γ_c chains, can be formed. Each receptor complex mediates downstream signal transduction through JAK1 and/or JAK3 and different STAT molecules. Tyr-phosphorylated STAT dimers regulate transcription of specific cytokine-sensitive genes. IL-15, IL-2, IL-7 and IL-9 signal through STAT 5, IL-4 signals through STAT 6 while IL-21 signals through STAT 3 (Leonard et al., 1995). IL-15 is required for NK cell homeostasis; however studies have shown that high levels of IL-15 can also activate NK cells (Lucas et al., 2007, Marcais et al., 2014). IL-2 has been shown to promote NK cell proliferation, cytotoxicity and, to a limited extent, secretion of cytokines (Trinchieri, 1989). IL-2 and IL-15 have been shown to synergize with IL-12 to augment NK cell function *in vitro*, while IL-12/15 stimulation of NK cells *in vivo* improves inflammatory responses to tumour surveillance and virally infected cells (Nguyen et al., 2002, Fawaz et al., 1999, Gri et al., 2002, Comes et al., 2002, Biber et al., 2002). Transforming growth factor (TGF) β has been shown to negatively regulate NK cell function (Laouar et al., 2005).

NK cells can also be activated through their cell surface receptors, (see Fig. 1.1). For example, many viruses activate NK cells through the mouse Ly49H activation receptor that recognizes a cytomegalovirus-encoded ligand (m157) (Arase et al., 2002, Smith et al., 2002). NKp46 receptor has also been reported to interact with derived hemagglutinins from influenza and parainfluenza and Sendai viruses (Mandelboim et al., 2001, Arnon et al., 2001). The NK cell surface receptor FcgRIIIA otherwise known as CD16 is able to stimulate antibody-dependent cell cytotoxicity (ADCC) and production of cytokines upon binding to the Fc region of some IgG antibodies (Mandelboim et al., 1999). CD16 is a low affinity receptor coupled with CD3ζ and FcRγ (Lanier et al., 1991) signal transduction polypeptides and

intracytoplasmic immunoreceptors tyrosine-based activation motifs (ITAMs) (Vivier et al., 1991). The natural cytotoxic receptors (NKp46, NKp44 and NKp30) are also strong activators connected to ITAM-bearing CD3ζ, FcRγ, or DAP12 molecules (Moretta et al., 2001). NKp30 has number of other ligands it can bind to induce NK cell activation such as B7-H6 (Fiegler et al., 2013) and BAT3 that are released by DC (Simhadri et al., 2008).

NK cells have adopted the ability to use several of their activating receptors to detect increasing levels of self-molecules upon cellular stress (Bottino et al., 2005). For example, the NK2GD receptor interacts with various ligands (MICA/B) present at low levels in most tissues but which are upregulated upon cellular stress, for example after initiation of the DNA damage response (Raulet and Guerra, 2009). B7-H6 is a ligand expressed on tumour cells but not on healthy cells and it binds NKp30 cell surface receptors inducing NK cell activation. NK cells have a number of MHC (Major Histocompatibility Complex) class I inhibitory receptors namely killer immunoglobulin-like receptors (KIR) in humans and lectin-like Ly49 molecules in mice while CD94/NKG2A heterodimers are present in both species (Karlhofer et al., 1992). However, the human non-classical MHC class I molecule HLA-E is a ligand for both CD94/NKG2A and the activating receptor CD94/NKG2C (Wada et al., 2004). Moreover, studies have shown that KIR can also act as an activating receptor for NK cells by recognition of HLA-A (Liu et al., 2014). Inhibitory receptors mediate their activity by signalling through intracytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) (Vivier et al., 2004). Studies have shown that NK cells can detect a reduced amount of major histocompatibility complex (MHC) class I ("missing self"), that can occur in virally infected or transformed cells (Karre et al., 1986). Cells that are "in distress" that have down regulated MHC class 1 molecules and/or increased stress-induced self-molecules such as NKG2D ligands are selectively killed (Raulet and Guerra, 2009). NK cells spare healthy cells that

have sufficient levels of class I molecules and low levels of the stress-induced self-molecules.

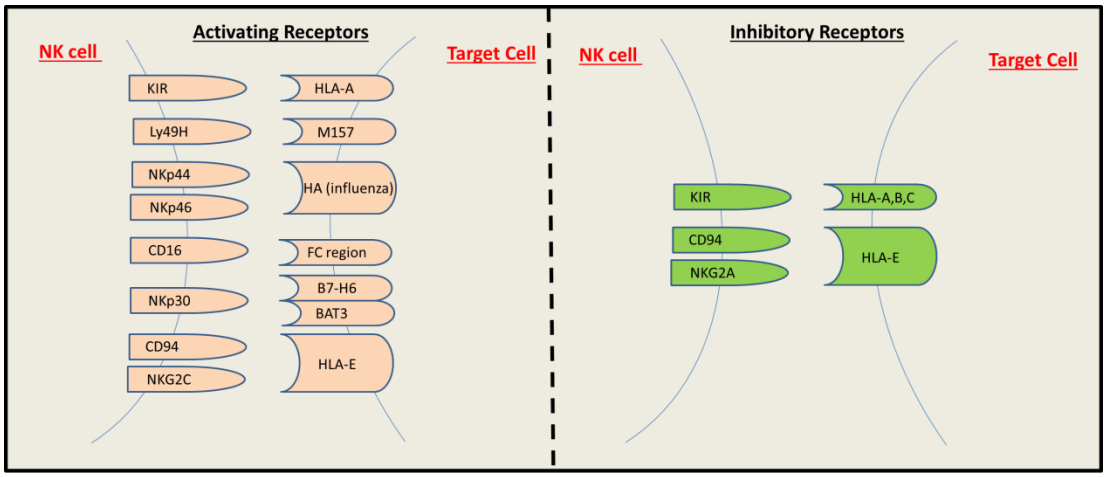

Figure 1.1. Natural Killer cell activating and inhibitory receptors. NK cells are constantly sensing the immune microenvironment for activation signals to help in the fight against virally infected or transformed cells. NK cells have a wide variety of activating receptors that can allows them to either sense signals sent from other immune cells or signals sent directly from virally infected or transformed cells. Moreover, NK cells also have a number of inhibitory receptors that preventing them from over activation and from attacking the body's own cells. Some of the NK cell's activating and inhibitory receptors are outlined in the above figure (Vivier et al., 2011).

1.1.1.5. Immune cell activation of NK cells

Numerous studies have shown that NK cells can interact with cells of both the innate and adaptive immune systems. In innate immunity macrophages (Mortier et al., 2009) and DCs (Gerosa et al., 2002, Fernandez et al., 1999, Ferlazzo et al., 2002, Piccioli et al., 2002, Andrews et al., 2003) interact with mature NK cells to modulate their functions. Moreover, DCs and macrophages are able to activate NK cells through the release of the

proinflammatory cytokines; IL-12 and IL18 (Fig 1.2) (Andrews et al., 2003). It has been suggested that DCs and macrophages can also activate NK cells through their expression of IL-15Rα (Koka et al., 2004, Mortier et al., 2009). DCs are able to cross-present IL-15 bound to their IL-15 receptor, resulting in increased cytolytic activity of NK cells. Furthermore, IL-15Rα synergizing with IL-12 has been shown to enhance IFNγ production by NK cells (Koka et al., 2004). Plasmacytoid DCs increase NK cell proliferation, cytotoxicity and IFNγ production through the secretion of type 1 interferon and IL-12 (Fig 1.2) (Dalod et al., 2002). Activated NK cells, which typically recognize MHC class I–negative targets, selectively kill MHC class I–expressing immature DCs. This recognition relies almost entirely on the NKp30 activating receptors. Mature DCs trigger the same receptor, but escape NK lysis by high MHC class I surface expression (Ferlazzo et al., 2002)

NK cells are generally considered part of the innate immune system. However, over the past few years, evidence has accumulated suggesting that NK cells have certain features characteristic of the adaptive immune system. More recently, it has been appreciated that NK cells can undergo clonal expansion and function in parallel with the adaptive immune system. NK cells reportedly mount antigen-specific recall responses against certain viruses. For instance a study by Daniels *et al* suggests a subset of NK cells express the Ly49H receptor, which binds to the MCMV protein m157. M157 is displayed on the surface of MCMV infected cells (Dokun et al., 2001, Daniels et al., 2001). Moreover, $Ly49H^+$ NK cells have been shown to undergo clonal expansion, and the proliferation was antigen-specific as infection with a MCMV mutant lacking m157 did not cause expansion of $Ly49H^+$ NK cells (Dokun et al., 2001, Sun et al., 2009, Bubic et al., 2004).

From these mouse studies, human NK cells were tested for their ability to mount recall responses. Studies done *in vitro* have shown that cytokine stimulation of human NK cells can heighten their responsiveness even after

the NK cell return to resting state, similar to what has been shown in mice (Romee et al., 2012). Moreover, NK cells that express the germline-encoded NKG2C receptor are typically absent or rare in the blood of healthy HCMV-seronegative individuals, but appeared in increased numbers in patients undergoing acute HCMV infection, and then diminish partially in frequency after the resolution of the acute phase (Guma et al., 2004, Lopez-Verges et al., 2011, Foley et al., 2012, Della Chiesa et al., 2012). Interestingly, this increase in NKG2C^{+} NK cells was not observed in other infections, such as HSV-2 infection (Bjorkstrom et al., 2011). Indeed numerous recent studies have shown that certain subsets of NK cells, such as FcRγ-deficient NK cells, undergo epigenetic alterations to maintain memory like NK cells (Lee et al., 2015, Schlums et al., 2015). Human NK cells also exhibit memory-like properties, induced by preactivation with IL-12, IL-15, and IL-18. These cells later exhibit enhanced functionality upon restimulation (Cooper et al., 2009). Recent research shows that IL-12, IL-15, and IL-18 preactivation induces a rapid and prolonged expression of CD25, resulting in a functional high-affinity IL-2 receptor that confers responsiveness to picomolar concentrations of IL-2 (Leong et al., 2014). Numerous studies have shown that NK cells can also respond to key cytokines associated with adaptive immunity such as IL-2 produced by T lymphocytes (Sun et al., 2011). Additionally, a key mechanism through which regulatory T cells control NK cell responses is through sequestering IL-2 (Kerdiles et al., 2013, Sitrin et al., 2013, Gasteiger et al., 2013).

1.1.2. Role in the Immune system

NK cells play a critical role in the clearance of infection as well as tumour surveillance. As well as their involvement in protection against viruses and tumours NK cells are able to regulate other immune cells in multiple ways.

1.2.1.1. Anti-tumour NK cell immunity

Numerous *in vivo* and *in vitro* studies have reported that tumour cells are recognized as NK cell targets (Trinchieri, 1989). Murine NK cells play a major role in the *in vivo* rejection of transplanted tumours, involving the presence or absence of NK cell receptor ligands expressed on tumour cells (Stewart and Vivier, 2008). Absence or reduced levels of MHC class I expression can leave tumour cells at risk for NK cell-mediated killing. Examples include the RMA/S MHC class 1-deficient mouse lymphoma cells (Karre et al., 1986)) or tumour cells with increased expression of NKG2D ligands (for example, H60, Rae1β, Rae1δ, Rae1γ, Mult-1 (Diefenbach et al., 2001, Cerwenka et al., 2001)) as well as CD70, the CD27 ligand (Kelly et al., 2002). NK cell-mediated killing of tumour cells induces the subsequent maturation of tumour-specific T cell responses to the parental tumour cell. This process occurs when cellular debris from tumour cells killed by NK cells is processed by antigen presenting cells (APCs) which in turn present antigens on MHC molecules to naïve T cells (Diefenbach et al., 2001, Kelly et al., 2002). This observation has caused increased interest as it has huge potential for NK cell-mediated immunotherapeutics. Other studies have shown that the NKG2D pathway has a protective role in the development of methylcholanthrene-induced sarcomas (Smyth et al., 2005). In addition to their endogenous protective duty in tumour models, NK cells are also mediators of the anti-tumour effects of numerous cytokines, including IL-2, IL-12, IL-18 and IL-21 (Stewart and Vivier, 2008).

Immunotherapy with NK cells has been limited by the inability to obtain sufficient numbers of pure NK cells suitable for manipulation and expansion. However numerous studies have developed strategies were NK cells are expanded *ex vivo* before adoptive transfer procedures (Lim et al., 2013, Klingemann and Martinson, 2004, Berg et al., 2009). Interestingly, certain preactivated NK cells provide a better immunotherapeutic response against tumours. IL-12/15/18 preactivated NK cells were shown to persist with

sustained effector function *in vivo* leading to substantially reduced growth of established mouse tumours (Ni et al., 2012). NK cells express CD16 on their surface which enables them to recognize Ab-coated target cells and trigger NK cell-mediated ADCC (antibody-dependent cell-mediated cytotoxicity), resulting in rapid NK-cell activation and degranulation (Borghaei et al., 2009, Sconocchia et al., 1997). Multiple alterations to antibody structure, including class switching, humanization and point mutations to reduce complement activation, have been generated to increase NK-cell ADCC function while reducing antibody-induced toxicity (Alderson and Sondel, 2011, Sorkin et al., 2010). Numerus examples exist where ADCC has resulted in clearance of tumour cells. For instance, A CD19-specific mAb with increased CD16-binding affinity significantly increased NK cell-mediated ADCC, thus efficiently clearing malignant B cells in cynomolgus macaques *in vivo* (Horton et al., 2008, Zalevsky et al., 2009).

1.2.1.2. Anti-viral NK cell immunity

Mouse model studies have shown that NK cells control numerous viral infections including; herpes simplex virus-1, influenza and ectromelia poxvirus (Lee et al., 2007). The effects of NK cells in viral infections have been best studied in murine models of herpesvirus and murine cytomegalovirus (MCMV). There studies showed an increased susceptibility or resistance after NK cell depletion or adoptive transfer, respectively. In addition, mice with decreased IFNγ production were also more susceptible to MCMV infection (Lee et al., 2007, Scalzo et al., 2007). In C57/BL6 mice, MCMV infected cells are specifically recognized by the interaction between a CMV-encoded cell surface molecule, m157 and the activating NK cell receptor Ly49H (Smith et al., 2002, Arase et al., 2002). MCMV have acquired mechanisms of evasion further highlighting the importance of NK cells during infections. The mechanism of evasion includes the avoidance of NK cell stimulation by down regulating NKG2D ligands (MCMV m145, m152, m155) and the expression of MCMV-encoded decoy ligands including MHC class I

homologs that stop NK cell function (MCMV m144) (Vidal and Lanier, 2006). The role of NK cells in immunopathology has become of major interest over the past number of years. NK cell depleted mice have been shown to have accelerated pathologies when infected Theiler's virus, to induce, encephalitis, or coxsackie B3, inducing myocarditis (Paya et al., 1989, Fairweather et al., 2001). Mouse NK cells accelerate the initiation of $CD8^+$ T cell responses and dampen the type 1 IFN-dependent immunopathology induced by uncontrolled virus diffusion (Robbins et al., 2007). These studies have shown that acquired or genetic deficiencies in cell-mediated cytotoxicity are often associated with HLH-like syndromes. These syndromes involve over activation of macrophages and they develop upon microbial infection in mice and in humans (Jordan et al., 2004). NK cell cytotoxicity seems to be involved in destroying overstimulated macrophages (van Dommelen et al., 2006). Overall NK cells play a key role in the direct eradication of viruses while also decreasing the risk of developing inflammatory disorders by removing activated immune cells.

1.2.1.3. Regulation of immune responses by NK cells

Various subsets of the innate and adaptive system are able to enhance NK cell activation and function. However, NK cells also act as regulatory immune cells with an impact on other cell types including T cells, B cells and DCs. NK cells encounter DCs in secondary lymphoid tissue organs and peripheral tissues and exert their effects in two distinct ways (Walzer et al., 2005, Moretta et al., 2006, Degli-Esposti and Smyth, 2005). NK cells can destroy immature DCs in humans and in mice and thus influence DC homeostasis, but also potentially limiting DC-based vaccination efficacy (Piccioli et al., 2002, Hayakawa et al., 2004). Alternatively, the killing of target cells by NK cells can result in the cross-presentation of antigen from apoptotic NK cell targets by DC subsets. This NK cell-mediated cytotoxicity can induce potent antigen-specific adaptive immunity involving $CD8^+$ T cells, $CD4^+$ T cells and immunoglobulin G (Krebs et al., 2009). NK cells rapidly eliminate donor-

derived DCs present in the graft in mouse allogeneic solid organ transplantation settings, which indirectly suppresses alloreactive effector T cell maturation (Yu et al., 2006). Alternatively NK cells can promote the maturation of DCs through the release of IFNγ and TNFα, which in turn can lead to the activation of NK cells through IL-12 (Fig 1.2) (Walzer et al., 2005, Moretta et al., 2006, Degli-Esposti and Smyth, 2005). In a similar manner NK cells can promote macrophage activation (Vivier et al., 2008).

NK cells can directly act on B and T cells and help to direct the adaptive immune response. In an inflamed lymph node, $CD4^+$ T helper type 1 (T_H1) cells can be primed by NK cell production of IFNγ (Fig 1.2) (Martin-Fontecha et al., 2004, Morandi et al., 2006). Alternatively NK cells can destroy activated T cells, which express insufficient levels of MHC class I molecules (Lu et al., 2007). Blockage of the CD94-NKG2A inhibitory receptors leads to NK cell cytotoxicity against $CD4^+$ T cells, an approach that could be adapted to stop $CD4^+$ T cell-dependent autoimmunity (Lu et al., 2007). A recent study identified a mutant NK cell with increased resistance to viral infections because of the presence of hyperresponsive NK cells. They determined that a loss-of-function mutation in the Ncr1 gene encoding the activating receptor NKp46 led to this phenotype (Narni-Mancinelli et al., 2012). Down-regulation of NK cell activity by NKp46 was associated with the silencing of the Helios transcription factor in NK cells. However, the study also showed that NKp46 was critical for the subsequent development of antiviral and antibacterial T cell responses, which suggests that the regulation of NK cell function by NKp46 allows for the optimal development of adaptive immune responses (Narni-Mancinelli et al., 2012). Soderquest and colleagues recently showed that NK cells inhibited the formation of memory antigen-specific $CD8^+$ T cells. NK cell deletion resulted in a significantly higher number of memory Antigen-specific $CD8^+$ T cells, leading to more effective control of tumors carrying model antigens (Soderquest et al., 2011). NK cell-based anti-inflammatory regulation of the immune system has been suggested to play a protective role

in multiple autoimmune disorders (Johansson et al., 2005). Further evidence to support this hypothesis comes from studies showing NK cell suppression of autoreactive B lymphocytes (in Fas-deficient mice) as removal of NK cells rapidly increases the severity of autoimmunity both *in vivo* and *in vitro* (Takeda and Dennert, 1993).

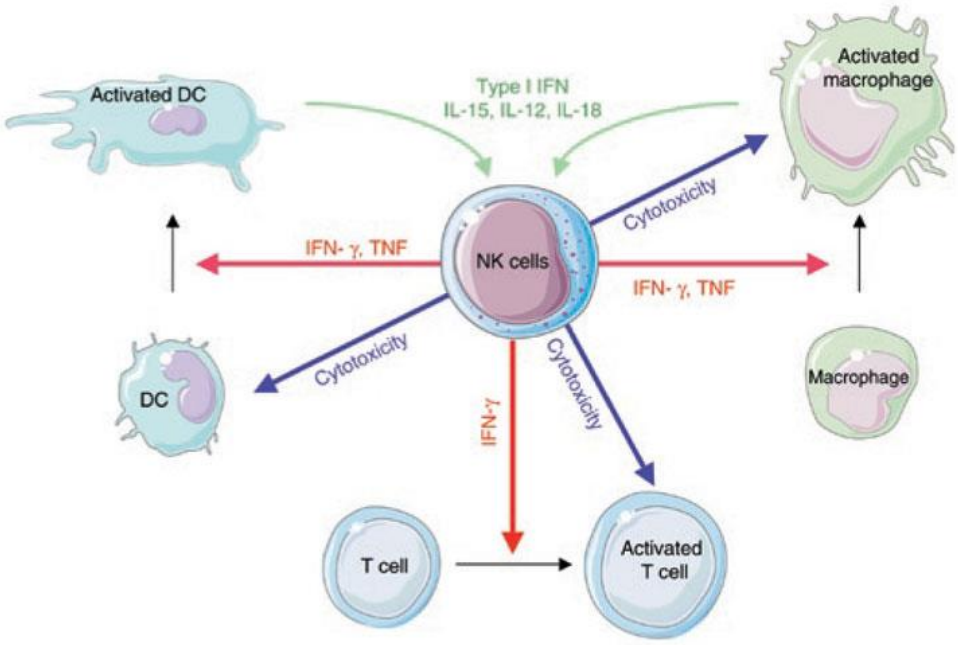

Figure 1.2. NK cell interactions with other cells in the immune system. NK cells are highly regulated by other cells in the immune system. It has been shown that macrophages and DCs have the ability to prime NK cells, while NK cells too have the ability to influence other immune cells. IFNγ a key effector molecule produced by NK cells has the ability to induce the activation of multiple innate and adaptive immune cells (as shown above), in conjunction with TNFα (Vivier et al., 2008).

1.2. Immunometabolism

It has been widely established that sufficient access to appropriate nutrients is central to controlling the function of any cell. Throughout the life cycle of a cell its metabolic needs change, whether it be for growth, proliferation or

even to die. In recent years, it has become clear that regulating the uptake of nutrients by immune cells is an essential requirement for controlling their function and numbers (Pearce and Pearce, 2013).

1.2.1. Immune cell metabolism facilitates diverse cellular activities

During an immune response lymphocytes can encounter different environments containing diverse conditions of nutrient and oxygen availability. In addition, in response to activation, immune cells often dramatically change their functional activities. Indeed, lymphocytes change from relatively inert cells to cells engaging in robust growth and proliferation, in addition to their production of large quantities of effector molecules such as cytokines or antibodies. Therefore activated lymphocytes encounter significant metabolic pressures that are effectively managed by immune cells due their ability to dynamically reprogramme their cellular metabolism.

1.2.1.1. Cellular metabolism for ATP synthesis vs biosynthesis

Adenosine triphosphate (ATP) is the key molecule that provides energy for cellular processes. Maintaining cellular ATP levels is essential for bioenergetic homeostasis and the survival of all cells, including lymphocytes. Glycolysis and oxidative phosphorylation (OxPhos) are two metabolic pathways that together efficiently convert glucose, the principal fuel source for mammalian cells, into ATP. Glucose is metabolised to pyruvate by glycolysis, which involves a series of enzymatic steps producing 2 molecules of ATP per molecule of glucose. Pyruvate can then be fully oxidised to CO_2 by the Krebs cycle, following its transport into the mitochondria. The Krebs cycle generates reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2). These two key reducing agents fuel OxPhos by feeding electrons into complex I and II of the electron transport

chain respectively, leading ultimately to the reduction of O_2 to H_2O. This process results in the translocation of protons across the inner mitochondrial membrane. The proton gradient that is generated is used to drive ATP synthase, an enzyme that converts ADP to ATP, to generate a net of approximately 36 molecules of ATP per molecule of glucose (Fig 1.3). In most cells glycolysis and the Krebs cycle are interconnected when pyruvate is converted to acetyl-CoA, which enters the Krebs cycle (Pearce and Pearce, 2013). To varying degrees cells have the potential to metabolize other substrates such as fatty acids via β–oxidation or glutamine via glutaminolysis to replenish the Krebs cycle and fuel OxPhos.

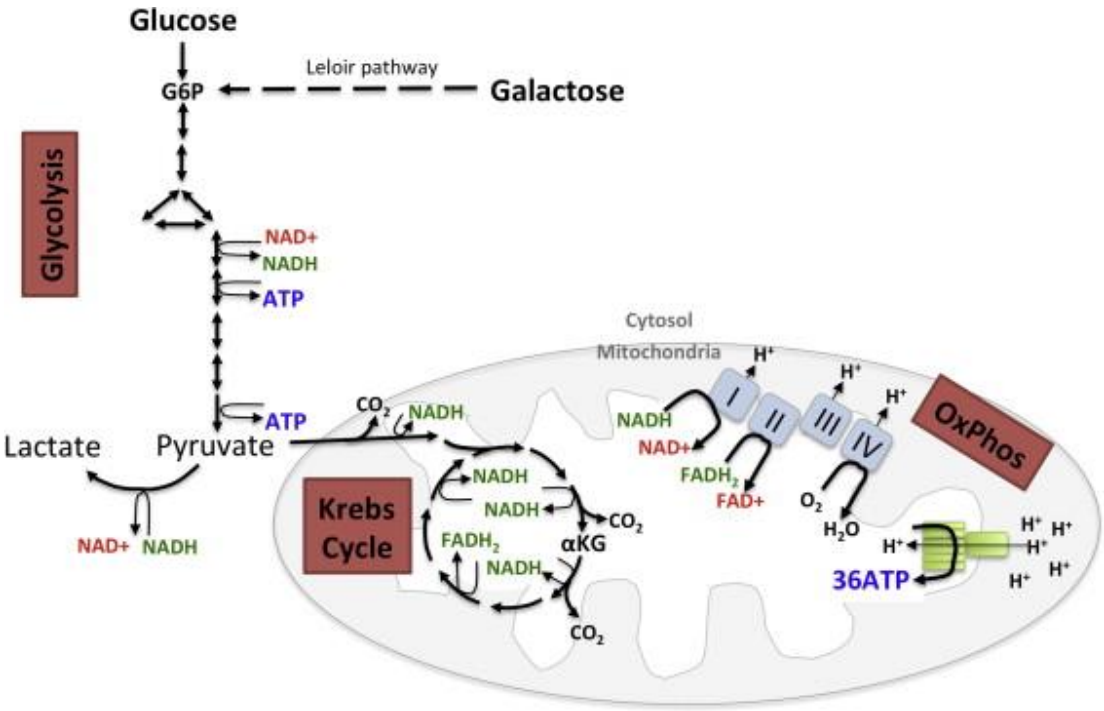

Figure 1.3. ATP production by glycolysis and oxidative phosphorylation.

Glucose is an important fuel for the generation of ATP to satisfy the bioenergetics demands of the cell. The first step of glycolysis involves the phosphorylation of glucose by hexokinase to generate glucose-6-phosphate (G6P). Glycolysis then proceeds as a series of enzymatic reactions that ultimately generate the final product, pyruvate. Depending on cellular activity, pyruvate can have multiple fates in the cell, one of which is to be

completely oxidised to CO_2 in the mitochondria for the efficient production of ATP. Alternatively, under anaerobic conditions, pyruvate can be converted to lactate which is secreted out of the cell. Pyruvate is also converted to lactate during aerobic glycolysis, a metabolic signature adopted by cells engaging robust growth and proliferation. The alternative fuel galactose can be metabolised by the Leloir pathway to generate G6P that is then further metabolised by glycolysis (Donnelly and Finlay, 2015).

Relatively inert or quiescent cells that are not engaging in cellular growth or proliferation have little demand for biosynthetic processes other than for basic housekeeping purposes. For this reason ATP alone is largely sufficient to sustain these cells. Usually, normal tissue is well vascularised and replete with nutrients and oxygen. However, during an immune response, conditions in the local immune microenvironment can often be significantly less accommodating due to competition for nutrients, oxygen and growth factors. Under hypoxic conditions cells can maintain ATP levels by engaging in anaerobic glycolysis, increasing glucose uptake and glycolytic flux and converting the pyruvate generated to lactate. A common feature of proinflammatory immune cells is that they adopt a distinct metabolic signature termed 'aerobic glycolysis'. Aerobic glycolysis involves high rates of glycolysis, but rather than shuttling the glucose derived pyruvate into the mitochondria for OxPhos, a large proportion of the pyruvate is converted to lactate in the cytosol before it is secretion from the cell. This is similar to what is done by cells under anaerobic conditions when OxPhos is not possible. However, during aerobic glycolysis there is an abundant supply of oxygen and ATP can still be efficiently made via OxPhos, yet pyruvate is still preferentially converted to lactate (Vander Heiden et al., 2009).

Aerobic glycolysis is adopted by cells engaging in robust growth and proliferation because it provides the biosynthetic precursors that are essential for the synthesis of nucleotides, amino acids and lipids (Hume et al., 1978, Wang et al., 1976). Many of the intermediates of the glycolytic pathway

act as a source of carbon that feeds into a range of biosynthetic pathways. For instance, glucose-6-phosphate, which is generated by the first step of glycolysis, can be used by the pentose phosphate pathway to generate ribulose-5-phosphate which in turn can be used for the synthesis of nucleotides (Fig 1.4). Therefore, for cells engaged in aerobic glycolysis the primary function of glucose has shifted from a fuel to generate energy, i.e. ATP, to a source of carbon that can be used for biosynthetic purposes (Hume et al., 1978).

If aerobic glycolysis is adopted to facilitate an increase in biomass it seems rather wasteful to secrete large quantities of lactate, a source of carbon. However, the secretion of lactate is essential to maintain high rates of glycolysis for a number of reasons. Firstly, glycolysis can generate pyruvate much faster than the mitochondria and the Krebs cycle can use it. Intuitively, this will lead to a build-up of pyruvate resulting in product inhibition of the final enzyme of glycolysis, pyruvate kinase. Through a knock-on effect upstream in the glycolytic pathway, the net result would be an inhibition of glycolytic flux. Therefore, one reason to convert pyruvate to lactate is to prevent a build-up of pyruvate and allow glycolysis to operate at a higher rate than OxPhos (Vander Heiden et al., 2009). Secondly, NAD^+ is an essential cofactor for the enzymatic reaction catalysed by the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). High rates of glycolysis will result in all NAD^+ being converted to its reduced form, NADH. In the absence of NAD^+, GAPDH activity is inhibited. The enzyme that converts pyruvate to lactate, lactate dehydrogenase, also oxidizes NADH to NAD^+, thus regenerating cytoplasmic NAD^+ levels to sustain GAPDH activity and glycolytic flux (Vander Heiden et al., 2009). Glucose metabolism can be programmed for efficient ATP synthesis or to enable biosynthetic processes, and these distinct metabolic settings are important in facilitating the diverse activities of naïve, effector and regulatory lymphocyte subsets.

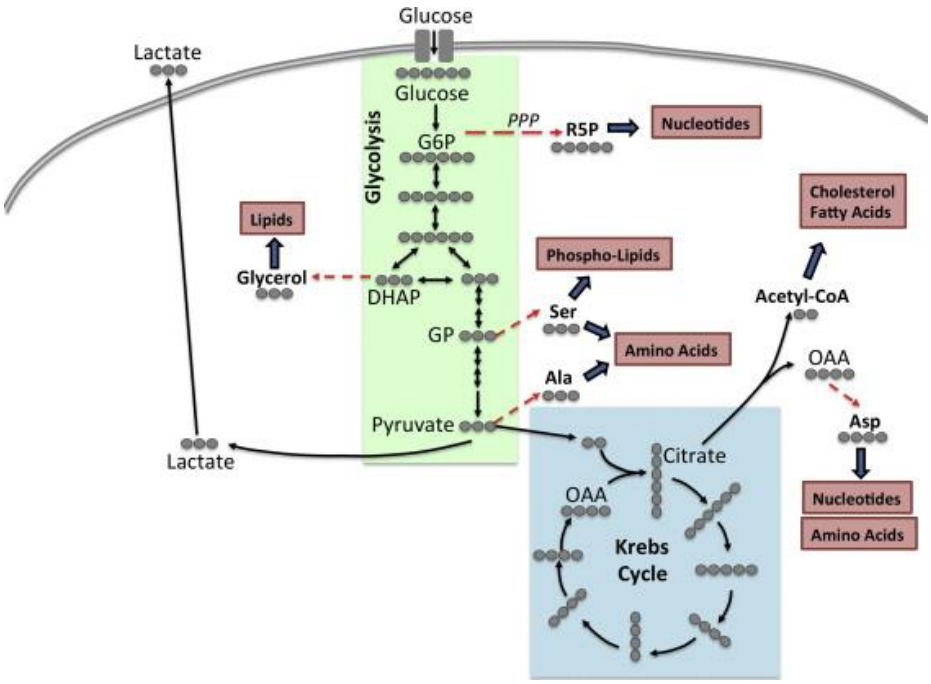

Figure 1.4. Glucose can be used as a source of carbon for biosynthetic pathways. Cells that are engaged in robust growth and proliferation, or indeed the production of large amounts of biomolecules for secretion, have a large biosynthetic demand. Glucose taken into the cell via glucose transporters can be converted through glycolysis into various biomolecules that serve as carbon sources for various biosynthetic pathways. Glucose-6-phosphate (G6P) can enter the pentose phosphate pathway (PPP) to generate ribulose-5-phosphate (R5P) that is used for nucleotide synthesis. Dihydroxyacetone phosphate (DHAP) can be converted to glycerol, which is used in the synthesis of lipids. 3-phosphoglycerate (GP) can be converted to the amino acid serine (Ser) for protein synthesis, and the production of phospholipids and other amino acids. Pyruvate can be converted to the amino acid alanine (Ala) for protein synthesis and the production of other amino acids. Glucose can also be converted into acetyl-CoA and oxaloacetate (OAA) via citrate in the Krebs cycle. Acetyl-CoA is used for the production of cholesterol and fatty acids for lipid synthesis. OAA can be converted to the amino acid aspartate (Asp) for protein synthesis, and the generation of nucleotides and other amino acids (Donnelly and Finlay, 2015).

1.2.1.2. T cell metabolism meets function

Immune cell activation is accompanied by substantial changes in cellular activities. Metabolic changes occurring in lymphocytes are best characterised in T cells. For example, a shift from oxidative to glycolytic glucose metabolism is central to the successful differentiation of a naïve $CD8^+$ T cell into a functional CTL (Finlay et al., 2012). Relatively inert naïve T cells that are not engaging in cellular growth or proliferation have little demand for biosynthetic processes other than for basic housekeeping purposes. For this reason ATP alone is largely sufficient to sustain these cells. Following TCR activation T cells undergo extensive changes in function that are reflected by equally dramatic metabolic adjustments including increased rates of glucose uptake and glycolysis, producing large amounts of lactate (Wang et al., 2011a). This is accompanied by increased expression of important nutrient receptors including; CD71 (transferrin receptor), glucose transporter glut 1 and amino acid transporters (Jacobs et al., 2008, Kelly et al., 2007, Fox et al., 2005a, Marko et al., 2010, Vander Heiden et al., 2009). Activated T cells engage in robust cellular growth and significantly increase in size before initiating rapid cellular proliferation (Wang et al., 2011a, Fox et al., 2005a). In support of these cellular activities is the provision of sufficient biomolecules, (amino acids, nucleotides, lipids) to feed the biosynthesis of new cellular components. Therefore, over the lifespan of a T cell the requirement of cellular metabolism changes from primarily generating ATP, in naïve T cells, to the generation of sufficient ATP plus large amounts of biomolecules for the generation of biomass, in activated T cells.

Studies have identified that different immune cell subsets adopt different metabolic profiles. $CD8^+$ T cells have the ability to generate long lasting antigen-specific memory T cells with distinct metabolic demands in comparison to their activated effector counterparts, in that they rely on mitochondrial OxPhos for development and persistence (Vander Heiden et al., 2009). As well as sustaining the glycolytic switch, IL-2 has been shown to

control the transcriptional program that determines $CD8^+$ T cell differentiation and promotes effector CTL differentiation at the expense of memory cell formation (Kalia et al., 2010, Pipkin et al., 2010, Macintyre et al., 2011). The balance between effector and regulatory $CD4^+$ T cells is critical in maintaining a balance between protective immunity and regulation of inappropriate inflammation (Kronenberg and Rudensky, 2005). Therefore, it is striking to see that $CD4^+$ T cell have distinct metabolic phenotypes between effector T cell subsets (T_H1, T_H2 and T_H17) and Treg cells (Michalek et al., 2011a). Regulatory T (Treg) cells predominantly use mitochondrial OxPhos for development and survival (Michalek et al., 2011a), while the development of T helper 17 (T_H17) relies on increased expression of glut 1 as well as elevated levels of glycolysis and glucose uptake (Shi et al., 2011). However, Tregs are not metabolically inactive as they exhibited increased mitochondrial membrane potential, oxidized lipids at a higher rate and also have elevated glucose metabolism compared to naïve T cells (Michalek et al., 2011a).

Studies have shown that limiting the availability of glucose and so the rate of glycolysis in activating T cells has a substantial impact on their growth and proliferation as well as the expression of key effector molecules such as IFNγ, perforin and granzymes (Finlay et al., 2012, Jacobs et al., 2008). While changes in metabolic profiles in T cells are well documented, it is only becoming evident how these completely distinct metabolic phenotypes impact on their differentiation and function. Recent studies have introduced the concept that metabolic pathways in immune cells may have a more direct and fundamental role in determining immune cell function (Ho et al., 2015, Chang et al., 2013, Tannahill et al., 2013).

While the exact mechanisms underlying glycolytic regulation of T cell function are still largely unknown, there is evidence that glycolytic enzymes can have additional roles in the cell that are independent of their enzymatic

activity. This has been 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 IFNγ-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 glyceraldehdye-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 IFNγ and IL-2 mRNA, thus providing a direct link between the rate of glycolysis and $CD4^+$ T cell effector function (Chang et al., 2013). This study suggested 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 IFNγ and IL-2 mRNA and repress their translation (Chang et al., 2013). However, the mRNA of other effector molecules regulated by glycolysis, such as granzyme B, do not contain AU-rich regions in their 3'UTR arguing that there are additional mechanisms linking glycolysis to lymphocyte function. Indeed, GAPDH is not the only metabolic enzyme that acts as a RNA binding protein. Interestingly, this protein can switch between functions based on the presence or absence of iron, with iron starvation promoting IRP1 and RNA binding (Hentze et al., 2004). In fact, a surprisingly large number of metabolic enzymes have mRNA binding capabilities. A systematic and unbiased proteomics approach identified over 800 proteins

that interact with mRNA (Castello et al., 2012). Included in these proteins are numerous glycolytic enzymes (GAPDH, pyruvate kinase, lactate dehydrogenase, enolase and aldolase) as well as enzymes involved in other aspects of cellular metabolism such as the Krebs cycle and lipid synthesis. This research revealed the abundant potential for metabolic pathways to directly impact upon cellular functions through controlling mRNA and thus protein expression. The various metabolic profiles of immune cells result in different levels of important metabolites, which are now emerging to play a role in T cell function. The glycolytic intermediate phosphoenolpyruvate (PEP) has been recently reported to be important in sustaining TCR signalling and T cell effector functions. PEP inhibits Ca^{2+} uptake into the endoplasmic reticulum resulting in elevated cytoplasmic Ca^{2+} levels and NFAT (Nuclear factor of activated T-cells) signalling (Ho et al., 2015). Mitochondrial reactive oxygen species (mROS) are also important for maximal TCR signal transduction. T cells that cannot produce mROS fail to activate nuclear NFAT, produce IL-2 or engage in proliferative expansion (Sena et al., 2013). Therefore, there are clear direct links between metabolism and immune cell function being identified with the potential for more as the research in immunometabolism progresses.

1.2.1.3. Regulation of T cell energy metabolism

Numerous studies have started to explore the mechanisms responsible for the changes in metabolism that occur as T cells are activated. A key transcription factor involved in T cell metabolism is c-myc. The oncogenic transcription factor c-myc plays a role in multiple human cancers and regulates numerous genes important in cell cycle and metabolism. C-myc has been shown to regulate glucose and glutamine metabolism and mitochondrial biogenesis. More specifically it binds to the promoter of multiple glycolytic genes including glut 1, lactate dehydrogenase A (LDHA), PKM2 and hexokinase and others to induce their expression (Dang et al., 2009). In T cells, c-myc is induced during activation and promotes T cell

growth and entry into the cell cycle (Lindsten et al., 1988, Kelly et al., 1983, Wang et al., 2011a, Chou et al., 2014). A c-myc-dependent global metabolic transcriptome drives metabolic reprogramming in activated T cells, resulting in activation-induced glycolysis and glutaminolysis (Wang et al., 2011a).

Another transcription factor with an important role in regulating cellular metabolism in T cells is HIF (Hypoxia-inducible factor) 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) (Stroka et al., 2001). HIF binds to hypoxia response elements (HRE) in target genes to promote their transcription. Many HIF target genes are associated with glycolysis (Pawlus and Hu, 2013). There are three isoforms of the HIF alpha subunit; HIF1α, HIF2α and HIF3α. All isoforms are similar in their binding sites and function (Pawlus and Hu, 2013). Moreover, cells negative for one isoform have shown a compensatory increase in other isoforms; further supporting their shared homology (Gu et al., 1998). The transcription factor HIF1α has also been implicated in the regulation of T cell metabolism. While the initial glycolytic switch induced in response to antigenic stimuli is regulated by c-myc, a recent study has reported that HIF1α is required for the long term commitment to elevated glucose uptake and glycolysis in CTLs (Fig 1.5) (Finlay et al., 2012). T cells deficient for HIF1α fail to sustain increased levels of glycolysis in the presence of IL-2. Furthermore, migratory patterns of T cells in the absence of HIF1α, reflect those of a non-glycolytic naive T cell; in response to IL-2 stimulation CD62L expression on T cells remains high in the absence of HIF1α activity (Finlay et al., 2012). HIF1α-dependent glycolytic pathway also orchestrates a metabolic checkpoint for differentiation of T_H17 and Treg cells. Thus blocking HIF1α induced aerobic glycolysis inhibited T_H17 development while promoting Treg generation (Shi et al., 2011). Moreover, the aryl-hydrocarbon receptor (AhR), in combination with HIF1α controls glycolytic metabolism in Tr1 regulatory T cells. Indeed, AhR is important for

Tr1 regulatory T cell differentiation and directly regulates the expression of IL10 and IL-21 cytokines in these cells (Mascanfroni et al., 2015, Apetoh et al., 2010). In addition, AhR promotes T_H17 differentiation while negatively impacting upon Treg differentiation. It is also required for the production of the T_H17 cytokines IL17 and IL-22 (Kimura et al., 2008, Veldhoen et al., 2008, Quintana et al., 2008).

In $CD8^+$ T cells there are additional layers of complexity in the regulation of aerobic glycolysis. The transcription factors IRF4 and AP4 also have important roles for initiating and sustaining glycolysis, respectively. IRF4 is induced by antigen receptor stimulation and promotes the expression of HIF1α mRNA. IRF4 deficient $CD8^+$ T cells fail to undergo glycolytic reprogramming, while AP4 deficient $CD8^+$ T cells initially up-regulate glycolysis normally but fail to sustain this aerobic glycolysis for the duration of the T cells response (Chou et al., 2014, Man et al., 2013). Mice deficient for AP4 show a significant decrease in the expression of MYC target genes, such as Hexokinase 2 (Chou et al., 2014). Additionally, mice lacking AP4 display a decreased clearance of the West Nile Virus (WNV) due to compromised T cell activation (Chou et al., 2014). Negative regulators of glycolytic reprogramming also impact upon T cell metabolism. It has been shown in $CD4^+$ T_H1 T cells that the transcription factor Bcl-6 competes for DNA binding with HIF1α and c-Myc at the promoters of glycolytic genes, thus inhibiting glycolytic programming (Oestreich et al., 2014).

During the resolution phase of T cell response, memory T cells develop with distinct metabolic characteristics including decreased glycolysis and switching to lipid oxidation. Central to the immunological protection provided by memory T cells is the ability to respond rapidly to subsequent infections. In this respect, metabolic responsiveness is important for recall responses and memory T cells have the ability to induce aerobic glycolysis much more rapidly compared to naïve T cells (Gubser et al., 2013, van der

Windt et al., 2013). While mTORC1 is central to controlling glucose metabolism in effector T cells, discussed below, mTORC1 activity must be low to allow Tregs and memory T cells to differentiate and reprogramme towards oxidative metabolism. AMPK and TRAF6 are important in establishing oxidative metabolism in these T cells (Michalek et al., 2011a, Pearce et al., 2009, Rolf et al., 2013).

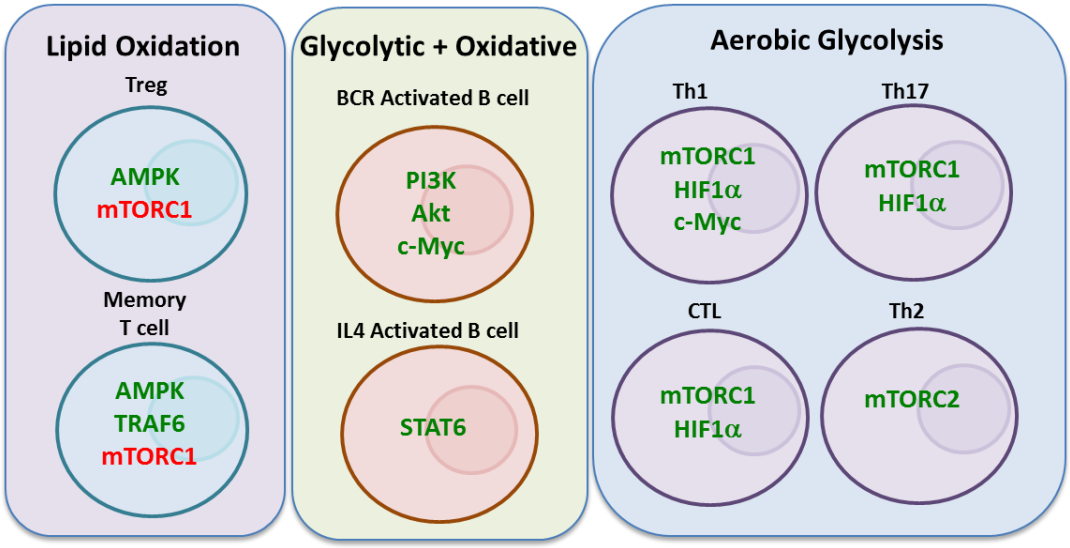

Figure 1.5. Metabolic signatures of different lymphocyte subsets.

Immune cells are able to adopt distinct forms of metabolism to supply the cell with energy, in the form of ATP. Interestingly, specific cell types adopt specific forms of metabolism to do this. Furthermore, there is a wide array of transcriptional regulators that promote (green) or inhibit (red) the type of metabolism an immune cell engages in.

1.2.1.4. B cells energy metabolism

While a considerable amount is known about T cell metabolism comparatively little is known about B cell metabolism. Thus the relationship between B cell metabolism and their function is only starting to emerge. A

number of studies have shown that after activation B cells increase their glucose uptake and induction of glycolysis (Doughty et al., 2006, Dufort et al., 2007). Upon B cell receptor (BCR) stimulation, B lymphocytes also transition from relatively inert cells into highly metabolically demanding effector cells engaging in clonal expansion and the production of antibodies. B cells increase the surface expression of the glucose transporter Glut1 and the rate of glycolysis, converting glucose to lactate (aerobic glycolysis), but interestingly studies have also shown that they also substantially increase the rate of OxPhos (Caro-Maldonado et al., 2014, Doughty et al., 2006). While in TCR activated T cells there is a pronounced shift in metabolism towards glycolysis, BCR activated B cells show a balanced increase in glycolysis and OxPhos (Caro-Maldonado et al., 2014). B cells also engage in elevated glycolysis in response to other stimuli such as LPS-mediated stimulation of Toll-like receptor 4 (TLR4) and IL4 stimulation (Caro-Maldonado et al., 2014, Dufort et al., 2007).

Interestingly, it appears that different B cell stimuli can promote glycolysis by distinct mechanisms. In BCR-stimulated B cells, increased rates of glycolysis require PI3-kinase/Akt and PKCβ signalling (Doughty et al., 2006, Blair et al., 2012). Additionally, the transcription factor c-Myc, but not HIF1α, is required for BCR-induced glycolysis (Caro-Maldonado et al., 2014). However, IL4-induced glycolysis in B cells is independent of PI3-kinase activity, but instead appears to rely upon STAT6 signalling. However, the molecular mechanisms linking STAT6 to glycolysis are currently unknown (Dufort et al., 2007). Interestingly, mTORC1 (mammalian target of rapamycin complex 1) is not involved in controlling glycolysis in B cells, which is in contrast to that seen in other lymphocytes (Doughty et al., 2006). A recent study showed that limiting glycolysis with 2-DG disrupts B cell proliferation and the production of antibodies (Caro-Maldonado et al., 2014). Therefore, as seen in T cells, elevated glycolysis is important for B-cell function.

1.2.1.5. NK cells energy metabolism

To date nothing is known regarding the changes in NK cell metabolism that occur during activation. Therefore this study aims to provide an insight into NK cell metabolism and the role it plays in NK cell function.

1.2.2. Immune cell lipid metabolism

There is also significant interest in understanding the role for lipid metabolism in determining the fate and function of immune cells. Lipids have important functions as energy stores, in signal transduction and as structural components of cell membranes (Lochner et al., 2015). One major component of lipids is fatty acids. Fatty acid metabolism encompasses the breakdown (oxidation) and synthesis of lipids. Cells can accumulate fatty acids through uptake of fatty acids from external environment or through *de novo* synthesis from acetyl-CoA. (Lochner et al., 2015). The cytosolic acetyl-CoA used for lipid synthesis pathways is supplied by the export of citrate from the mitochondria and the Krebs cycle. Cytosolic citrate is then cleaved into Acetyl-CoA and oxaloacetate. As both glucose and glutamine feed into the Krebs cycle both these fuels can be a source of carbon for lipid synthesis (Fig 1.6).

Fatty acid synthesis begins with the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA, which is catalyzed by acetyl-CoA carboxylase 1 (ACC1). The next step is performed by FA synthase (FASN), that catalyzes the condensation of acetyl-CoA and malonyl-CoA to produce the 16-carbon saturated FA palmitate and other saturated long-chain FAs (LCFAs). Saturated LCFAs can be further modified by elongases or desaturases to form more complex lipids, which are then used for the synthesis of phospholipids, triglycerides, and cholesterol esters, or for the acylation of proteins. In the synthesis of triglycerides, fatty acids combine with glycerol. Interestingly, glycerol can be formed using dihydroxyacetone phosphate (DHAP), which is

an intermediate of glycolysis (Fig 1.6). Cholesterol synthesis also consists of a number of enzymatic steps starting again with acetyl-coA.

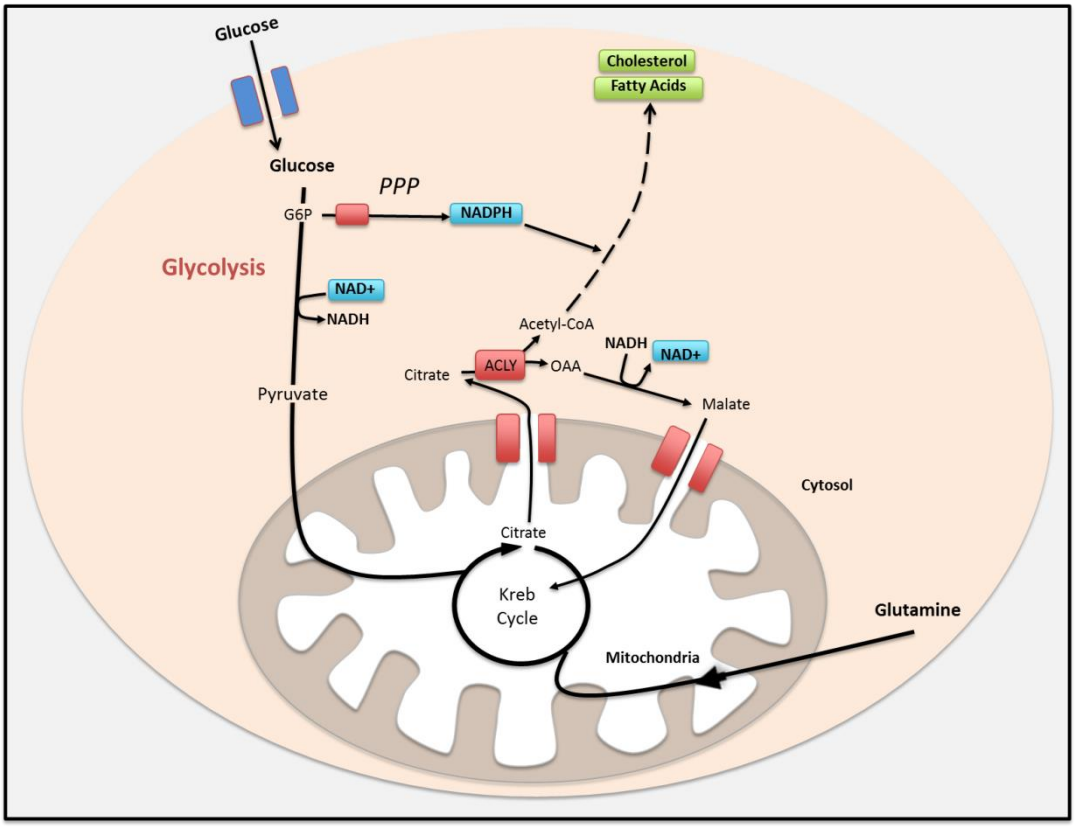

Figure 1.6. Glucose and Glutamine can fuel lipid synthesis. Both glucose and glutamine derived carbon can fuel the Krebs cycle and subsequently lipid synthesis as citrate is exported out of the mitochondria and converted to Acetyl-CoA by ATP citrate lyase (ACLY). This reaction is also generates oxaloacetate which can then be metabolized to malate, producing NAD^+ required for glycolysis, which replenishes the Krebs cycle. Glucose-6-phosphate (G6P) from glycolysis is shuttled into the pentose phosphate pathway (PPP). Indeed for every G6P molecule two NADPH, required for lipid synthesis, is generated by reducing $NADP^+$ in the PPP.

Lipids can also be broken down via β-oxidation, a process primarily taking place in the mitochondria. Fatty acids cannot enter the mitochondria unless they are attached to a component called carnitine. Carnitine

palmitoyltransferase 1A connects carnitine to fatty acids so they can cross the inner membrane of mitochondria. Once these fatty acids are inside mitochondria carnitine is removed. β-oxidation involves the sequential removal of 2-carbon units at the β-carbon position of a fatty acyl-CoA molecule. Each round in the β-oxidation cycle yields one molecule of acetyl-CoA, which can be directly shuttled into the Krebs cycle and further oxidized to drive OxPhos and ATP production.

1.2.2.1. T cell lipid metabolism

The initiation of cellular lipid synthesis is a crucial part of T cell activation and directly links glucose metabolism with *de novo* lipid synthesis (Berod et al., 2014) as described in section 1.2.2. Indeed, inhibition of acetyl-coA carboxylase 1 (ACC1), the enzyme that catalyzes the first step of fatty acid and cholesterol an important lipid synthesis, prevents the development of T_H17 cells. Instead it promotes the maturation of anti-inflammatory Treg cells (Berod et al., 2014). T_H17 development depends on the glucose dependent-*de novo* fatty acid synthesis pathway for synthesis of phospholipids needed for cellular membrane formation. Alternatively, Treg cells take up exogenous fatty acids for this purpose (Berod et al., 2014). Furthermore inhibition of ACC1 was shown to decrease $CD8^+$ T cell proliferation and blastogenesis and cell survival (Lee et al., 2014).

Fatty acids are important for T cell signal transduction. Palmitoylation of proteins is an important post-translational modification that controls protein membrane localization and is required for T cell activation (Arcaro et al., 2000). Palmitolyation of LAT (linker for activation of T cells) is selectively disrupted in anergic T cells (Hundt et al., 2009, Hundt et al., 2006), suggesting that protein palmitolyation is a selectively regulated process involved in T cell activation. Palmitolyation of proteins Fyn, STAT3 and AMPK is involved in T_H17 differentiation (Berod et al., 2014).

T cells can also use lipids to fuel ATP production following β-oxidation (fatty acid oxidation; FAO). FAO is of particular importance for supplying ATP in Tregs and memory T cells. Indeed, the generation of memory $CD8^+$ T cells is dependent on FAO (Pearce et al., 2009, van der Windt et al., 2012). Recently, O'Sullivan and colleagues showed that memory $CD8^+$ T cells do engage in *de novo* fatty acid synthesis; however, they store it in lysosomes as triglycerides and use this source of lipid to fuel FAO (O'Sullivan et al., 2014). While effector $CD4^+$ T cells tend to adopt a glycolytic phenotype, the generation of Tregs is dependent on FAO. Inhibition of CPT1a, required for FAO, with etomoxir disrupts Treg development (Michalek et al., 2011a).

1.2.2.2. B cell lipid metabolism

It is now recognized that like T cells B cells engage glucose metabolism upon activation for efficient effector functions and proliferation. Lipid metabolism is emerging as an important metabolic process for T cell differentiation and function. However, very little is known about whether B cells engage in lipid metabolism upon activation and whether it is required for their effector responses. One study by Dufort and colleagues showed that LPS stimulated B cells increase *de novo* lipid synthesis in a manner dependent on ATP citrate lyase (ACLY) activity (Dufort et al., 2014). Moreover, inhibition of ACLY resulted in decreased proliferation and antibody production in B cells (Dufort et al., 2014). The role lipid metabolism plays in B cell function is still an area that has yet to be explored fully.

1.2.2.3. Regulation of lipid metabolism in lymphocytes

The transcription of genes required for cholesterol and fatty acid synthesis are regulated by transcription factors called sterol regulatory element binding proteins (SREBPs) (Horton et al., 2002). SREBP is an integral membrane protein composed of 3 isoforms encoded by 2 genes; SREBP1a,

SREBP1c and SREBP2 (Tontonoz et al., 1993, Brown and Goldstein, 1997). SREBP1a and SREBP1c are generally recognized as being responsible for the expression of fatty acid synthesis genes, while SREBP 2 is known to regulate cholesterol synthesis genes. Each isoform contains an N-terminal and a C-terminal domain. The N-terminal domain is a basic helix-loop-helix leucine zipper transcription factor. The C-terminal domain forms a rigid complex with SCAP, which functions as the sterol sensing component of this system. Between the C and N terminal domains are multiple membrane spanning regions. SREBP also regulates the expression of key genes involved in the oxidative PPP and the generation of the co-enzyme NADPH (Shimomura et al., 1998), ensuring sufficient reducing equivalents to meet anabolic demands.

1.2.2.4. Regulation and activation of SREBP

In sterol-replete cells SCAP binds tightly to cholesterol in the ER membrane and undergoes a conformational change that encourages it to bind to the ER-resident protein INSIG (for Insulin induced gene) (Peng et al., 1997, Matsuda et al., 2001). This interaction retains the SREBP-SCAP complex in the ER (Rawson, 2003). When sterol levels are low, INSIG and SCAP no longer bind. Then, SCAP undergoes a conformational change that exposes a portion of the protein ('MELADL') that signals it to be included as cargo in the COPII vesicles that move from the ER to the Golgi apparatus. In these vesicles, SCAP, dragging SREBP along with it, is transported to the Golgi where two sequential proteolytic events, mediated by site 1 (S1P) and site 2 (S2P) proteases, release the N-terminal transcription factor domain from the golgi membrane into the cytosol (Rawson, 2003). The N-terminal domain is subsequently transported to the nucleus as a dimer by importin β (Lee et al., 2003). Once in the nucleus, SREBP induces the expression of target genes by binding to sterol regulatory elements (SRE) sequences in their promoter

regions. The resulting increase in expression of lipid synthesis genes results in increased sterols, that feedback to inhibit SREBP activation (Fig 1.7).

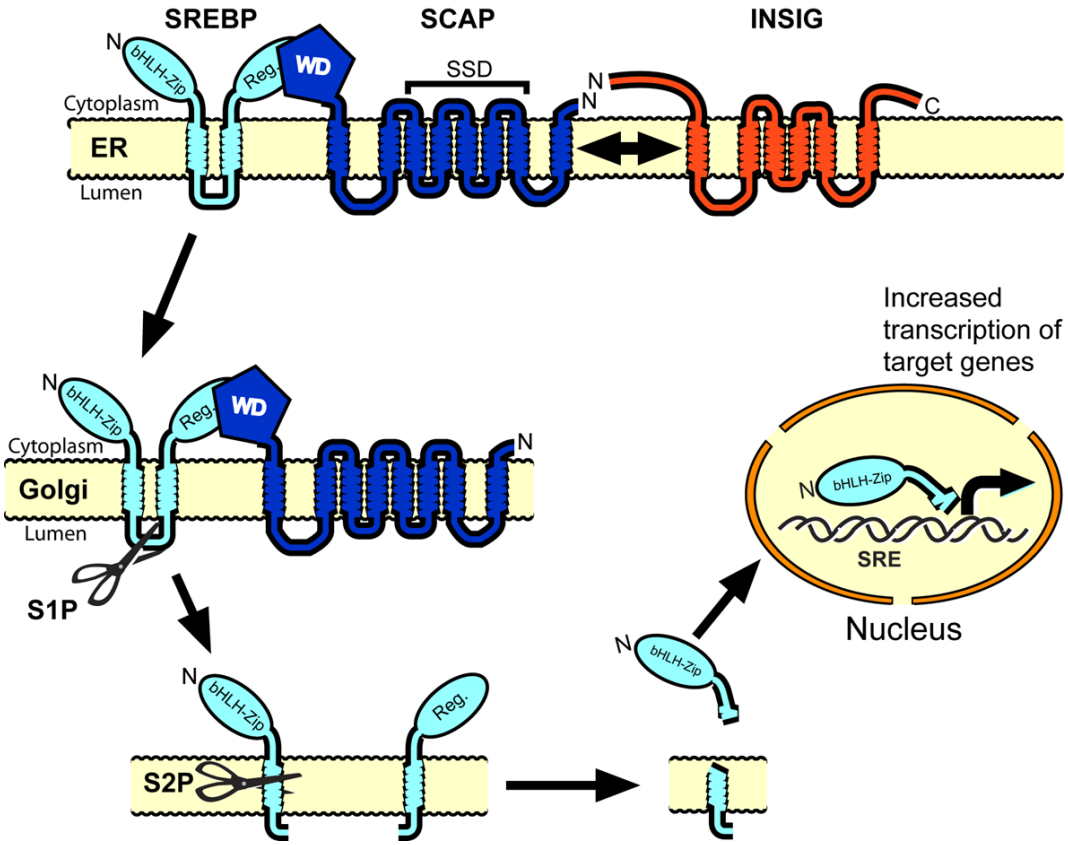

Figure 1.7. SREBP regulation and activation. SREBP activation is tightly regulated by sterol levels within the cell. The figure above outlines the detail described in section 1.2.2.4 (Rawson 2009).

1.2.2.5. SREBP activity controls T cell function

SREBP regulated lipid metabolism is also crucial in T cell growth and activation. In T cells, antigenic stimulation is followed by an increase in SREBP activity, fatty acid and cholesterol synthesis (Bensinger et al., 2008). Changes in lipid metabolism are essential for effector T cell activation and function. T cells are one of the main contributors to an aberrant immune response resulting in autoimmunity. Recent studies have shown that inhibiting lipid synthesis has also resulted restoring T cell tolerance in EAE

models (Bensinger et al., 2008, Xu et al., 2009, Cui et al., 2011). Indeed, inhibition of SREBP activity results in reduced cellular growth in mitogen-stimulated T cells and arrest in G0/G1 of cell cycle before being subjected to apoptosis-independent cell death. Homeostatic proliferation and antigen-specific viral immunity is also compromised with decreased SREBP activity (Kidani et al., 2013). Loss of SREBP function doesn't only impact lipid synthesis, but also prevents metabolic reprogramming towards a glycolytic phenotype in $CD8^+$ T cells following activation (Kidani et al., 2013).

1.3. Mammalian target of rapamycin (mTOR)

Rapamycin was first identified for its anti-fungal activity and purified from the bacterium streptomyces hygroscopicus (Dobashi et al., 2011). Rapamycin was subsequently found to have immunosuppressive effects and to suppress cell proliferation (Vignot et al., 2005). Rapamycin in a complex with FKBP12 (FK506 binding protein of 12KDa) binds to a 289KDa protein TOR (Target of Rapamycin) (Brown et al., 1995, Brunn et al., 1997). The mammalian homologue of TOR was identified and named mammalian Target of Rapamycin (mTOR). The evolutionarily conserved mTOR protein kinase which belongs to the phosphoinositol 3-kinase (PI3K)-related kinase family (Keith and Schreiber, 1995) plays a fundamental role in the coordination of metabolism, cell growth, protein synthesis and proliferation (Soliman, 2011, Laplante and Sabatini, 2009, Howell and Manning, 2011, Yecies and Manning, 2011, Soliman, 2005). mTOR serine/threonine kinase also functions as a molecular sensor of metabolism and cellular homeostasis and incorporates environmental signals by changing the cellular metabolic processes (Soliman, 2005) (Foster and Fingar, 2010).

mTOR forms two protein complexes, mTORC1 and mTORC2 and has important roles in regulating immunological systems. mTORC1, in particular, has emerged as a central regulator of immune responses. mTORC1 activity regulates a wide range of immune cells in both the innate and adaptive arms

of immunity (Powell et al., 2012). It is becoming clear that mTORC1-regulated cellular metabolism is crucial to the immunoregulatory function of this kinase complex. mTORC1 is linked to the control of glycolysis through two transcription factors, c-Myc and HIF1α, each of which promote the expression of multiple glycolytic enzymes and glucose transporters. mTORC1 activity has also been linked to the control of lipid synthesis through the SREBP transcription factor. Moreover, evidence is emerging that mTORC1-dependent metabolism plays a fundamental role in dictating immune cell differentiation and function. 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., 2012).

1.3.1. mTOR complexes

mTOR forms two distinct multi-subunit complexes, mTOR complex 1 (mTORC1-rapapmycin sensitive complex) which contains mTOR, raptor, mLST8, PRAS40, and deptor and mTOR complex 2 (mTORC2-rapamycin insensitive complex) which contains mTOR, rictor, mLST8, mSIN1, protor, and deptor (Wullschleger et al., 2006). Studies have a elucidated a large amount of the molecular detail in regards to the upstream signalling of mTORC1 (Laplante and Sabatini, 2009), however, mTORC2 upstream signalling is relatively undiscovered (Sparks and Guertin, 2010).

The exact function of the all the components making up mTORC1 still remains elusive. However, certain roles have been recognized, Raptor has been suggested to affect mTORC1 activity by regulating complex assembly and recruiting substrates for mTOR (Hara et al., 2002, Kim et al., 2002a). PRAS40 and Deptor are negative regulators of mTORC1 (Peterson et al., 2009, Sancak et al., 2007, Vander Haar et al., 2007), with PRAS40 acting as a direct inhibitor of substrate binding (Wang et al., 2007). In mTORC2 there is suggestion though that Rictor and mSIN1 stabilize each other thus

establishing the structural foundation of the complex (Frias et al., 2006, Jacinto et al., 2006). As in mTORC1, Deptor has been reported to inhibit mTORC2 (Peterson et al., 2009). mLSTB has been reported to be required for the stability and function of mTORC2 (Guertin et al., 2006).

1.3.2. mTORC1 signalling pathway

mTORC1 senses signals from numerous signalling pathways such as growth factors, energy, stress, mitogens and amino acids, particularly leucine and glutamine (Schriever et al., 2013). One of the most important regulators of mTORC1 activity is the tuberous sclerosis complex (TSC), which is a heterodimer that comprises TSC1 and TSC2. TSC1/2 functions as a GTPase-activating protein (GAP) for the small Ras-related GTPase Rheb (Ras homolog enriched in brain). The active, GTP-bound form of Rheb, located on the lysosomal surface, directly interacts with mTORC1 to stimulate its activity (Long et al., 2005) (Sancak et al., 2007). As a Rheb-specific GAP, TSC1/2 negatively regulates mTORC1 signalling by converting Rheb into its inactive GDP-bound state (Fig 1.8) (Garami et al., 2003, Stocker et al., 2003, Nobukini and Thomas, 2004, Kwiatkowski et al., 2002, Tee et al., 2003, Inoki et al., 2003). When a growth factor, such as insulin, binds to its extracellular surface receptor, insulin receptor (IR), it activates PI3K and Ras signalling pathways. The stimulation of these pathways increases the phosphorylation of TSC2 by Akt (Inoki et al., 2002, Potter et al., 2002), extracellular-signal-regulated kinase 1/2 (ERK1/2) (Ma et al., 2005), and/or p90 ribosomal S6 kinase 1 (RSK1) (Roux et al., 2004), and leads to the destabilization of TSC1/2 and thus to the activation of Rheb and mTORC1. AKT also regulates mTORC1 by phosphorylating PRAS40, thereby blocking PRAS40-mediated inhibition of mTORC1 (Nascimento et al., 2010). Amino acids activate Rag (Ras-realted GTPases), which in turn bind to Raptor and promote the relocalization of mTORC1 from discrete locations throughout the cytoplasm to a perinuclear region that contains its activator Rheb (Sancak et al., 2008).

This permits prolonged mTOR-Rheb interactions, thereby enhancing mTOR activity in response to amino acid uptake (Ogmundsdottir et al., 2012, Cang et al., 2013). If a cell becomes energy deprived, AMP-activated protein kinase (AMPK) is switched on. 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). In this setting, mTORC1 activity is inhibited by AMPK phosphorylation and stabilization of TSC2 (Inoki et al., 2006). AMPK can also phosphorylate raptor to inhibit mTORC1 activity (Gwinn et al., 2008). Recently, multiple studies have shown signals from the immune microenvironment including the ligation of co-stimulatory molecule CD28, and cytokines such as IFNγ and interleukins including IL-1, IL-2, IL-4, and IL-12 stimulate signalling through the PI3K-Akt-mTORC1 axis (Powell et al., 2012).

1.3.3. mTORC1: a master regulator of cell growth and metabolism

Lipid Synthesis

The role of mTORC1 in regulating lipid synthesis, which is required for cell growth and proliferation, is beginning to be appreciated. It has been demonstrated that mTORC1 induces the activity of SREBP Sterol regulatory element binding protein (SREBP) (Porstmann et al., 2008) and of peroxisome proliferator-activated receptor-γ (PPARγ) (Fig 1.8) (Kim and Chen, 2004). These two transcription factors have been shown to control the expression of genes encoding proteins involved in fatty acid and cholesterol synthesis. Inhibition of mTORC1 with rapamycin decreases the expression and the transactivation activity of PPARγ (Kim and Chen, 2004). mTORC1 can regulate SREBP expression and activity in multiple ways, which will be discussed in section 1.3.5. Additionally, mTORC1 induces the phosphorylation of lipin-1 (Huffman et al., 2002), a phosphatidic acid (PA) phosphatase that is involved in glycerolipid synthesis and in the coactivation

of many transcription factors linked to lipid metabolism, including PPARγ, PPARα, PGC1-α and SREBP.

Protein synthesis

mTORC1 positively controls protein synthesis, which is required for cell growth, through multiple mechanisms. mTORC1 directly phosphorylates the eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and the p70 ribosomal S6 Kinase 1 (S6K1) (Fig 1.8). Phosphorylated 4E-BP1 is prevented from binding to eIF4E, thus allowing eIF4E to promote cap-dependent translation (Richter and Sonenberg, 2005). Phosphorylation of S6K1 results in the activation of S6K1 activity and the phosphorylation of multiple S6K1 substrates that promote the initiation of cap-dependent translation and translational elongation. These S6K1 targets include aly/REF-like target (SKAR), programmed cell death 4 (PDCD4), eukaryotic elongation factor 2 kinase (eEF2K) and ribosomal protein S6 (Ma and Blenis, 2009).

Energy metabolism

mTORC1 has been implicated in the control of energy metabolism by enhancing the translation of hypoxia-inducible factor 1α (HIF-1α), a transcription factor that regulates numerous glycolytic genes and glucose transporters (Zhong et al., 2000, Hudson et al., 2002). mTORC1 can also promote cMYC protein expression to induce glucose metabolism (Wang et al., 2011b). In addition, mTORC1 also regulates mitochondrial metabolism and biogenesis. Inhibition of mTORC1 by rapamycin was shown to lower mitochondrial membrane potential, oxygen consumption and cellular ATP levels in mammalian cells (Schieke et al., 2006). mTORC1 can also promote the expression of numerous genes encoding proteins involved in oxidative metabolism (Chen et al., 2008). mTORC1 also has a role in mitochondrial biogenesis through controlling the transcriptional activity of PPARγ coactivator 1 (PFC1-α) (Cunningham et al., 2007).

Autophagy

Autophagy is a catabolic process that is important in organelle degradation and protein turnover. When nutrient become depleted, the degradation of organelles and protein complexes via autophagy provides biological material to sustain anabolic processes such as protein synthesis and energy production. Studies have shown that mTORC1 signalling is a negative regulator of autophagy (Codogno and Meijer, 2005). mTORC1 phosphorylates and inhibits protein complexes composed of unc-51-like kinase 1 (ULK1) and autophagy-related gene 13 (ATG13) that are involved in the initiation of autophagy (Fig 1.8) (Ganley et al., 2009, Hosokawa et al., 2009, Jung et al., 2009).

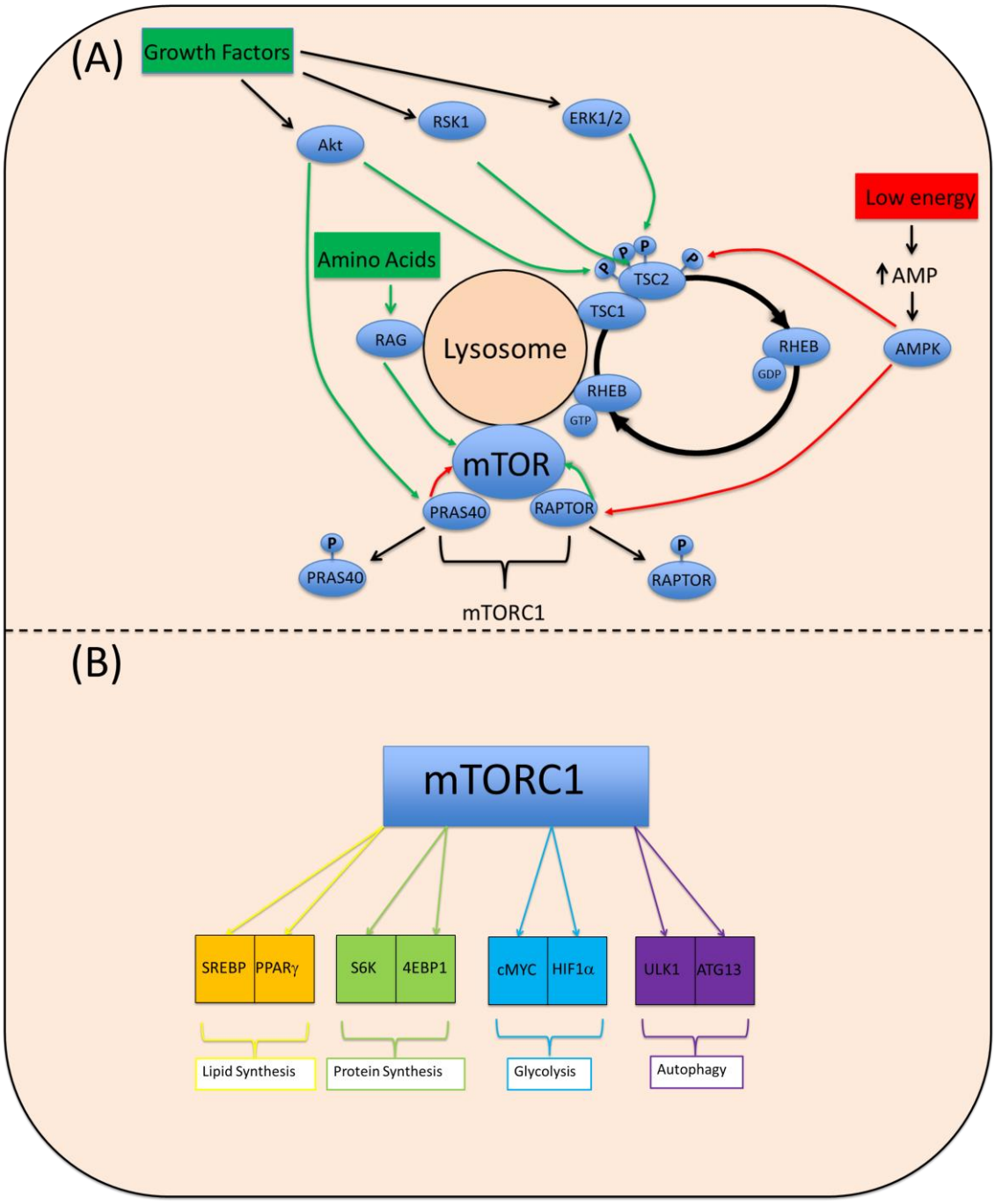

Figure 1.8. mTORC1 integrates environmental signals to control cellular processes. (A) mTORC1 activity is controlled by microenvironmental cues including growth factors, amino acids and the energy status of the cell. The TSC1/2 complex negatively regulates mTORC1 activity through inhibition of Rheb. There is a wide array of factors that promote (green) or inhibit (red) mTORC1 activity as explained in section

1.3.2. (B) mTORC1 signalling promotes anabolic cellular processes such as lipid synthesis, protein synthesis and glycolysis through the activation of downstream signalling molecules SREBP & PPARγ, S6K1 & 4EBP1, HIF1a & cMYC respectively. mTORC1 also functions as an inhibitor of autophagy through the phosphorylation of ULK1 & ATG13.

1.3.4. mTORC1 integrates immune cell energy metabolism and immune cell function

T-cell activation and tolerance are tightly regulated to ensure effective elimination of foreign antigen while maintaining immune tolerance to self-antigens. Central to immune homeostasis is maintaining a balance between effector, memory and regulatory T cells subsets. Ultimately the effector versus regulatory/memory fate of activated T cells is dictated by a multitude of cues received from the immune microenvironment thus ensuring appropriate T cell responses. In this respect, the mTORC1 kinase complex is acutely sensitive to these cues, being differentially activated by the various cytokines responsible for directing the discrete T cell differentiation pathways while also sensing a range of cellular metabolic parameters such as amino acid and glucose availability and the cellular energy status (Delgoffe et al., 2009). Recent studies demonstrate that mTORC1 signalling is crucial in controlling differentiation of $CD4^+$ Th cell lineages (T_H1, T_H17, Treg), in addition to controlling $CD8^+$ T cell differentiation into effector or memory cells, and the control of T cell trafficking patterns (Sinclair et al., 2008, Finlay and Cantrell, 2011, Powell and Delgoffe, 2010, Sauer et al., 2008, Delgoffe et al., 2011b). Therefore, the level of mTORC1 activity can be viewed as a sensitive indicator of the nature of the surrounding microenvironment that then directs the fate of the activated T cell appropriately. This is exemplified by the study by Stephen Cobbold *et al*, 2009, which demonstrated that amino acid depletion leading to inhibition of mTORC1 promotes infectious tolerance via the generation of surplus Treg cells (Cobbold et al., 2009).

While progress has been made in understanding how mTORC1 can direct T cell differentiation and function, much of the molecular detail is yet to be fully characterized. It is clear, however, that T cell function is closely linked to the elevated metabolic state of activated T cells. mTORC1 has well described roles in non-immune cells in controlling multiple metabolic pathways and it is now becoming clear that mTORC1 regulated cellular metabolism is central to its functions in immune cells. Indeed, mTORC1 activity is required to maintain elevated glycolysis in multiple T cell subsets (Finlay et al., 2012, Shi et al., 2011). Studies have elucidated mTORC1 regulated glycolysis is due to its control of multiple metabolic transcription factors including HIF1α and c-myc (Shi et al., 2011, Wang et al., 2011a, Finlay et al., 2012, Sinclair et al., 2013). Indeed, T cell-specific deletion of the gene encoding the negative regulator of mTORC1, TSC2, resulted in the generation of highly glycolytic and potent effector $CD8^+$ T cells. In contrast, $CD8^+$ T cells deficient in mTORC1 activity due to loss of RHEB failed to differentiate into effector cells but retained memory characteristics, such as decreased glycolysis and increased longevity (Pollizzi et al., 2015). Studies have shown that direct inhibition of elevated glycolysis in $CD8^+$ T cells with 2DG results in a similar phenotype with the generation of long lasting memory T cells instead of effector CTLs (Sukumar et al., 2013). Therefore, regulation of immune cell metabolism is integrally linked to the important immunoregulatory functions of mTORC1.

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.

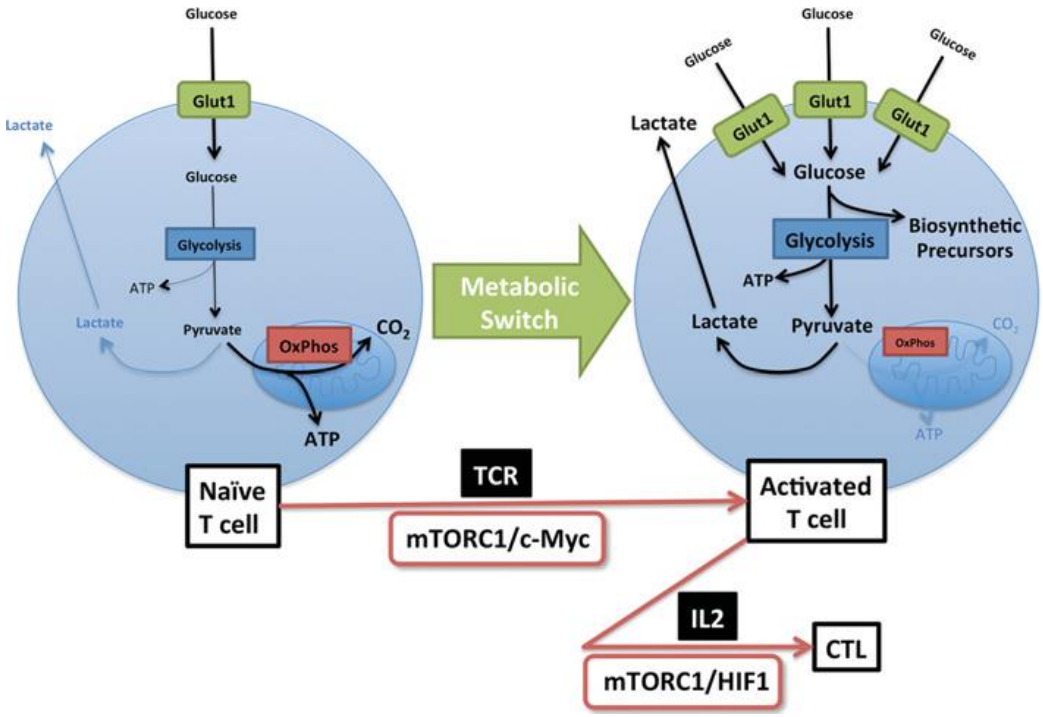

Figure 1.9. mTORC1 controls T cell glucose metabolism. Naive T-cells take up relative low quantities of glucose, which is mostly metabolized to CO_2 via mitochondrial OxPhos for the efficient generation of ATP. A shift from oxidative to glycolytic glucose metabolism involving an increase in the expression of Glut1 and thus glucose uptake is essential for successful activation of naïve T cells. mTORC1/c-myc signalling promotes this metabolic shift in response to TCR stimulation, mTORC1 signalling through HIF1α sustains CTL glycolytic metabolism in response to IL-2 (Finlay, 2013).

1.3.5. mTORC1/SREBP signalling axis

Recent studies have shown that mTORC1 regulated SREBP activity is required for *de novo* lipid synthesis (Quinn and Birnbaum, 2012). As previously explained in section 1.2.2.4 SREBP undergoes a complex activation process. mTORC1 has been shown to act at multiple stages of SREBP activation in different cell types. Active mTORC1 does this in multiple ways:

1) Downstream substrates of mTORC1 are required for increased SREBP activity, increased expression of target genes and lipid synthesis (Duvel et al., 2010). S6K1 which is directly phosphorylated by mTORC1 is required for increased SREBP activity (Duvel et al., 2010). In addition, the mRNA cap-binding protein eIF4E, involved in promoting translation initiation, is activated by mTORC1 signalling and has been shown to be required for SREBP protein expression in multiple tumour cells (Luyimbazi et al., 2010).

2) mTORC1 induces SREBP activity through preventing lipin-1 from translocating into the nucleus. Nuclear Lipin-1 has been shown to decrease SREBP activity (Peterson et al., 2011).

3) Nuclear SREBP is also rapidly degraded through the ubiquitin-proteasome pathway. Sundgvist and colleagues showed that the ubiquitin ligase Fbw7 degrades SREBP when it is located in the nucleus. The translocation of Fbw7 to the nucleus is dependent on its phosphorylation by GSK-3 (Sundqvist et al., 2005). GSK-3 activity is inhibited by Akt.

To date nothing is currently known about the regulation of SREBP in NK cells.

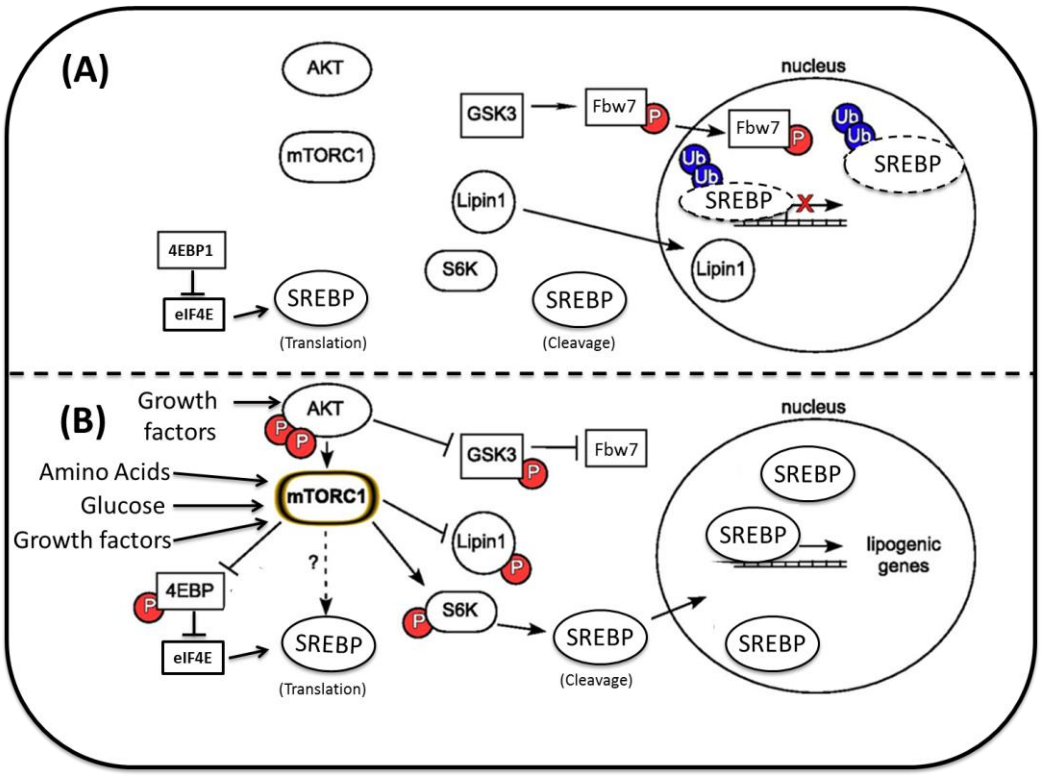

Figure 1.10. mTORC1 regulates SREBP activity at multiple levels. (A) mTORC1 is inactive, resulting in inhibition of SREBP at different stages of its activation. (B) Upon mTORC1 activation SREBP activity is promoted leading to the transcription of lipogenic genes (Quinn and Birnbaum, 2012).

Aims and objectives

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 thesis 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 & Methods

2.1. Materials

2.1.1. Chemicals/Reagents/Cytokines

2-Mercaptoethanol, Albumin from bovine serum, Ethanol, Phosphate buffered saline tablets, 2-Deoxglucose, Glucose, Galactose, dNTPs, NAD/NADH quantification colourimetric kit, 1,2,3-benzenetricarboxylic acid, FK866 hydrochloride, paraformaldeyde, oligomycin, fluoro-carbonyl cyanide phenylhdrazone, rotenone, antimycin and Tris(hydroxymethyl)aminomethane, 99+% were all 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. Poly (I:C) was purchased from Invivogen. C57BL/6J mice were purchased from Harlan. Glucose-free medium, Dulbecco's phosphate buffered saline, RPMI medium 1640 (1X) [+]L-Glutamine, Pen-Strep, Mem vitamin solution (100X), Insulin-Transferrin-Selenium-G Supplement (100X) were purchased from Biosciences. IL-15 was purchased from Peprotech. IL-2 was purchased from NCI preclinical repository. IL-12 and isolation were purchased from Miltenyi Biotech. Foetal Bovine Serum was purchased from Labtech International. Golgi plug, Cytofix/Cytoperm, Perm/Wash reagent and Celltak were purchased from BD Pharmingen. Methanol and Absolute Alcohol were purchased from the school stores. phorbol 12,13-dibutyrate (PdBu) was purchased from Calbiochem. RNeasy RNA purification mini kit was purchased from qiagen. qScript cDNA synthesis kit was purchased from quanta. iQ SYBR Green was purchased from VWR. Seahorse Media was purchased from Seahorse Biosciences. ATP assay determination kit and ^{14}C labelled Acetate were purchased from Perkin Elmer. IL-18 was obtained from GlaxoSmithKline (GSK). BMS303141 was purchased from R&D. PF429242 was purchased from Adooq Biosciences.

2.1.2. Equipment

Pipettes, Pipette tips, filtered tips, Cell culture strainers, 2ml disposable syringes Tissue culture dishes (6, 12 and 24 well) and Tissue culture flasks (25cm^2 75cm^2 175cm2) were obtained from Fisher. AB-1900 High sided low profile PCR plates (natural - 25 plates/box), 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 from *Becton Dickinson*. FlowJo software was obtained from TreeStar. 7900HT fast rtPCR machine was obtained from Applied Biosciences. XF 24-well microplate was from Seahorse Bioscience. LS Macs purification columns and Quadro Macs cell separator were from Miltenyi Biotec. Scintillation Counter was from Perkin Elmer. ^{3}Prime Thermal Cycler and Dri-Block DB-2D were purchased from Techne. Grant JB Series Water Bath was obtained from Amlab Services. Power Source 300 V was obtained from VWR International. Steri-Cycle CO_2 Incubator from Thermo Forma. Heraeus Fresco 21 and Heraeus Pico 17 Centrifuges from Thermo Scientific. Legend RT Centrifuge from Sorvall. 1500 Standard Fumehood from Phoenix Controls Corporation. Bioair Topsafe Fumehood from Crowthorne HiTec Services. Bright-Line Hemacytometer from Hausser Scientific. Nanodrop Spectrophotometer ND-1000 from Labtech International.

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 the *in vivo* experiments mice were injected IP with 200μg of Poly (I:C) in PBS (Invivogen) +/- 0.6mg/kg rapamycin (Fisher Scientific) or 1g/kg 2-deoxyglucose (Sigma). Mice were sacrificed after 24 hours, spleens harvested and NK cells analyzed.

2.1.4. 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. Flow cytometry antibodies PE-Cy7 CD69 (H1.2F3), PerCP-Cy5.5 CD69 (H1.2F3), APC-Cy7 CD25 (PC61), APC IFN-g (XMG1.2) and PE-Cy7 IFN-γ (XMG1.2) were purchased from BD pharmingen. 2NBDG was purchased from Invitrogen. S6 ribosomal protein (Ser235) was incubated along with a secondary antibody PE-conjugated donkey anti-rabbit immunoglobulin G purchased from Jackson ImmunoResearch for Flow cytometry analysis.

Antibody (Flow cytometry)	Source	Dilution	Location
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	1/200	Extracellular
PE-Cy7 CD69 (H1.2F3)	BD Pharmingen	1/100	Extracellular
PerCP-Cy5.5 CD69 (H1.2F3)	BD Pharmingen	1/200	Extracellular
APC-Cy7 CD25 (PC61)	BD Pharmingen	1/100	Extracellular
APC CD71 (R17217)	eBioscience	1/200	Extracellular
PE CD98 (RL388),	eBioscience	1/100	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. Antibodies

2.2. Methods

2.2.1. Preparation of Buffers

Most of the buffers used during experimentation are listed in Table 2.2; others are mentioned in text as necessary.

2.2.2. Cell Culture

All cell culture media were pre-warmed to 37^{o}C except for media used to isolate splenocytes from the spleen.

2.2.2.1. Natural Killer (NK) cell culture

The spleen was extracted from the mouse, placed in a 70 µm nylon cell strainer, which is furthered placed in a well in a 6 well cell culture plate contain 5 ml ice cold media. The spleen is minced with a plunger from a 1ml syringe and the cell suspension is filtered through the cell strainer and spun down and supernatant decanted. Erythrocytes were lysed with 2 ml sterile ddH2O for 3 sec, and adding 10 ml of RPMI media to stop the lysis. Cells were spun down again and resuspend at a concentration of 2x10^{6} cells/ml, fed with IL-15 (25ng/ml, Peprotech) and incubated at 37°C for 6 days. On day 4, IL-15 (25ng/ml) was re-added and the cells cultured for a further 2 days. On day 6, cultured NK cells were maintained in low dose of IL-15 (5ng/ml, Peprotech) or were stimulated for 18 hours with the cytokines; IL-2 (20ng/ml, NCI preclinical repository), IL-12 (10ng/ml, *Miltenyi Biotech*), IL-18 (25ng/ml, GSK) and inhibitors; 2-deoxy-D-glucose (Sigma), rapamycin (20nM, Fisher), BMS303141 (50µM, R&D), FK866 (10µM, Sigma), BTA(1mM, Sigma) PF429242 (10µM, AdooQ Biosciences) or 25-HC (1µg/ml, Sigma). 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 NK cells were cultured in

supplemented glucose-free medium (see Table 2.2) containing dialysed FCS with glucose or galactose added at 10mM.

Buffers/Media	Composition
Cell Culture Media	RPMI medium (+) L-Glutamine, FCS (10%), PenStrep (1%), 2-mercaptoethanol (55μM)
RNA lysis Buffer	RTL buffer (Qiagen), 10% 2-mercaptoethanol
Facs Buffer	Dulbecco's phosphate buffered saline, Cell culture media (20%)
Macs Buffer	To make up 1L: 800mls ddH_2O, 4 PBS tablets, 3.2mls EDTA (1M)
Celltak	For 20 wells: 13ul Cell-Tak, 4180.5ul Sodium Bicarbonate (0.1M), 6.5ul NaOH (1M)
Supplemented glucose free cell culture media	10% Dialayzed FCS, 2mM Glutamine, 1mM Sodium Pyruvate, 1x Vitamin Cocktail, 1x Selenium/Insulin Cocktail, 50μM β-mercaptoethanol and 1% Penicillin/Streptomycin
Ear digest Buffer	25mM NaCl, 50mM Tris/HCl, 0.1% SDS
DNA loading buffer	50% glycerol, 20mg Bromophenol blue
50X TAE buffer	2M Tris, 17.4M acetic acid, 64mM EDTA
2% Agarose Gel	2g Agarose, 100ml 1X TAE, 20μl Nancy[520]

Table 2.2. Buffers/Media

Inhibitor concentrations were determined after a comprehensive literature review and titrations to find the optimal concentrations that were not toxic. To determine if FK866 is having its desired effect NAD^+ levels should be measured. To determine if BTA and BMS are inhibiting the citrate malate shuttle a lipid synthesis assay can be undertaken as it is well documented that citrate derived acetyl-coA is needed for de novo lipid synthesis.

Compound	Stock concentration	Final concentration
IL-15	20ug/ml	25ng/ml
IL-2	20µg/ml	20ng/ml
IL-12	50µg/ml	10ng/ml
IL-18	25µg/ml	25ng/ml
Rapamycin	1mM	20nM
Oligomycin	2mM	2µM
2-DG	1M	0.5mM-30mM
Galactose	1M	10mM
Glucose	1M	10mM
FCCP	1mM	0.5µM
Rotenone	1mM	100nM
Antimycin	5mM	4µM
2NBDG	1M	10mM
PF429242	100mM	10µM
Cholesterol	10mg/ml	10µg/ml
25-Hydroxycholesterol	10mg/ml	1µg/ml
FK866	100mM	10µM
BMS303141	50mM	50µM
BTA	1M	1mM
TOFA	1mg/ml	5µg/ml
C75	10mg/ml	10µg/ml

Table 2.3. Cell culture Stimulants

2.2.3. RNA Manipulation

2.2.3.1. RNA isolation

Following stimulation 1 x 10^6 cells were counted using a haemocytometer, spun down in a centrifuge at 300g for 3 minutes and resuspended in zero volume. The cells were lysed in 600µl RTL buffer containing 1% 2-Mercaptoethanol. 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).

2.2.3.2. cDNA synthesis (RT)

1µg of isolated RNA were made up to 16 µl with ddH20 and added to 4 µl of qScript cDNA Supermix (5x) (Quanta BioSciences), which contains the reverse transcriptase enzyme required to carry out the reaction. The reverse transcription was carried out in Techne's ^{3}Prime Thermal Cycler at the following incubation times:

5 minutes at 25 ^{o}C
30 minutes at 42 ^{o}C
5 minutes at 85 ^{o}C
Hold at 4 ^{o}C

2.2.3.3. Primer design

Primers for quantitative real time PCR (qRT-PCR) where designed using the Primer BLAST tool located on the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)

Rplp0 forward: 5'-CATGTCGCTCCGAGGGAAG-3'
Rplp0 reverse: 5'-CAGCAGCTGGCACCTTATTG-3'

LDHa forward: 5'-CTGGGAGAACATGGCGACTC-3'
LDHa reverse: 5'-ATGGCCCAGGATGTGTAACC-3'
Glut1 forward: 5'-GGAATCGTCGTTGGCATCCT-3'
Glut1 reverse: 5'-CGAAGCTTCTTCAGCACACTC-3'
Hex2 forward: 5'-TCGCCTGCTTATTCACGGAG-3'
Hex2 reverse: 5'- TCGCCTGCTTATTCACGGAG -3'
SCD1 forward: 5'- TCACGACCCCACCTATCAG -3'
SCD1 reverse: 5'- CCAGAGCGCTGGTCATGTAG -3'
FASN forward: 5'- GGTATAGACGACGGGCACAG-3'
FASN reverse: 5'- GCCATTCCAGGTAAATGGGC-3'
HMGCoS forward: 5'- CATTGGGCGGCTAGAAGTTG-3'
HMGCoS reverse: 5'- ACCAGAGCATATCGTCCATCC -3'
ACAT2: 5'- CCAGAGCGACAAGATGAATGC -3'
ACAT2 reverse: 5'- CCACAACCTGCCGTCAAGA-3
IFNγ forward: 5'- ACGCTACACACTGCATCTTG-3'
IFNγ reverse: 5'- GTCACCATCCTTTTGCCAGTTC-3'
ACLY forward: 5'- CCCCAAGATTCAGTCCCAAGTC -3'
ACLY reverse: 5'- GATCCCCAGTGAAAGGGTAGAC -3'
Slc25a1 (CiC) forward: 5'- CTTCCTGGGGTGCCTATGTG-3'
Slc25a1 (CiC) reverse: 5'- CATTTAAGGCCAATGGCGGG-3'

2.2.3.4. 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 10μl reaction containing: 0.5μl cDNA, 0.4ul (10pmol/μl) sense and antisense primer, 5μl SYBR Green supermix and 3.7μl nuclease free water. The plate was sealed with ABsolute qPCR seal (MSC) and run under the following conditions;

Stage 1

95°C for 3 minutes

Stage 2 (x40)

95°C for 20 seconds

57°C for 45 seconds

Stage 3

95°C for 15 seconds

55°C for 1 minute

95°C for 15 seconds

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

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

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

2.2.4. Flow cytometry analysis

2.2.4.1. Extracellular staining

Following stimulations approximately $2x10^5$ cells were transferred to labelled FACS tubes which were subsequently filled with FACS buffer. The cells were spun down at 300g for 3 minutes and the pellets resuspended in zero volume. Cells were stained for 20 min at 4°C with saturating varying concentrations of antibody (see Table 2.1). Live cells were gated according to their forward scatter and side scatter. Data were acquired on a FACS Canto (*Becton Dickinson*) and analysed using FlowJo software (TreeStar).

2.2.4.2. Intracellular staining

For intracellular (IFNγ, Granzyme B and TNFα) cytokine staining, endocytosis was blocked using golgi plug (*BD*) for four hours at the end of stimulations. Subsequently cells were stained with extracellular antibodies as outlined above. The cells were washed in FACS buffer spun down at 300g for 3 minutes. The cells were then fixed and permeabilized using Cytofix/Cytoperm reagent (BD Pharmingen) as per manufacturer's instructions. Following fixation and permeabilization the cells were washed in 1x permwash and spun down at 300g for 3 minutes. Subsequently the cells were stained with intracellular antibodies for 20 minutes and washed again in 1x permwash. Data were acquired on a FACS Canto (*Becton Dickinson*) and analysed using FlowJo software (TreeStar).

2.2.4.3. Phospho-S6 ribosomal protein intracellular staining.

Following stimulation cells were washed in FACS buffer and fixed in 0.5% paraformaldeyde at 4°C for 15 minutes. Following this they were further washed in phosphate-buffered saline (PBS), and permeabilized with 90% methanol (30 min, -20°C). After being washed again, cells were blocked with 0.5% bovine serum albumin in PBS (10 min, room temperature) and then incubated with primary anti-phospho-S6 ribosomal protein Ser 235/236 (Cell Signalling Technologies) for 30 min at RT. Cells were washed and then incubated with PE-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch) for 30 min at RT in the dark. Samples were washed and data acquired on a FACS Canto flow cytometer. As a positive control, maximal phospho-S6 staining was achieved with the pharmacological stimulation of cells using phorbol 12, 13-dibutyrate (PdBu) (Calbiochem) (20 ng/ml, 20 min, 37°C). As a negative control, rapamycin (20nM, 20 min, 37°C) was used to inhibit S6 phosphorylation.

2.2.4.4. 2NBDG Glucose uptake assay

$3x10^6$ splenocytes or $0.5x10^6$ cultured cell 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), 55μM β-mercaptoethanol (Sigma) and 1% Penicillin/Streptomycin (Invitrogen/Biosciences), at a concentration of $6x10^6$cells/ml. After a 15 minute incubation period at 37°C, cells were re-suspended in 1 ml of supplemented Glucose-free media with 50 μM 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 and analysed by flow cytometry.

2.2.4.5. CFSE labelling

$10x10^6$ Cells were pelleted in a 15ml falcon at 300g for 3 minutes. The cells were subsequently washed in FCS free media, while the pellet remained undisturbed. The media was removed and the pellet resuspended in 1ml FCS free media. 1ml of 2X CFSE (20μM) for a final concentration of 10μM was added. The cells were incubated at 37ºC for 12 minutes with occasional inverting. Finally, the cells were topped up with media containing 10% FCS, spun down at 300g for 3 minutes and put into the relevant culture.

2.2.5. Metabolic Analysis

2.2.5.2. Metabolic Seahorse analysis

A XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) simultaneously interrogates the two major energy producing pathways of the cell–

mitochondrial respiration and glycolysis in a microplate, in real-time. It determines the real-time *in vitro* oxygen consumption rate (OCR), and Extracellular acidification rate (ECAR), in order to assess cellular functions such as oxidative phosphorylation and glycolysis of NK cells cultured under various conditions. MACS purified NK cells were stimulated in their relevant conditions for 18hr. To get the seahorse plate ready for the addition of cells 200µl celltak (BD Pharmingen) (See Table 2.2) was added and incubated at room temperature for at least 20 minutes and washed off with sterile ddH_2O. Following 18hr stimulation NK cells were centrifuged at 300g, the supernatant was decanted and the cells were washed in 2-3mls of pre-warmed seahorse media (containing 10mM Glucose). The cells were spun down again and resuspended at a concentration of $7.5x10^6$ cells/ml in seahorse media supplemented with relevant treatments. 100µl ($0.75x10^6$ cells) media was added to each well and centrifuged with the break off at 300g for 3 minutes. The seahorse plate was incubated in a non-CO_2 incubator for 20 minutes. Following incubation wells were topped up with 500µl seahorse media containing relevant treatments. The seahorse plate was incubated for a further 40 minute. While the plate was incubating the inhibitors were made up and loaded into the relevant injection ports. Final inhibitor concentrations were as follows; oligomycin (2µM), fluoro-carbonyl cyanide phenylhdrazone (FCCP 0.5µM), rotenone (100nM) plus antimycin (4µM) and 2DG (30mM). Sequential measurements of ECAR and OCR following the addition of the inhibitors oligomycin (2µM), fluoro-carbonyl cyanide phenylhdrazone (FCCP 0.5µM), rotenone (100nM) plus antimycin (4µM) and 2DG (30mM) allows for the accurate calculation of the oxygen consumption due to OxPhos and the proton production due to glycolysis. First we must consider the role each of these inhibitors play.

Determining Glycolytic rates:

First we must consider the role each of these components play:

- Glucose (Glycolysis Substrate)
- Oligomycin (ATP Synthase Inhibitor)
- 2 Deoxy-D-glucose (Glycolysis Inhibitor)

Figure 2.1 below details the process of how to calculate the rate of glycolysis as well as the glycolytic capacity of a cell using the seahorse metabolic analyser.

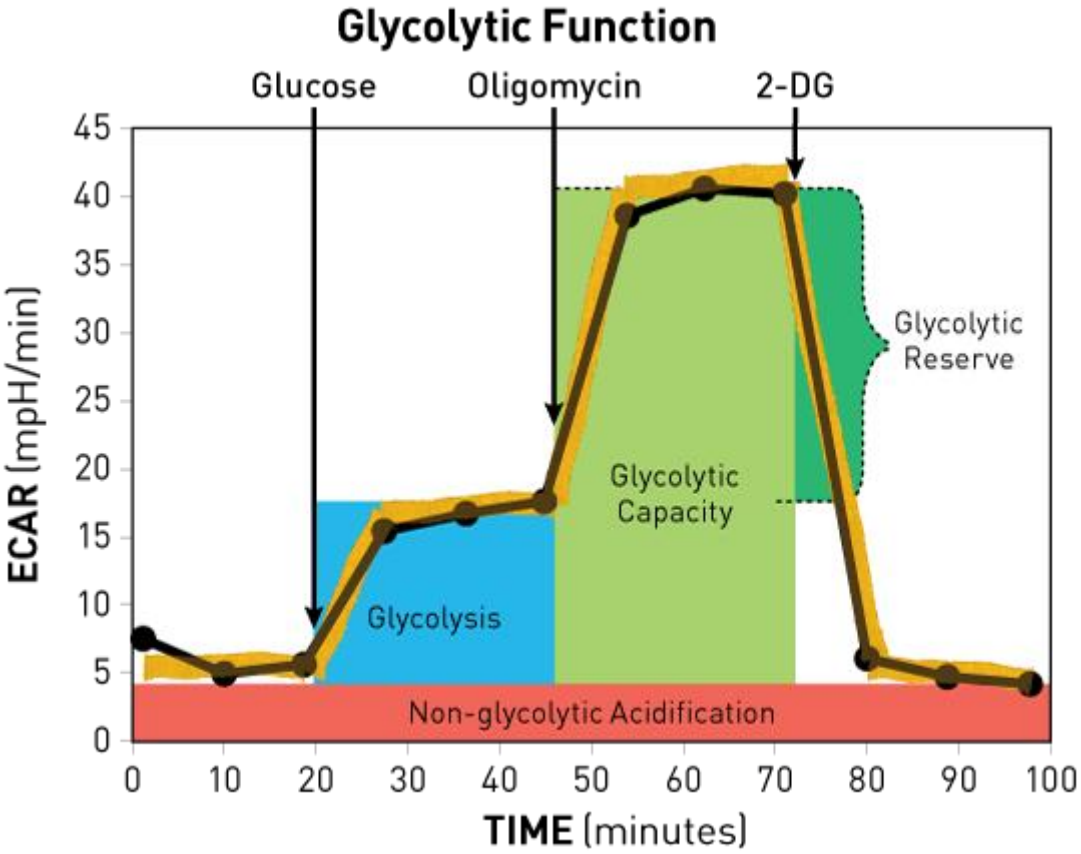

Figure 2.1. Determining Glycolytic rates using the Seahorse metabolic flux analyser.

Basal rates of glycolysis can be determined by the change in acidification in the media over time by release of protons during glycolysis. Oligomycin inhibits ATP synthase thus blocking OxPhos and increasing Glycolysis to maintain ATP production, this allows us to determine the maximum amount of glycolysis any given cell can undergo i.e. its glycolytic capacity. Finally 2DG, a direct glycolytic inhibitor blocks all glycolysis and thus any change in acidification of the media can be determined to be from a process other than glycolysis. This allows us to calculate the actual rates of glycolysis.

Determining OxPhos rates:

First we must consider the role each of these components play:

- Oligomycin (ATP Synthase Inhibitor)
- FCCP (Electron Transport Chain Accelerator)
- Antimycin A (Electron Transport Chain inhibitor A)
- Rotenone (Electron Transport Chain inhibitor B)

Figure 2.2 below details the process of how to calculate the rate of OxPhos of a cell using the seahorse metabolic analyser.

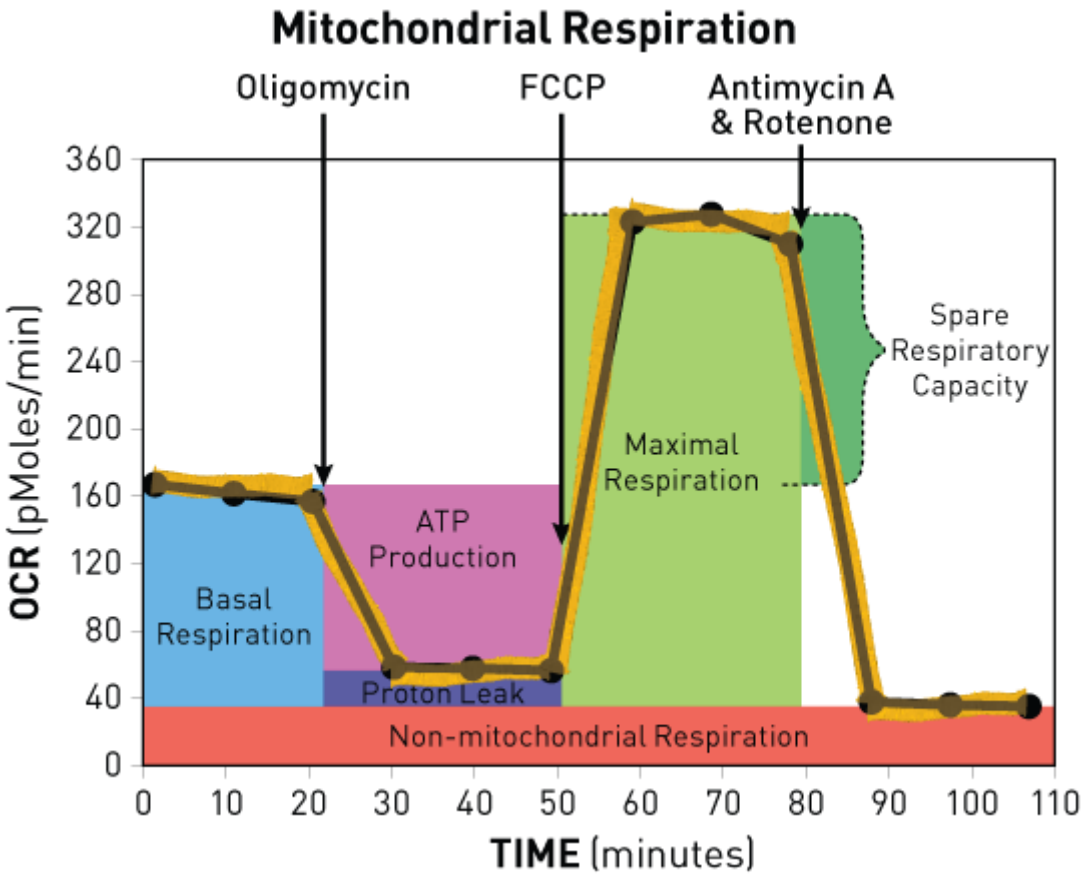

Figure 2.2. Determining OxPhos rates using the Seahorse metabolic flux analyser.

Basal rates of OxPhos can be determined by the amount of oxygen being consumed over time. While Oligomycin inhibits ATP synthase FCCP uncouples it which will dissipate the gradient of protons across the inner

mitochondrial membrane and thus maximising OxPhos. Antimycin A and Roteone inhibit the Electron transport chain thus any residual oxygen consumption can be discarded. This allows us to calculate the actual rates of OxPhos.

2.2.5.3. ATP assay

For ATP analysis, NK cells were MACS purified and seeded in a 96 well plate $1x10^7$ cells/ml (100,000 cells in 100μls). Following this they were stimulated with various cytokine and inhibitor combinations, for 18hours in triplicate. Cells were lysed and relative ATP levels in mM per 10^6cells were determined using and ATP assay determination kit (Perkin Elmer) as per the manufacturer's instructions. 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.

2.2.5.4 Lipid synthesis assay

Cells were spun down at 300g for 3 minutes; the supernatant was discarded before the samples were spun down once more to tighten the pellet in order to reduce the number of cells lost. The pellets were not resuspended but were washed with 10 ml of FCS free media before being spun down again. The supernatant was discarded and the pellet resuspended in fresh FCS free

RPMI media supplemented with LPDS. Cells were resuspended at a concentration of $4x10^6$ cells/ml and 250ul of cells were plated out into a 24 well plate. The plate was transferred to a hot lab. In the hot lab, 250 µl of prewarmed media (+LPDS) with 2-^{14}C acetate was added to each well, resulting in a final volume of 500 µl. At this point the plate was incubated in a radioactive incubator for four hours. Following incubation, the cells were transferred back to the hot lab where they were washed with ice cold PBS and spun down in 15 ml falcon tubes. The supernatant was discarded and the washing step repeated before the cells were lysed using 800ul 0.5% Triton X-100. 2ml of methanol was then added followed by a short vortexing step followed by the addition of 2ml of chloroform. Tubes were vortexed for two minutes followed by the addition of 1 ml ddH_2O and a further vortexing for 1 minute. The samples were all spun down for 15 minutes at 1000 rpm and the lower organic layer removed by careful pipetting into scintillation vials. The samples were then left in the fume hood for 2 days to allow the chloroform to evaporate. Once the chloroform had evaporated completely, 3 ml of Ultima Gold LSC cocktail was added to each scintillation vial to re-dissolve the lipids. The samples were then analysed on a scintillation counter, which quantified their radioactive content. The resulting dpm (disintegrations per minute) values were then converted to nmol of acetate incorporated per million cells.

2.2.5. Genotyping

45µl of ear lysis buffer was added to each ear punch along with 5µl of 10mg/ml proteinase K in 1.5ml eppendorfs. The eppendorfs were subsequently placed on the heating block for 4 hours at 55^{o}C followed by a final 7 minutes at 100^{o}C. After this time 50µl of ddH_2O was add per eppendorfs and samples were spun down at 17,000g for 2 minutes. DNA samples can be stored at 4^{o}C until use. Before using Template for the PCR reaction the samples underwent a 1 in 5 dilution in DEPC water. For CRE PCR 1µl of template was added to 24µl of Master mix, while for the SCAP PCR 1µl of Template was added to 19µls of Master mix (See Table Below for

appropriate master mixes). Genotyping PCRs were carried out as described below.

Primers;

CRE-forward: CGGTCGATGCAACGAGTGATGAGG

CRE-Reverse: CCAGAGACGGAAATCCATCGCTCG

SCAP-Forward: GCTCTGCGCATCCTATCCAATTCCC

SCAP-Reverse: CAGCCGGCAAGTAACAAGGGATCCG

Components (MM)	CRE (μl)	SCAP(μl)
5X Buffer	5	4
$MgCl_2$	2.5	1.6
25mM dNTPs	1	0.4
Forward Primer (10pmol/μl)	0.5	2
Reverse Primer (10pmol/μl)	0.5	2
GoTaq	0.1	0.2
DEPC water	14.4	10.2

Table 2.4: Genotyping Master Mixes.

CRE PCR:

94 °C	Hold	Have hot ready to go then skip to
94 °C	5min	
94 °C	1 min	
63 °C	2 min	X 35
72 °C	3 min	
72 °C	5 min	
4 °C	Hold	

SCAP PCR:

94 °C	Hold	Have hot ready to go then skip to

94 °C	3 min	
94 °C	30 Sec	
68 °C	45 Sec	X 35
72 °C	30 Sec	
72 °C	2 min	
4 °C	Hold	

2.2.7. Statistical Analysis

GraphPad Prism 6.00 for Macintosh (GraphPad Software) was used for statistical analysis. The data was tested for normality before undergoing statistical analysis. The one-way ANOVA test was used when comparing more than two data sets with a Tukey multiple comparisons test. A students T-test was used when there were only 2 data sets to compare. Column statistics and a one-sample t test was used to calculate *P* values with the theoretical mean set to 1.00 of one of the data sets.

Chapter 3- mTORC1-dependent metabolic reprogramming is a prerequisite for IL-2/12 stimulated NK cell effector function.

3.1. Introduction

mTORC1 is a serine/ threonine kinase with roles in regulating immunological systems. mTORC1 activity has been ascribed important roles in the regulation of a wide range of immune cells in both the innate and adaptive arms of immunity (Powell et al., 2012). It is becoming clear that mTORC1-regulated cellular metabolism is crucial to the immunoregulatory function of this kinase complex in immune cells. Indeed, new evidence is emerging that metabolism plays a fundamental role in dictating immune cell differentiation and function. In 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., 2012). mTORC1 regulated glycolysis is also linked to normal differentiation of effector $CD4^+$ T cells (Shi et al., 2011). However, the role played by 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 antiviral and antitumor immune responses. Although NK cells are critical in early immune responses, they also regulate the ensuing adaptive immune response through the release of cytokines that modulate the downstream immune response, notably IFNγ and TNFα, and by modulating DC numbers (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. In particular, 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 produced by T cells is critical for modulating NK cell activation in response to various pathogenic infections (Lee et al., 2012, Bihl et al., 2010, Kerdiles et al., 2013). 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.

3.2. IFNγ production is linked to metabolic activity of NK cells in vivo.

Natural killer (NK) cells are a cytotoxic lymphocyte critical to the innate immune system helping to fight viral infections and tumours. Poly I:C is a synthetic dsRNA analogue, which is often used as a mimic of viral infection and as such has the ability to activate NK cells *in vivo* (Miyake et al., 2009, Schmidt et al., 2004). To investigate whether NK cell activation is associated with changes in NK cell metabolism, mice were injected with poly (I:C) and splenocytes isolated after 24 hours for analysis. Poly (I:C) efficiently activated $CD3^{-}NK1.1^{+}NKp46^{+}$ NK cells (Fig. 3.1) as measured by increased expression of the glycoprotein CD69, an early activation marker (Fig. 3.2a). *In vivo* analysis show that Poly (I:C) activated NK cells increase in size based on FSC which reflects increased cell growth (Fig. 3.2b). Increased cell size suggests these cells have increased metabolic activity and increased rates of cell growth. If this were the case we predicted that these cells would have increased rates of nutrient uptake. A key cellular nutrient for multiple effector immune cell subsets is glucose. To investigate whether activation of NK cells *in vivo* is indeed linked to increased nutrient uptake we measured glucose uptake using a fluorescently labeled 2-deoxyglucose analogue; NBDG, which can be taken up by the cell but cannot be metabolized. As expected we saw that NK cells with increased forward scatter had elevated rates of glucose uptake (Fig. 3.3a). As glucose is taken into the cell by passive diffusion down a concentration gradient, elevated glucose uptake would imply increased expression of glucose transporters. As well as increasing their uptake of glucose, the bigger NK cells also increased the expression of other important nutrient receptors including CD71, required for iron delivery from transferrin to cells, and CD98, a component of the L-amino acid transporter (Fig. 3.3b). Overall there is an increase in expression of CD71 and CD98 with Poly (I:C) treatment compared to the PBS vehicle control (Fig 3.3c).

As the activated NK cells had increased metabolism, we hypothesized that there may be a direct link between metabolic activity and NK cell function. To address this, we investigated the production of IFNγ by NK cells activated *in vivo.* In response to poly (I:C), activated NK cells consistently induce the expression of IFNγ (Fig. 3.4a). Further detailed analysis of the NK cells that were producing IFNγ led us to the finding that the IFNγ producing cells were the NK cells with increased cell size and thus increased metabolic activity (Fig. 3.4b-c). A cut off point for an increase in cell size was determined based on the unactivated PBS treated NK cells (Fig 3.4a). Analysis of FSC^{high} NK cells showed a significant increase in IFNγ in Poly (I:C) activated cells (Fig 3.4b). A comparison of the big NK cells and the small cells of the Poly (I:C) activated NK cells reveals a substantial shift in the production of IFNγ with the big metabolically active NK cells producing more IFNγ (Fig 3.4c). Together, this data demonstrates that NK cells activated *in vivo* with Poly (I:C) increased the uptake of key nutrients to promote cell growth and increased cell mass. Strikingly it appears that key effector molecules such as the production of IFNγ are restricted to these metabolically active big NK cells.

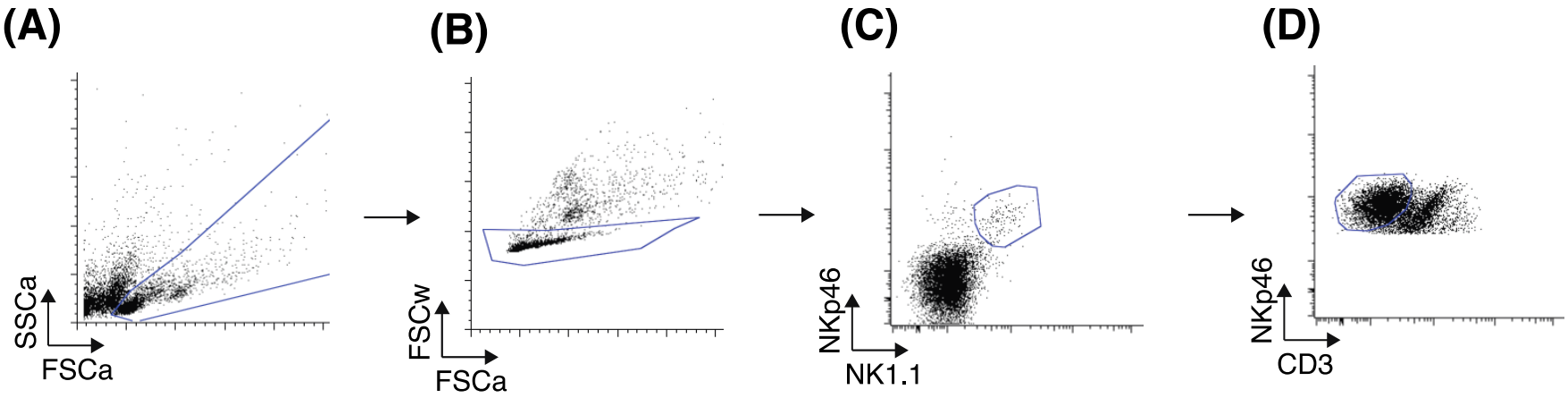

Fig 3.1. Gating strategy for isolating NK cells from splenocyte population. (A-D) NK cells are isolated from other splenocytes based on the following gating strategy. Live cells are distinguished from dead cells using Forward and Side Scatter area (a). Doublets are then excluded from the live cell population based on their increased forward-scatter width (b). Total NK cells were isolated using two NK cell markers; NKp46 and NK1.1 (c). Finally NKT cells were excluded from the population with a CD3 marker (d). All data was acquired on the FACS canto and are representative of at least N=3.

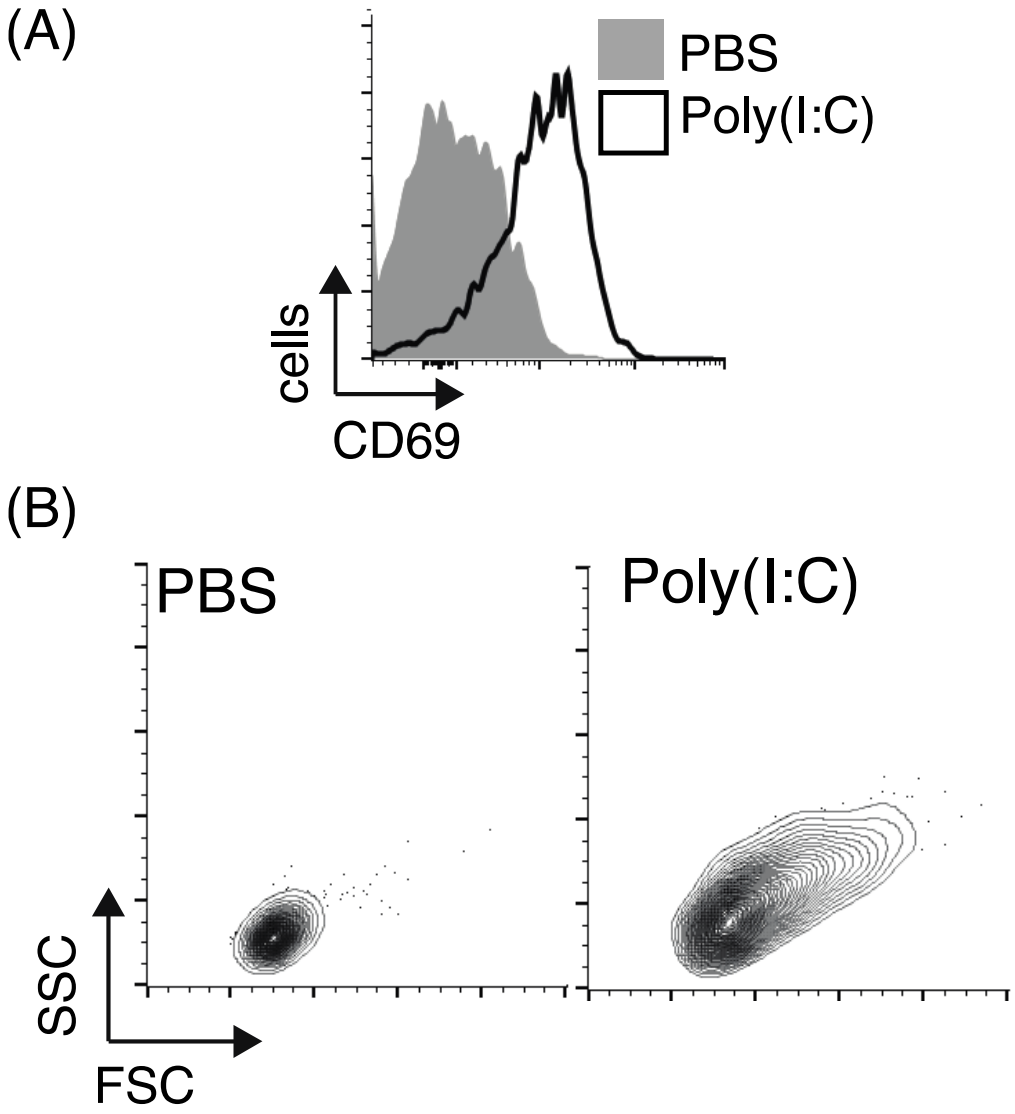

Figure 3.2. Poly (I:C) activated NK cells increase in cell size. Mice were administered PBS or 200μg Poly(I:C) by peritoneal injection and spleens harvested after 24 hours. Splenocytes were isolated and subjected to flow cytometry staining to determine expression of CD69 (a) and cell size based on forward-side scatter (b) of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of 10 mice for each condition.

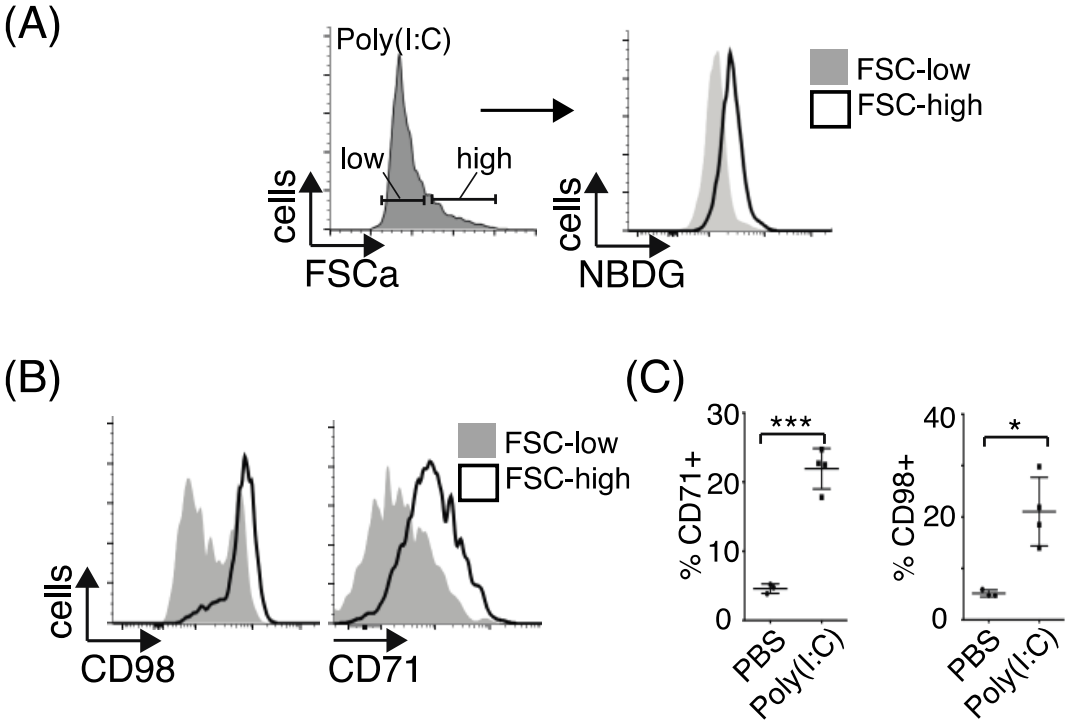

Figure 3.3. Poly (I:C) activated NK cells undergo changes in metabolism. (A-C) Mice were administered PBS or 200μg Poly (I:C) by peritoneal injection and spleens harvested after 24 hours. Splenocytes were isolated and subjected to Flow cytometry staining to determine CD71 and CD98 (b,c) and glucose uptake based on 2NBDG (c) of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of 10 mice for each condition. Data is mean +/- S.E.M of 10 mice for each condition (c) or representative of 10 mice (a and b). (* $p<0.05$, ***$p<0.001$).

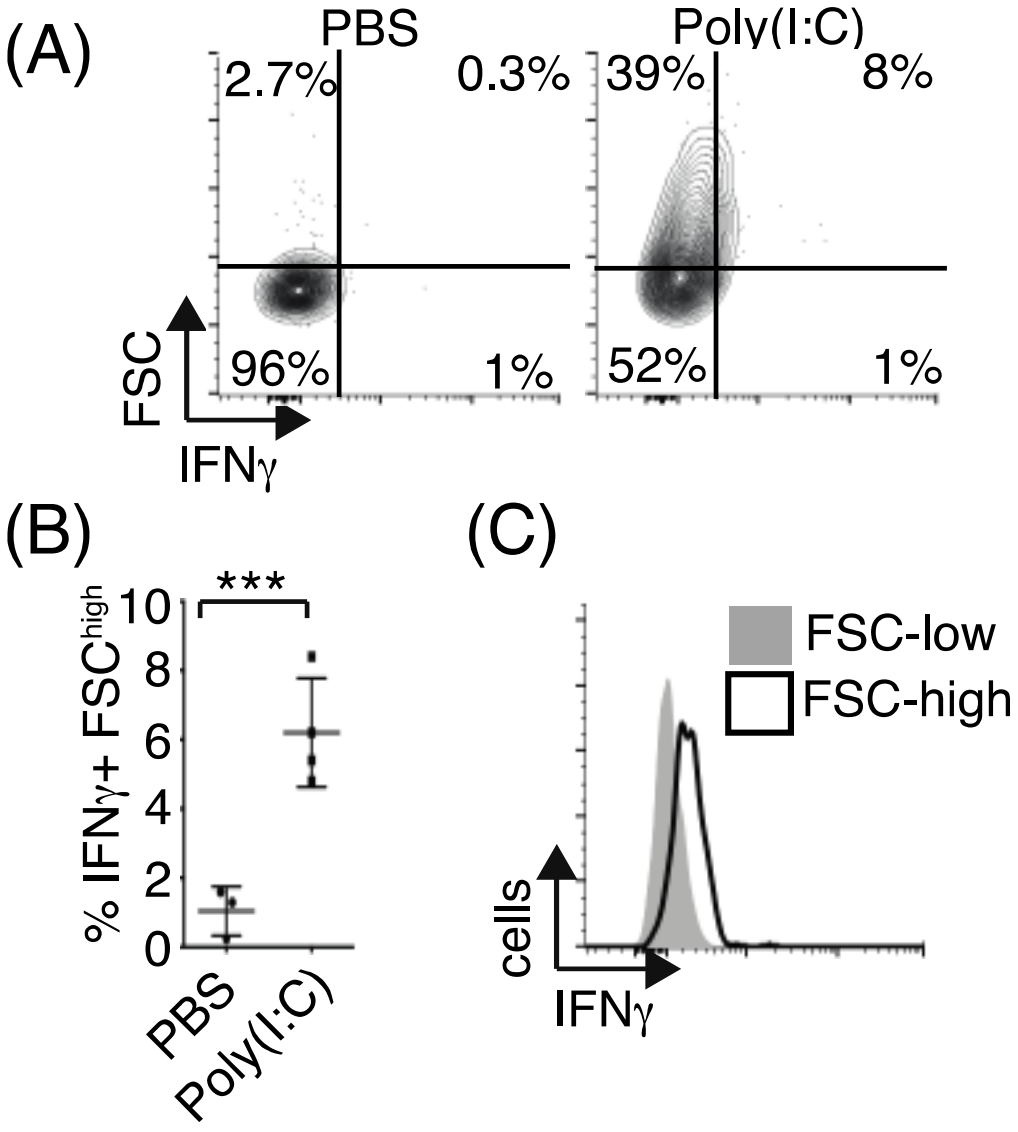

Figure 3.4. IFNγ production is primarily linked to metabolically active Poly (I:C) activated NK cells. (A-C) Mice were administered PBS or 200μg Poly (I:C) by peritoneal injection and spleens harvested after 24 hours. Splenocytes were isolated and subjected to Flow cytometry staining to determine IFNγ expression of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of 10 mice for each condition. Data is mean +/- S.E.M of 10 mice for each condition (b) or representative of 10 mice (a and c). (***p<0.001)

3.3. IL-2/12 activated NK cells undergo a metabolic shift involving increased glycolytic levels and metabolic reprogramming.

To accurately characterize the metabolic changes occurring following NK cell activation requires detailed metabolic analyses, which require cell numbers that cannot realistically be generated and isolated from *in vivo* models. We therefore validated an *ex vivo* culture system (Zhao and French, 2012) whereby splenocytes are cultured in low dose IL15 (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). We consistently observed an increase in NK numbers (30 fold increase in 7 days) resulting in relative enrichment of NK cells from 2 to 70% of cultured cells (Fig. 3.5a-b). While IL-15 has been shown to have the ability to activate NK cells at high doses (Walzer et al., 2005, Trinchieri, 1989), it has also been shown to have an essential role in the survival of mature NK cells (Koka et al., 2003). Indeed, this culture process provided large numbers of "primed" NK cells that responded robustly to subsequent stimulation, with distinct increases in cell size, CD69 expression, and IFNγ production, as expected (Fig 3.6a-c). Given that NK cell activation can be closely linked to T cell responses and the observations that T cell– derived IL-2 and the innate cytokine IL-12 are important for optimal NK cell activation in response to various infections (Lee et al., 2012, Bihl et al., 2010), the effect of supplementing poly (I:C)-stimulated splenocytes with IL-2 and IL-12 was investigated. Poly (I:C) plus IL-2/12 stimulated NK cells had significantly enhanced levels of IFNγ production compared with poly(I:C) stimulation alone, both in terms of the frequency of $IFN\gamma^+$ NK cells and the amount of IFNγ protein expressed per NK cell, as measured by the MFI of IFNγ staining (Fig 3.7). Indeed, consistent with observations of NK cells activated *in vivo*, IL-2/12 stimulation of cultured NK cells resulted in increased glucose uptake (Fig. 3.8a), and increased CD71 and CD98 expression (Fig. 3.8b-c). Detailed metabolic analysis of these cytokine activated NK cells was conducted using the 'Seahorse" metabolic flux

analyzer which can be used to measure the rates glycolysis and mitochondrial oxidative phosphorylation (OxPhos). We found that IL-2 but not IL-12 increased the rate of glycolysis, metabolizing glucose to produce lactate, i.e. aerobic glycolysis (Fig. 3.9a). However, it was also apparent that the combination of IL-2 and IL-12 resulted in a synergistic increase in NK cell glycolysis (Fig. 3.9a). This synergy likely reflects IL-12 induced expression of the high affinity IL-2 receptor subunit CD25 (Fig 3.10a) (Lee et al., 2012), which would result in enhanced IL-2 signalling. Increased IL-2 signalling can be seen with the synergistic increased in pS6 that is reflective of increased mTORC1 activity (Fig. 3.10b). IL-2/12 stimulation also increases the rate of oxidative phosphorylation (OxPhos) (Fig. 3.9b) but overall there is a shift in balance of glucose utilization from oxidative metabolism to glycolysis (Fig. 3.9c).

Increased aerobic glycolysis might simply reflect increased flux of glucose metabolized to lactate rather than to CO_2 via oxidative phosphorylation. Alternatively, the observed metabolic changes might involve reprogramming of the core glycolytic machinery as reflected by increased expression of glucose transporters and glycolytic enzymes. To distinguish between these possibilities the glycolytic capacity (max rate of glycolysis possible) of IL-2/12 activated NK cells was measured. The glycolytic capacity can be determined by measuring the rate of glycolysis following the addition of the OxPhos inhibitor oligomycin. In the absence of ATP synthase within the mitochondria, there is a compensatory increase in the rate of glycolysis to the maximum rate permitted by the glycolytic machinery available to maintain energy homeostasis. IL-2/12 stimulated NK cells showed a substantial increased glycolytic capacity (Fig. 3.11a) indicating that there was increased glycolytic machinery available in activated NK cells. Indeed, there was increased expression of key components of the glycolytic pathway including the glucose receptor Glut1, and the glycolytic enzymes Hexokinase 2 (Hex2), and Lactate dehydrogenase A (Ldha) in IL-2/12 stimulated NK cells (Fig.

3.11b). Therefore, cytokine stimulated NK cells undergo metabolic reprogramming to allow for elevated levels of glycolysis.

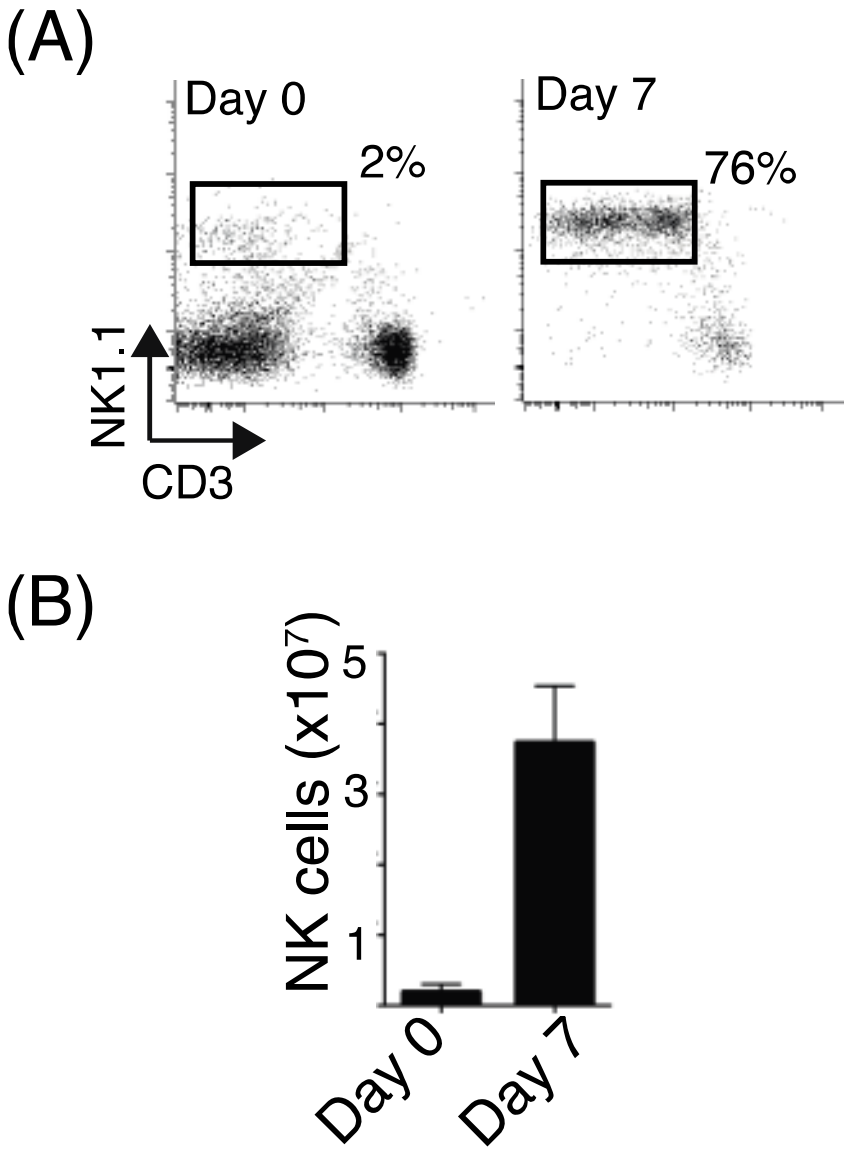

Figure 3.5. NK cells can be expanded ex vivo in low dose of IL-15. (A-B) Splenocytes were isolated from a spleen, counted and subjected to flow cytometry analysis. The remainder was cultured in IL-15 (25ng/ml) for 6 days. Cells were harvested on day 6, counted and FACS analysis performed to assess NK cell numbers and purity. Analysis of the frequency (a) and numbers (b) of NK1.1+ NKp46+ CD3- NK cells in splenocytes analyzed directly ex vivo (day 0) and following 6 days culture in 25ng/ml IL15 can be seen.

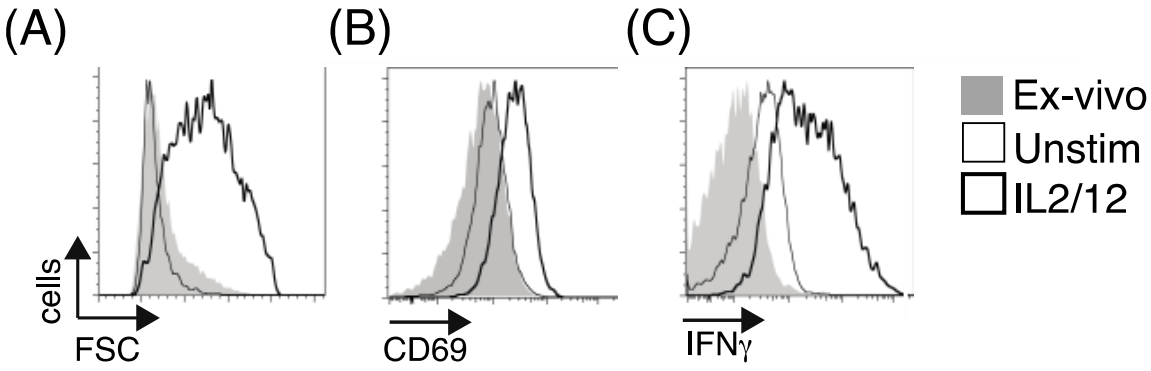

Figure 3.6. NK cells expanded in IL-15 remain inactive. (A-C) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 overnight and subjected to flow cytometry analysis to determine expression of CD69 (b), IFNγ (c) as well as cell size based on forward-side scatter (a) of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of at least N=3.

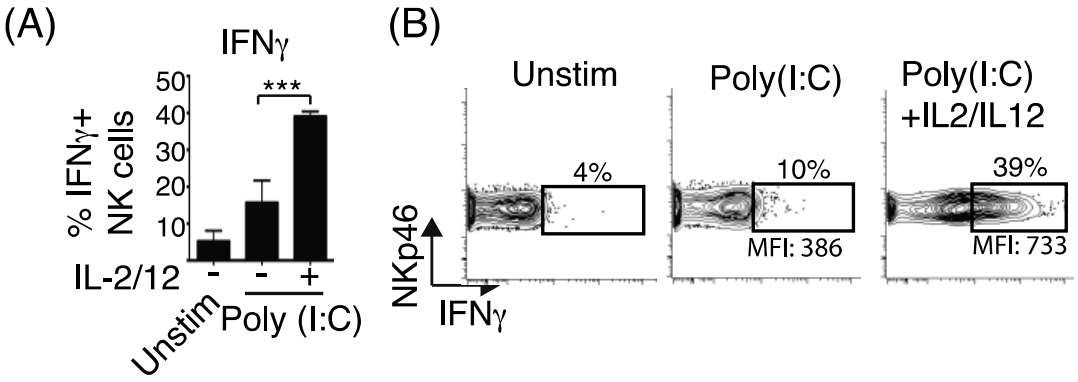

Figure 3.7. IL-2 and IL-12 enhance Poly (I:C) induced IFNγ production in NK cells. (A-B) Splenocytes were isolated from a spleen and stimulated ex vivo with Poly (I:C) +/- a combination of IL-2 and IL-12 or left unstimulated in IL-15 (5ng/ml) for 18hrs. Stimulated cells were subjected to flow cytometry analysis where expression of IFNγ of NK1.1+ NKp46+ CD3- NK cells was determined. Data is mean +/- S.E.M of N=3 (a) and representative dot plot (b). (***p<0.001).

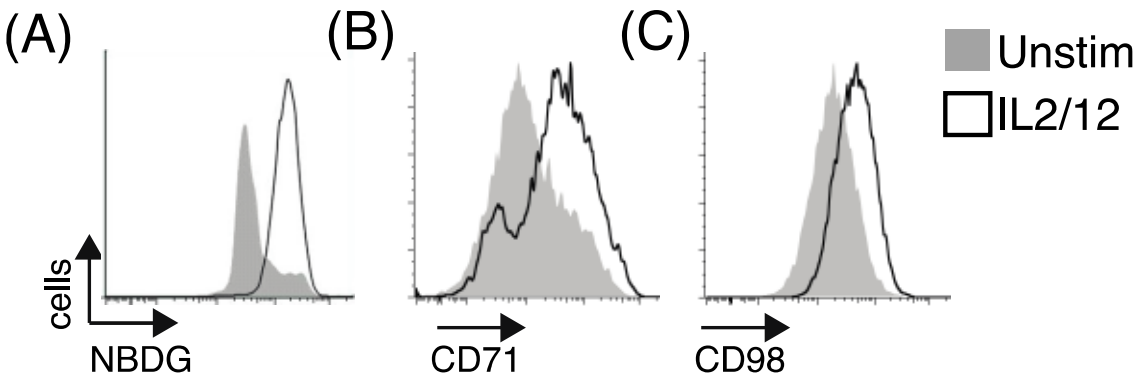

Figure 3.8. IL-2/12 simulates NK cell metabolism. (A-C) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 overnight and subjected to flow cytometry analysis to determine the expression of CD71 (b), CD98 (c) as well as glucose uptake (a) of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of at least N=3.

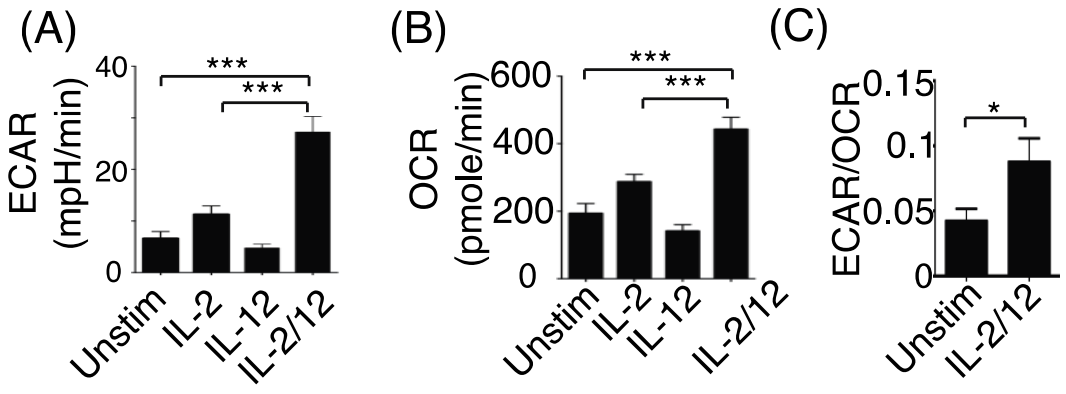

Figure 3.9. IL-2/12 stimulates NK cell energy metabolism in a synergistic manner. (A-C) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2, IL-12 or a combination of IL-2/12 overnight. Stimulated NK cells underwent detailed metabolic analysis using the Seahorse metabolic Flux analyzer to determine the glycolytic (a) and Oxidative Phosphorylation (b) levels as well as the overall metabolic shift (c). Synergy was determined using the synergy index tool. Data is mean +/- S.E.M of N=3 (*$p<0.05$, ***$p<0.001$).

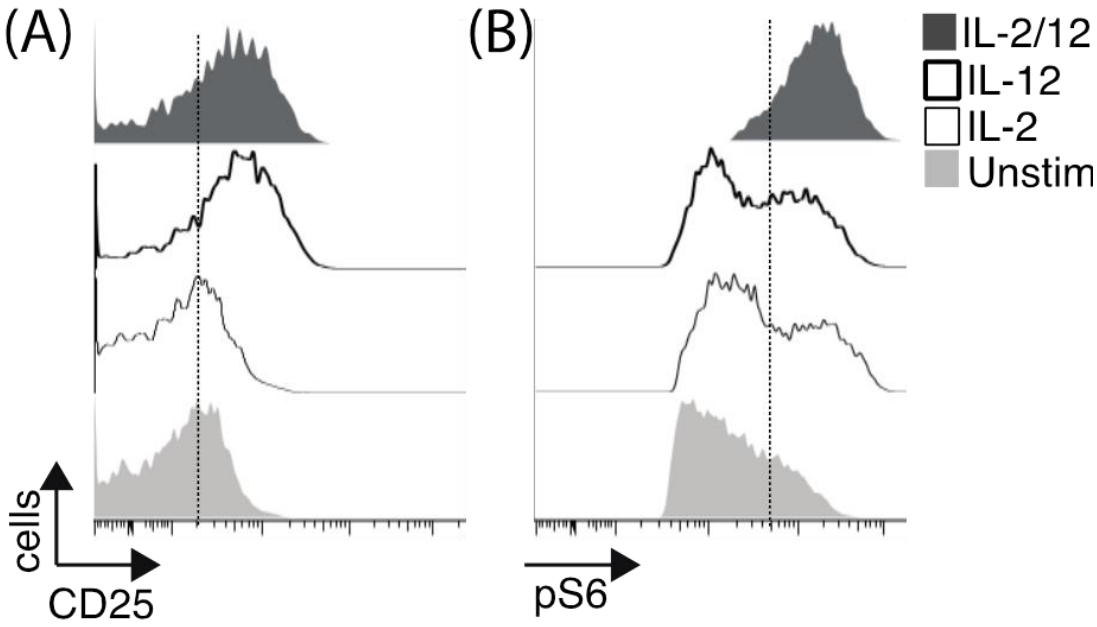

Figure 3.10. IL-12 induces expression of the high affinity IL-2 receptor subunit CD25. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2, IL-12 or a combination of IL-2/12 overnight. Stimulated NK cells were subjected to flow cytometry analysis to determine the expression of CD25 (a) and pS6 (b) of NK1.1+ NKp46+ CD3- NK cells. Data is representative of at least N=5.

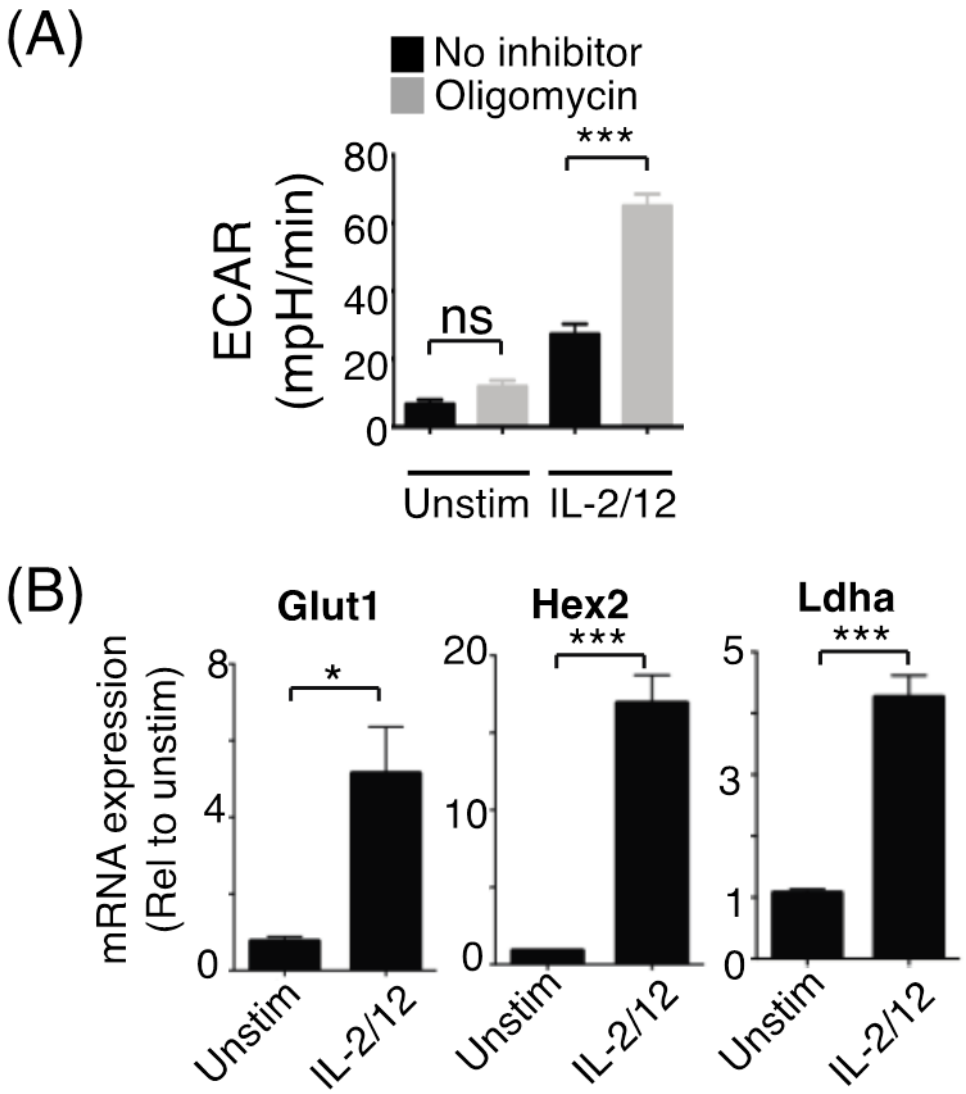

Figure 3.11. IL-2/12 simulation induces metabolic reprogramming in NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 overnight. Stimulated NK cells underwent detailed metabolic analysis using the Seahorse metabolic Flux analyzer, which determined their glycolytic capacity (a). Stimulated NK cells were also subjected to RT-qPCR and expression levels of Glut1, Hex2 and Ldha (b) were determined. Data is mean +/- S.E.M of N=3. (ns=not significant, * $p<0.05$, ***$p<0.001$).

3.4. Elevated glycolysis is required for IL-2/12 stimulated IFNγ production

The discovery of a clear metabolic shift accompanying cytokine stimulation of NK cells prompted the question as to whether this elevated metabolic state is required for key NK cell effector molecules. A series of experiments were designed to assess the effect of limiting NK cell metabolism in activated NK cells with respect to NK cell functional outputs. Initial experiments sought to disrupt elevated rates of glycolysis using low doses of the glycolytic inhibitor 2-deoxyglucose (2DG) as previously described (Sukumar et al., 2013). High doses of 2DG (10mM) are toxic as they exert a complete block on glycolysis and compromise cellular energy homeostasis as evidenced by decreased ATP levels (Fig. 3.12). However, low doses of 2DG (0.5-1mM) that are predicted to exert a partial glycolytic block do not compromise cellular ATP levels and are not toxic to NK cells (Fig. 3.12). Cultured NK cells were activated in the presence of low doses of 2DG; NK cell activation and the expression of NK cell effector molecules IFNγ and granzyme b were assessed after 18 hours. While 2DG treated NK cells were activated in response to IL-2/12 as measured by increased expression of CD69 and CD25 (Fig 3.13a), they failed to achieve the cellular growth required to increase cell mass (Fig. 3.13b). Thus, IL-2/12 activated NK cells in the presence of 2DG are small cells compared to those activated in the absence of 2DG. Strikingly, limiting the rate of glycolysis inhibits the production of IFNγ and granzyme b in IL-2/12 stimulated NK cells (Fig. 3.14). Together this data demonstrates that elevated levels of glycolysis are required for activation induced NK cell growth and acquisition of effector function.

A second complementary approach was also used to limit the rate of glycolysis in activated NK cells. Galactose is a carbon fuel source that is metabolized by glycolysis following 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 acts to limit the overall rate of glycolysis. Galactose is not toxic to NK cells and they are able to maintain

cellular ATP levels comparable to NK cells stimulated in glucose (Fig 3.15b). NK cells cultured in galactose up-regulated CD69 expression in response to IL-2/12 stimulation as expected (Fig. 3.16a). However, NK cells cultured in galactose could not promote cellular growth and increased cell size in response to IL-2/12 stimulation (Fig. 3.16b). Metabolic analysis confirmed that galactose did not support elevated rates of glycolysis; however, the cells had normal rates of OxPhos (Fig. 3.17a-b). Furthermore, galactose fed cells did not undergo a metabolic shift, and primarily utilize oxidative glucose metabolism (Fig 3.17c). Limiting the rate of glycolysis by providing NK cells with galactose rather than glucose as their primary fuel source inhibits IL-2/12 induced IFNγ and granzyme b production (Fig 3.18). Combined, this data clearly demonstrates an integral link between the rate of NK cell glycolysis, NK cell growth and effector molecules.

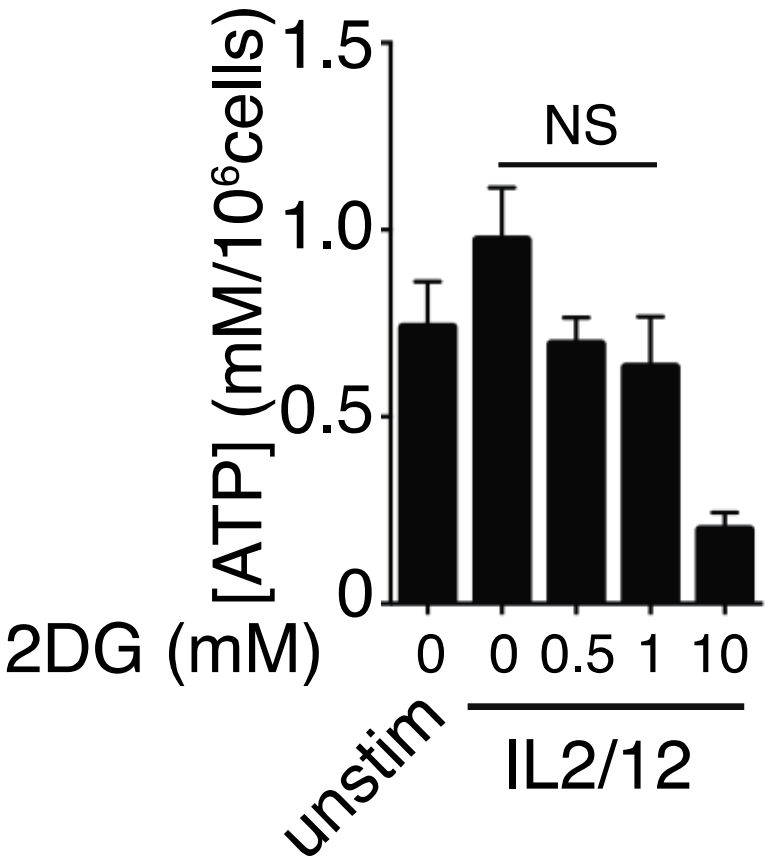

Figure 3.12. Inhibition of elevated glycolysis in IL-2/12 stimulated NK cells has no effect on ATP homeostasis. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- 2DG overnight. Stimulated NK cells were subjected to metabolic analysis to measure their ATP levels. Data is mean +/- S.E.M of N=3. (ns=not significant).

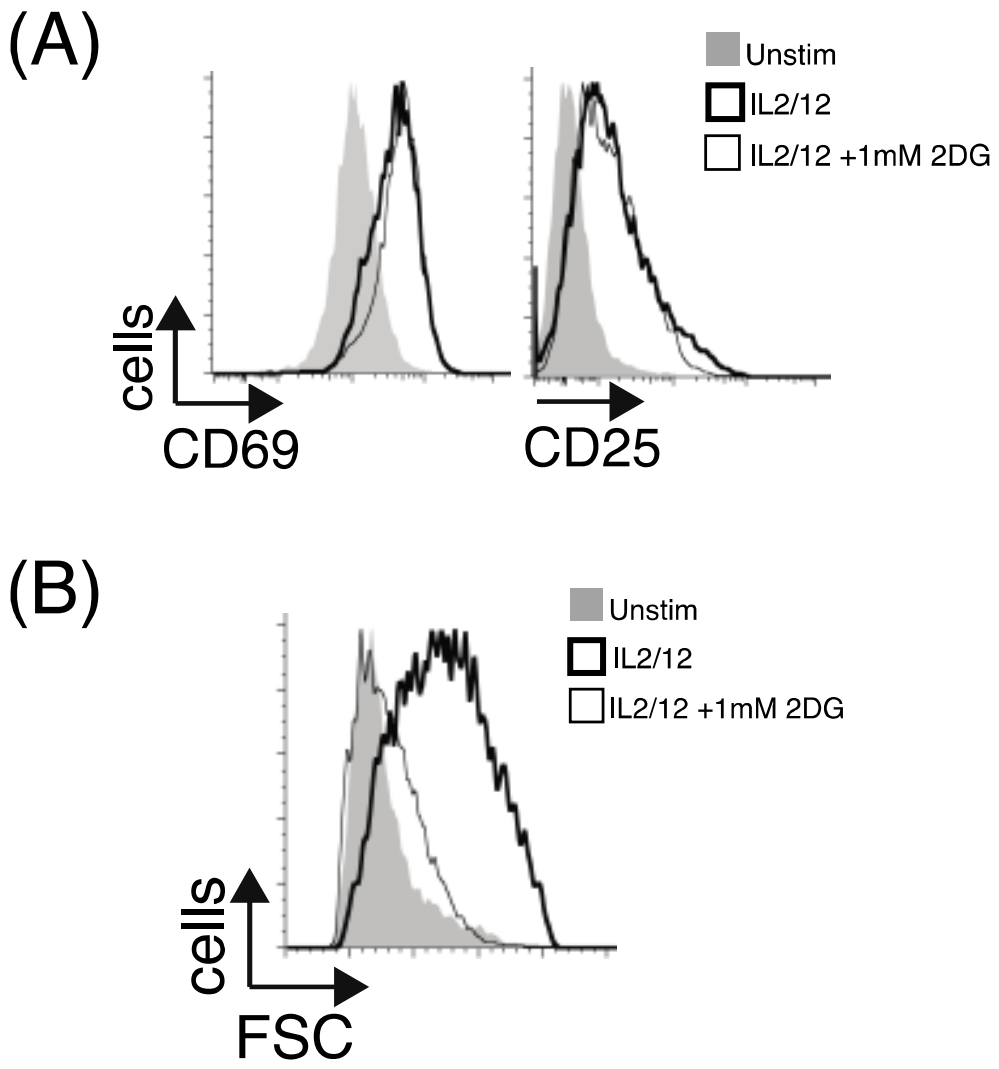

Figure 3.13. 2DG treated NK cells are unable to engage in cellular growth while activating normally with IL-2/12. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- 2DG overnight and subjected to flow cytometry analysis to determine the expression of CD69 and CD25 (a) and size based on Forward-side scatter (b) of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of at least N=3.

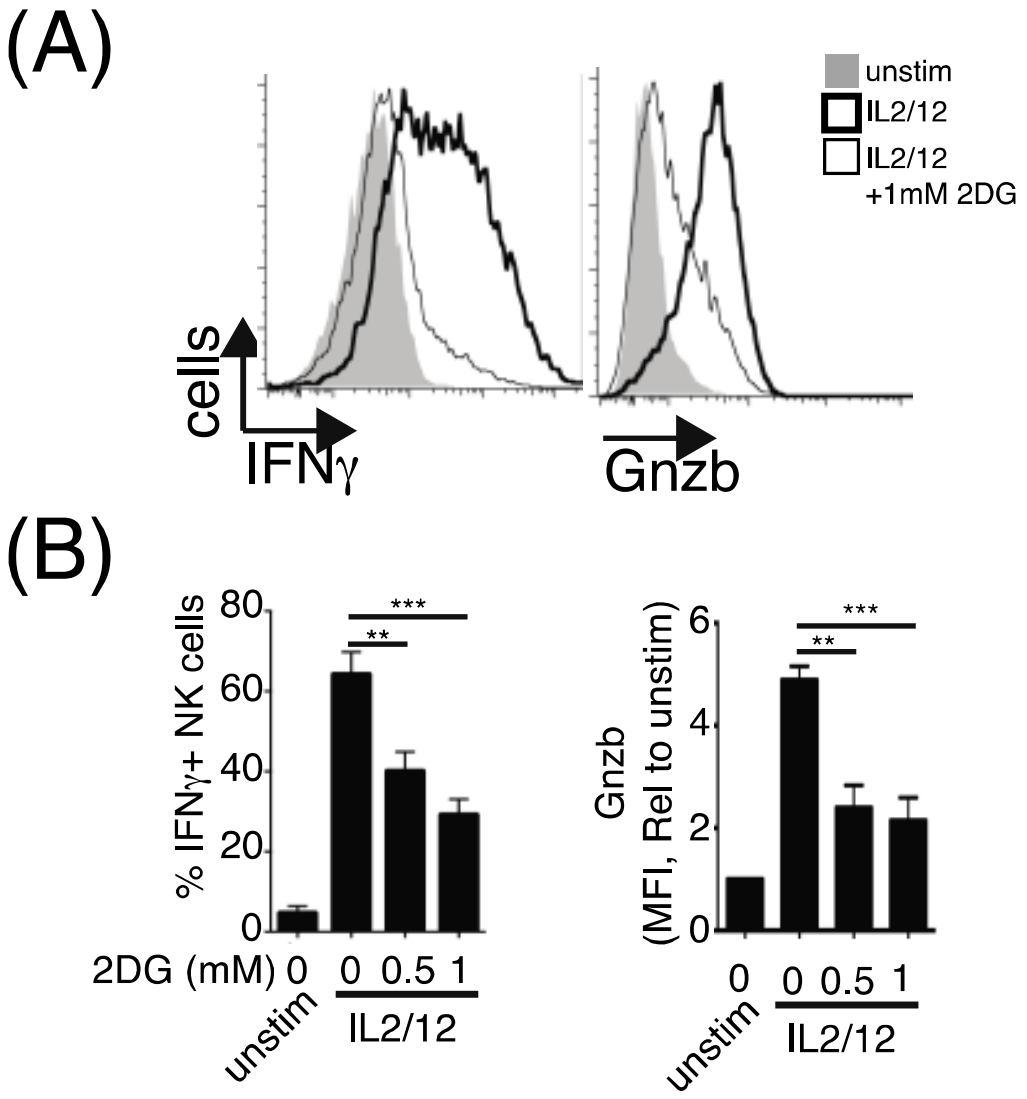

Figure 3.14. Inhibition of elevated Glycolysis with 2DG treatment partially inhibits IL-2/12 induced increase in IFNγ and Granzyme B Production. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- 2DG in a dose response (0mM, 0.5mM and 1mM) overnight and subjected to flow cytometry analysis to determine IFNγ and granzyme b expression of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of at least N=3 or is mean +/- S.E.M of N=3. (** $p<0.01$, ***$p<0.001$).

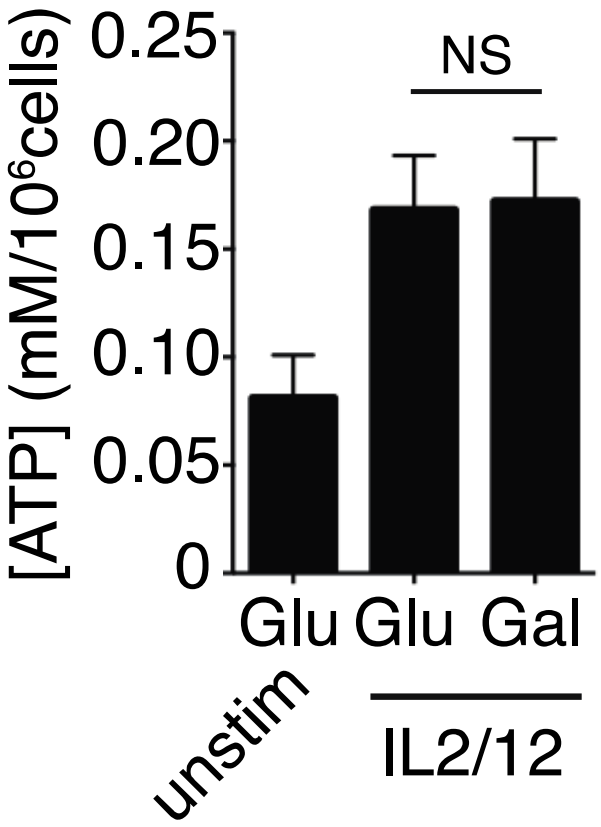

Figure 3.15. Inhibition of elevated glycolysis in IL-2/12 stimulated NK cells has no effect on ATP homeostasis. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 in glucose or galactose containing media overnight. Stimulated NK cells were subjected to metabolic analysis to measure their ATP levels. Data is mean +/- S.E.M of N=3. (ns=not significant).

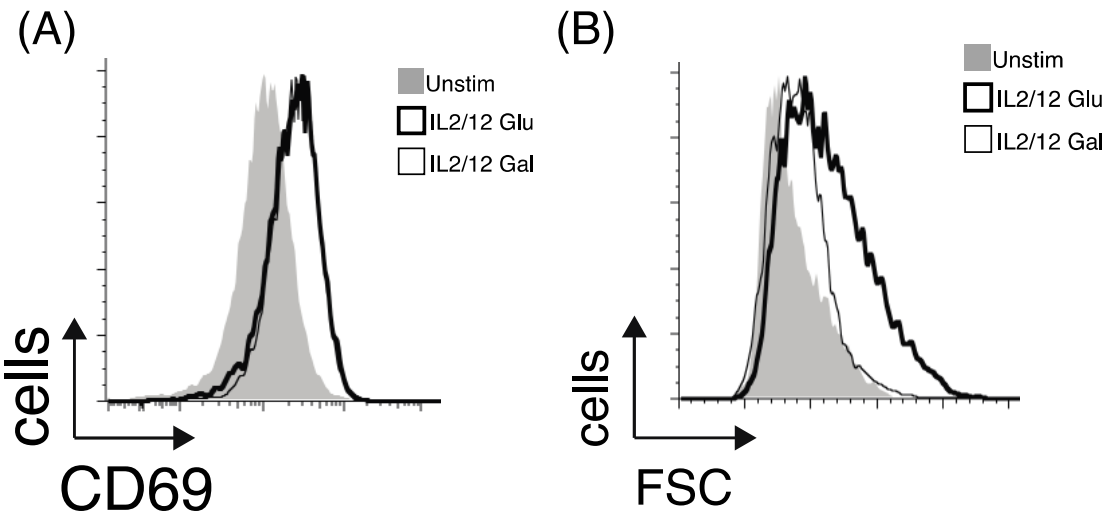

Figure 3.16. NK cells cultured in Galactose are unable to engage in cellular growth while activating normally. Expanded NK cells were maintained in IL15 (5ng/ml) or stimulated with IL-2/12 in the presence of glucose or galactose as a fuel source overnight and subjected to flow cytometry analysis to determine the expression of CD69 (a) as well as cell size based of forward-side scatter (b) of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of at least N=3.

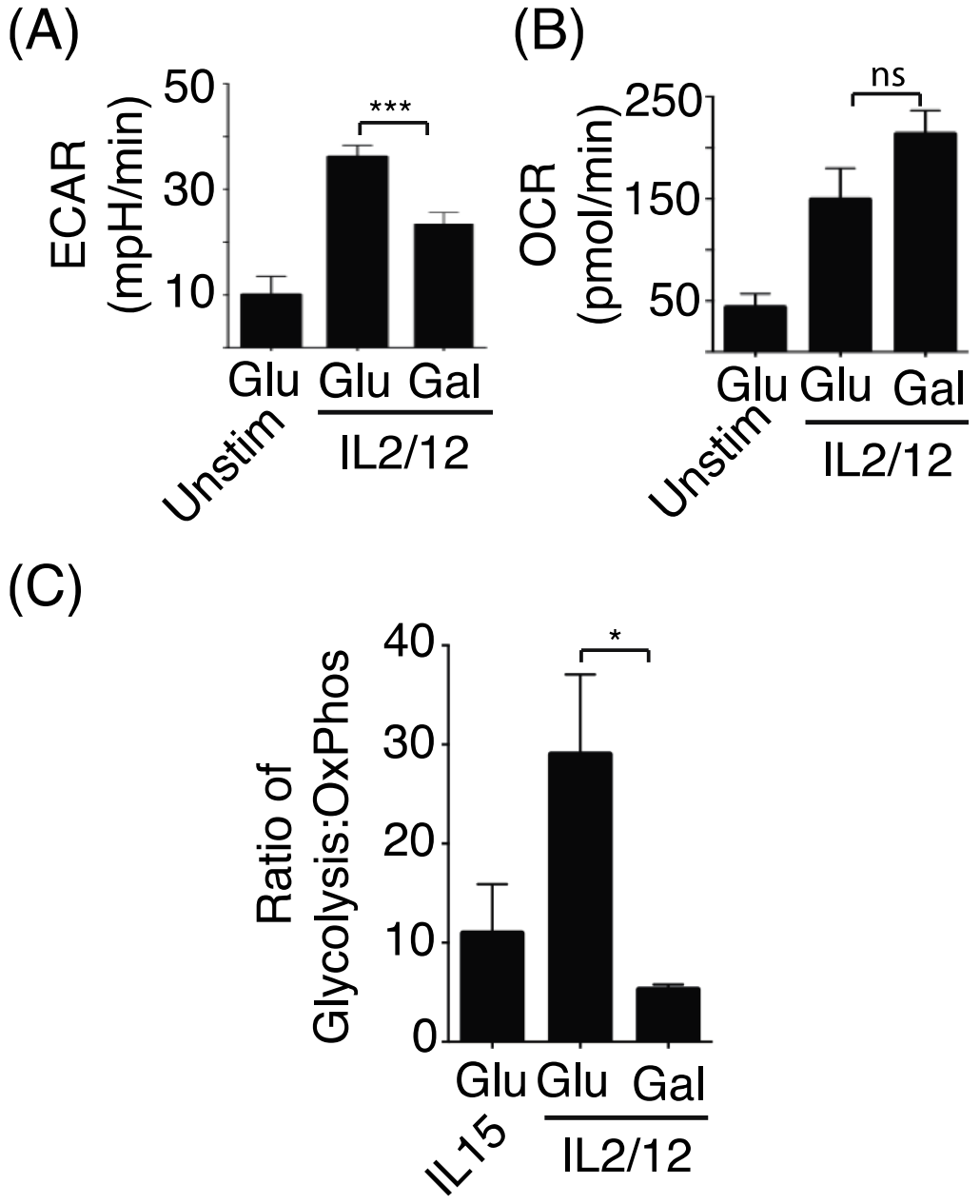

Figure 3.17. Inhibition of IL-2/12 induced elevated glycolysis through culturing cells in galactose. Expanded NK cells were maintained in IL15 (5ng/ml) or stimulated with IL-2/12 in the presence of glucose or galactose as a fuel source overnight and subjected to detailed metabolic analysis using the Seahorse metabolic Flux analyzer, which determined the levels of glycolysis (a) and oxidative phosphorylation (b). Data is mean +/- S.E.M of N=2 (ns=not significant, *p<0.05, ***p<0.001).

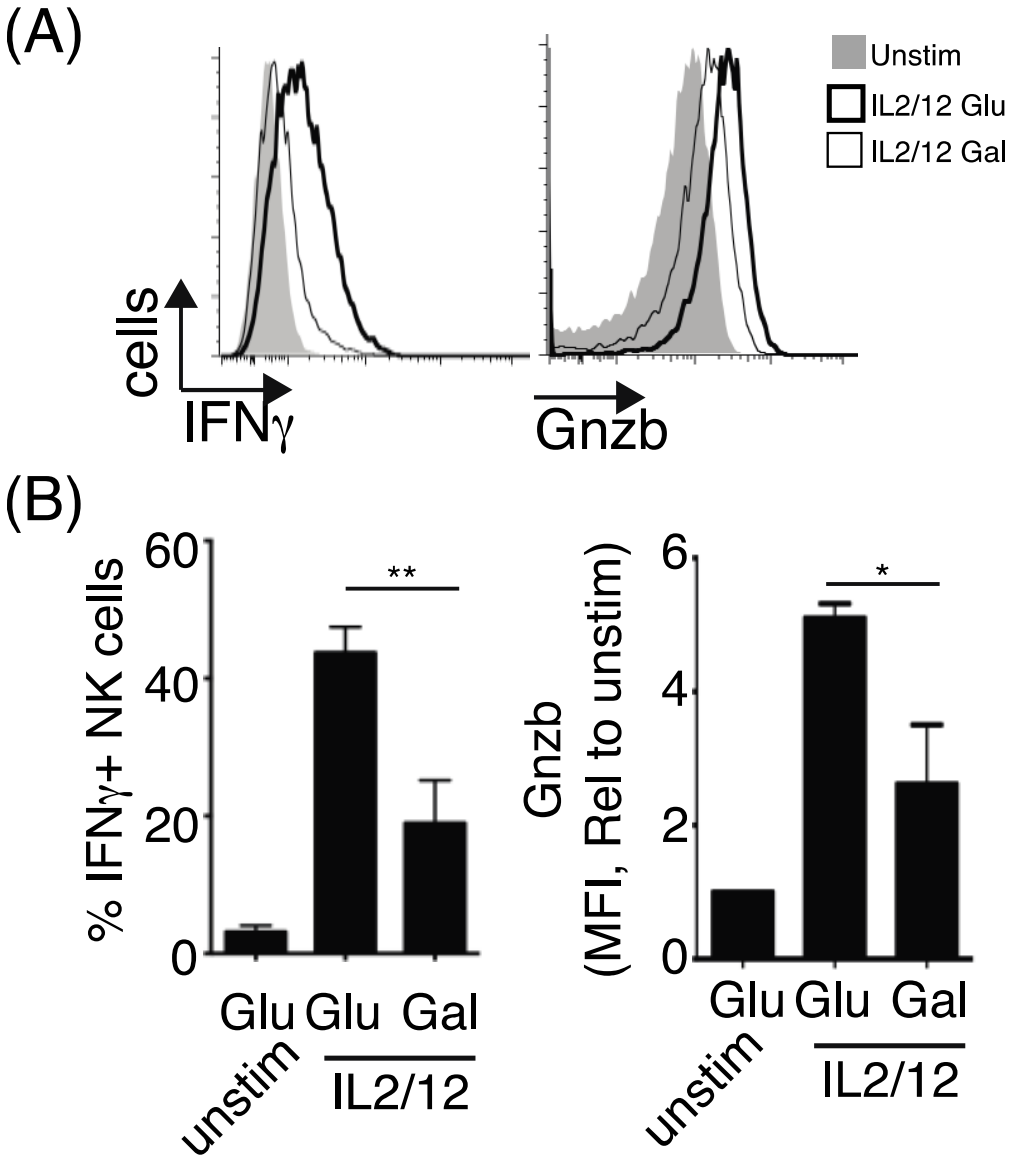

Figure 3.18. Elevated glycolysis is required for IFNγ and Granzyme b Production in IL-2/12 stimulated NK cells. (A-B) Expanded NK cells were maintained in IL15 (5ng/ml) or stimulated with IL-2/12 in the presence of glucose or galactose as a fuel source overnight and subjected to flow cytometry analysis to determine the expression of IFNγ and granzyme B of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of at least N=3 (a) or is mean +/- S.E.M of N=3. (* $p<0.05$, ** $p<0.01$).

3.5. mTORC1 regulates glucose uptake and glycolysis in IL-2/12 activated NK cells

In considering the key mechanisms required for elevated metabolism in activated NK cells it was striking that IL-2/12 stimulated NK cells dramatically increased mTORC1 (mammalian target of rapamycin complex 1) signalling. The level of mTORC1 activity can be assessed through the analysis of the levels of phosphorylated S6 ribosomal protein on Ser235/6 (pS6) and phosphorylated S6 Kinase on Ser389 (pS6K). S6 is phosphorylated by pS6 kinase which is in turn is directly activated by mTORC1. Upon IL-2/12 stimulation NK cells rapidly increase mTORC1 activity as seen by increased phosphorylation of S6 (Fig 3.10b). The serine/threonine kinase mTORC1 has well defined roles in controlling the metabolic activity of other lymphocyte populations, most notably CD8+ CTL. Therefore, it was reasoned that mTORC1 activity would be important for the observed elevated metabolism in activated NK cells. Rapamycin was used to assess the role of mTORC1 on the metabolism and function of cytokine activated NK cells. NK cells treated with rapamycin upregulated the activation markers CD69 and CD25 normally in response to IL-2/12 (Fig. 3.19a) but in the absence of mTORC1 activity NK cells failed to engage cellular growth and remained small (Fig. 3.19b). Interestingly, rapamycin treatment also inhibited glucose uptake (Fig 3.20c) suggesting a decrease in glucose metabolism, as well as inhibiting the expression of CD71, and CD98 (Fig 3.20a-b). Consistent with decreased glucose uptake rapamycin treatment inhibited the rate of glycolysis in IL-2/12 activated NK cells (Fig. 3.21a). In contrast, the rate of OxPhos in IL-2/12 stimulated NK cells is insensitive to rapamycin treatment (Fig. 3.21b), thus demonstrating a specific role for mTORC1 for promoting glycolytic metabolism. Furthermore, the observed metabolic switch observed upon IL-2/12 stimulation is inhibited with rapamycin treatment (Fig 3.21c). The glycolytic capacity of IL-2/12 stimulated NK cells is also decreased by rapamycin treatment (Fig. 3.22a) suggesting that mTORC1 activity is required for increased expression of NK cell glycolytic machinery. Indeed, the

expression of the glucose transporter Glut1 and the glycolytic enzymes Ldha and Hex2 were decreased in rapamycin treated IL-2/12 activated NK cells (Fig. 3.22b). This data reveals mTORC1 is a key regulator of NK cell glycolytic activity. Given our data has described a key role for NK metabolism in the regulation of key NK cell effector molecules, rapamcyin mediated inhibition of NK metabolism might be expected to impact upon NK cell function. Indeed, IFNγ and granzyme B production in NK cells stimulated with IL-2/12 stimulation, in the presence of rapamycin, was substantially decreased (Fig. 3.23). However, mTORC1 activity is not required for universal NK cell effector function as rapamycin treatment had no effect on TNFα production (Fig 3.24). Accompanying the decrease in frequency of NK cells producing IFNγ decreasing glycolysis or rapamycin treatment also resulted in a small but significant decrease in the amount of IFNγ being produced per $IFN\gamma^+$ NK cell, determined by MFI (Fig 3.25a). Together, this data shows that mTORC1 is critical for IL-2/12 induced metabolic reprograming in NK cells.

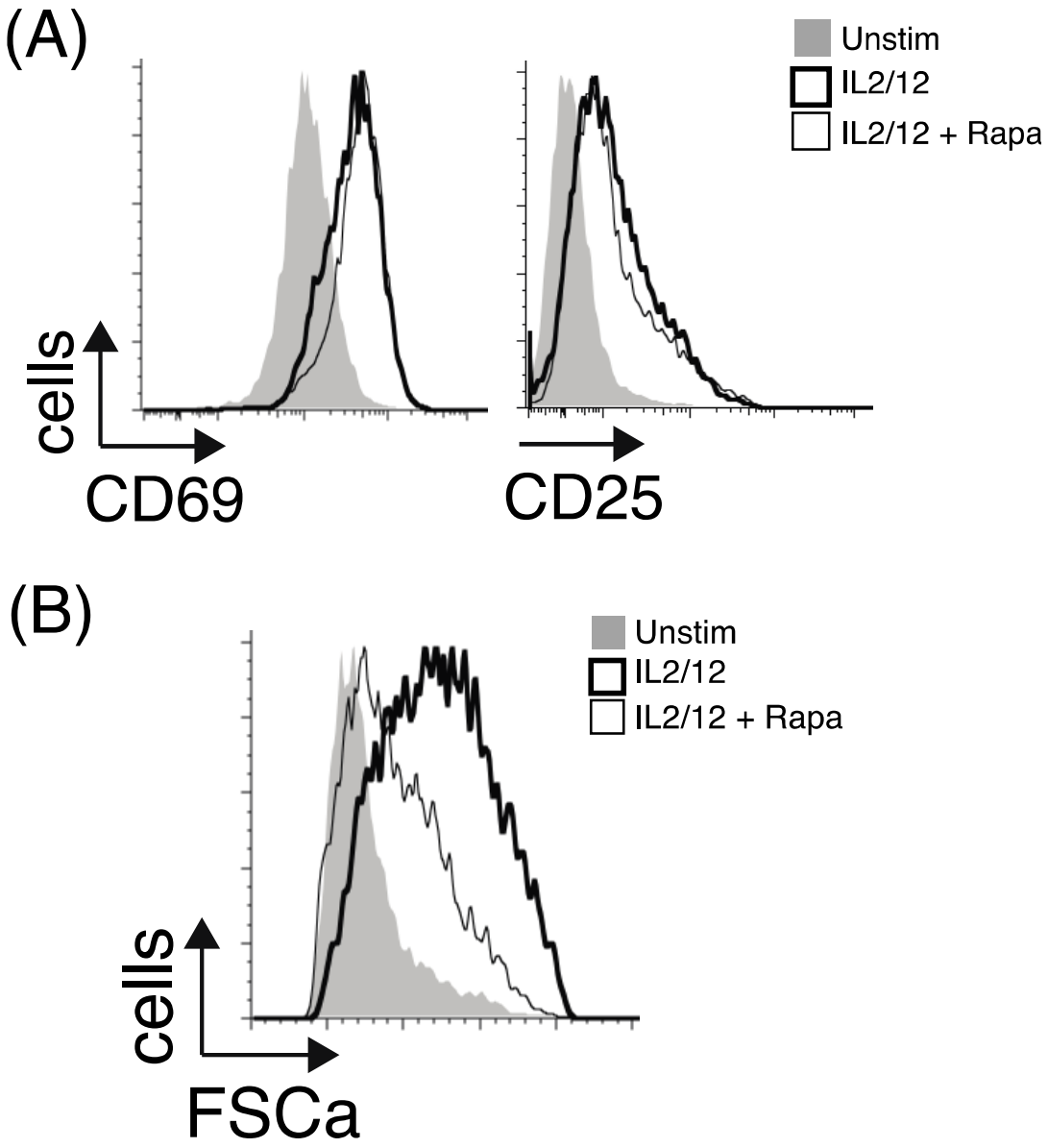

Figure 3.19. Rapamycin treatment inhibits cellular growth in IL-2/12 activated NK cells. Expanded NK cells were maintained in IL-15 or stimulated with IL-2/12 +/- Rapamycin overnight and subjected to flow cytometry analysis to determine the expression of CD69 and CD25 (a) as well as NK cell size (b) of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of at least N=3.

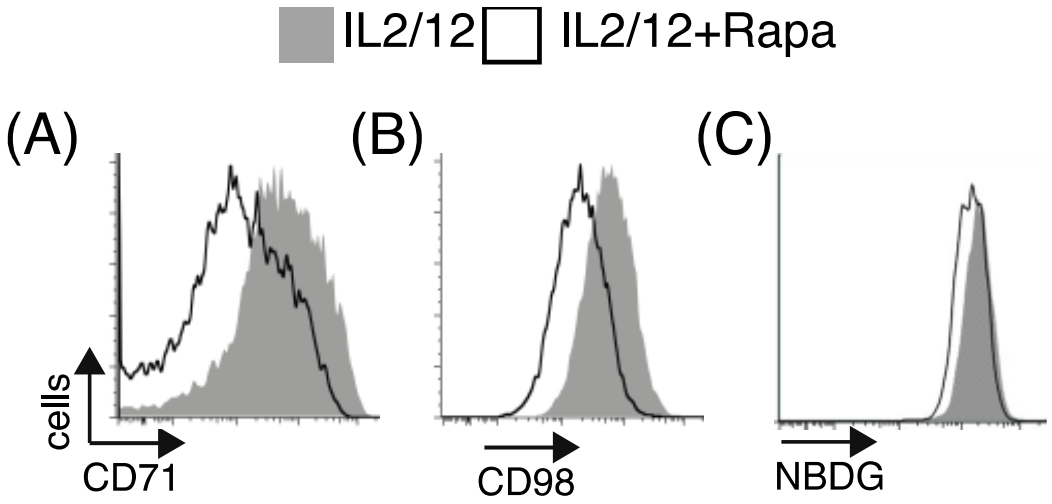

Figure 3.20. mTORC1 regulates glucose uptake and key nutrient receptors expression in activated NK cells. (A-C) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- Rapamycin overnight and subjected to flow cytometry analysis to determine the expression of CD71 (a), CD98 (b) as well as glucose uptake (c) of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of at least N=3.

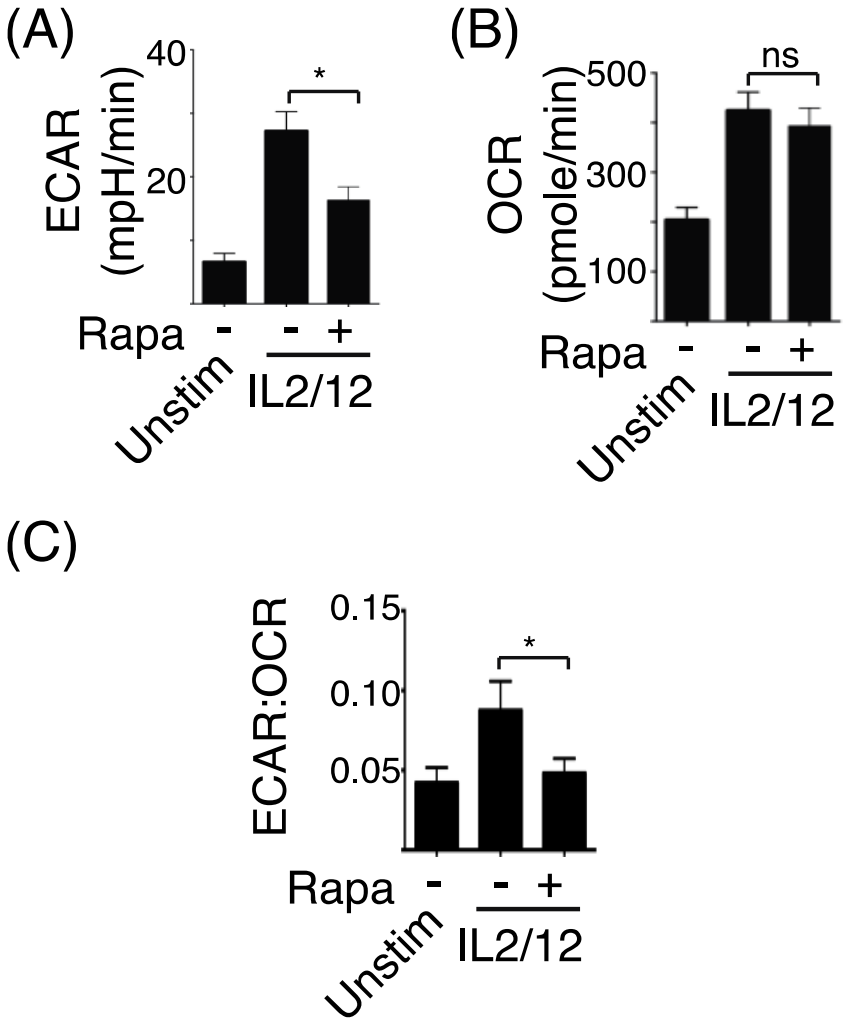

Figure 3.21. Rapamycin treatment prevents IL-2/12 increase in glycolysis levels. (A-C) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- Rapamycin overnight and subjected to detailed metabolic analysis using the Seahorse metabolic Flux analyzer, which determined the levels of glycolysis (a) Oxidative Phosphorylation (b) as well as the relative ratio between glycolysis (ECAR) and oxidative phosphorylation (OCR) (c). Data is mean +/- S.E.M of N=3 (ns=not significant, *$p<0.05$).

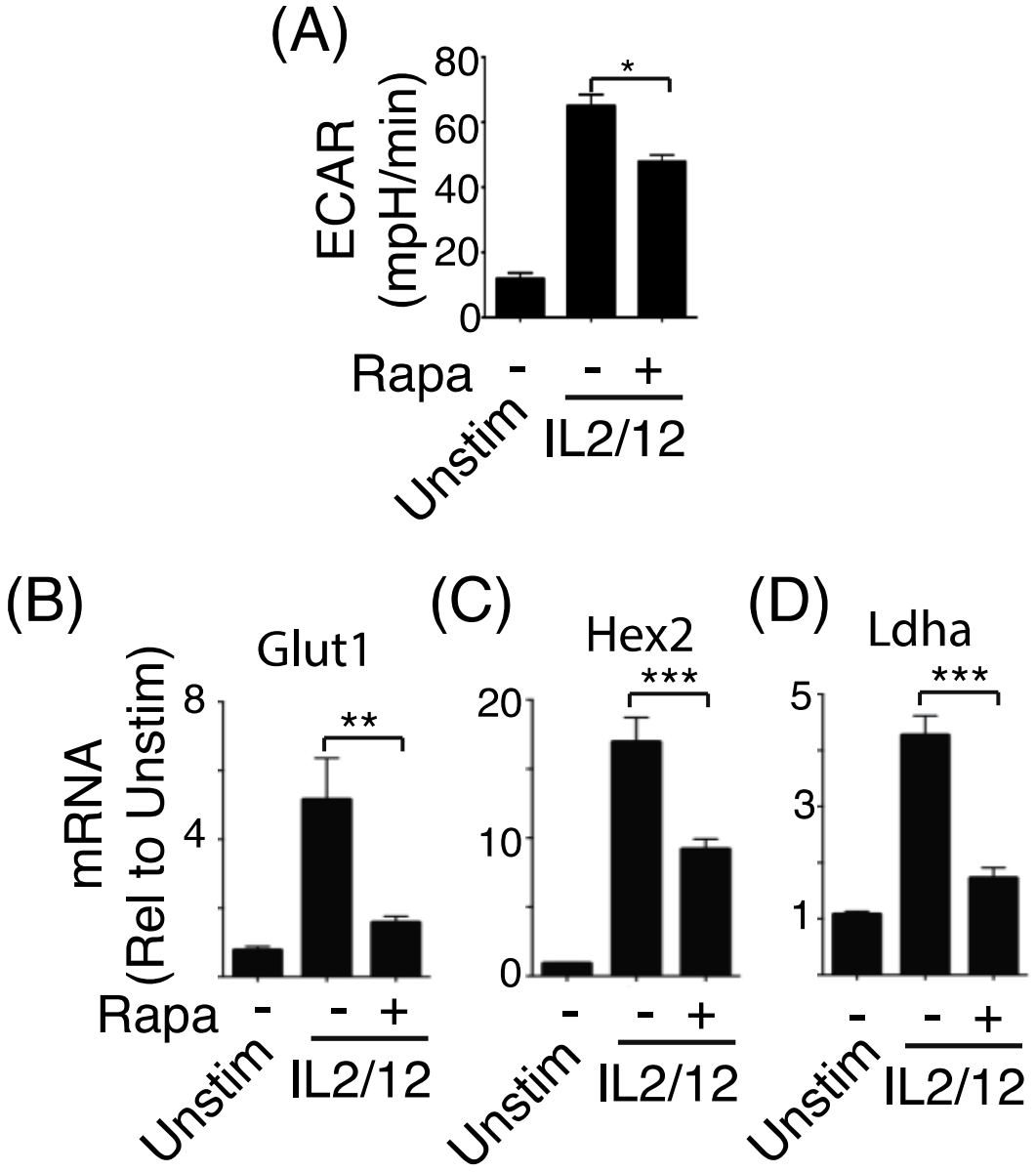

Figure 3.22. Rapamycin treatment prevents IL-2/12 induced metabolic reprogramming. (A-D) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- Rapamycin overnight. Stimulated NK cells were subjected to metabolic analysis using the seahorse metabolic flux analyzer to determine the level of glycolysis (a). Cells were also subjected to RT-qPCR and expression levels of Glut1, Hex2 and Ldha were determined (b-d). Data is mean +/- S.E.M of N=3. (** $p<0.01$, ***$p<0.001$).

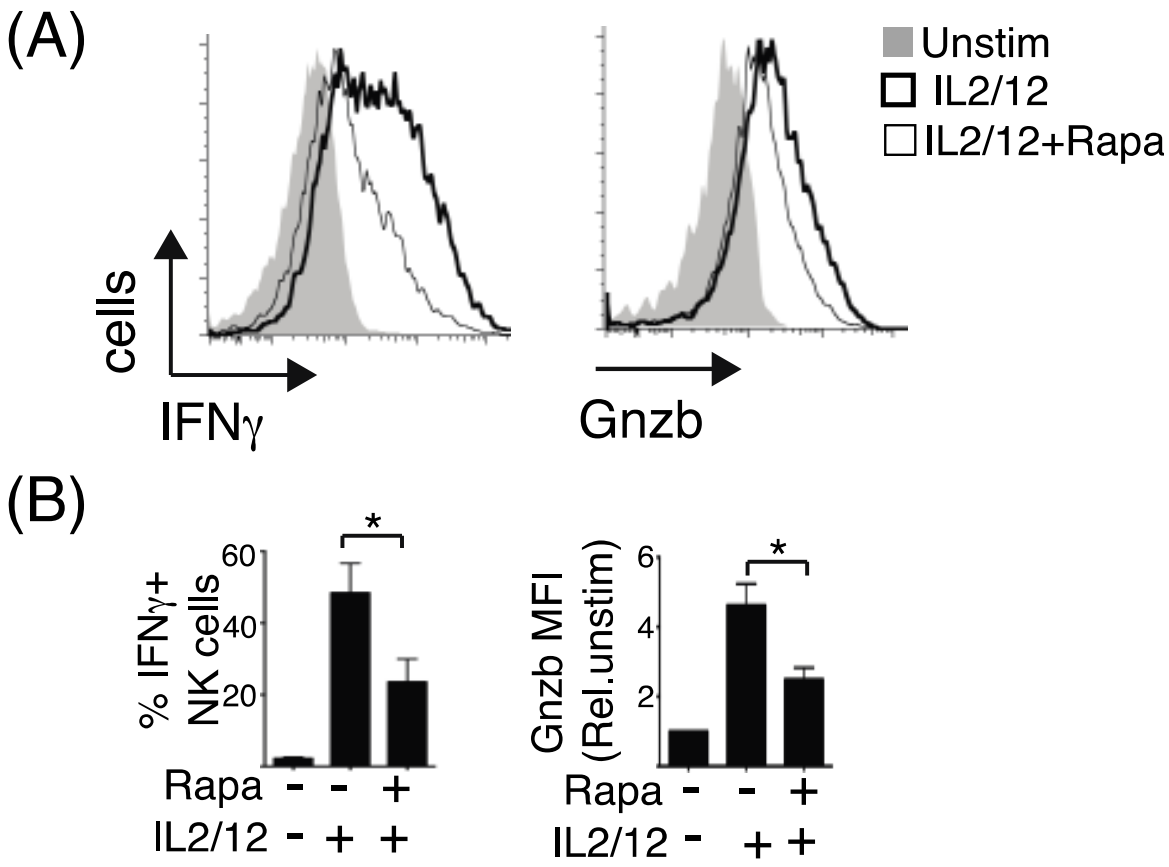

Figure 3.23. Inhibition of elevated Glycolysis with Rapamycin treatment inhibits IL-2/12 induced increase in IFNγ and Granzyme b Production. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- Rapamycin overnight and subjected to flow cytometry analysis to determine the expression of IFNγ and granzyme b of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of at least N=3 (a) or is mean +/- S.E.M of N=3 (b). (*$p<0.05$).

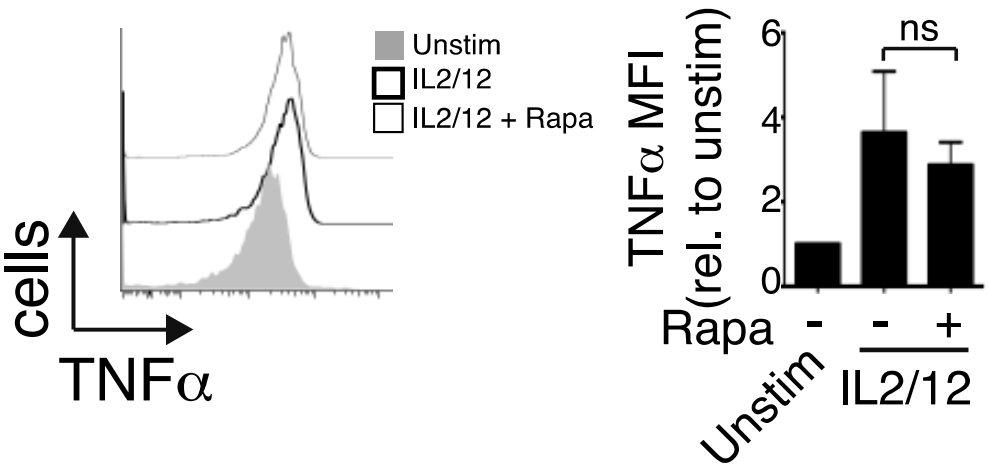

Figure 3.24. Rapamycin does not significantly inhibit TNFα production in IL-2/12 stimulated NK cells. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- Rapamycin overnight and subjected to flow cytometry analysis to determine the expression of TNFα of NK1.1+ NKp46+ CD3- NK cells. The data shown above is representative of at least N=3 or is mean +/- S.E.M of N=3. (ns=not significant).

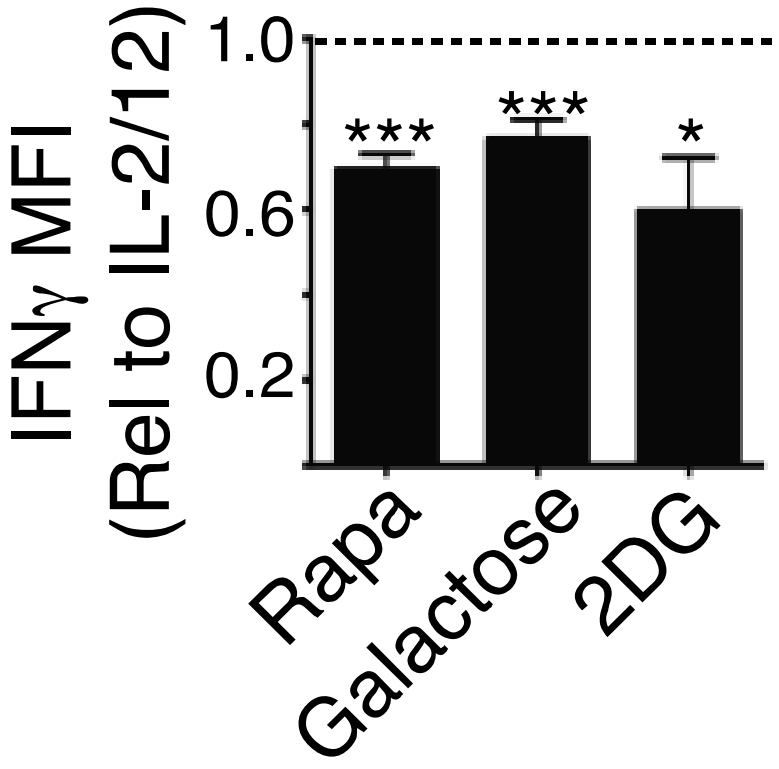

Figure 3.25. mTORC1-dependent glycolysis determines the amount of IFNγ being produced by IFNγ+ NK cells. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- Rapamycin/2-DG overnight. Alternatively NK cells were stimulated in galactose instead of glucose. Expression of IFNγ based on MFI of NK1.1+ NKp46+ CD3- NK cells was determined by flow cytometry. The data shown above is mean +/- S.E.M of N=3. (*$p<0.05$, ***$p<0.001$).

3.6. In vivo disruption of NK metabolism inhibits NK cell effector functions

Our *in vitro* data clearly demonstrates that NK cell function is closely linked to cellular glycolysis. To confirm that metabolism is linked to NK cell function *in vivo*, we used two separate approaches to disrupt the rate of glycolysis in NK cells *in vivo,* and observe the impact on the NK cell response to poly(I:C). Two complementary approaches were taken to disrupt the rate of glycolysis *in vivo*, namely rapamcyin and 2DG administration. It was reasoned that rapamycin treatment would block mTORC1 dependent up-regulation of glycolysis in activated NK cells while 2DG would act as a direct glycolytic inhibitor. mTORC1 is highly active in Poly (I:C) activated NK cells as demonstrated by an increase in pS6 expression (Fig 3.26). Mice receiving poly(I:C)/rapamycin or poly(I:C)/2DG injections activated NK cells at comparable levels to poly(I:C) alone as measured by expression of CD69 (Fig. 3.27). As predicted, inhibition of mTORC1 and direct inhibition of glycolysis with 2DG disrupted the metabolic activity of poly(I:C) activated NK cells, as evidenced by decreased NK cell size (Fig. 3.28a-b) and decreased glucose uptake (Fig. 3.29).

The predicted differential actions of rapamycin and 2DG were confirmed through analysis of CD71 and CD98 expression on activated NK cells. Consistent with the role for mTORC1 in the metabolic reprogramming of activated NK cells rapamycin disrupted the up-regulation of CD71 and CD98 on activated NK cells (Fig. 3.30a-b). In contrast, there was no significant effect of 2DG on the expression of CD71 and CD98 on activated NK cells, which is consistent with 2DG acting as a direct inhibitor of NK cell glycolysis (Fig. 3.30a-b). Therefore, the data suggests that rapamycin or 2DG can disrupt the glycolytic activity of activated NK cells *in vivo*. Crucially, poly(I:C) induced IFNγ production in activated NK cells was substantially inhibited by both rapamycin and 2DG (Fig. 3.31a-b). However, Poly(I:C) induced TNFα expression is not inhibited by rapamycin or 2DG treatment suggesting that

elevated glycolysis is not required for all NK cell effector molecules and confirming that rapamycin or 2DG did not globally block NK cell function (Fig 3.32a-b). Therefore, this data confirms *in vivo* that mTORC1 and elevated glycolysis are required for the normal effector molecules of activated NK cells.

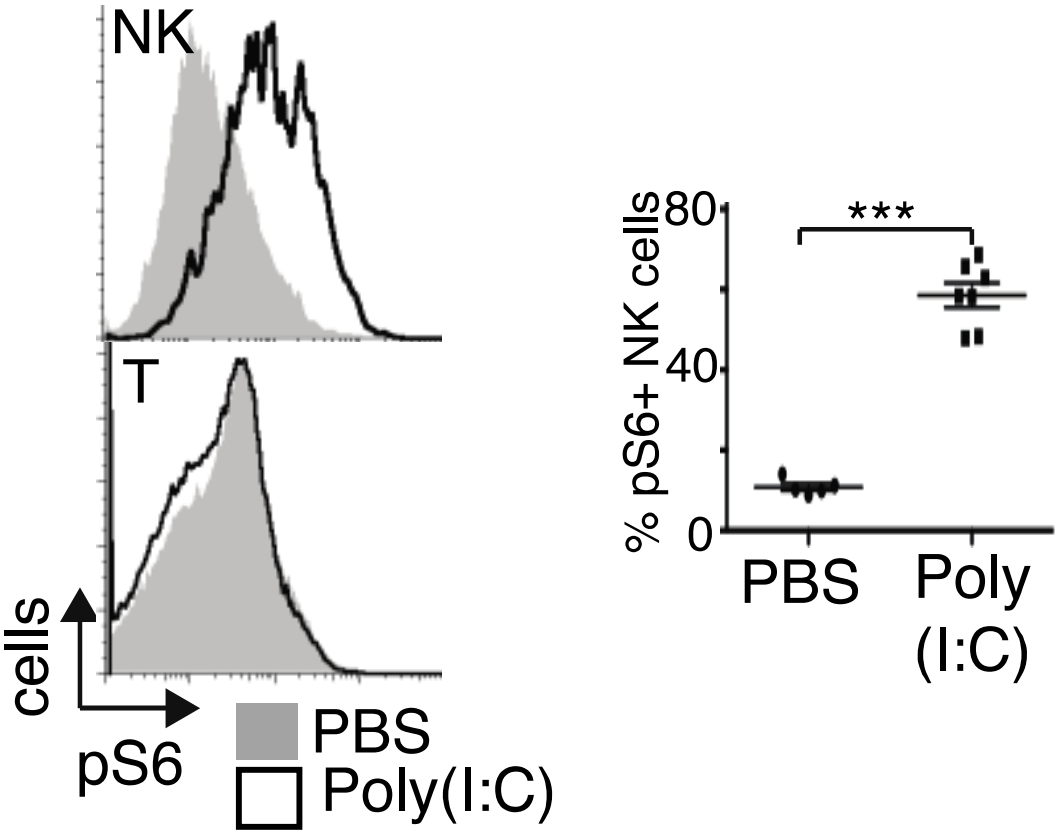

Figure 3.26. Poly (I:C) mTORC1 activity in vivo in NK cells. Mice were administered PBS or 200μg Poly(I:C) by peritoneal injection and spleens harvested after 24 hours. Splenocytes were isolated and NK1.1+ NKp46+ CD3- NK cells were analyzed for pS6 expression using flow cytometry. $CD3^+$ cells (T cells) were also analyzed for pS6 expression. The data shown above is mean +/- S.E.M of N=7 and representative histograms. (***$p<0.001$)

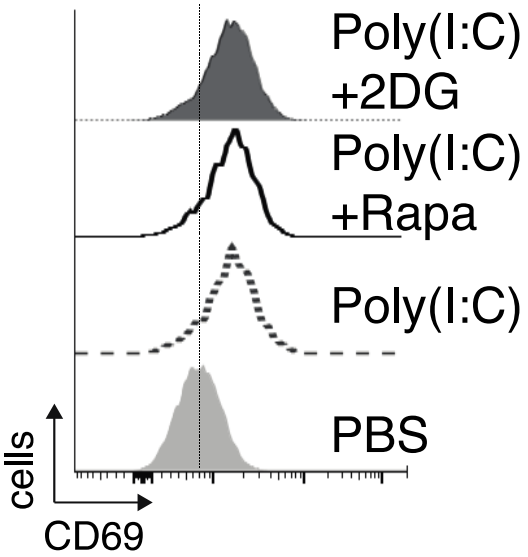

Figure 3.27. 2DG and Rapamycin treatment have no effect on Poly (I:C) activation of NK cells in vivo. Mice were administered PBS, 200μg Poly(I:C) alone or in combination with rapamycin (0.6mg/kg) or 2DG (1g/kg) with by peritoneal injection and spleens harvested after 24 hours. Splenocytes were isolated and NK1.1+ NKp46+ CD3- NK cells were analyzed for CD69 expression using flow cytometry. Data is representative of 8-10 mice for each condition.

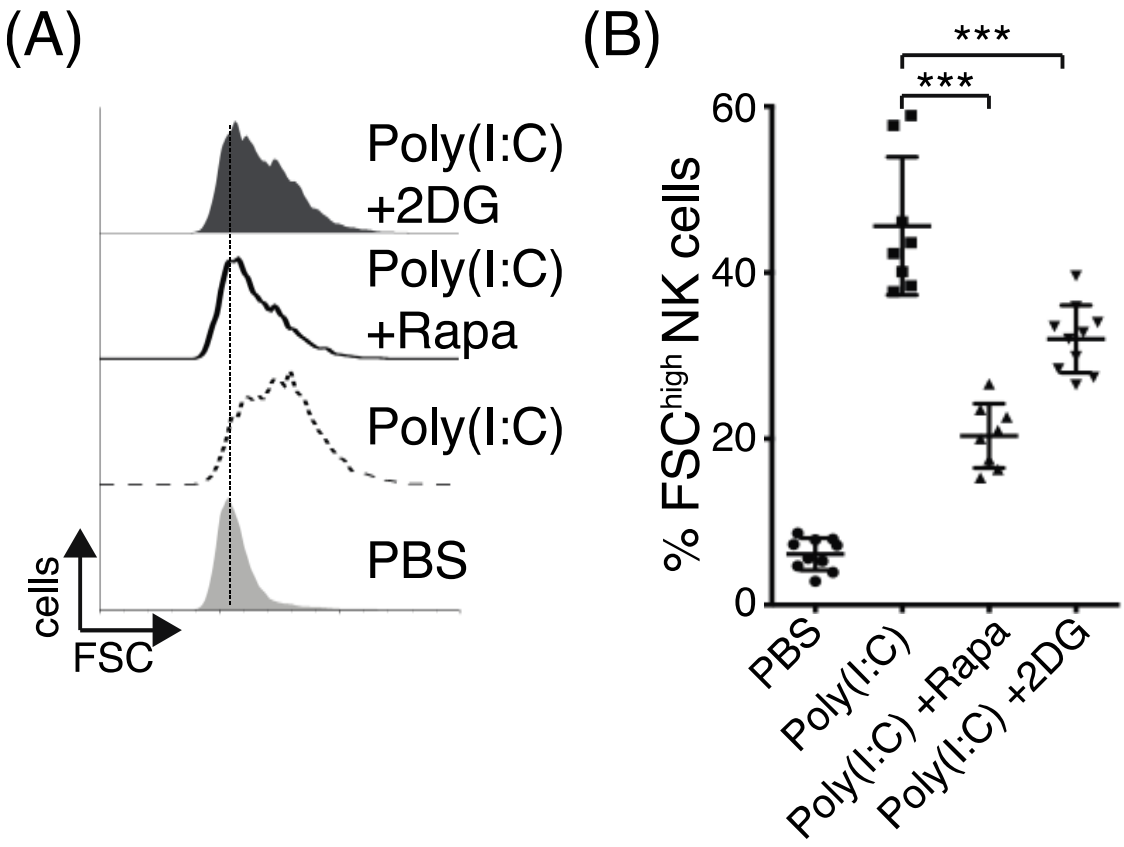

Figure 3.28. 2DG and Rapamycin treatment inhibit Poly (I:C) induced increase in cell size of NK cells in vivo. (A-B) Mice were administered PBS, 200μg Poly(I:C) alone or in combination with rapamycin (0.6mg/kg) or 2DG (1g/kg) with by peritoneal injection and spleens harvested after 24 hours. Splenocytes were isolated and NK1.1+ NKp46+ CD3- NK cells were analyzed for cell size based onforward-side scatter using flow cytometry. All data is mean +/- S.E.M (b) or representative (a) of 8-10 mice for each condition. (***p<0.001)

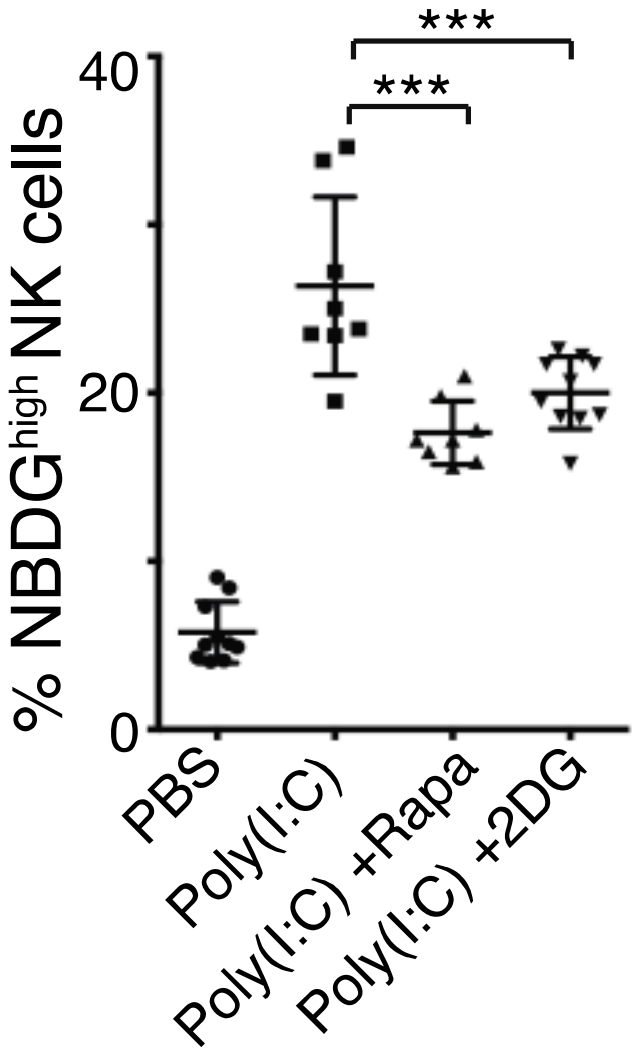

Figure 3.29. 2DG and Rapamycin treatment inhibit Poly (I:C) induced glucose uptake in NK cells in vivo. Mice were administered PBS, 200μg Poly(I:C) alone or in combination with rapamycin (0.6mg/kg) or 2DG (1g/kg) with by peritoneal injection and spleens harvested after 24 hours. Splenocytes were isolated and NK1.1+ NKp46+ CD3- NK cells were analyzed for 2NBDG levels as a measure of glucose uptake using flow cytometry. All data is mean +/- S.E.M of 8-10 mice for each condition. (***$p<0.001$)

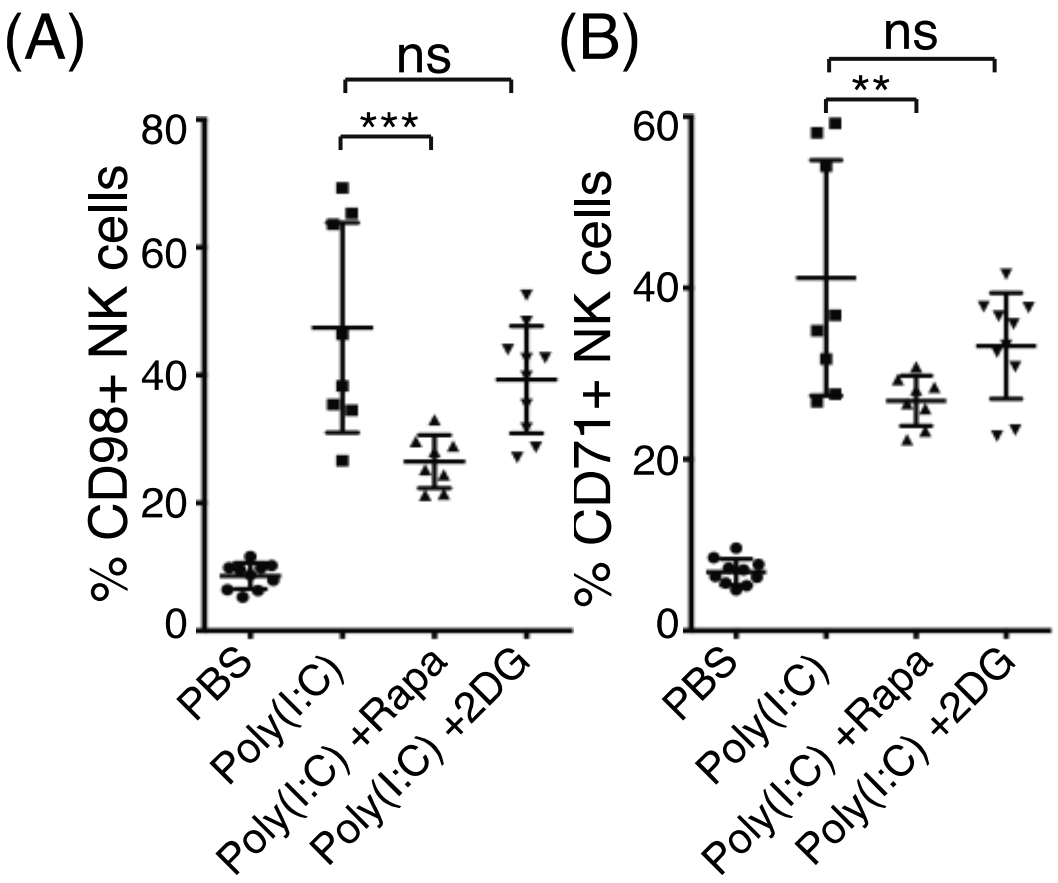

Figure 3.30. Rapamycin but not 2DG treatment inhibit Poly (I:C) induced expression of nutrient receptors CD71 and CD98 in NK cells in vivo. (A-B) Mice were administered PBS, 200μg Poly(I:C) alone or in combination with rapamycin (0.6mg/kg) or 2DG (1g/kg) with by peritoneal injection and spleens harvested after 24 hours. Splenocytes were isolated and NK1.1+ NKp46+ CD3- NK cells were analyzed for CD98 (a) and CD71 (b) expression using flow cytometry. The data is mean +/- S.E.M of 8-10 mice for each condition. (ns=not significant, **p<0.01) ***p<0.001).

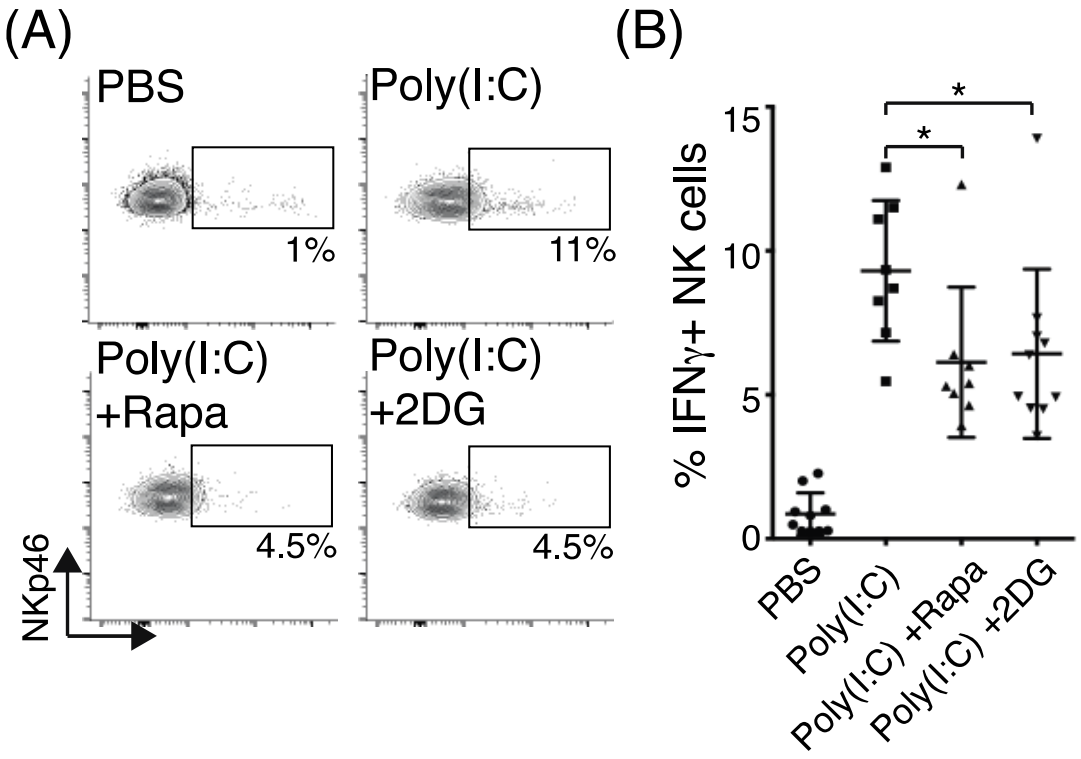

Figure 3.31. 2DG and Rapamycin treatment inhibit Poly (I:C) induced expression of IFNγ in NK cells in vivo. (A-B) Mice were administered PBS, 200μg Poly(I:C) alone or in combination with rapamycin (0.6mg/kg) or 2DG (1g/kg) with by peritoneal injection and spleens harvested after 24 hours. Splenocytes were isolated and NK1.1+ NKp46+ CD3- NK cells were analyzed for expression of IFNγ using flow cytometry. All data is mean +/- S.E.M (b) or representative (a) of 8-10 mice for each condition. (*$p<0.05$)

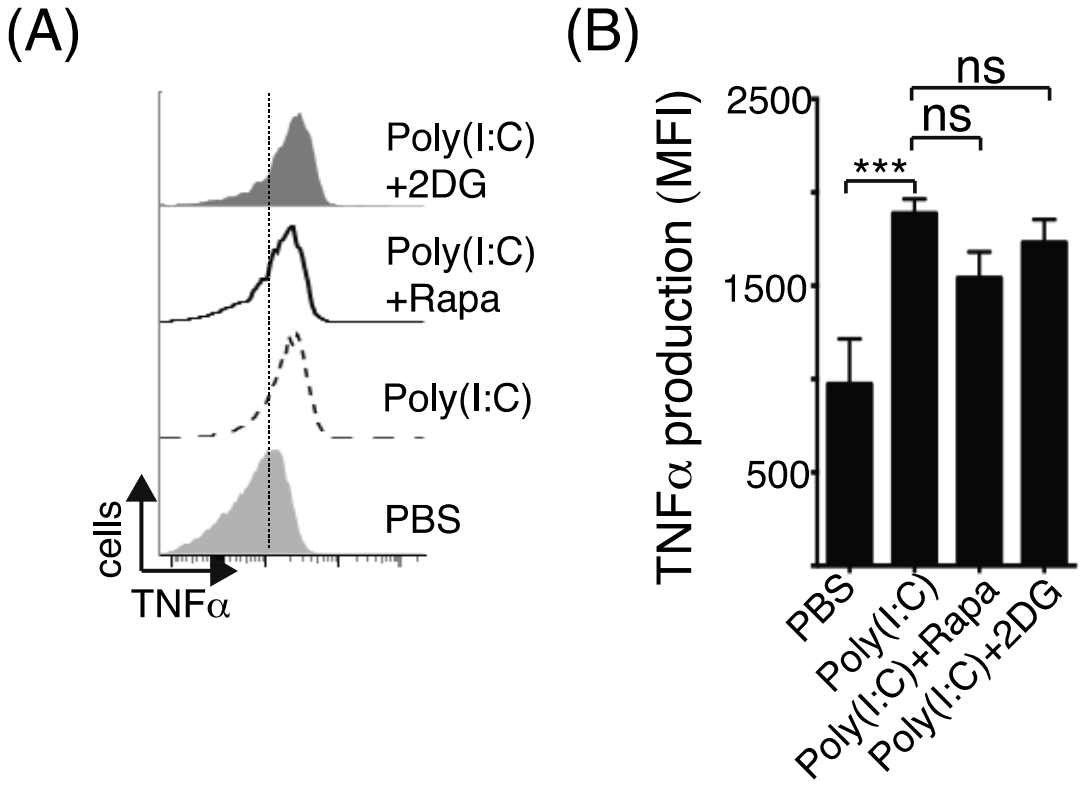

Figure 3.32. 2DG and Rapamycin treatment have no effect on Poly (I:C) induced expression of TNFα in NK cells in vivo. (A-B) Mice were administered PBS, 200μg Poly(I:C) alone or in combination with rapamycin (0.6mg/kg) (a) or 2DG (1g/kg) (b) with by peritoneal injection and spleens harvested after 24 hours. Splenocytes were isolated and NK1.1+ NKp46+ CD3- NK cells were analyzed for expression of TNFα using flow cytometry. All data is mean +/- S.E.M of 5 mice for each condition. (ns=not significant, ***p<0.001).

3.7. Discussion

NK cells are critical effector cells in the immune response to virally infected or transformed cells. Significant progress has been made in terms of identifying how NK cells recognise their targets and how they are regulated at a cellular level by cytokines. Here we demonstrate the fundamental contribution that metabolism makes to NK cell immune function. We have successfully measured and defined changes in NK cell metabolism both *in vitro* and *in vivo.* We have shown that NK cells are dynamically regulated by multiple cytokines in vitro. In response to IL-2/12 cytokine stimulation, activated NK cells dramatically increase glucose uptake, and the rates of both glycolysis and OxPhos, in a synergistic manner. Overall, there is a shift towards preferentially metabolising glucose to lactate, a metabolic process called aerobic glycolysis.

This increase in metabolism coincided with complete metabolic reprogramming with an observed increase in glucose uptake and expression of key glycolytic genes. The metabolic profile of activated NK cells resembles that of what is seen in proinflammatory effector T cell subsets (Finlay et al., 2012, Michalek et al., 2011a, Shi et al., 2011). Thus, stimulation of a T cell through the T cell receptor induces elevated rates of glucose uptake and glycolysis (Wang et al., 2011a), maintained by various cytokines as T cells differentiate into effector $CD4^+$ or $CD8^+$ T cell subsets. In contrast regulatory or memory T cells decrease rates of glycolysis and revert to utilizing oxidative glucose and lipid metabolism to maintain energy homeostasis (Michalek et al., 2011a, Shi et al., 2011, van der Windt et al., 2012) demonstrating that different immune cell subsets adapt their metabolism to meet their function.

Previous work by others and observations made in our laboratory led us to the hypothesis that cellular metabolism has a role in dictating NK cell

function. In order to test this hypothesis we developed a couple of strategies to limit the increase in glycolysis in cultured 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 2DG, an inhibitor of hexokinase. In support of our hypothesis, a key finding from this study is that NK cell glycolysis is integrally linked to key NK cell effector molecules including the production of IFNγ and granzyme B. It is interesting that in CTLs, cells with many analogous roles to NK cells, glycolytic metabolism is also essential for normal effector molecules. 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 IFNγ 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 IFNγ, that is, IFNγ 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 $IFN\gamma^+$ effector T cells (Michalek et al., 2011a). In an analogous fashion this report demonstrates that elevated glucose uptake and glycolysis is required for robust IFNγ 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 reprogamming 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 molecules *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 T_H1 and T_H17 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 generated 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 glucose uptake and glycolysis, and cell growth, which are essential for the acquisition of NK cell effector molecules; IFNγ and granzyme B production. Interestingly, TNFα expression is not inhibited by rapamycin *in vivo* or *in vitro* suggesting that elevated glycolysis is not required for all NK cell effector molecules and confirming that rapamycin or 2DG did not globally block NK cell function. Additionally, inhibition of mTORC1 (induced with high dose of IL-15), in NK cells inhibits the expression of effector molecules key for NK cell function, namely IFNγ and granzyme B (Marcais et al., 2014, Nandagopal et al., 2014). Furthermore, a recent study suggested that the mTORC1 inhibitor rapamcyin can inhibit the production of IFNγ in human NK cells (Neudoerfl et al., 2013). While mTORC1 appears to be required for glucose metabolism in T cells and NK cells this is not the case for all lymphocytes as mTORC1 is not involved in 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 IFNγ 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-κB 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 I (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.

Numerous studies have started to explore the mechanisms responsible for the changes in metabolism that occur as T cells are activated. A key transcription factor involved in T cell metabolism is c-myc. C-myc plays a role in multiple human cancers and regulates numerous genes important in cell cycle and metabolism. Moreover, c-myc has been shown to regulate glucose and glutamine metabolism and mitochondrial biogenesis. More specifically it binds to the promoter of multiple glycolytic enzymes and glucose transporters, including glut 1, lactate dehydrogenase A (LDHA), PKM2 and hexokinase to induce their expression (Dang et al., 2009). In T cells, c-myc is induced following TCR activation and promotes T cell growth and entry into the cell cycle (Lindsten et al., 1988, Kelly et al., 1983, Wang et al., 2011a). A c-myc-dependent global metabolic transcriptome drives metabolic reprogramming in activated T cells, resulting in activation-induced glycolysis and glutaminolysis (Wang et al., 2011a).

The transcription factor HIF-1α has also been implicated in the regulation of T cell metabolism. While the initial glycolytic switch induced in response to

antigenic stimuli is regulated by c-myc, HIF-1α is required for the long term commitment to elevated glucose uptake and glycolysis in CTLs (Finlay et al., 2012). In addition, HIF-1α-dependent glycolytic pathway orchestrates a metabolic checkpoint for differentiation of T_H17 and Treg cells. Thus blocking HIF-1α induced increase in glycolysis inhibited T_H17 development while promoting Treg generation (Shi et al., 2011). Ongoing work in our laboratory suggests that HIF-1α is dispensable for metabolic reprogramming in NK cells though a role for c-myc has not been investigated yet.

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 molecules. 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 IFNγ and IL-2 (Chang et al., 2013). Similarly, when the signalling molecules that promote elevated glycolysis in CTLs were disrupted, i.e. mTORC1 or HIF1α, 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 rapamcyin treated CTLs or HIF1-null 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. Although, glycolysis is not universally required for all NK cell functions as there was no observed decrease in TNFα production. Suggesting that glycolysis has a more direct role in regulating the effector molecules of activated lymphocytes.

This has been 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 IFNγ-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 glyceraldehdye-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 IFNγ and IL-2 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 IFNγ and IL-2 mRNA and repress their translation (Chang et al., 2013). In 2DG-treated NK cells, as well as cells cultured in galactose, there is evidence of a

decrease in IFNγ being produced by IFNγ$^+$ NK cells consistent with the model described by Chang *et al*. This decrease in IFNγ levels can also be seen in NK cells treated with rapamycin. While there is a significant decrease in IFNγ production the differences are small compared to those reported in CD4$^+$ T cells. 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-1β 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 tumour microenvironments, which will be discussed in chapter 6.

Chapter 4-SREBP activity is required for elevated glycolysis in cytokine stimulated NK cells by maintaining NAD^+ via the citrate-malate shuttle.

4.1. Introduction

In addition to its role in providing energy in the form of ATP, aerobic glycolysis allows the cell to shuttle glucose-derived carbons into biosynthetic precursors required for the synthesis of proteins, nucleic acids, and lipids (Wang et al., 1976, Macintyre et al., 2014). Increasing the biosynthesis of important biomolecules is an essential prerequisite for the increase in cellular growth and the rapid proliferation that follows the differentiation of naïve T cells into effector T lymphocytes. A core anabolic cellular pathway that increases during metabolic reprogramming of effector T lymphocytes is the lipid-biosynthetic pathway (Chen et al., 1975). Indeed lipid biosynthesis is essential for T cell proliferation and clonal expansion (Chakrabarti and Engleman, 1991). Disruption of sterol homeostasis has been shown to inhibit cell-cycle progression, survival and effector molecules of T lymphocytes (Bensinger et al., 2008, Michalek et al., 2011b, Chakrabarti and Engleman, 1991, Geyeregger et al., 2009).

Sterol regulatory element binding proteins (SREBPs) are basic helix-loop-helix-zip transcription factors that have key roles in promoting lipid biosynthesis. Additionally, SREBP is also emerging as an important regulator of immune cell responses. In fact, SREBP can directly impact upon T cell function. In $CD4^+$ T cells the isoform SREBP1c is involved in TH17 differentiation where it directly binds to the promoter of IL-17 and inhibits gene expression (Cui et al., 2011). In $CD8^+$ T cells, inhibition of SREBP activity inhibits proliferation and blastogenesis (Kidani et al., 2013). mTORC1 signalling has been linked to the activation of SREBP transcription factors in other cell types. mTORC1 induced glycolysis has an important role for NK cell function, described in chapter 3. Therefore, experiments were next designed to investigate whether mTORC1 controls SREBP activity and lipid synthesis in cytokine stimulated NK cells and if so whether this impacts on NK cell function.

4.2. Cytokine induced lipid synthesis in NK cells is dependent on SREBP activity.

To investigate whether NK cell activation is linked to changes in lipid synthesis we measured the expression of genes involved in fatty acid and cholesterol synthesis. NK cells were stimulated with either IL-2/12 or IL-2/12/18 and subjected to RT-qPCR analysis. NK cells upregulate their expression of fatty acid synthase (FASN) and stearoyl-CoA desaturase 1 (SCD1) following IL-2/12 stimulation and to a greater degree following stimulation with IL-2/12/18 (Fig 4.1a). Both of these genes are part of the fatty acid synthesis pathway. Furthermore, NK cells also upregulate genes involved in cholesterol synthesis pathway; HMG-CoA synthase (HMGCoS) and acetyl-CoA acetyltransferase 2 (ACAT2) following stimulation with IL-2/12 and IL-2/12/18 (Fig 4.1a). To determine whether the observed increased expression in lipid synthesis genes translates to changes in lipid synthesis we measured the rate of incorporation of radiolabeled C^{14} acetate into celluar lipid. The amount of acetate being incorporated into lipid significantly increases with IL-2/12 stimulation and further increases with the addition of IL-18 (Fig.4.1b).

Sterol regulatory element binding protein (SREBP) is a major transcription factor involved in controlling lipid synthesis and is known to regulate the expression of most of the genes in this pathway including; FASN, SCD1, HMGCoS and ACAT2. To investigate whether SREBP activity is required for the cytokine-induced increases in the expression of these genes we used a pharmacological inhibitor of SREBP activation, PF429242. This molecule targets and inhibits site 1 protease (SIP) which is required for cleaving SREBP and releasing the active transcription factor from the golgi (Fig 4.2)(Hawkins et al., 2008). A second approach to inhibit SREBP activity involved the use of the oxysterol 25-Hydroxycholesterol (25HC). 25HC prevents SREBP from trafficking from the ER to the golgi and in doing so blocks SREBP processing and activation (Fig 4.2) (Adams et al., 2004). Both

PF429242 and 25HC treatments inhibits the expression of fatty acid and cholesterol genes in IL-2/12 and IL-2/12/8 stimulated NK cells (Fig 4.3a). Furthermore, inhibition of SREBP activity by both methods decreases acetate incorporation into cellular lipid in stimulated NK cells (Fig 4.3b). Combined this data shows that upon cytokine stimulation NK cells upregulate SREBP-dependent lipid synthesis.

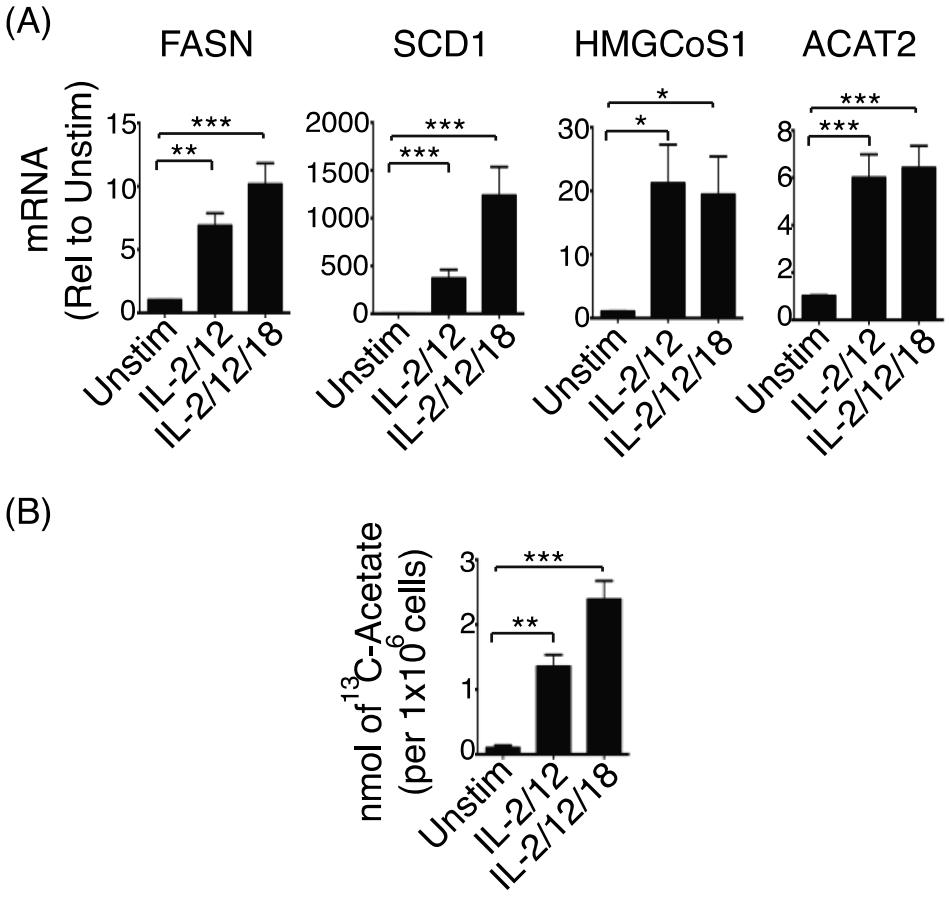

Figure 4.1. IL-2/12 and IL-2/12/18 simulation increases lipid synthesis in NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 overnight. (A) RT-qPCR analysis of the expression of FASN, SCD1, HMGcoS1 and ACAT2 in NK cells under conditions shown. (B) Analysis of the rate of lipid synthesis through measuring the incorporation of radiolabeled ^{13}C-acetate into cellular lipids incorporated. Data is mean +/- S.E.M of N=3. (*p<0.05, ** p<0.01, ***p<0.001).

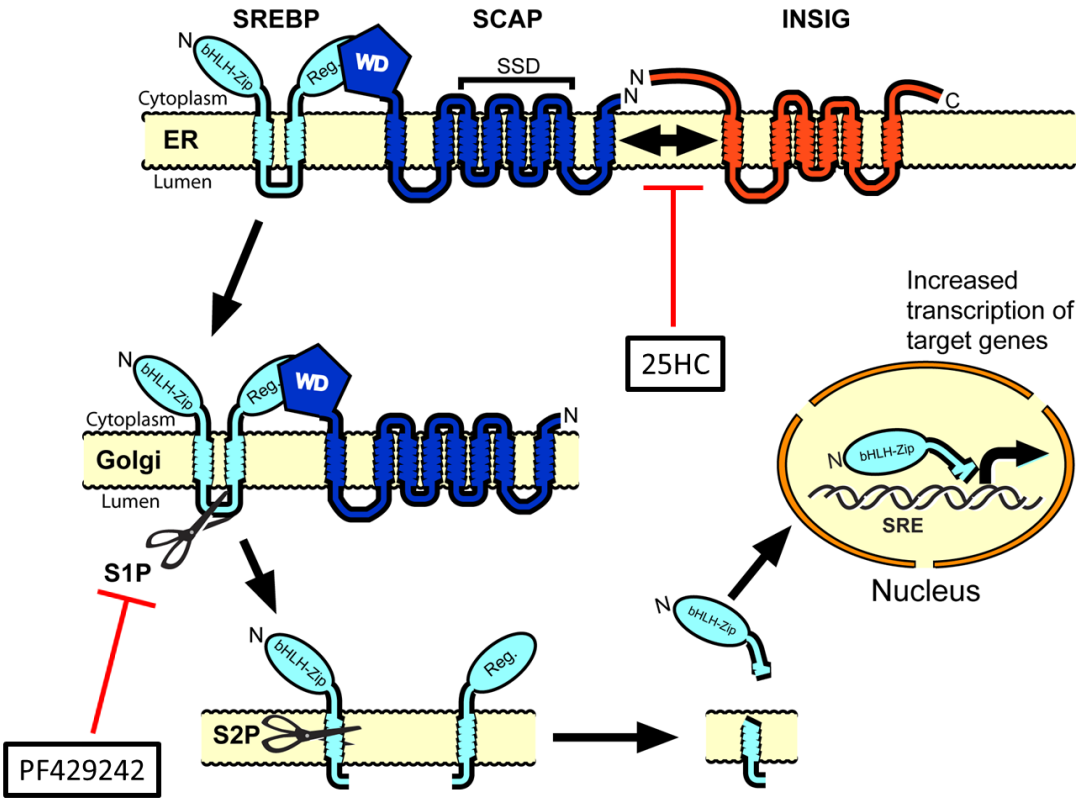

Figure 4.2. SREBP regulation and activation. SREBP undergoes a complex activation process as described in section 1.2.3.4. 25HC inhibits SREBP activation by enhancing the binding of SCAP with INSIG, which retains SREBP in the ER. PF429242 is an inhibitor of site 1 protease, required for cleaving and activating SREBP.

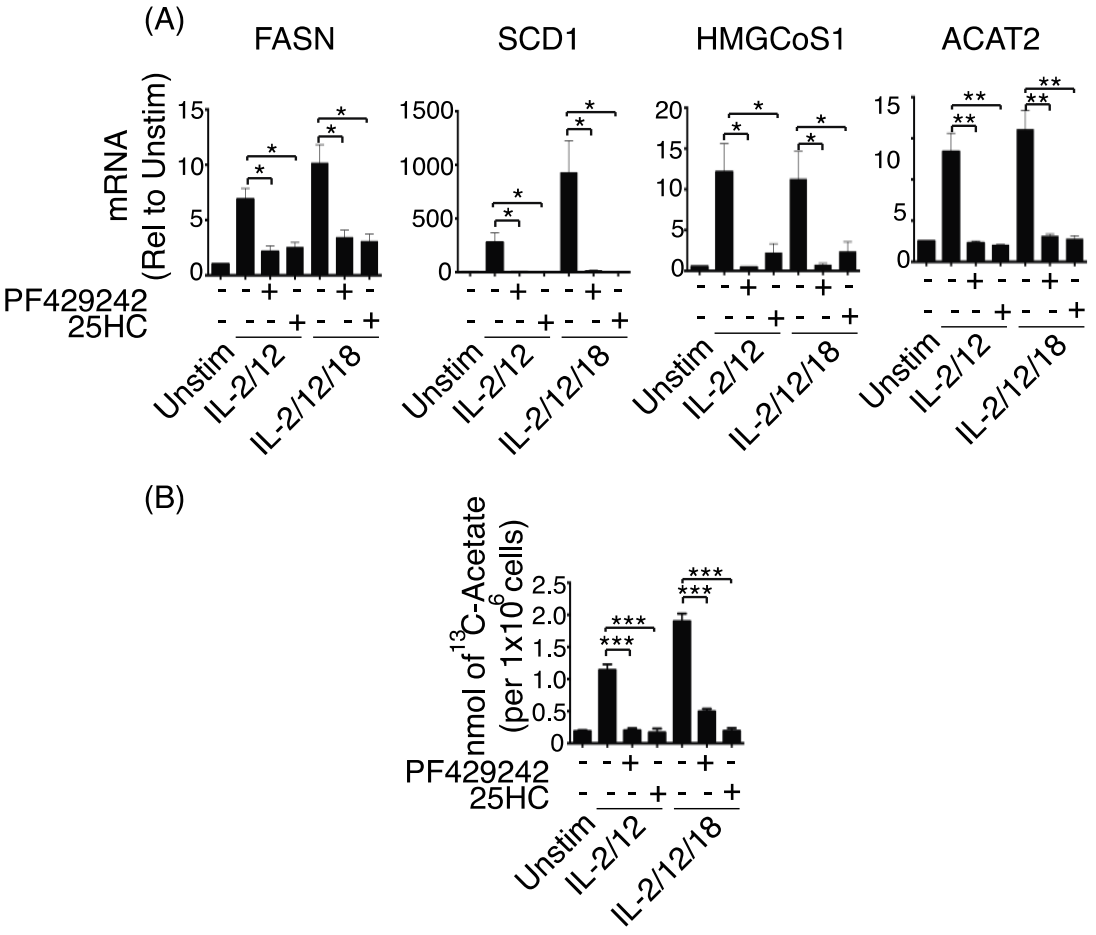

Figure 4.3. SREBP activity is required for lipid synthesis in IL-2/12 and IL-2/12/18 stimulated NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- PF429242 (10μM) or 25-Hydroxycholoesterol (25HC, 1μg/ml) overnight. (A) RT-qPCR of the expression of FASN, SCD1, HMGcoS1 and ACAT2. (B) Analysis of the rate of lipid synthesis through measuring the incorporation of radiolabeled ^{13}C-acetate into cellular lipids incorporated. Data is mean +/- S.E.M of N=3 (* $p<0.05$, ** $p<0.01$, ***$p<0.001$).

4.3. SREBP activity is required for IL-15 induced homeostatic proliferation and blastogenesis in cytokine stimulated NK cell

Fatty acid and cholesterol synthesis is important for making lipid bilayers and so we predicted that SREBP activity would be important for NK cell growth and proliferation. To test this hypothesis we assessed NK cell growth and proliferation in response to various cytokines in the presence or absence of SREBP activity. IL-15 is known to be an important cytokine for homoeostatic NK cell proliferation. To investigate whether SREBP activity is required for IL-15 induced homeostatic proliferation we cultured CFSE labeled splenocytes for 7 days in IL-15 (25ng/ml) with and without PF429242. PF429242 was readded once on day 3 of the culture. On day 7 NK cell proliferation was analyzed based on the dilution of CFSE staining. PF429242 treated cells proliferated substantially less than untreated NK cells (Fig 4.4a). A second complimentary approach was taken to inhibit SREBP activity. SCAP (SREBP cleavage activating protein) is an integral membrane protein located in the endoplasmic reticulum (ER) and bound to SREBP. Cells lacking SCAP cannot activate SREBP (Matsuda et al., 2001) Mice containing loxP sites that flank exon 1 of the SCAP gene (Matsuda et al., 2001) were imported and crossed to mice expressing a tamoxifen-inducible cre recombinase (Fig 4.5). The deletion of SCAP can be induced in NK cells from these mice upon addition of 4-OH tamoxifen. Similarly, to the PF429242 treated cells, NK cells lacking SCAP expression are unable to proliferate normally in response to IL-15 (Fig 4.4b).

Lipid synthesis is also critically important for cell growth. We investigated the role for SREBP activity in cytokine stimulated NK cell blastogenesis by analyzing forward scatter by flow cytometry. Importantly inhibition of SREBP has no effect on NK cell activation, as demonstrated by normal expression of CD69 in cytokine stimulated NK cells treated with or without PF429242/25HC (Fig 4.6). We found that inhibition of SREBP activity decreased NK cell blastogenesis in IL-2/12 but more so in IL-2/12/18

stimulated NK cells (Fig 4.7a). Furthermore, deletion of SCAP also inhibited cytokine induced NK cell blastogenesis (Fig 4.7b). Combined this data demonstrates a critical role for SREBP activity in NK cell proliferation and growth following cytokine stimulation.

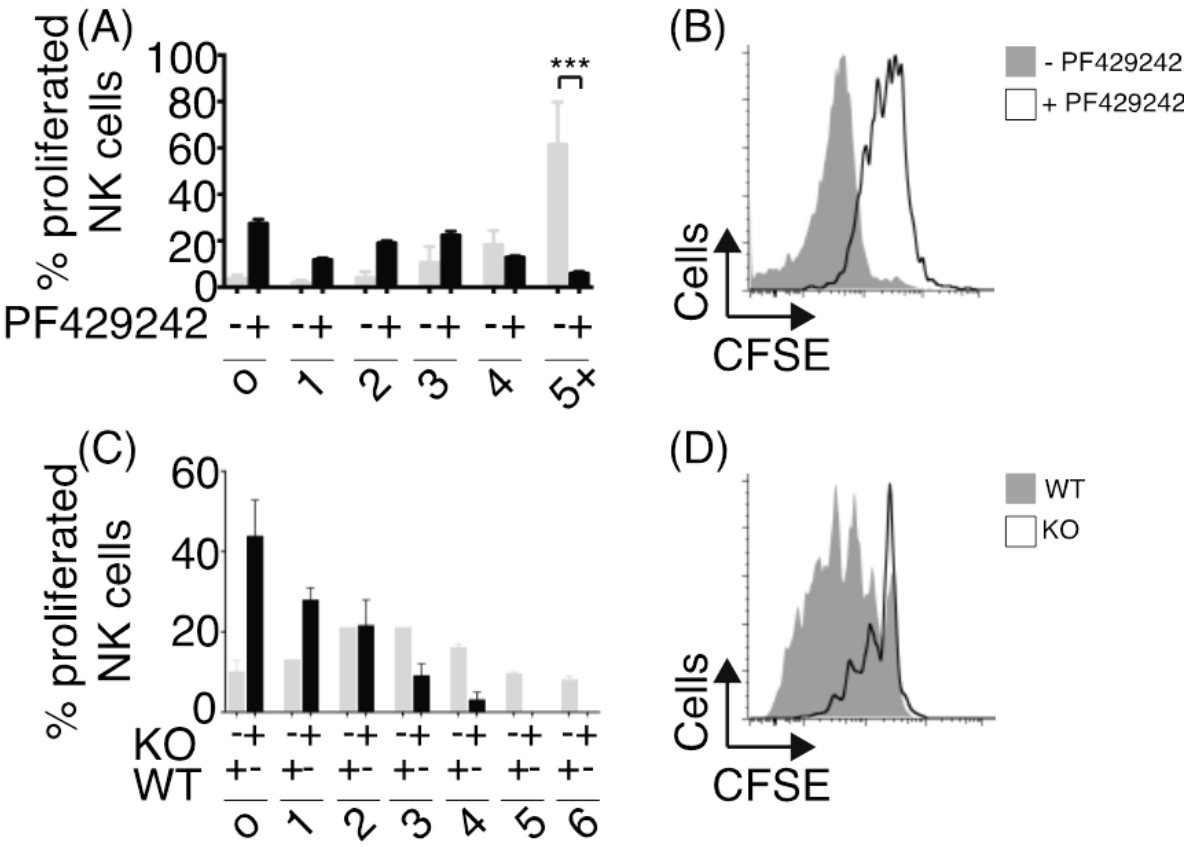

Figure 4.4. SREBP activity is required for IL-15 induced homeostatic proliferation. (A-B) Splenocytes were isolated from a spleen, labelled with CFSE and cultured in IL-15 (25ng/ml) +/- 10μM PF429242 for 7 days. 10μM PF429242 was readded on Day 3. Proliferation was measured by the dilution of CFSE staining. (C-D) Splenocytes were isolated from a spleen of either a WT ($SCAP^{wt/wt}$ Tamox-cre) or KO ($SCAP^{fl/fl}$ Tamox-cre) mice, labelled with CFSE and cultured in IL-15 (25ng/ml) with 0.6μM 4-Hydroxytamoxifen for 6 days. 0.6μM 4-Hydroxytamoxifen was readded on Day 3. Proliferation of NK1.1+ NKp46+ CD3- NK cells was determined based on the dilution of CFSE staining. Data shown is mean +/- S.E.M of N=3 (a,c) and representative histograms (b,d), (***$p<0.001$).

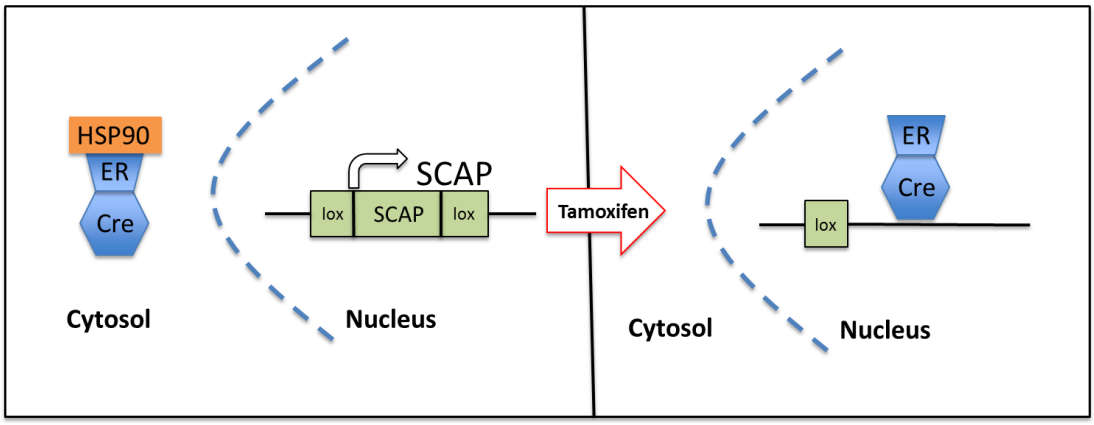

Figure 4.5. Tamoxifen-inducible cre recombinase system. These mice express a cre-recombinase-estrogen receptor fusion protein that is maintained in the cytoplasm through interactions with HSP90. The estrogen receptor is modified so that it too does not bind to endogenous ligands. However, when 4-OH tamoxifen 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 exon 1 of the SCAP gene.

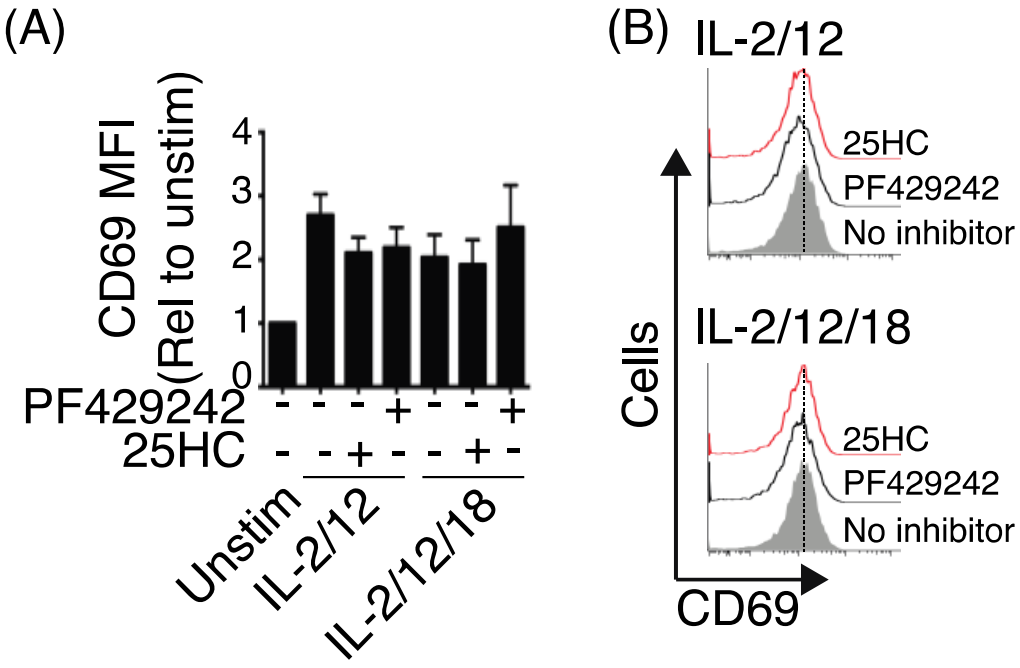

Figure 4.6. Inhibition of SREBP activity has no effect on NK cell activation. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- PF429242 (10μM) or 25HC (1μg/ml) overnight. Stimulated NK cells were subjected to flow cytometry analysis, expression levels of CD69 of NK1.1+ NKp46+ CD3- NK cells was determined using the FACS canto. Data shown is mean +/- S.E.M of N=5 (a) and representative histograms (b).

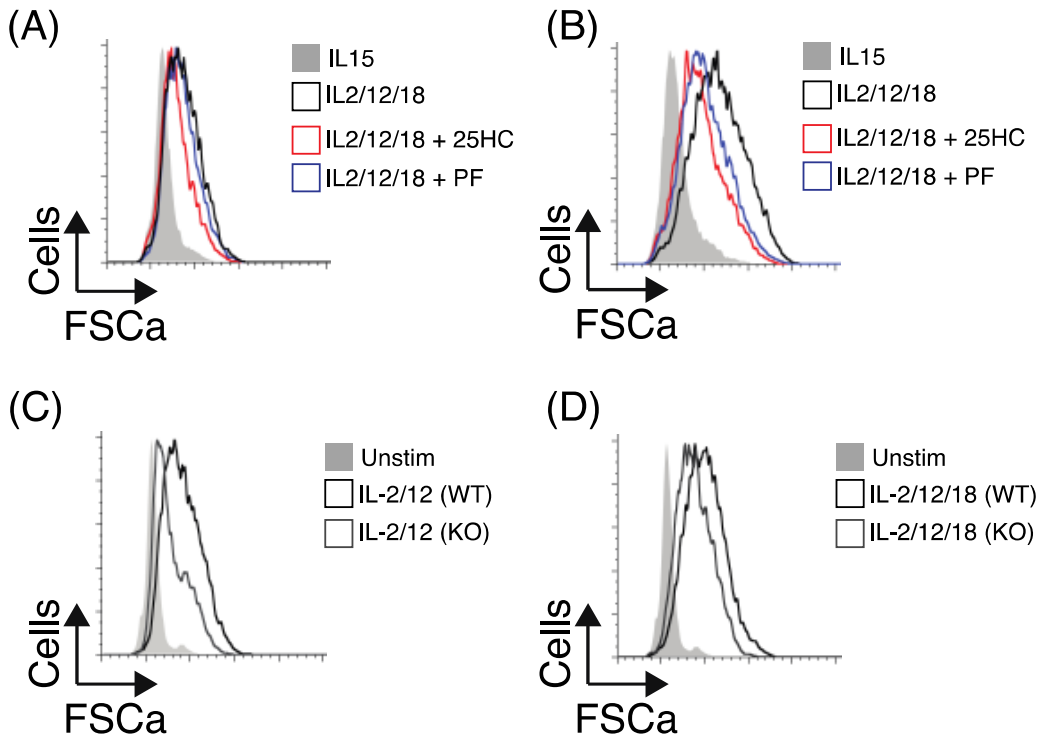

Figure 4.7. SREBP activity is required for blastogenesis in IL-2/12 and IL-2/12/18 stimulated NK cells. (A-B) Splenocytes were isolated from a spleen and cultured in IL-15 (25ng/ml) for 6 days. Cells were harvested on day 6 and either maintained in IL-15 (5ng/ml) or stimulated with a combination of IL-2/12 or IL-2/12/18 +/- PF429242 (10μM) or 25HC (1μg/ml) overnight. (C-D) Splenocytes were isolated from a spleen of either a WT ($SCAP^{wt/wt}$ Tamox-cre) or KO ($SCAP^{fl/fl}$ Tamox-cre) mouse and cultured in IL-15 (25ng/ml) with 0.6μM 4-Hydroxytamoxifen for 6 days. 0.6μM 4-Hydroxytamoxifen was readded on Day 3. Cells were harvested on day 6 and either maintained in IL-15 (5ng/ml) or stimulated with a combination of IL-2/12 or IL-2/12/18. (A-D) Stimulated NK cells were subjected to flow cytometry analysis, cell size of NK1.1+ NKp46+ CD3- NK cells was assessed based on forward light scatter. Data shown is representative of N=3

4.4. SREBP activity is required for IFNγ and Granzyme B production in IL-2/12 and IL-2/12/18 stimulated NK cells

SREBP activity is critical for regulating effector T cell blastogenesis and their effector responses (Lochner et al., 2015). Our data shows SREBP activity is important for NK cell blastogenesis. We next investigated whether perturbations in SREBP activity might impact upon NK cell effector responses. Interestingly, NK cells stimulated with IL-2/12/18 produce significantly more IFNγ than IL-2/12 stimulated NK cells. Furthermore, inhibition of SREBP activity with PF429242 and 25HC decreases the percentage of IFNγ^{+} NK cells when stimulated with IL-2/12 (Fig 4.8a-b). However, decreased SREBP activity has no effect on the percentage of IFNγ^{+} NK cells when stimulated with IL-2/12/18 (Fig 4.8a-b). Moreover, inhibition of SREBP activity decreased the amount (mean fluorescent intensity, MFI) of IFNγ being produced by IFNγ^{+} NK cells (Fig 4.8c). Our previous data shows that granzyme B production is induced upon IL-2/12 stimulation. Interestingly, IL-2/12/18 stimulated NK cells further increase granzyme B production (Fig 4.9a, c). Inhibition of SREBP activity resulted in a decrease in granzyme B production in IL-2/12 and IL-2/12/18 stimulated NK cells (Fig 4.9b, d). However, cytokine induced TNFα expression is not significantly affected by SREBP inhibition (Fig 4.10).

The effect of SCAP deletion on NK cell function was also investigated. To date IFNγ and granzyme B expression has only been analyzed twice in two SCAP$^{fl/fl}$ x Tamox-Cre mice. Granzyme B and IFNγ expression in unstimulated NK cells, maintained in IL-15 (5ng/ml), wasn't dependent on the presence of the SCAP gene. However, preliminary experiments show that cytokine stimulated SCAP$^{fl/fl}$ x Tamox-Cre NK cells treated with 4-OH express significantly less granzyme B (Fig 4.11). IFNγ expression was dramatically reduced in one of the replicates, however the there was no observed decrease in the other replicate (Fig 4.12). Our data shows that SREBP is required for blastogenesis (Fig 4.7). In these experiments with Scap$^{fl/fl}$ x Tamox-Cre splenocytes

stimulated with cytokines there were 2 clear populations of small and big cells. Previous experience with the Tamox-Cre system in the laboratory has revealed that incomplete gene deletion can be an issue. We considered that the small NK cells may deleted Scap while the large NK cells may still contain the Scap gene. Indeed, the small NK cells had substantially less IFNγ and granzyme B compared to the big NK cells. Scap deletion in small versus big NK cells needs to be formally proven. Together this data describes how SREBP activity not only is required for NK cell growth and proliferation but also is a prerequisite for NK cells effector molecules.

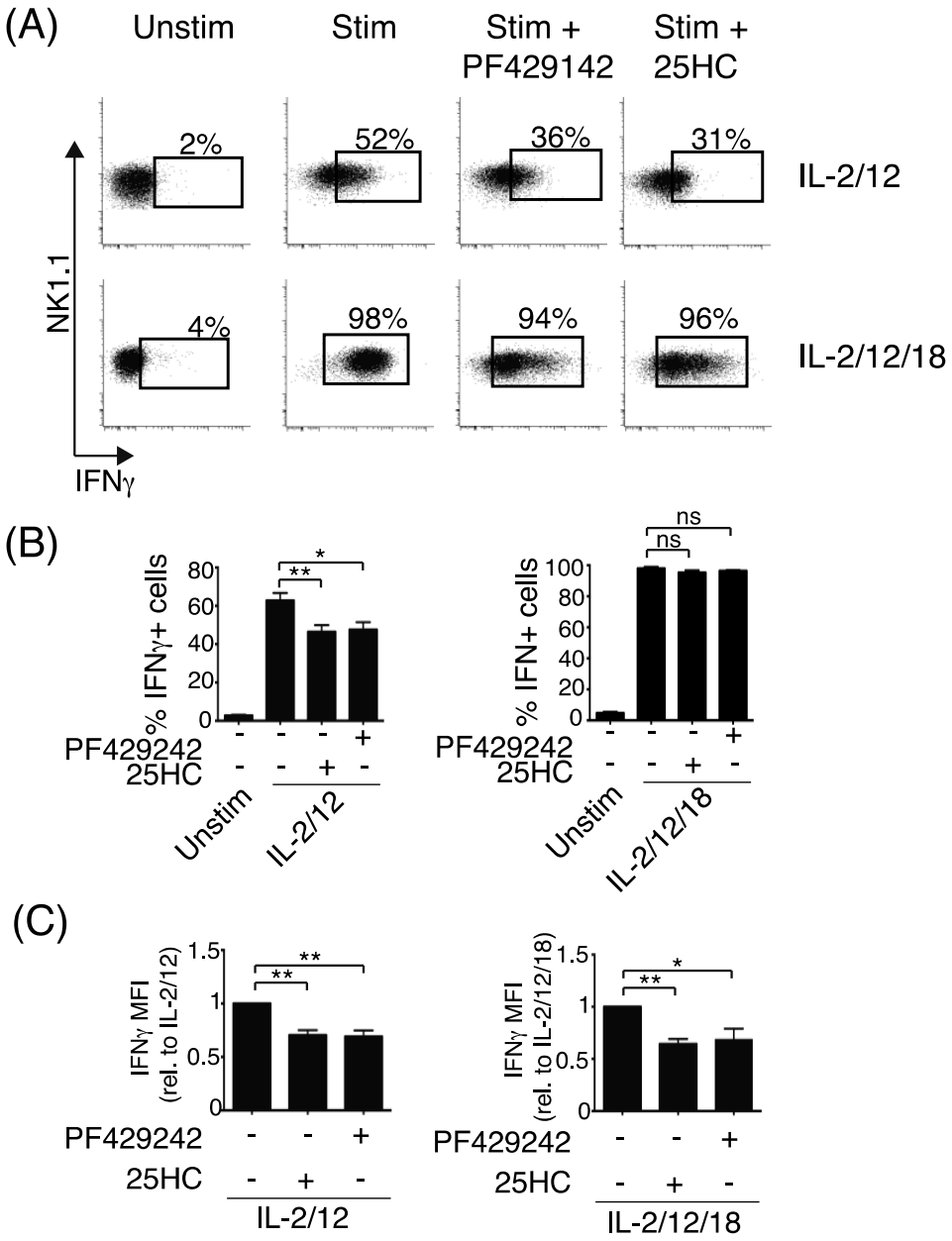

Figure 4.8. SREBP activity is required for IFNγ expression in cytokine stimulated NK cells. (A-C) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- PF429242 (10μM) or 25HC (1μg/ml) overnight. The percentage of IFNγ+ NK1.1+ NKp46+ CD3- NK cells was determined based on unstimulated control NK cells using flow cytometry. Data shown is mean +/- S.E.M of N=10 (b-c) and representative dot plots (a). (ns=not significant, *p<0.05, **p<0.01).

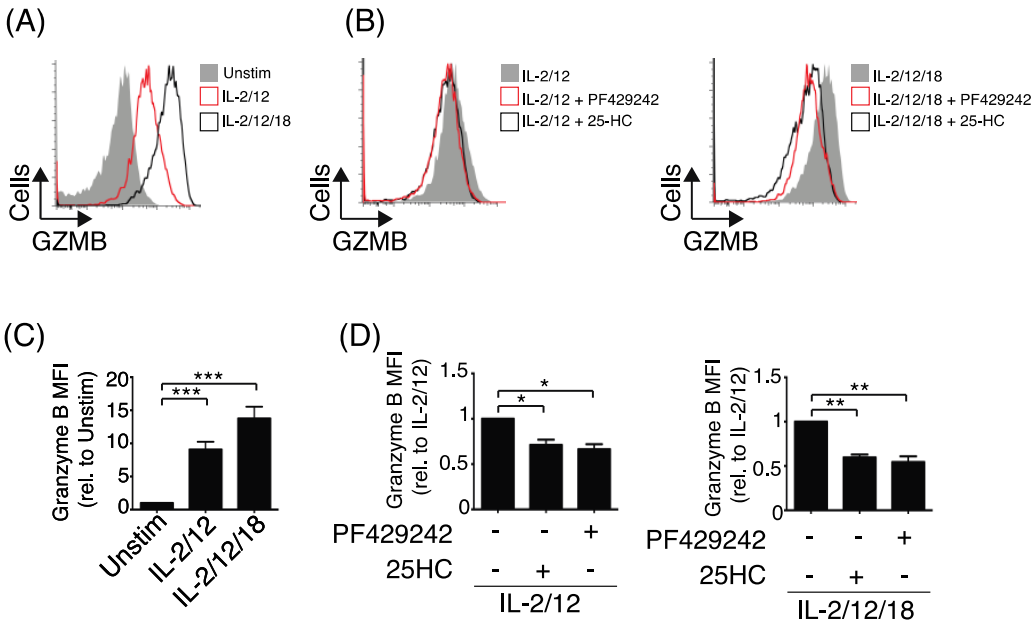

Figure 4.9. SREBP activity is required for Granzyme B production in cytokine stimulated NK cells. (A-D) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- PF429242 (10μM) or 25HC (1μg/ml) overnight. Granzyme B expression of NK1.1+ NKp46+ CD3- NK cells was determined using flow cytometry. Data shown is mean +/- S.E.M of N=10 (c-d) and representative dot plots (a-b) (*p<0.05, **p<0.01, ***p<0.001).

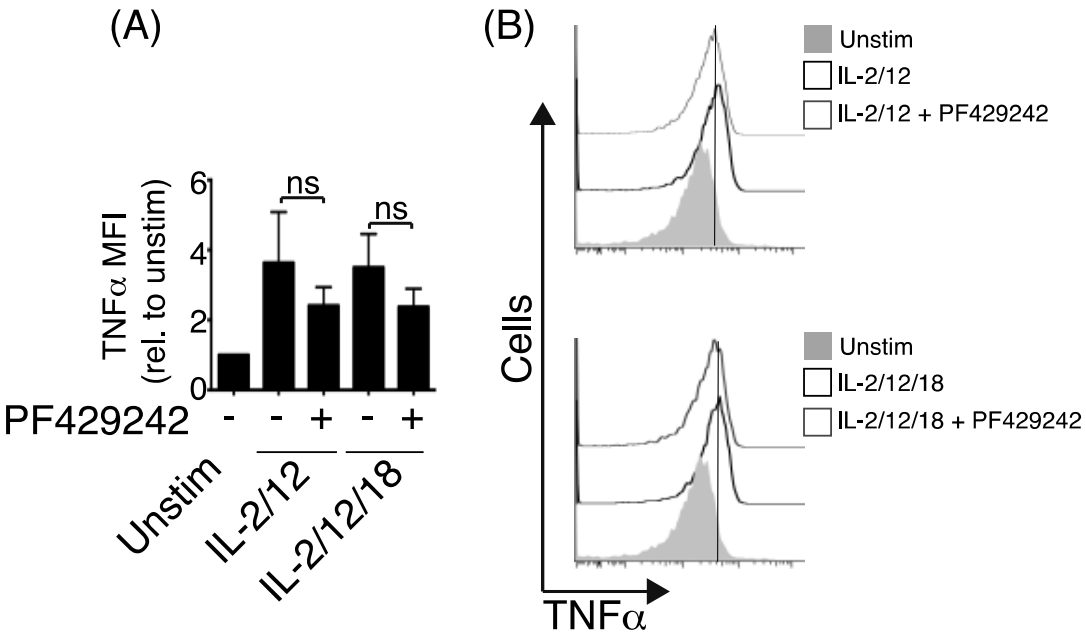

Figure 4.10. SREBP activity is not required for TNFα production in cytokine stimulated NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- PF429242 (10μM) overnight. TNFα expression was determined by MFI of NK1.1+ NKp46+ CD3- NK cells using flow cytometry. Data shown is mean +/- S.E.M of N=3 (a) and representative histograms (b) (ns=not significant).

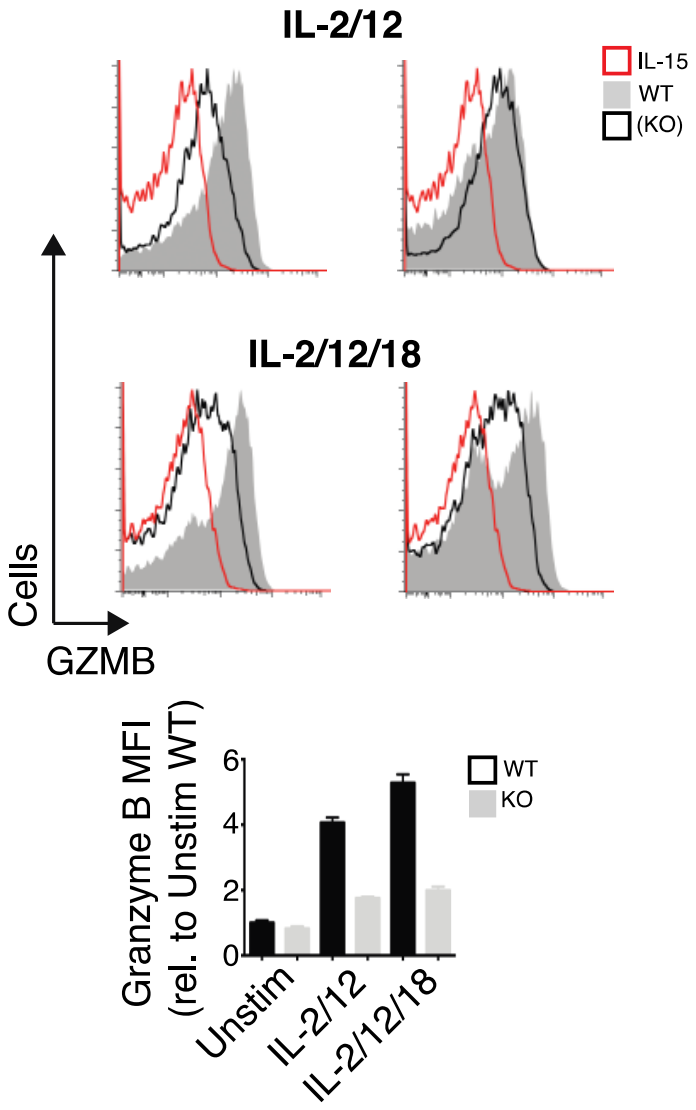

Figure 4.11. Granzyme B production is inhibited in cytokine stimulated NK cells in the absence of SCAP expression. Splenocytes were isolated from a spleen of either a WT ($SCAP^{wt/wt}$ Tamox-cre) or KO ($SCAP^{fl/fl}$ Tamox-cre) mouse and cultured in IL-15 (25ng/ml) with 0.6μM 4-Hydroxytamoxifen for 3 days. Cells were harvested on day 3 and either maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 overnight. Granzyme B expression of NK1.1+ NKp46+ CD3- NK cells was determined using flow cytometry. Data shown is N=2. Both histograms are shown along with the combined data.

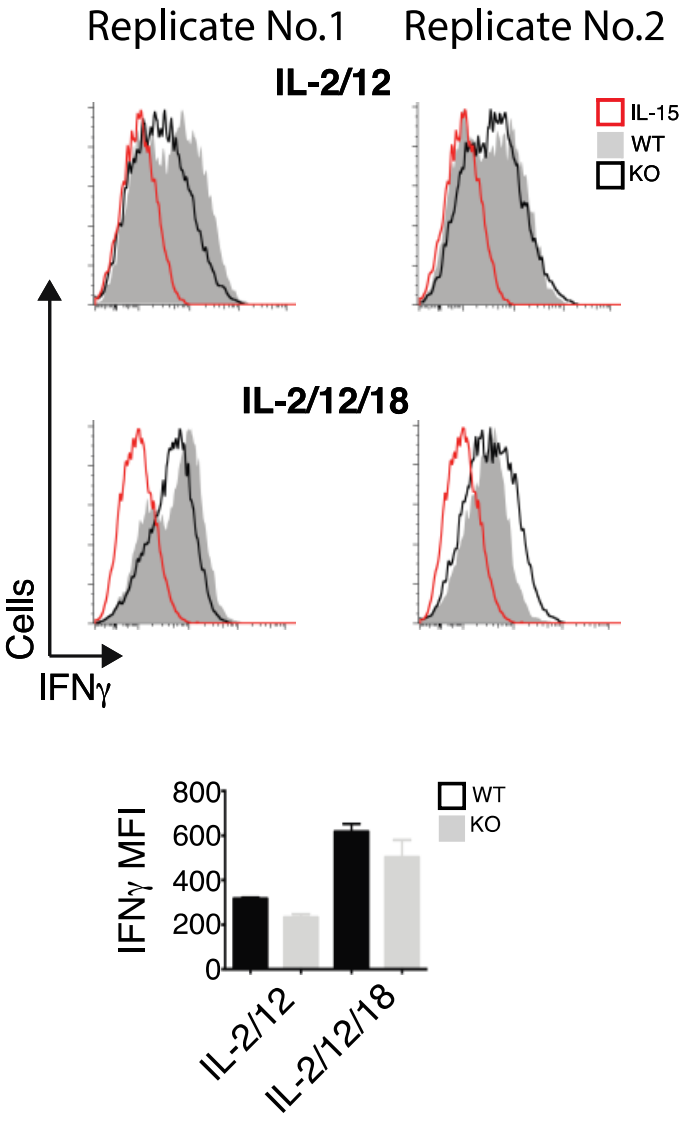

Figure 4.12. IFNγ production is inhibited in cytokine stimulated NK cells in the absence of SCAP expression. Splenocytes were isolated from a spleen of either a WT ($SCAP^{wt/wt}$ Tamox-cre) or KO ($SCAP^{fl/fl}$ Tamox-cre) mouse and cultured in IL-15 (25ng/ml) with 0.6μM 4-Hydroxytamoxifen for 3 days. Cells were harvested on day 3 and either maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 overnight. IFNγ expression of NK1.1+ NKp46+ CD3- NK cells was determined using flow cytometry. Data shown is N=2. Both histograms are shown along with the combined data.

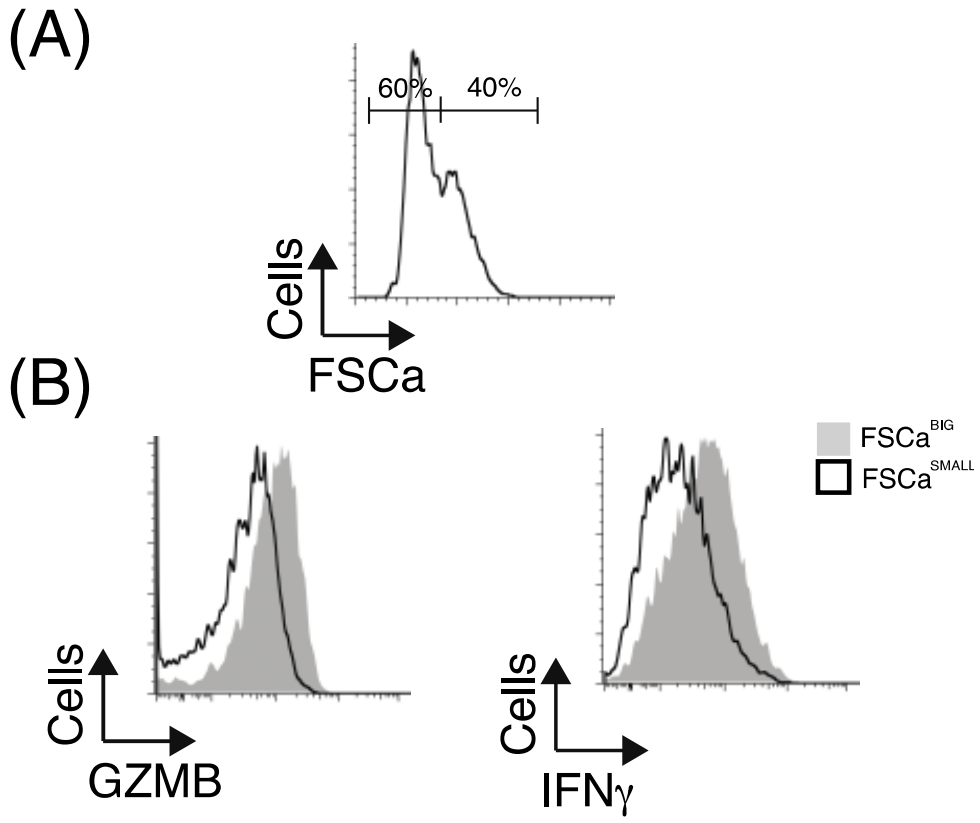

Figure 4.13. Tamoxifen doesn't inhibit SCAP expression in every cell. IFNγ and Granzyme B expression are reduced in the cells where SCAP is decreased. Splenocytes were isolated from a spleen of a $SCAP^{fl/fl}$ Tamox-cre mouse and cultured in IL-15 (25ng/ml) with 0.6μM 4-Hydroxytamoxifen for 3 days. Cells were harvested on day 3 stimulated with IL-2/12 overnight. IFNγ and Granzyme B expression of $FSCa^{BIG}$ vs $FSCa^{SMALL}$ NK1.1+ NKp46+ CD3- NK cells was determined using flow cytometry. Data shown is representative of N=2.

4.5. SREBP activity is required for elevated glycolysis and OxPhos in cytokine stimulated in NK cells

Previously we have shown that NK cell glycolysis is linked to NK cell effector responses. SREBP deficient $CD8^+$ T cells fail to increase glycolysis upon TCR stimulation (Kidani et al., 2013). Therefore we hypothesized that SREBP is required for NK cell function because it is needed for NK cell glucose metabolism. To test this we measured the rates of glycolysis and OxPhos in cytokine stimulated NK cells in the presence and absence of SREBP inhibitors PF429242 and 25HC. The data show that PF429242 and 25HC treatment inhibits IL-2/12 and IL-2/12/18 induced NK cell glycolysis (Fig 4.14a). Furthermore, SREBP inhibition also resulted in decreased OxPhos levels (Fig 4.14b). Overall, inhibition of SREBP activity decreased NK cell energy metabolism. Inhibition of SREBP did not reduce the expression of Hexokinase 2 (Fig 4.15a), Ldha (Fig 4.15b) or the glucose transporter Glut 1 (Fig 4.15c). However, oligomycin treatment did not result in increased rates of glycolysis in NK cells where SREBP was inhibited (Fig 4.16). Therefore cytokine stimulated NK cells with reduced SREBP activity are already operating at their glycolytic capacity. Given that the data shows the glycolytic machinery is intact in cells lacking SREBP activity, the data argues that another factor is limiting the rate of NK cell glycolysis. This is suggestive that another important component for elevated glycolysis in our cytokine activated NK cells is absent. Combined this data states that SREBP activity is linked to NK cell glycolysis and OxPhos. Moreover, SREBP activity is not required for metabolic reprogramming and SREBP activity must control glycolysis and OxPhos in cytokine stimulated NK cells through an alternative mechanism.

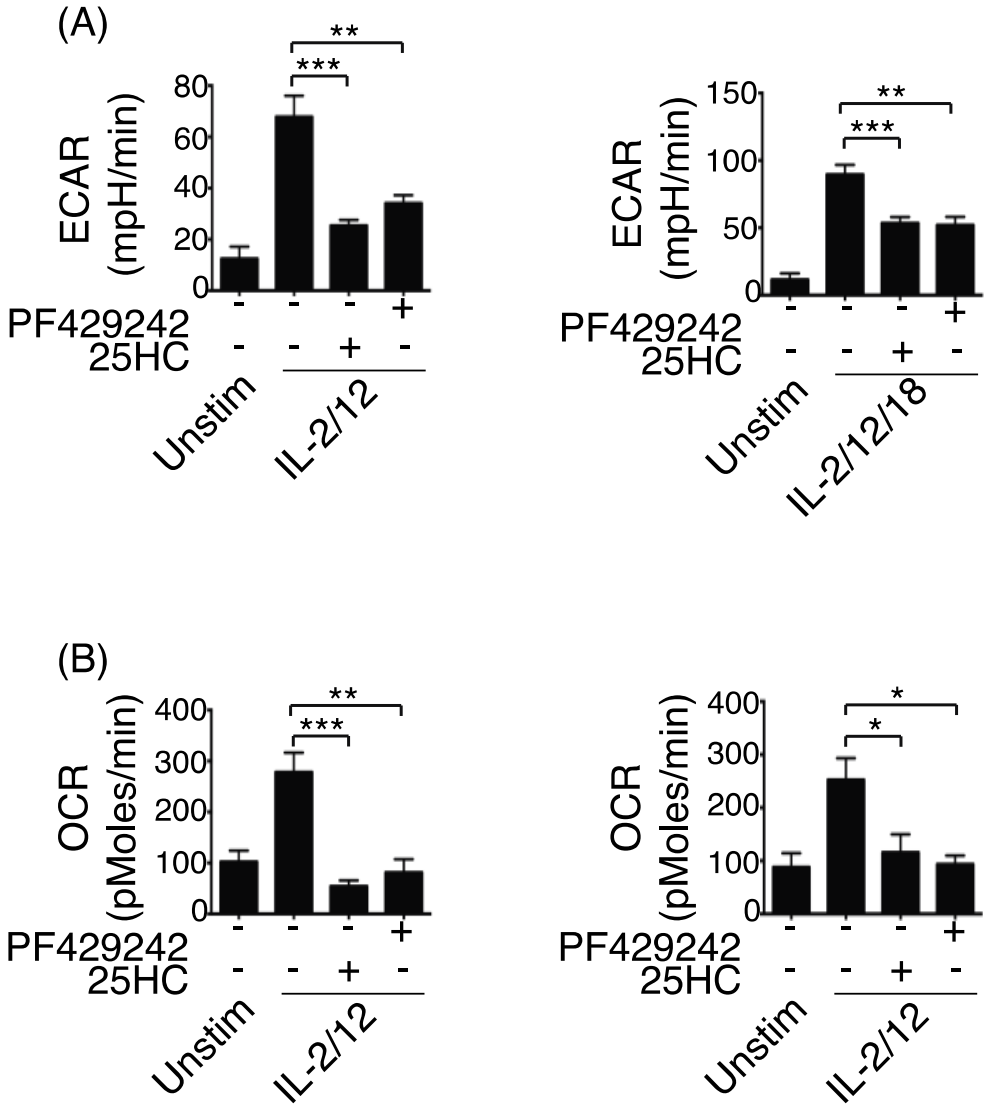

Figure 4.14. PF429242 and 25HC treatment inhibits cytokine stimulated NK cell glycolysis and OxPhos. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- PF429242 (10μM) or 25HC (1μg/ml) overnight. Stimulated NK cells underwent detailed metabolic analysis using the Seahorse metabolic Flux analyzer, which determined the glycolytic (a) and Oxidative Phosphorylation (b) levels of the cell. Data shown is mean +/- S.E.M of N=3 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

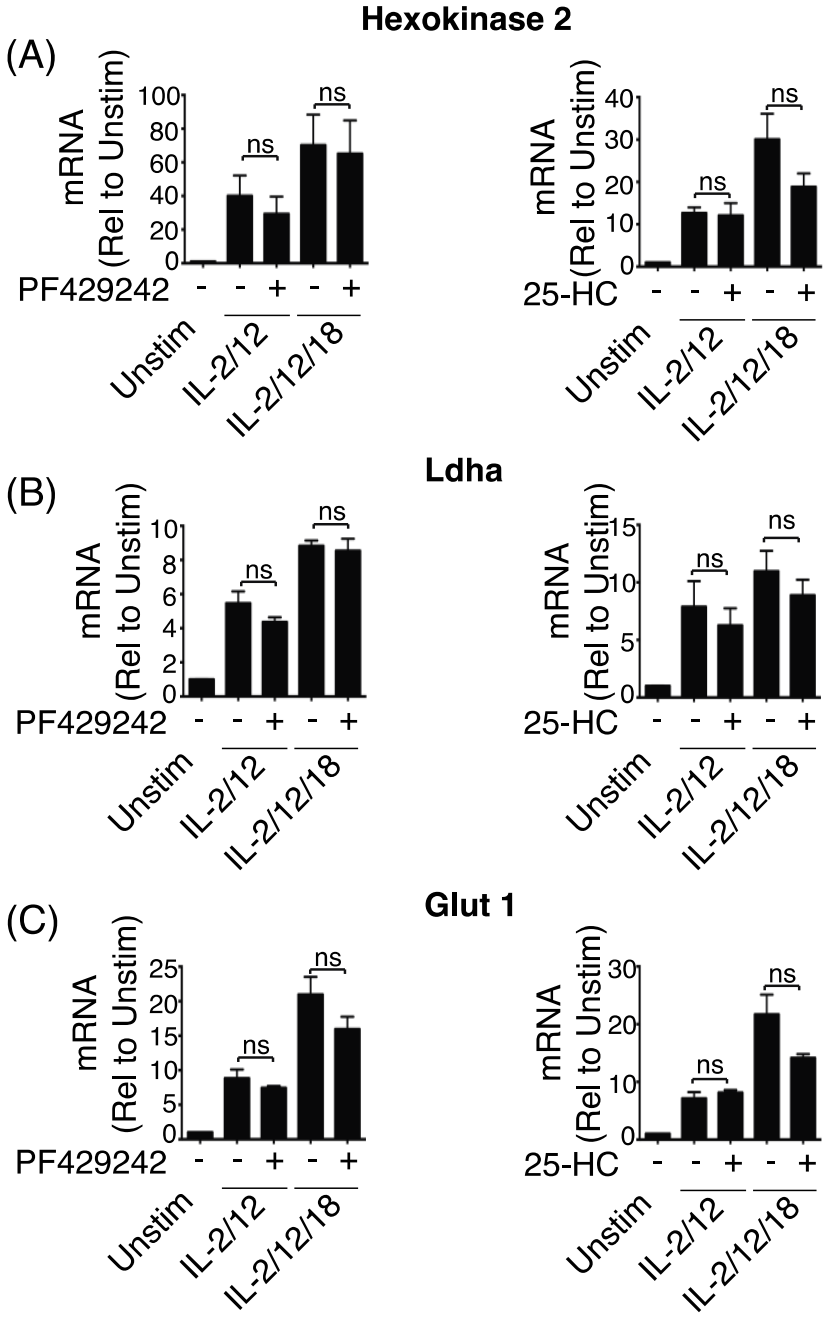

Figure 4.15. SREBP Activity is not required for the expression of glycolytic genes in cytokine stimulated NK cells. (A-C) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- PF429242 or 25HC overnight. RT-qPCR analysis of the expression of hexokinase 2 (a), Ldha (b) and Glut1 (c) carried out under conditions shown. Data is mean +/- S.E.M of N=3 (ns=not significant).

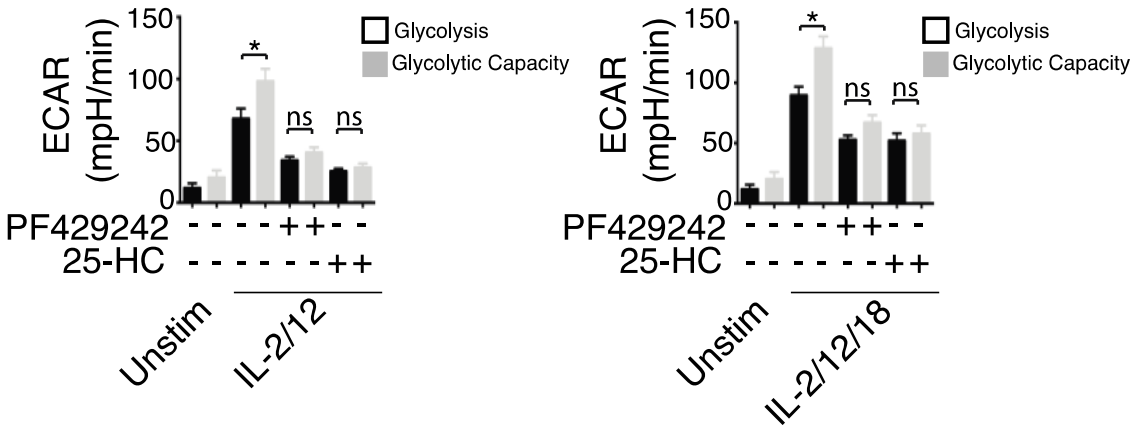

Figure 4.16. NK cells lacking SREBP activity are operating at maximal glycolytic capacity. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- PF429242 (10μM) or 25HC (1μg/ml) overnight. Stimulated NK cells underwent detailed metabolic analysis using the Seahorse metabolic Flux analyzer, which determined the glycolytic levels of the cell before (glycolysis) and after the addition of oligomycin (glycolytic capacity) during the assay. Data shown is mean +/- S.E.M of N=3 (ns=not significant, * $p<0.05$).

4.6. NK cell effector functions do not require cytokine induced lipid synthesis

The best characterized role for SREBP is its function in lipid synthesis. When sterol levels within the cell are low SREBP is activated to restore lipid biosynthesis and lipid homeostasis. Hence, we decided to investigate if directly targeting lipid biosynthesis resulted in decreased effector molecules in cytokine stimulated NK cells. Pharmacological inhibitors were used to inhibit Fatty acid synthesis alone, using C75 an inhibitor of FASN, or both fatty acid synthesis and cholesterol synthesis, using TOFA an inhibitor of ACC). Both C75 and TOFA decreased the incorporation of radiolabeled acetate into cellular lipid in cytokine stimulated NK cells (Fig 4.17). C75 and TOFA do not disrupt NK cell activation as judged by the level of the activation marker CD69 (Fig 4.18). The data shows C75 and TOFA treatment has no effect on granzyme B (Fig 4.19a-b) or IFNγ (Fig 20-21) production. Additionally, direct inhibition of lipid synthesis with C75 and TOFA did not decrease glycolysis or OxPhos in IL-2/12 or IL-2/12/18 stimulated NK cells (Fig 4.22). In fact the data suggests that TOFA treatment significantly increases glycolytic levels in IL-2/12/18 stimulated NK cells (Fig 4.22a). OxPhos levels are also slightly elevated with TOFA treatment; however this did not reach significance (Fig 4.21b). Combined this data shows that SREBP control of lipid synthesis is not required for elevated NK cell glycolysis and OxPhos, nor is it required for NK cell effector molecules.

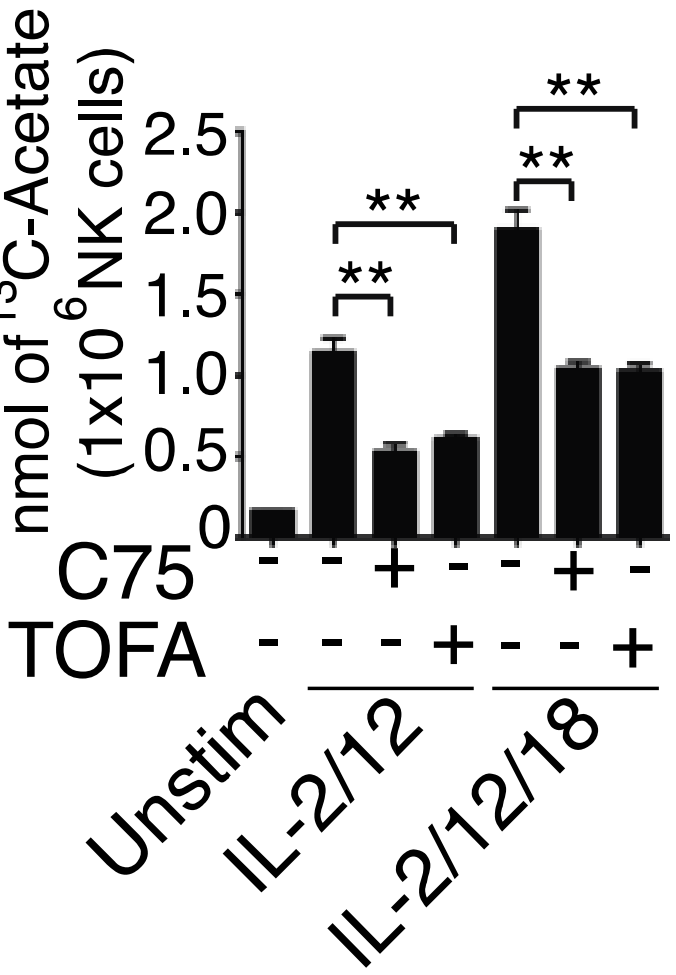

Figure 4.17. C75 and TOFA treatment inhibits lipid synthesis in IL-2/12 and IL-2/12/18 simulated NK cells. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- C75 or TOFA overnight. Analysis of the rate of lipid synthesis through measuring the incorporation of radiolabeled ^{13}C-acetate into cellular lipids incorporated was carried out. Data is mean +/- S.E.M of N=2. (** $p<0.01$).

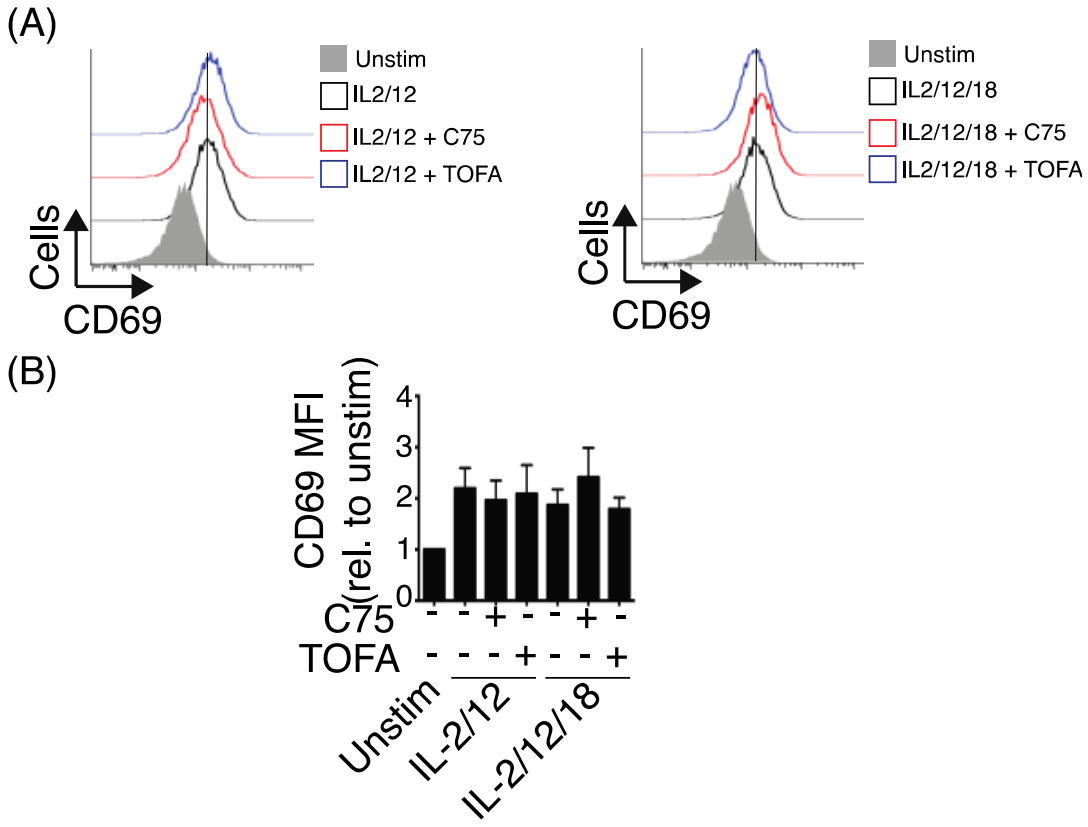

Figure 4.18. Inhibition of lipid synthesis with C75 and TOFA has no effect on NK cell activation. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with a combination of IL-2/12 or IL-2/12/18 overnight. Expression of CD69 of NK1.1+ NKp46+ CD3- NK cells was determined through Flow cytometry. Data shown is mean +/- S.E.M of N=4 (b) and representative histograms (a).

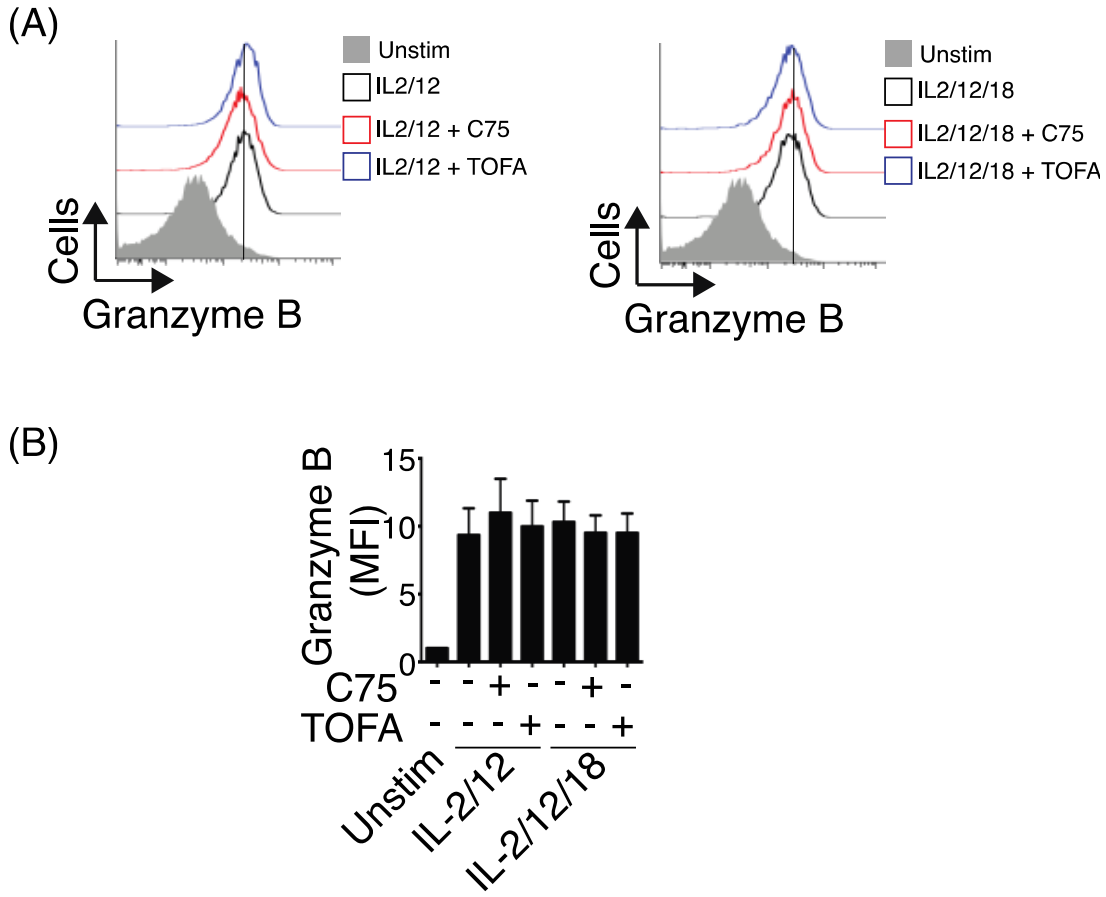

Figure 4.19. Lipid synthesis is not required for Granzyme B production in cytokine stimulated NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 overnight. Granzyme B expression of NK1.1+ NKp46+ CD3- NK cells was determined using Flow cytometry. Data shown is mean +/- S.E.M of N=4 (b) and representative histograms (a).

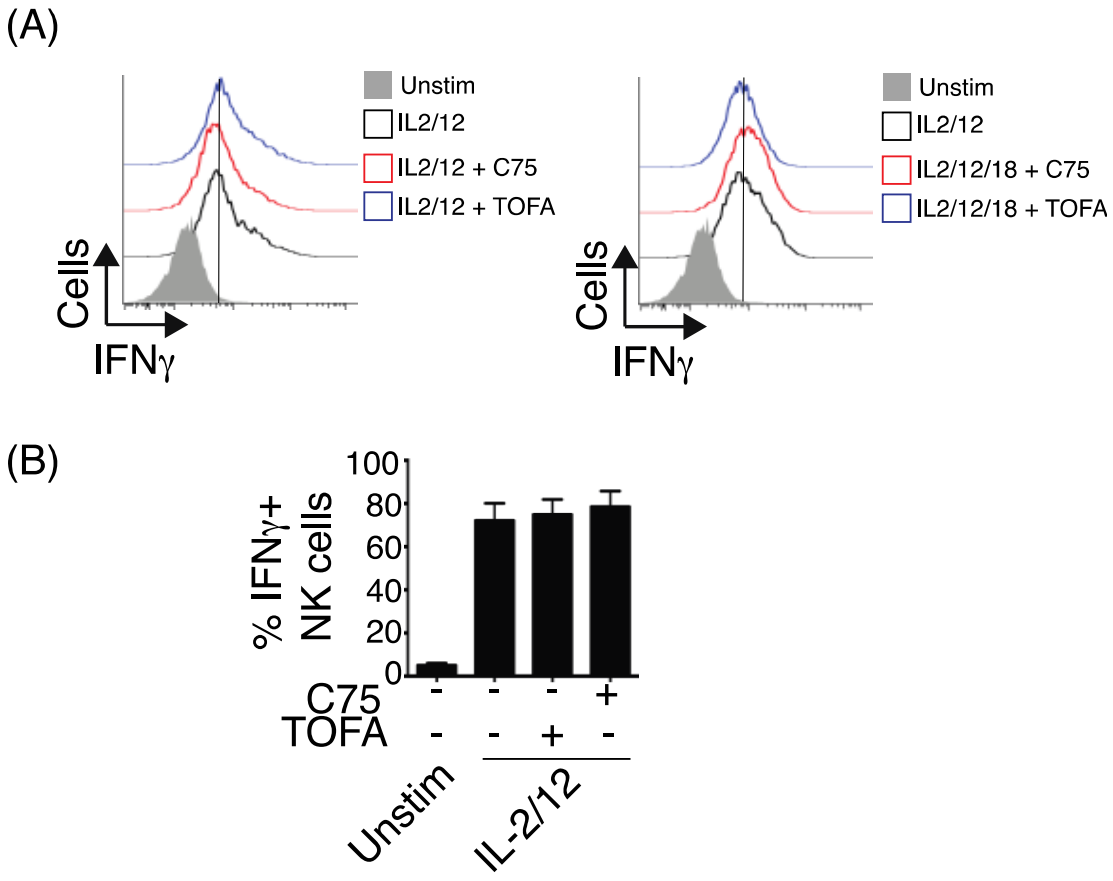

Figure 4.20. Lipid synthesis is not required for IFNγ expression in cytokine NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- C75 or TOFA overnight. The percentage of IFNγ⁺ NK1.1+ NKp46+ CD3- NK cells was determined based on unstim control NK cells using flow cytometry. Data shown is mean +/- S.E.M of N=4 (b) and representative histograms (a).

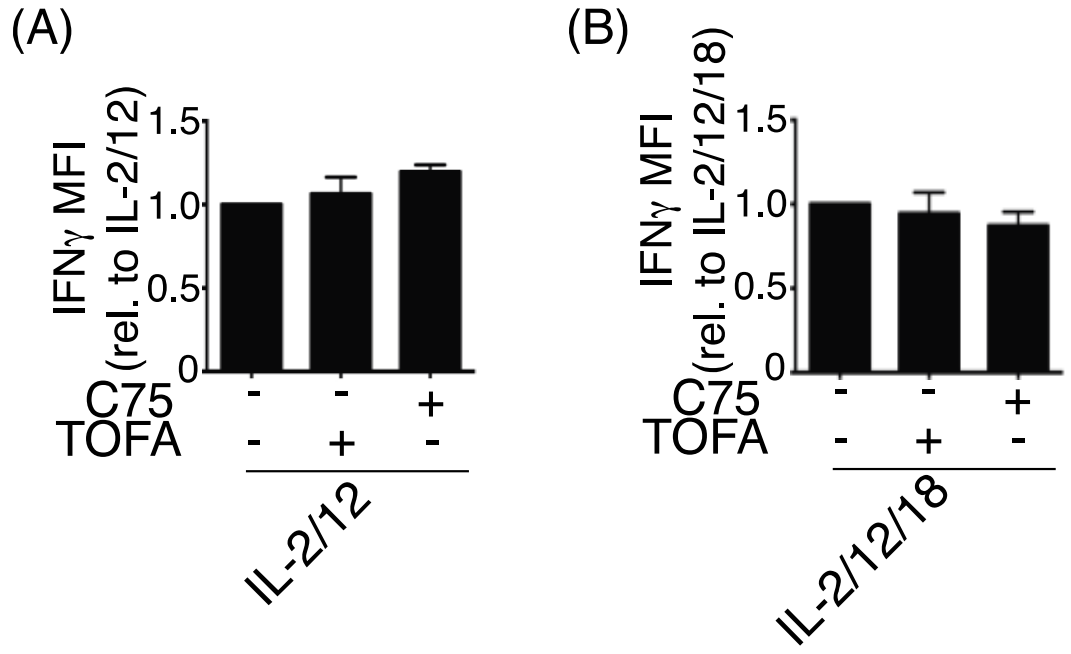

Figure 4.21. Lipid synthesis is not required for IFNγ production in cytokine stimulated NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- C75 or TOFA overnight. The amount of IFNγ being produced was determined by MFI of IFNγ+ NK1.1+ NKp46+ CD3- NK cells using Flow cytometry. Data shown is mean +/- S.E.M of N=4

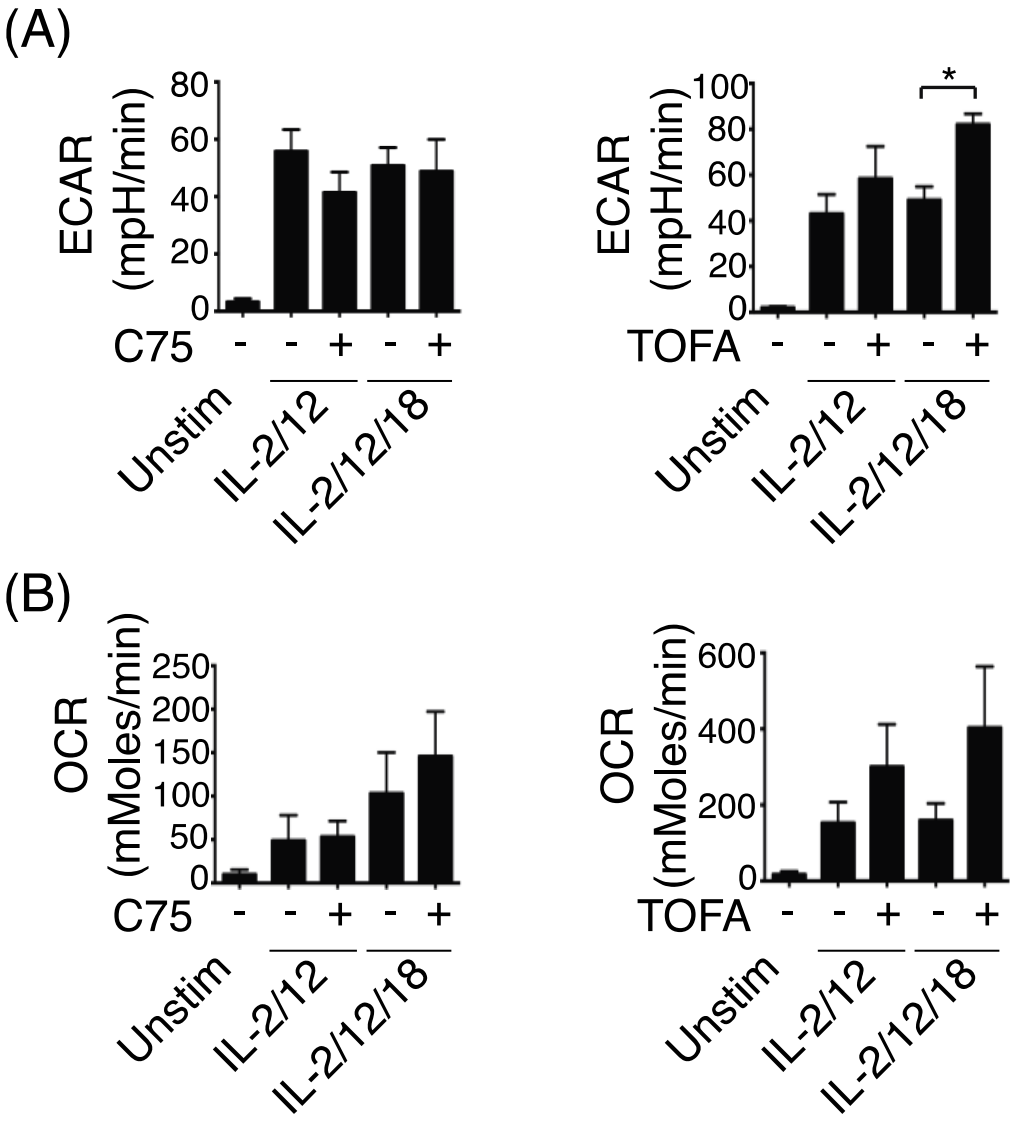

Figure 4.22. Lipid synthesis is not required for glycolysis or OxPhos in cytokine stimulated NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- C75 or TOFA overnight. Stimulated NK cells underwent detailed metabolic analysis using the Seahorse metabolic Flux analyzer, which determined the rates of glycolysis (a) and Oxidative Phosphorylation (b). Data shown is mean +/- S.E.M of N=3 (* $p<0.05$)

4.7. The Citrate-Malate shuttle is required for NK cell metabolism and function

Given that SREBP controlled lipid synthesis is not required for NK cell glucose metabolism and NK cell effector function, this suggests other SREBP target genes must be important. In other cells, SREBP is reported to control the expression of important proteins that supply the cytoplasm with the substrate for lipid synthesis, acetyl-CoA. These include the citrate/malate transporter (CiC), which transports citrate from the mitochondria to the cytosol, and ATP-citrate lyase (ACLY), that cleaves cytoplasmic citrate into acetyl-CoA and oxaloacetate. While acetyl-CoA is used for lipid synthesis, oxaloacetate is converted to malate, in a reaction that also oxidizes NADH to NAD^+, and the malate returns to the mitochondria. This is called the citrate malate shuttle and the net result is the export of acetyl-CoA and the oxidation of NADH to NAD^+ (Fig 4.23). Alternatively malate can be converted to pyruvate, generating NADPH an important cofactor for biosynthetic processes including lipid synthesis. Pyruvate can either enter the mitochondria and the Krebs cycle or be converted to lactate. Given that NAD^+ is essential for the activity of the glycolytic enzyme GAPDH and therefore glycolytic flux, we reasoned that SREBP dependent control of the citrate-malate shuttle and cytoplasmic NAD^+ may underpin SREBP dependent NK cell glycolysis and NK cell effector function. CiC and ACLY expression is increased with cytokine stimulation in a SREBP dependent manner (Fig 4.24-25). The data argues that cytokine stimulated NK cells engage the citrate-malate shuttle. Experiments were designed to pharmacologically inhibit the citrate-malate shuttle and the effect on NK cell metabolism and function ascertained. The citrate/malate transporter (CiC) and ATP-citrate lyase (ACLY) were inhibited using benzenetricarboxylic acid (BTA) and BMS303141, respectively. Importantly, inhibition of CiC and ACLY had no effect on NK cell activation based on the expression of the activation marker CD69 (Fig 4.26a-b). Inhibition of ACLY decreased cellular growth in cytokine stimulated NK cells (Fig 4.27). This is likely due to decreased cytoplasmic

acetyl-CoA leading to decreased lipid synthesis and decreased NK cell blastogenesis. However, cytokine stimulated NK cells treated with BTA exhibited similar cellular growth compared to untreated cells (Fig 4.27). Inhibition of CiC or ACLY decreased glycolysis (Fig 4.27a,c) and OxPhos (Fig 4.28b,d) in both IL-2/12 and IL-2/12/18 activated NK cells. Inhibition of ACLY disrupted granzyme B and IFNγ production in cytokine stimulated NK cells (Fig 4.29-31). However, inhibition of CiC only inhibited effector molecules in IL-2/12/18 stimulated NK cells. Additionally the reduction in IFNγ and granzyme B in IL-2/12/18 stimulated NK cells induced by BTA treatment was not as dramatic and more variable than what was observed with inhibition of ACLY. Therefore, there is a discrepancy between BTA mediated inhibition of NK cell metabolism and the lack of an effect on NK cell function. Importantly, experiments looking at growth and function of NK cells require BTA to inhibit CiC for 24 hours while the BTA inhibitor is readded prior to the seahorse extracellular flux analyses. This leads us to believe that BTA is not stable in culture for prolonged periods and that this underpins the observed discrepancy. Combined this data suggests SREBP control of the citrate-malate shuttle as a possible mechanism for controlling NK cell glycolysis, OxPhos and effector molecules.

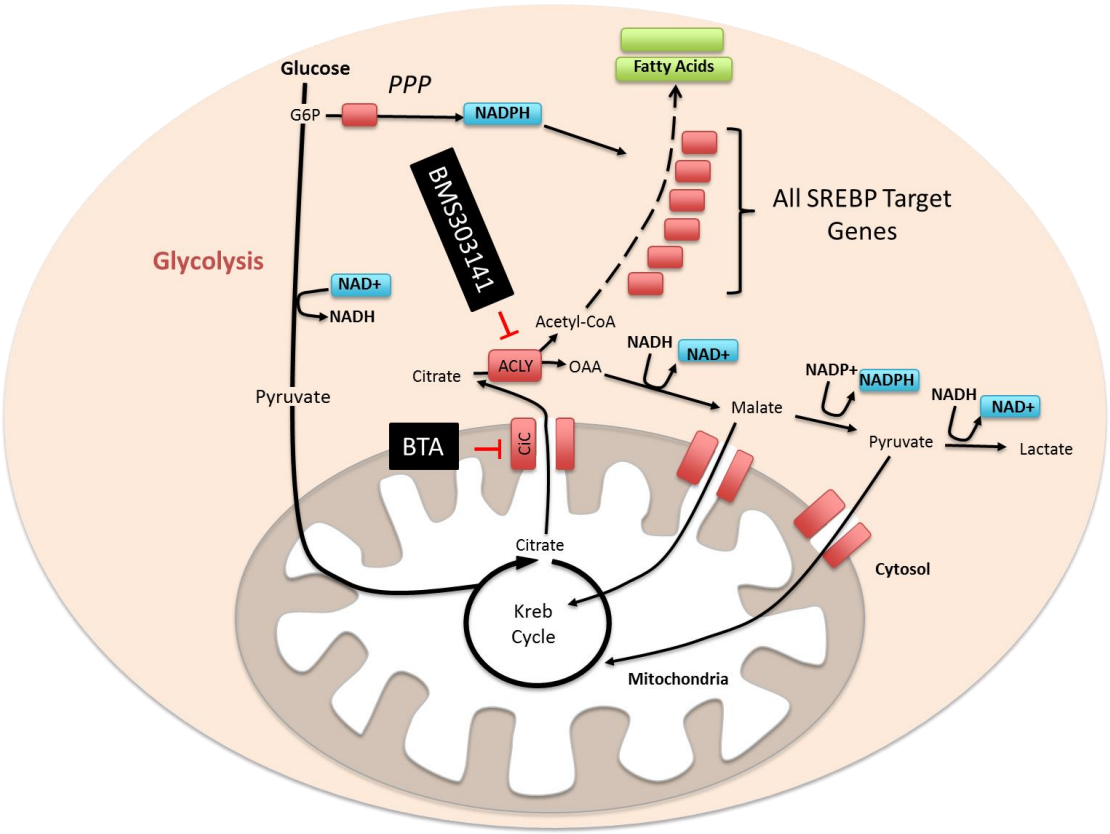

Figure 4.23. Citrate-Malate shuttle regenerates cytosolic NAD+. The end product of glycolysis, pyruvate enters the mitochondria and is converted to acetyl-CoA which enters the Krebs cycle. One metabolite formed in the TCA cycle is citrate. Citrate can leave the mitochondria through the mitochondrial citrate transporter (CiC). Citrate in the cytosol is cleaved by ATP-citrate lyase (ACLY), into acetyl-CoA and oxaloacetate. Acetyl-CoA is used for lipid synthesis, while oxaloacetate is converted to malate, in a reaction that also oxidizes NADH to NAD^+. Subsequently the malate returns to the mitochondria through the CiC. Furthermore, malate can be metabolized to pyruvate, in a NADPH generating reaction required for lipid synthesis, in the cytosol which in turn can be metabolized to lactate in a reaction that will also generate NAD^+. CiC and ACLY can be pharmacological inhibited by benzenetricarboxylic acid (BTA) and BMS303141, respectively.

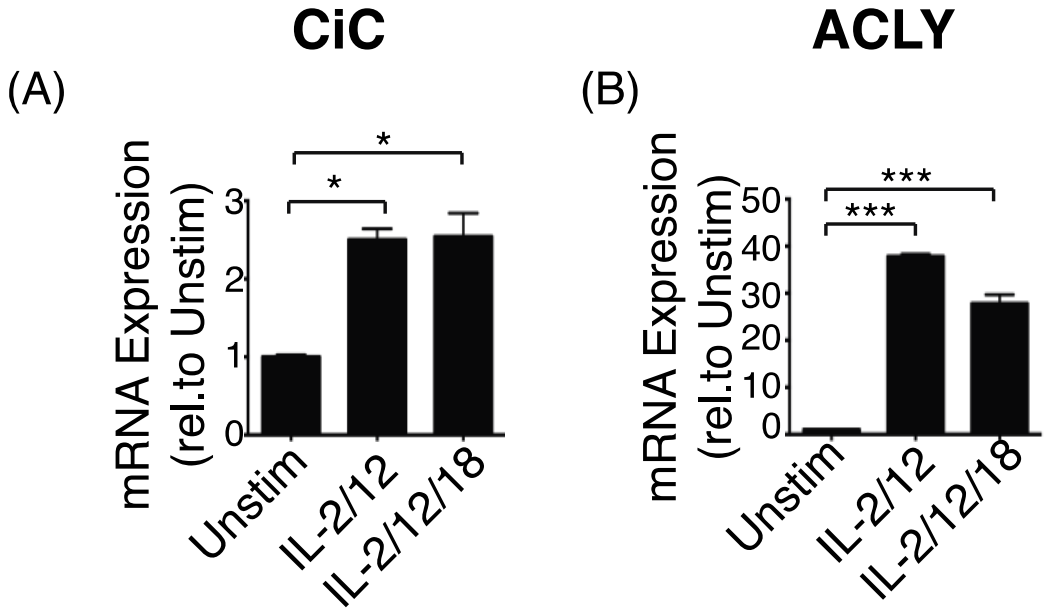

Figure 4.24. Cytokine stimulated NK cells increase the expression of ACLY and CiC. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 overnight. RT-qPCR analysis of the expression of CiC (a) and ACLY (b) of the conditions shown. Data is mean +/- S.E.M of N=3 (* $p<0.05$, *** $p<0.001$).

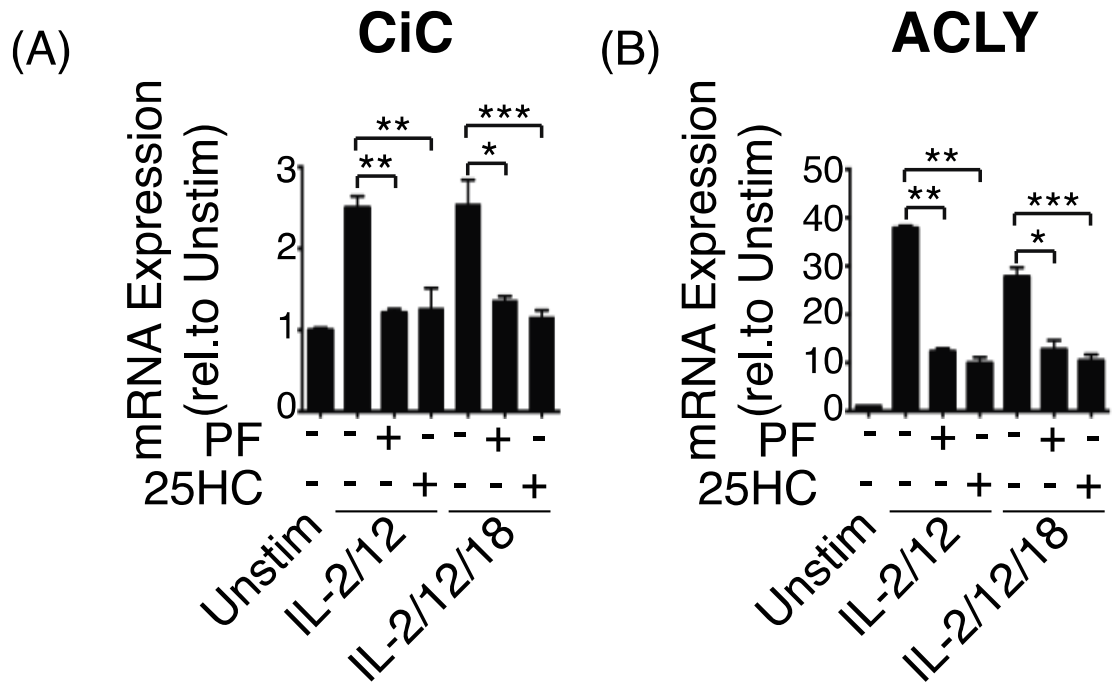

Figure 4.25. SREBP activity is required for the expression of CiC and ACLY in cytokine stimulated NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- PF429242 or 25HC overnight. RT-qPCR analysis of the expression of CiC (a) and ACLY (b) of the conditions shown. Data is mean +/- S.E.M of N=3 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

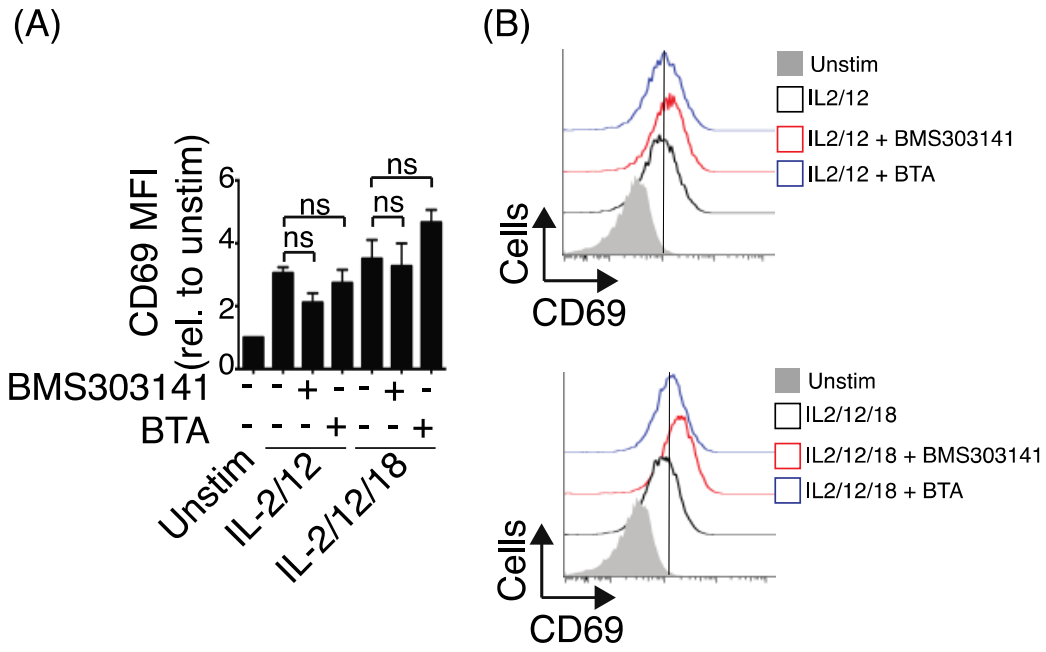

Figure 4.26. Inhibition of CiC or ACLY has no effect on NK cell activation. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- BTA or BMS303141 overnight. Expression levels of CD69 of NK1.1+ NKp46+ CD3- NK cells was determined using the Flow cytometry. Data shown is mean +/- S.E.M of N=4 (a) and representative histograms (b).

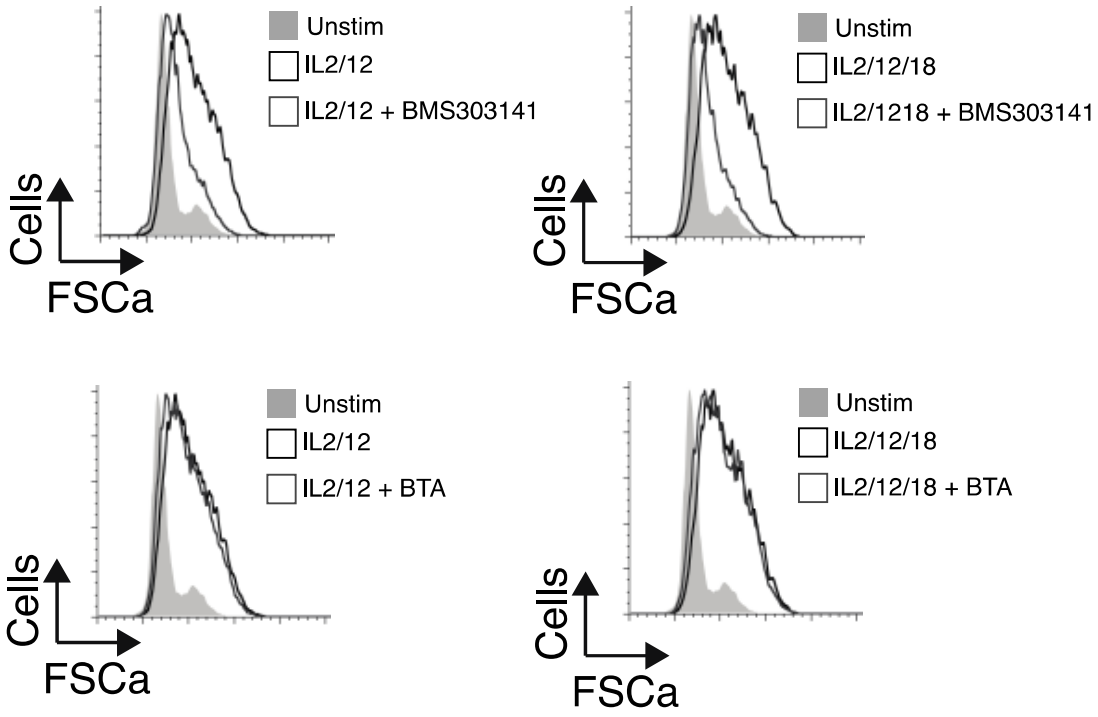

Figure 4.27. Inhibiton of ATP citrate lyase but not the mitochondrial citrate carrier decreases cytokine induced NK cell blastogenesis. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- BMS303141 or BTA overnight. NK cell size based on forward light scatter of NK1.1+ NKp46+ CD3- NK cells was determined using flow cytometry. Data shown is representative of N=4.

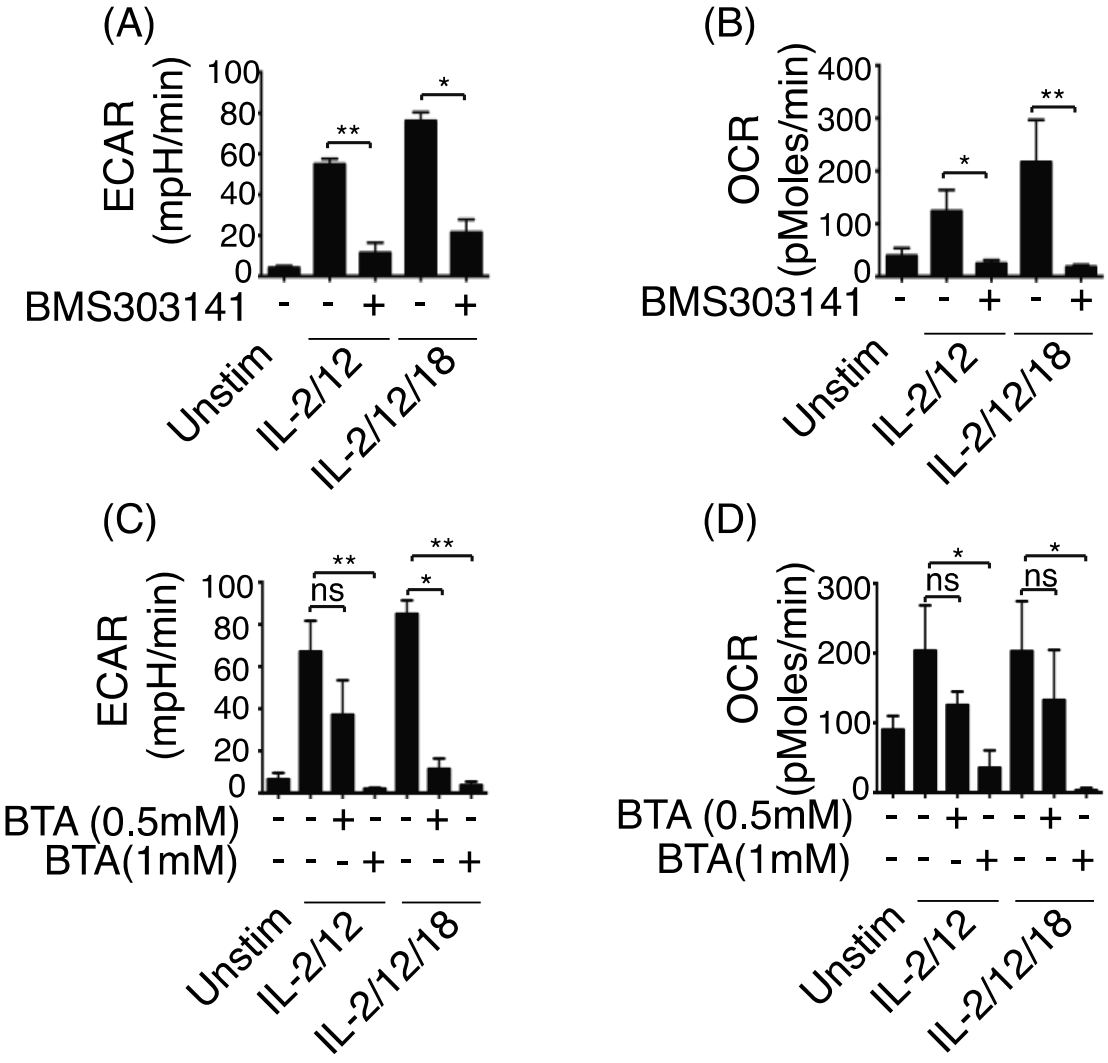

Figure 4.28. The citrate-malate shuttle is required to maintain glycolysis and OxPhos in cytokine stimulated NK cells. (A-D) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- BMS303141 or BTA overnight. Stimulated NK cells underwent detailed metabolic analysis using the Seahorse metabolic Flux analyzer which determined the glycolytic (a,c) and Oxidative Phosphorylation (b,d) levels of the cell. Data shown is mean +/- S.E.M of N=2 (ns=not significant, * $p<0.05$, ** $p<0.01$).

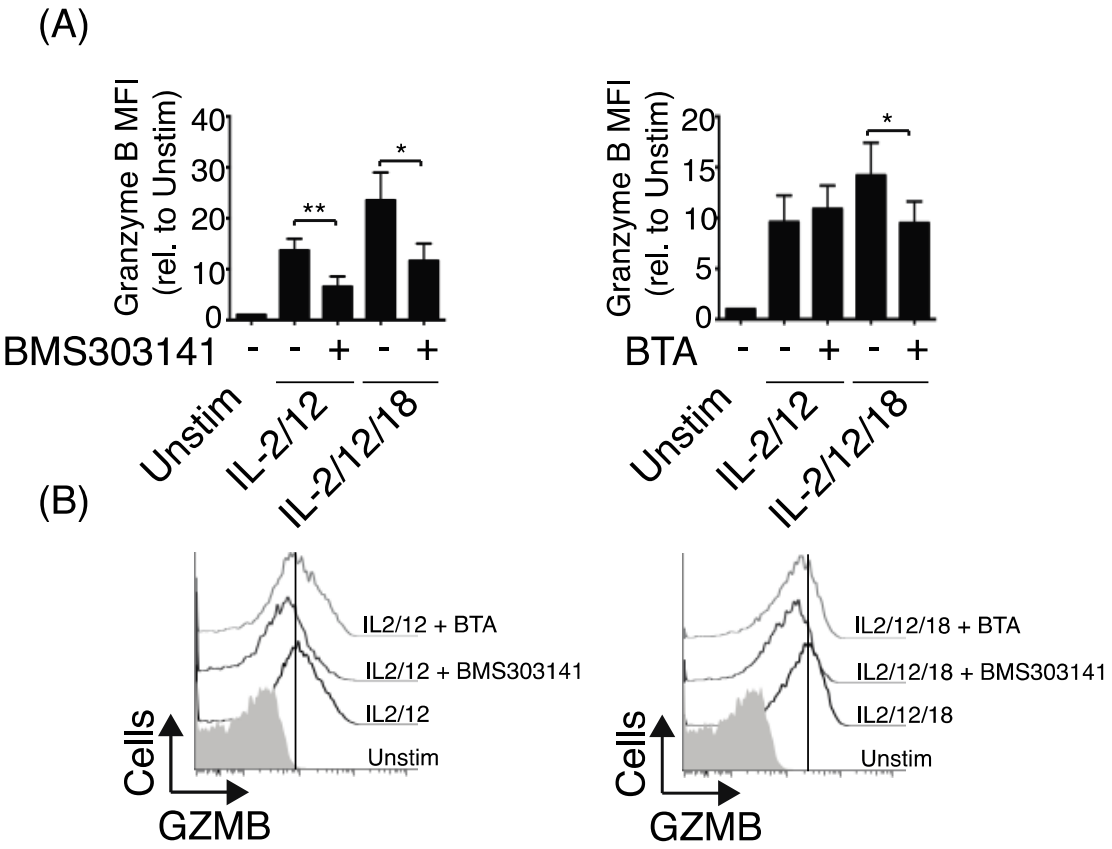

Figure 4.29. Inhibition of ACLY and CiC affects granzyme B expression in cytokine stimulated NK cells (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- BMS303141 or BTA. Granzyme B being expression was determined by the MFI of NK1.1+ NKp46+ CD3- NK cells using flow cytometry. Data shown is mean +/- S.E.M of N=4 (a) and representative histograms (b) (* $p<0.05$, ** $p<0.01$).

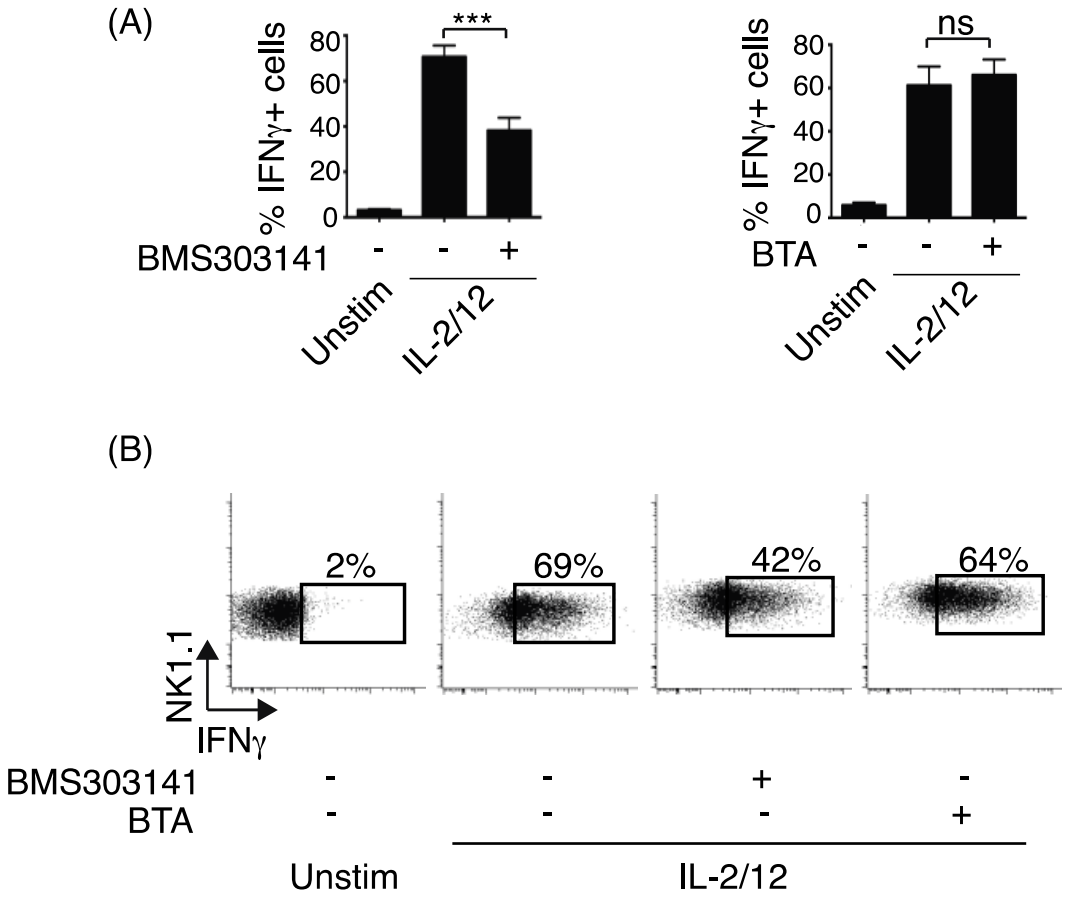

Figure 4.30. Inhibition of ACLY but not CiC decreases IFNγ expression in IL-2/12 stimulated NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- BMS303141 or BTA overnight. The percentage of IFNγ+ NK1.1+ NKp46+ CD3- NK cells was determined based on unstim control NK cells using flow cytometry. Data shown is mean +/- S.E.M of N=5 (a) and representative dot plots (b) (*** $p<0.001$).

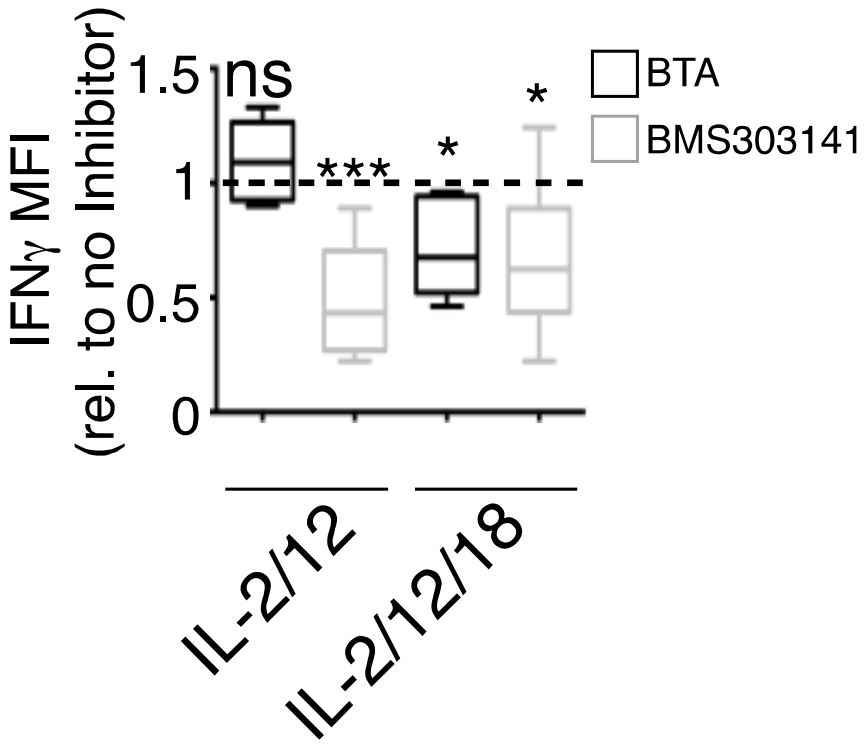

Figure 4.31. Inhibition of ACLY or CiC decreases IFNγ production in cytokine stimulated NK cells. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- BMS or BTA overnight. IFNγ expression was determined by MFI of IFNγ+ NK1.1+ NKp46+ CD3- NK cells using flow cytometry. Data shown is mean +/- S.E.M of N=5 (* $p<0.05$, *** $p<0.001$).

4.8. De novo NAD+ synthesis is required for cytokine induced NK cell maintaining energy metabolism and effector functions

The data argues that SREBP dependent upregulation of the citrate-malate shuttle is required for elevated glycolysis in cytokine activated NK cells. This is likely due to a role for the citrate malate shuttle in sustaining cytosolic NAD^+ through oxidizing NADH. Given that NAD^+ can also be used as a substrate for enzymes such as the sirtuins, it was hypothesized that *de novo* NAD^+ synthesis would also be important for elevated glycolysis in cytokine activated NK cells. Nictotinamide phosphoribosyltransferase (NAMPT) is an enzyme involved in the *de novo* generation of NAD^+. To investigate whether disrupting *de novo* NAD^+ synthesis reduces glycolysis in cytokine stimulated NK cells we targeted NAMPT with the inhibitor FK866. FK866 treatment blocked increased rates of glycolysis in IL-2/12 and IL-2/12/18 stimulated NK cells (Fig 4.32a-b). Moreover, cytokine stimulated NK cells treated with FK866 exhibited a slight reduction in cellular growth (Fig 4.33). Inhibition of NAMPT substantially reduced IFNγ production (Fig 3.34-35) in cytokine stimulated NK cells and also resulted in reduced granzyme B expression (Fig 4.36). Together, this data shows that NAD^+ is critical in maintaining glycolysis in cytokine stimulated NK cells and argues that SREBP activity is required for elevated glycolysis as it sustains cytosolic NAD^+ through the citrate-malate shuttle.

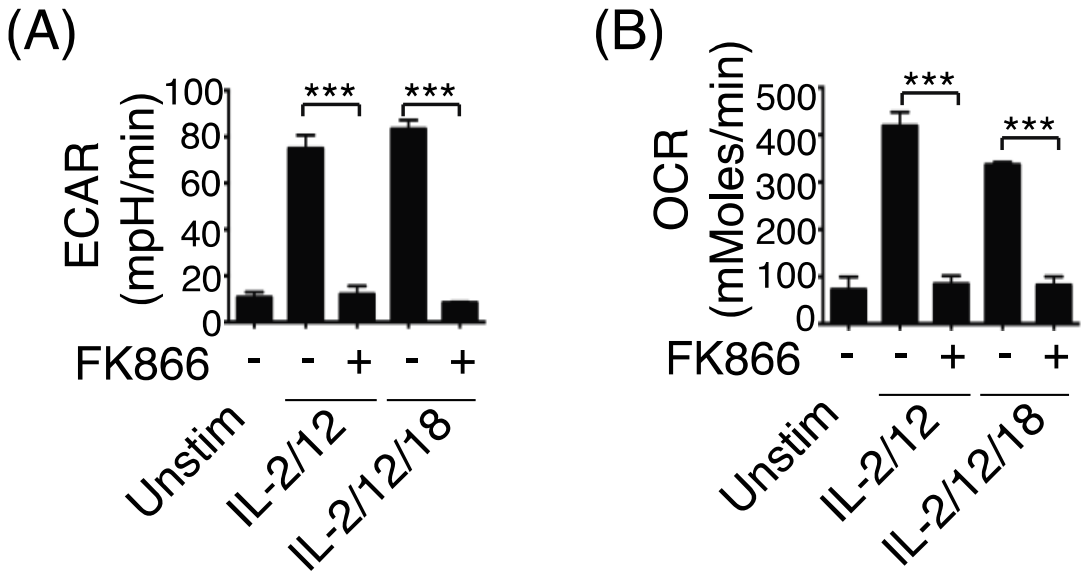

Figure 4.32. De novo NAD^+ is required to maintain glycolysis and OxPhos in cytokine stimulated NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- FK866 overnight. Stimulated NK cells underwent detailed metabolic analysis using the Seahorse metabolic Flux analyzer, which determined the glycolytic (a) and Oxidative Phosphorylation (b) levels of the cell. Data shown is mean +/- S.E.M of N=3 (*** $p<0.001$).

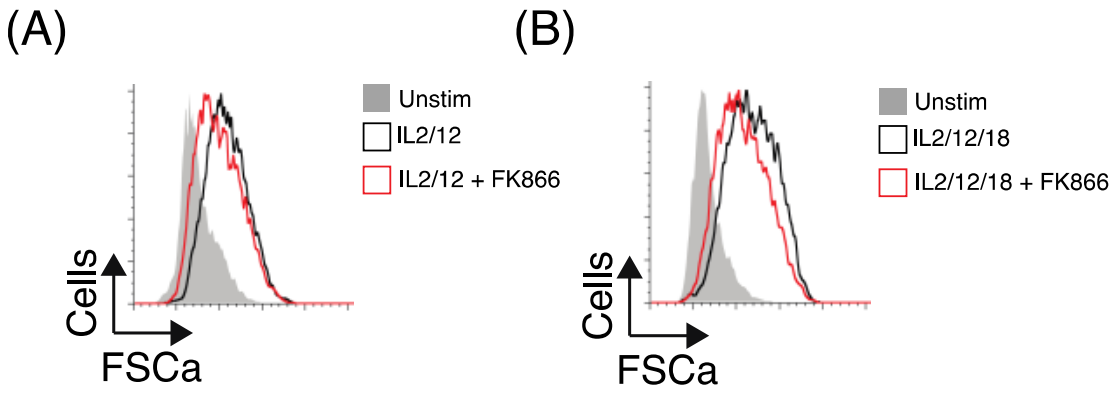

Figure 4.33. De novo NAD⁺ is required for maximal NK cell blastogenesis upon IL-2/12 or IL-2/12/18 cytokine stimulation. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 (a) or IL-2/12/18 (b) +/- FK866 overnight. NK cell size of NK1.1+ NKp46+ CD3- NK cells was determined based on forward light scatter using flow cytometry. Data shown is representative of N=4.

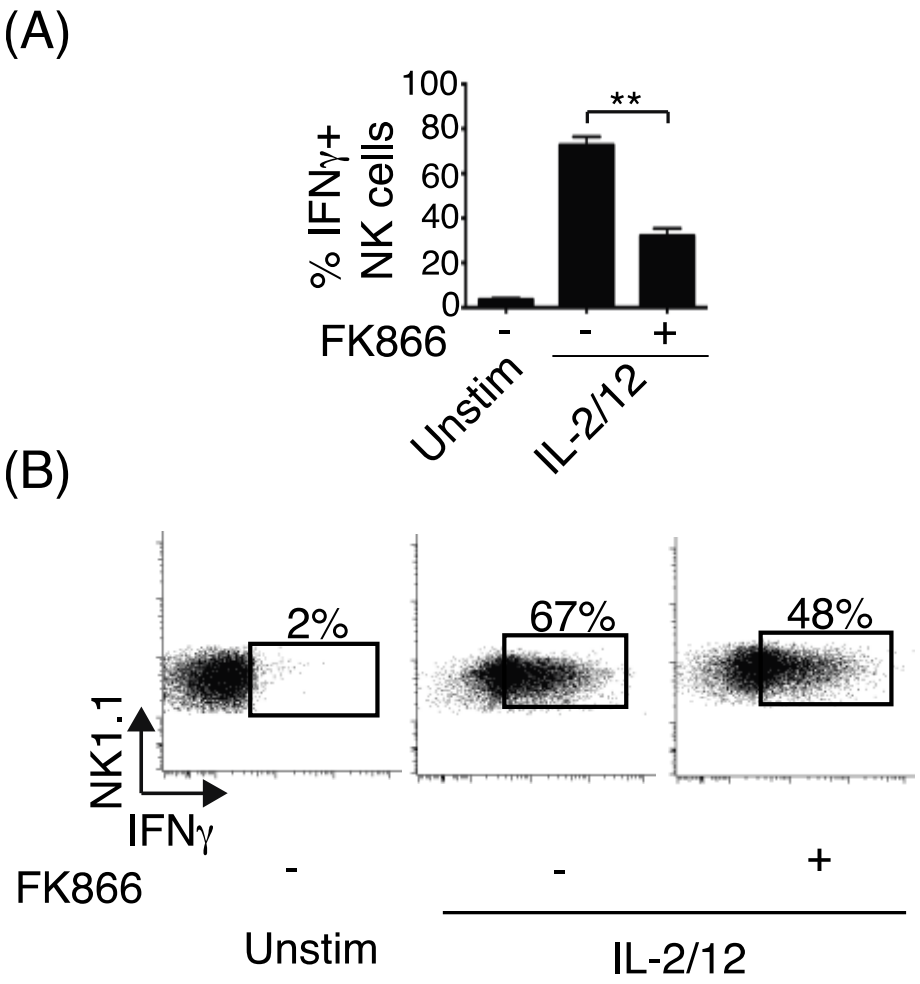

Figure 4.34. De novo NAD⁺ is required for IFNγ expression in IL-2/12 stimulated NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- FK866 overnight. Stimulated NK The percentage of IFNγ⁺ NK1.1+ NKp46+ CD3- NK cells was determined based on unstimulated control NK cells using flow cytometry. Data shown is mean +/- S.E.M of N=4 (b) and representative dot plots (a) (** $p<0.01$).

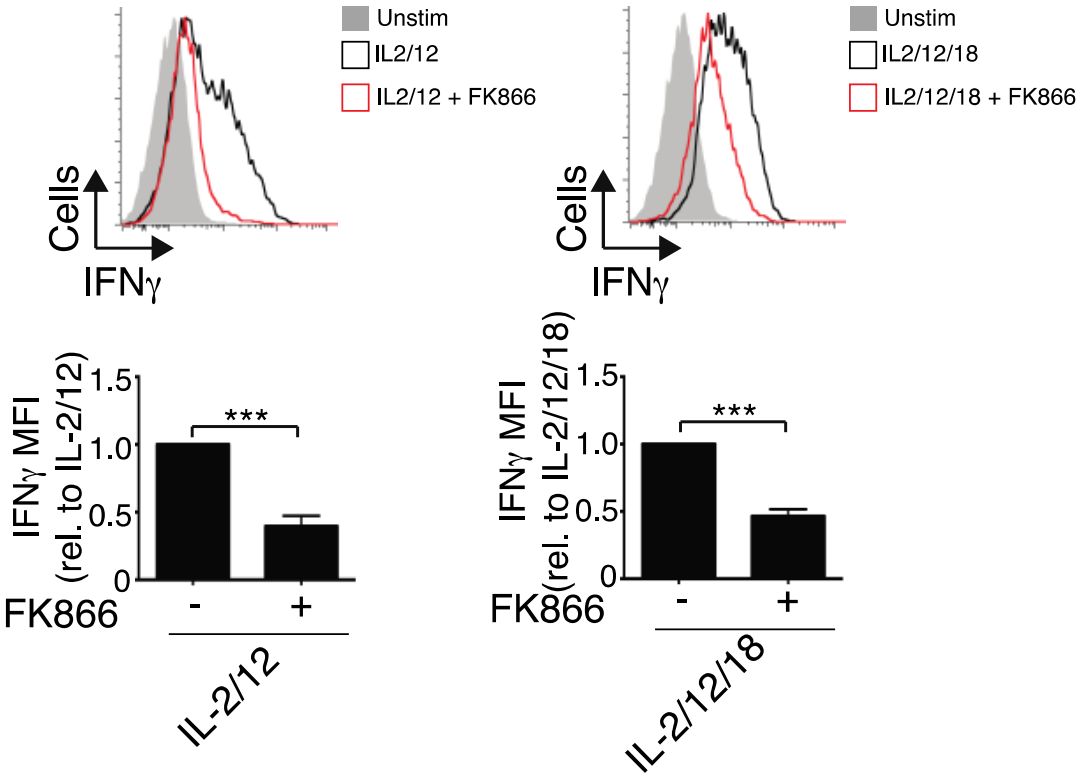

Figure 4.35. De novo NAD⁺ is required for IFNγ production in cytokine stimulated NK cells. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- FK866 overnight. The amount of IFNγ being produced was determined by MFI of IFNγ+ NK1.1+ NKp46+ CD3- NK cells using flow cytometry. Data shown is mean +/- S.E.M of N=4 (*** p<0.001).

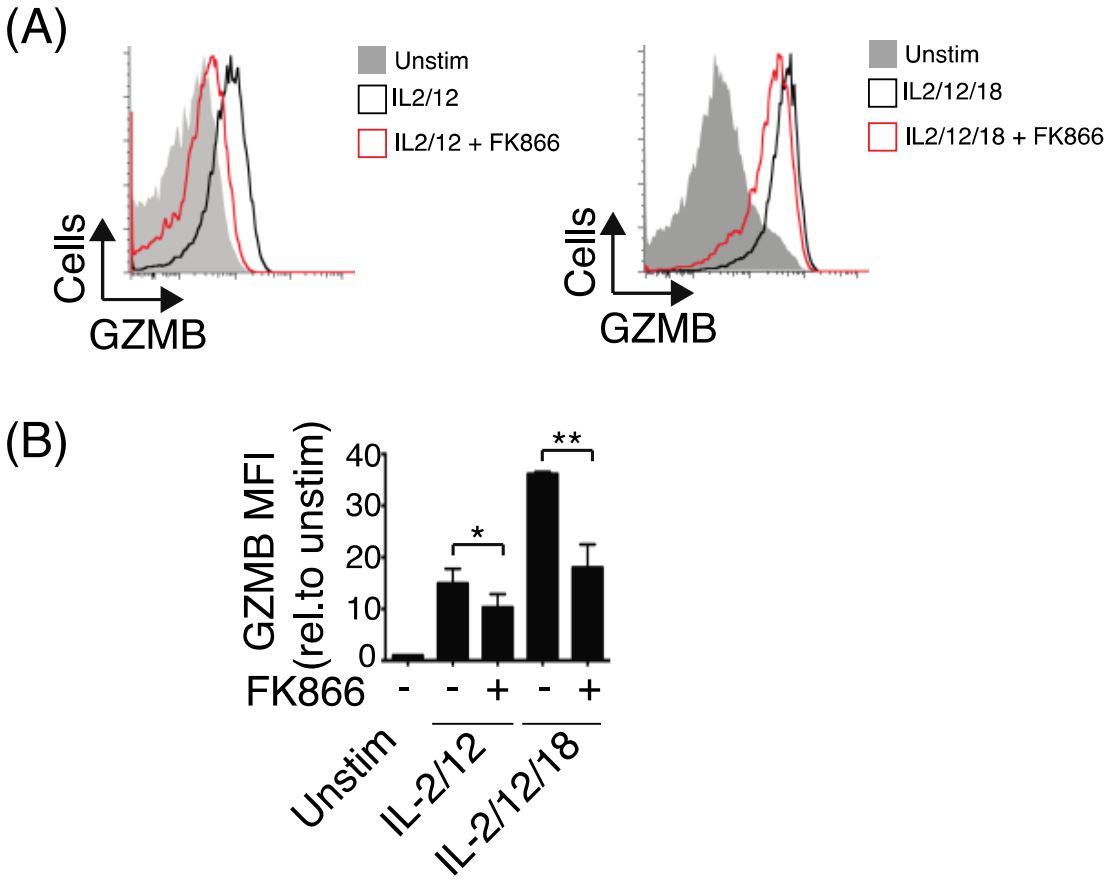

Figure 4.36. De novo NAD⁺ is required for Granzyme B production in cytokine stimulated NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 +/- FK866 overnight. The expression of Granzyme B was determined by MFI of NK1.1+ NKp46+ CD3- NK cells using flow cytometry. Data shown is mean +/- S.E.M of N=4 (b) and representative histograms (a) (* $p<0.05$, ** $p<0.01$).

4.9. Discussion

Glycolysis provides the biosynthetic precursors that are essential for the synthesis of proteins, nucleic acids and lipids (Hume et al., 1978). Pro-inflammatory immune cells have the ability to engage in robust cellular growth and rapid proliferation. Therefore, it is not surprising that multiple studies have shown T cells engaging in *de novo* lipid synthesis upon activation (Berod et al., 2014). Many of the enzymes involved in *de novo* lipid synthesis are under control of SREBP in other cell types. Furthermore, SREBP activity is required for *de novo* lipid synthesis in T cells (Bensinger et al., 2008). In order to test our hypothesis that SREBP is required for lipid synthesis in cytokine stimulated NK cells, we disrupted SREBP activity using various complimentary approaches. Multiple studies have suggested that SREBP1 and SREBP2 can compensate for each other when one protein is deleted (Shimano et al., 1996, Shimano et al., 1997). Therefore, we developed strategies to inhibit all SREBP isoforms. Firstly, we used the oxysterol 25-HC in order to keep the SREBP-SCAP complex bound to the ER-membrane resident protein INSIG. Secondly, we used a pharmacological inhibitor of S1P, PF429242, to prevent cleavage and activation of SREBP. As an alternative approach to pharmacologically inhibiting SREBP activation, we developed a genetically altered mouse model to decrease SREBP activity. The protein SCAP is essential for SREBP processing and activation. We crossed a mouse containing loxP sites that flanked the first exon of the SCAP gene (Matsuda et al., 2001) with a mouse expressing a tamoxifen-inducible CRE recombinase. The deletion of SCAP is induced in NK cells from these mice by the addition of 4-OH tamoxifen to the cultures. In support of our hypothesis, we showed that decreased SREBP activity resulted in decreased lipid synthesis, as a result of reduced expression of key lipid synthesis genes; FASN, HMGCoS, SCD1 and ACAT2.

IL-15 is an essential cytokine for NK cell homeostasis and proliferation, both *in vitro* and *in vivo* (Zhao and French, 2012). SREBP activity and lipid

synthesis has been shown to be important for proliferation of effector T cells (Bensinger et al., 2008). The data described herein show that NK cells also depend on SREBP activity for homeostatic proliferation in response to IL-15. Our data also shows that NK cells required SREBP activity for cellular growth following cytokine stimulation. This is a phenotype that is not unlike that observed in $CD8^+$ T cells (Bensinger et al., 2008). The requirement for SREBP in controlling NK cell proliferation and cellular growth is most likely due to SREBP-regulated lipid synthesis as TOFA and C75 treatment similarly resulted in decreased cellular growth upon cytokine stimulation. Indeed, inhibition of fatty acid synthesis was shown to decrease $CD8^+$ T cell proliferation, blastogenesis and cell survival (Lee et al., 2014). In addition, C75 treatment reduced CTL proliferation (O'Sullivan et al., 2014). Therefore, the requirement for SREBP activity for cell growth and proliferation was in line with what has been shown in T cells.

A key finding from this chapter was that SREBP activity is required to maintain NK cell effector molecules. SREBP has been reported to control discrete effector molecules of certain immune cells, such as IL-17 production by T_H17 cells (Cui et al., 2011). While glucose-driven *de novo* lipid synthesis is required for determining the fate of T_H17 cells (Berod et al., 2014) our data shows that lipid synthesis had no identified impact on NK cell function. In support of our previous findings that elevated glycolysis is required for NK cell effector molecules our data showed that inhibiting SREBP activity resulted in decreased glucose metabolism. In $CD8^+$ T cells SREBP activity is also required for elevated glycolysis (Kidani et al., 2013). Additionally, cytokine stimulated NK cells were unable to increase OxPhos in the absence of SREBP activity. Decreased SREBP activity also resulted in decreased OxPhos in $CD8^+$ T cells (Kidani et al., 2013). Direct inhibition of lipid synthesis had no effect on NK cell glycolysis or OxPhos in IL-2/12 or IL-2/12/18 stimulated NK cells. Therefore, SREBP is required for elevated glycolysis, OxPhos and effector molecules in NK cells independently of its effects on rates of lipid synthesis. Interestingly, SREBP activity isn't required

for metabolic reprogramming in cytokine stimulated NK cells, as there was no decrease in expression of glycolytic enzymes or glucose transporter with decreased SREBP activity. While Kidani *et al* state that SREBP is required for metabolic reprogramming of $CD8^+$ T cells, they never actually commented on the expression levels of the glycolytic machinery. Strikingly, when we inhibited OxPhos with the ATP synthase inhibitor, oligomycin, cytokine stimulated NK cells were unable to induce a compensatory increase in glycolysis to maintain ATP. Therefore, SREBP inhibited NK cells are already operating at their maximal glycolytic rate.

Given that SREBP controlled lipid synthesis is not required for NK cell glucose metabolism and NK cell effector function, another key finding from this study was that SREBP regulated citrate-malate shuttle is required to maintain glycolysis. One of the outputs of the citrate-malate shuttle is to oxidize cytosolic NAD^+, which is required for the activity of the glycolytic enzyme GAPDH. There is evidence in the literature that the citrate malate shuttle is important for the function of other immune cells. Inhibition of ACLY, a key component of the citrate-malate shuttle, in LPS stimulated B cells resulted in decreased antibody production (Dufort et al., 2014). Dufort and colleagues attributed this decrease in function of B cells down to impaired glucose-driven *de novo* lipid synthesis. However, it is interesting to note that LPS stimulated B cells treated with 2DG, the glycolytic inhibitor, had similar defects in function (Caro-Maldonado et al., 2014). In support of our hypothesis that the citrate-malate shuttle maintains glycolysis a key finding was inhibition of ACLY or CiC with BMS303141 and BTA, respectively, resulted in decreased glycolysis in cytokine stimulated NK cells. Similar, to inhibition of SREBP, decreased ACLY and CiC activity resulted in decreased OxPhos also. ACLY was also required for IFNγ and granzyme B production in cytokine stimulated NK cells. However, inhibition of CiC only resulted in a subtle decrease in function in NK cells when stimulated with IL-2/12/18. There are several possible reasons for the discrepancy in the effect of this

inhibitor on metabolism versus NK cell function. There is no information available in the literature on the stability of BTA, the inhibitor of CiC. For metabolic analysis the BTA was readded to the cells, in assay medium, a couple hours before glycolysis and OxPhos were measured. This was not the case for analysis of IFNγ and granzyme B expression carried out by flow cytometry. Therefore, BTA is potentially unstable when used for a long period of time. Future experiments involving the readdition of BTA to the cultures cells are required to fully elucidate the discrepancy.

SREBP activity is also required to sustain mitochondrial OxPhos in cytokine stimulated NK cells. While we have not determined the mechanism behind SREBP regulated OxPhos there are multiple potential factors controlling this observed phenotype. The potential ways by which SREBP activity regulates OxPhos are:

1). Our data shows that inhibition of SREBP induced citrate-malate shuttle results in decreased glycolysis leading to a decrease in pyruvate. As described in section 1.2.1.1, glycolysis and OxPhos are interconnected through pyruvate being metabolized to Acetyl-CoA which fuels the Krebs cycle. Therefore decreased pyruvate might result in a reduction in the levels of mitochondrial NADH. Indeed, inhibition of the citrate-malate shuttle would also directly result in a decrease of mitochondrial NADH. The malate that returns to the mitochondria is oxidized to oxaloacetate generating 1 molecule of NADH. However, to date the proportion of glucose-driven pyruvate entering the mitochondria is undetermined. Indeed, glutamine can also provide the fuel for rapidly dividing cells, such as lymphocytes (Nakaya et al., 2014). Lymphocytes consume glutamine at rates comparable to, or even higher than, glucose consumption (Ardawi and Newsholme, 1983, Ardawi, 1988) and mitogen-induced T cell proliferation in culture require high levels of glutamine (Yaqoob and Calder, 1997). Current metabolomic studies underway in the laboratory are trying to identify the proportion of glucose-

derived pyruvate that enters the mitochondria to fuel the Krebs cycle for ATP production.

2). *De novo* lipid synthesis is required for formation of cellular membranes. Studies have shown that upon activation lymphocytes increase mitochondrial mass and number (Walker et al., 2014). Decreased mitochondrial number may explain the observed decrease in OxPhos. However, it should be noted that SREBP activity did not regulate mitochondrial mass or explain the decrease in OxPhos in $CD8^+$ T cells (Kidani et al., 2013). Alternatively, O'Sullivan and colleagues showed that memory $CD8^+$ T cells engage in fatty acid synthesis solely to provide a source of energy via β-oxidation (O'Sullivan et al., 2014). However, we observed no decrease in OxPhos when we directly inhibited lipid synthesis with C75 and TOFA. Therefore, SREBP controlled OxPhos must be independent of lipid synthesis.

3). SREBP has also recently been shown to positively regulate the expression of multiple Krebs cycle enzymes; Aconitase, Isocitrate dehydrogenase, succinate-CoA ligase, succinate dehydrogenase complex subunit B and malate dehydrogenase (Reed et al., 2008). Inhibition of these enzymes would result in a reduction in the pool of mitochondrial NADH and an inhibition of OxPhos. In addition, SREBP can positively regulate the expression of Cytochrome C, a component of the ETC (Reed et al., 2008). Whether or not SREBP regulates these enzymes in cytokine stimulated NK cells will require some future experiments.

The importance of OxPhos in determining cytokine stimulated murine NK cell function has not been explored yet. However, a recent study has shown that IFNγ production via activation of NK cell receptors required glucose-driven OxPhos (Keppel et al., 2015). Moreover, recent findings in our lab have shown that OxPhos is required for effector molecules in human NK cells

(unpublished data). In summary, there are numerous ways by which SREBP can potentially regulate the TCA cycle and the ETC in cytokine stimulated NK cells. However, more detailed analysis is needed to fully elucidate the mechanism by which SREBP activity is required for efficient OxPhos in cytokine stimulated NK cells.

Apart from its role as a cofactor for various enzymes, NAD^+ is also a substrate for other cellular processes. For example, sirtuins require NAD^+ to carry out their role of removing acetyl groups from proteins. If cells are using NAD^+ as a substrate for such reactions they then need to replenish their NAD^+/NADH pool either via the NAD salvage pathway or *de novo* NAD^+ synthesis. The NAD^+ salvage pathway regenerates NAD^+ from nicotinamide. Nicotinamide phosphoribosyltransferase (NAMPT) is one of the key enzymes involved in the NAD^+ salvage pathway (Hasmann and Schemainda, 2003). Interestingly, in preclinical studies targeting tumour cells, inhibition of NAMPT with FK866 revealed that lymphocytes are the most effected non tumour cell, with long term treatment resulting in lymphopenia (Holen et al., 2008). Rongavaux and colleagues have shown in mice models that expression of NAMPT is required for B and T cell development (Rongvaux et al., 2008). Moreover, recent studies have suggested that NAMPT might be involved in T cell activation, as they dramatically upregulate its expression upon mitogenic stimulation (Berger et al., 1987, Rongvaux et al., 2002). We hypothesized that inhibition of NAMPT with FK866 would decrease cellular NAD^+ levels, resulting in decreased glucose metabolism and NK cell function. In support of our hypothesis, FK866 inhibition of NAMPT dramatically reduced rates of glycolysis in cytokine stimulated NK cells, which resulted in attenuated IFNγ and granzyme B production. It is interesting that inhibition of NAMPT with FK866 similarly completely abrogated IFNγ production in T cells (Bruzzone et al., 2009). Moreover, NAMPT inhibition and NAD^+ shortage have also been shown to decrease IL-1β, IL-6 and TNFα in macrophages and DCs (Busso et al., 2008, Van Gool et al., 2009).

During this study we have inhibited multiple pathways that are required to maintain cytosolic NAD^+ levels. Firstly, by inhibiting the citrate-malate shuttle and secondly by inhibiting NAMPT and the NAD^+ salvage pathway. Our data shows that inhibition of these pathways results in impaired NK cell effector molecules. This could be as a subsequent impact on elevated glycolysis; however, recent studies have suggested that NAD^+ itself may play a more direct role in regulating proinflammatory cytokine production, independent of glycolysis. The observed impact of FK866 on NK cell glycolysis and function suggests that NK cells are consuming NAD^+ and need to engage the NAD^+ salvage pathway to sustain the NAD^+/NADH pool and elevated glycolysis. As previously mentioned NAD^+ is a substrate for sirtuins. Van Gool and colleagues attributed a decrease in the proinflammatory cytokine TNFα production to reduced function of the NAD^+-dependent sirtuin Sirt6 (Van Gool et al., 2009). Sirt6-deficient mice develop lymphopenia as a result of a cell non-autonomous mechanism, thus suggesting a decrease in cytokines required to support T cell function (Mostoslavsky et al., 2006). Moreover, inhibition of Sirt6, which is upregulated upon activation, inhibited IFNγ expression in T cells (Bruzzone et al., 2009). Sirt6 is a stress induced protein deacetylase and mono-ADP ribosyltransferase enzyme. It is able to function in numerous cellular pathways, DNA repair, telomere maintenance, glycolysis and inflammation (Frye, 2000). The sirtuin family consists of seven members, which were originally identified as histone deacetylases, regulating gene expression through chromatin modifications. Moreover, sirtuins play an important role in maintaining cellular homeostasis by regulating energy status and stress resistance (Finkel et al., 2009). Accordingly, their activity is regulated by various stress conditions, such as hypoxia (Dioum et al., 2009, Lim et al., 2010, Bell et al., 2011) and nutrient availability (Rodgers et al., 2005).

The exact mechanism by which sirtuins regulate immune cell function hasn't been fully elucidated. However, studies have shown that Sirt6 can restructure the chromatin at the NFκβ promoter, allowing for enhanced transcription (Schug et al., 2010). Interestingly all cytokines involved in the differentiation of T_H1 and T_H2 cells are regulated by histone deacetylases (HDACs). One of the best examples of HDAC involvement in T cell differentiation is the epigenetic regulation of the IFNγ locus. Most of the changes in DNA methylation and acetylation of the IFNγ locus happen within a conserved non-coding sequence ~50Kb upstream and downstream of the IFNγ gene (Zhou et al., 2004). Similar to T cells, an array of histone acetylation domains exist in the promoter region of the IFNγ in NK cells (Chang and Aune, 2005).

In conclusion, herein we have shown that SREBP activity is essential for elevated levels of glycolysis in cytokine activated NK cells through maintaining the citrate-malate shuttle needed for sustaining cytosolic NAD^+.

Chapter 5-The role of mTORC1 and SREBP signalling in maintaining glycolysis in NK cells is dependent on the stimulus received

5.1. Introduction

The data in chapter 4 demonstrates that SREBP activity is important for elevated glycolysis and optimal effector molecules in cytokine stimulated NK cells. SREBP promotes the expression of key genes that are involved in the citrate-malate shuttle, which is important for sustaining a pool of cytosolic NAD^+ required for maintaining glycolytic flux. However, the molecular mechanisms controlling SREBP activity have not been characterized. mTORC1 has been shown to control SREBP activity in a variety of cell types. Indeed, recent studies have shown that TCR stimulated *de novo* lipid synthesis requires mTORC1 signalling and SREBP activity (Yang et al., 2013). Inhibition of mTORC1 activity results in decreased SREBP1 and SREBP2 activity in T cells (Yang et al., 2013, Kidani et al., 2013). Given that mTORC1 regulates SREBP in T lymphocytes, we hypothesized that mTORC1 would similarly control SREBP activity in cytokine stimulated NK cells.

5.2. mTORC1 controls SREBP activity in IL-2/12 stimulated NK cells but not IL-2/12/18 stimulated NK cells.

Experiments were designed to investigate the role for mTORC1 in regulating SREBP activity and lipid synthesis in cytokine stimulated NK cells. Rates of lipid synthesis were measured in cytokine activated NK cells in the presence and absence of rapamycin as a readout of SREBP activity. Rapamycin decreases the amount of radiolabelled acetate incorporated into cellular lipid in IL-2/12 stimulated NK cells (Fig 5.1). This initial data argues that IL-2/12 stimulated SREBP activity requires mTORC1 signalling. Therefore, the expression of SREBP target genes was analyzed. Inhibition of mTORC1 resulted in decreased expression of SREBP target genes; FASN, HMGCoS, CiC and ACLY (Fig 5.2). Together this data demonstrates that mTORC1 activity is required for IL-2/12 SREBP induced activity in NK cells. Experiments also investigated whether mTORC1 was required for IL-2/12/18 stimulated SREBP activity. Surprisingly, inhibition of mTORC1 activity with rapamycin did not significantly decrease lipid synthesis in IL-2/12/18 stimulated NK cells (Fig 5.3). Moreover, inhibition of mTORC1 did not decrease the expression of SREBP target genes (Fig 5.4). This data argues that IL-18 drives mTORC1 independent mechanisms to promote SREBP activity.

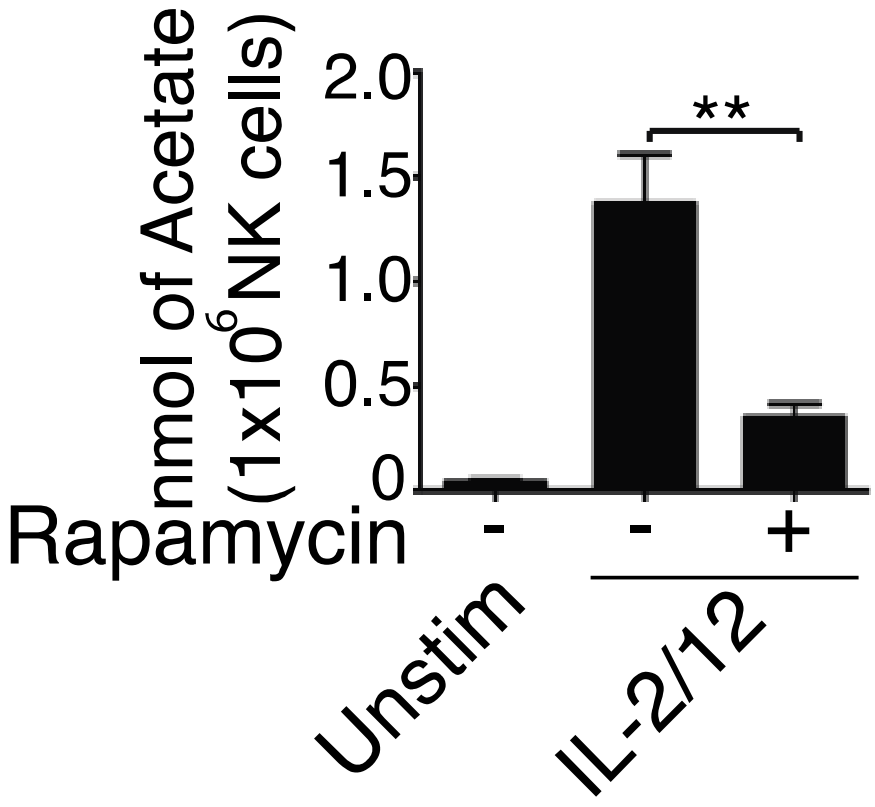

Figure 5.1. mTORC1 is required for IL-2/12 stimulated lipid synthesis Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- rapamycin overnight. The rate of lipid synthesis was investigated by measuring the incorporation of C^{14} labeled acetate into cellular lipid. Data is mean +/- S.E.M of N=3. (** $p<0.01$).

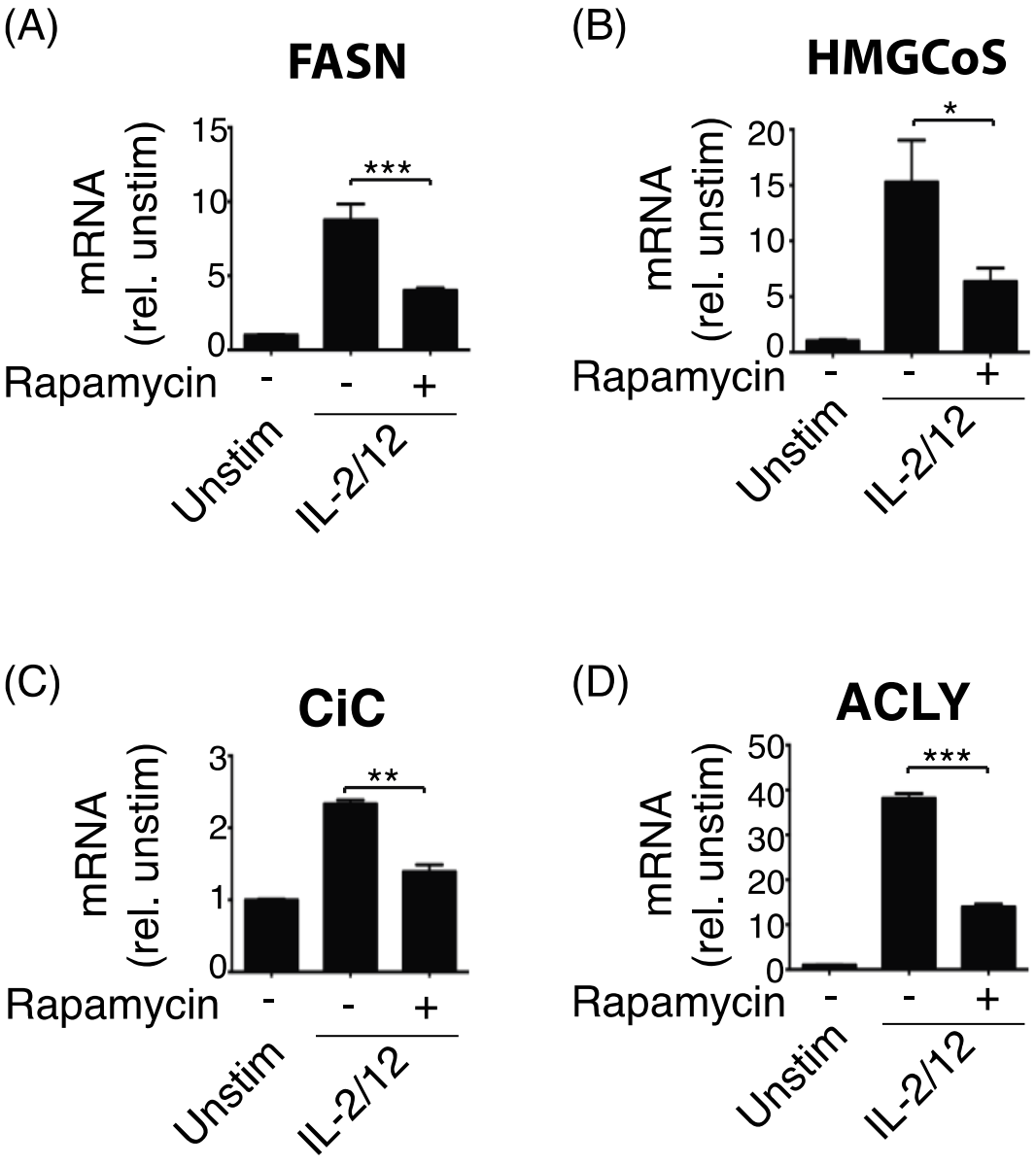

Figure 5.2. mTORC1 is required for SREBP activity in NK cells stimulated with IL-2/12. (A-D) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 +/- rapamycin overnight. RT-qPCR analysis of expression of FASN, HMGcoS1, CiC and ACLY were determined. Data is mean +/- S.E.M of N=3. (* $p<0.05$, ** $p<0.01$, ***$p<0.001$).

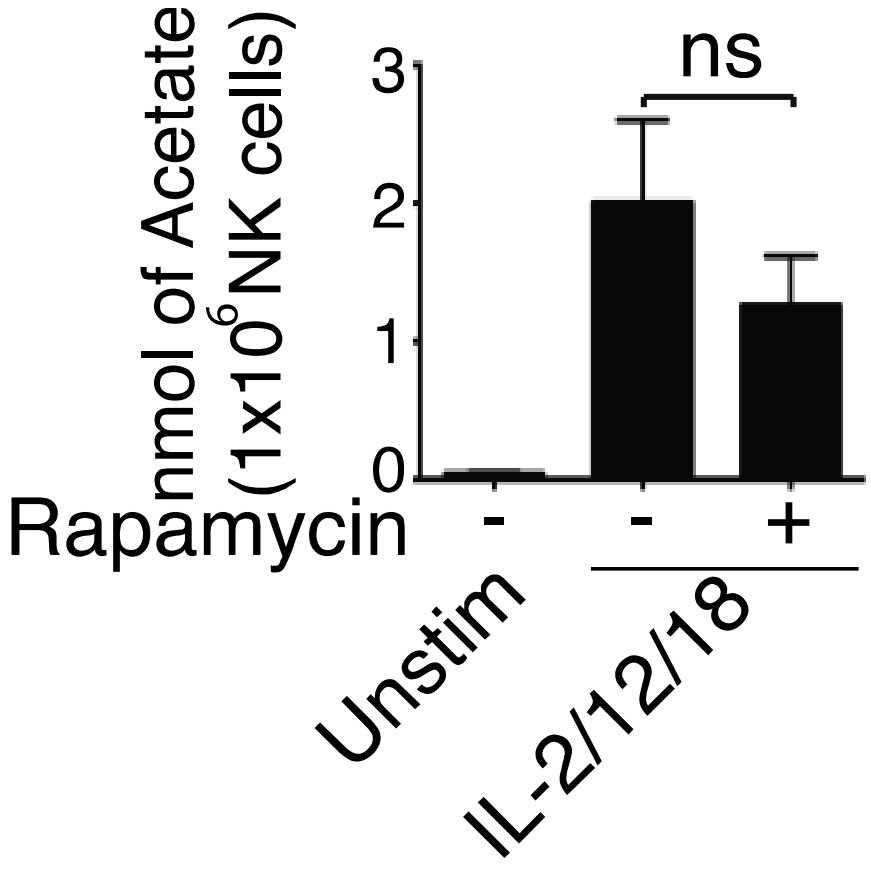

Figure 5.3. mTORC1 is not required for lipid synthesis in NK cells stimulated with IL-2/12/18. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12/18 +/- rapamycin overnight. The rate of lipid synthesis was investigated by measuring the incorporation of C^{14} labeled acetate into cellular lipid. Data is mean +/- S.E.M of N=3. (ns=not significant).

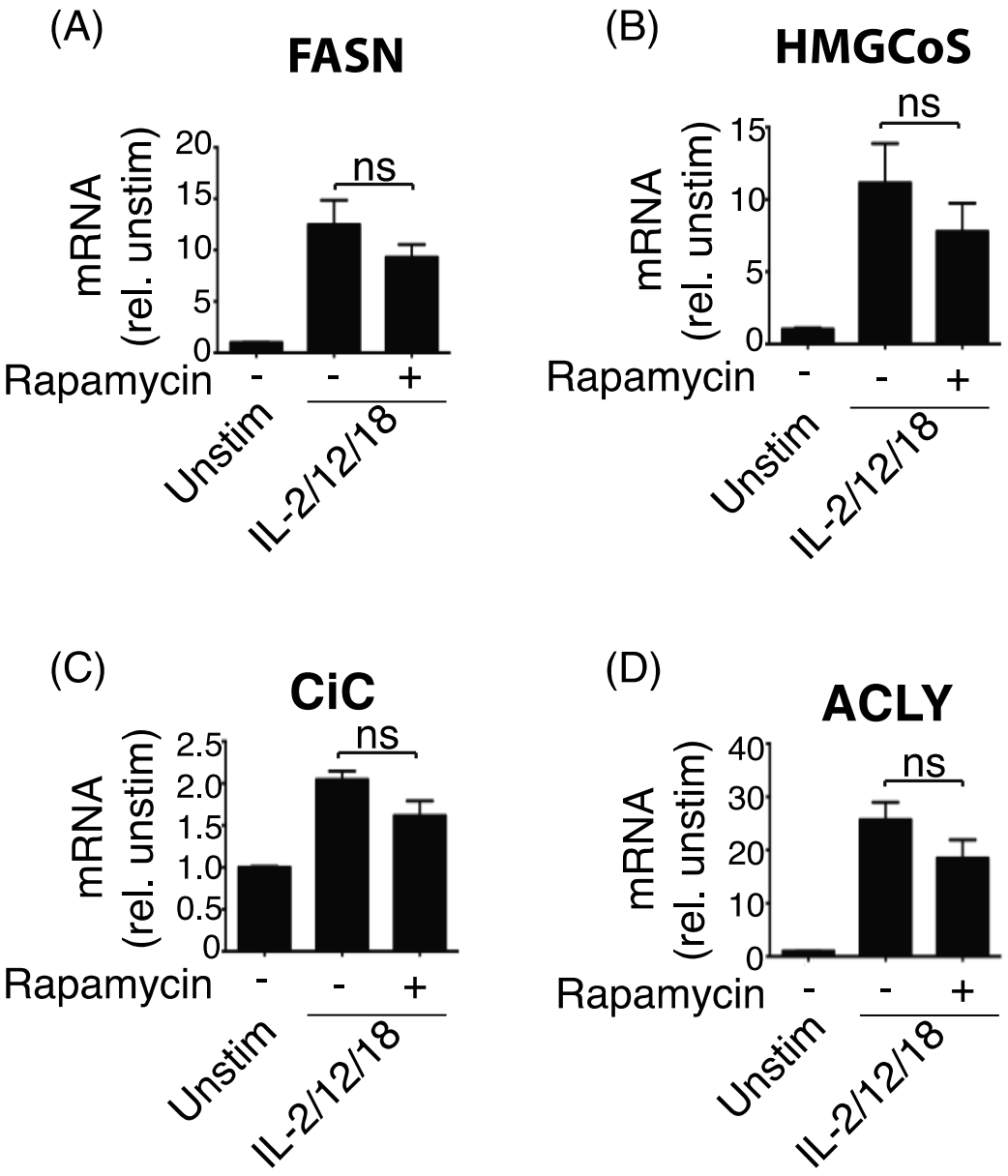

Figure 5.4. mTORC1 is required for SREBP activity in NK cells stimulated with IL-2/12. (A-D) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12/18 +/- rapamycin overnight. RT-qPCR analysis of expression of FASN, HMGcoS1, CiC and ACLY were determined.. Data is mean +/- S.E.M of N=3. (ns=not significant).

5.3. Metabolic reprogramming in NK cells stimulated with IL-2/12/18 is independent of mTORC1 activity.

The data shows that IL-2/12/18 stimulation promotes mTORC1 independent SREBP activity and lipid synthesis which raised the question whether other aspects of IL-2/12/18 stimulated NK cell metabolism require mTORC1 activity. It was previously shown that mTORC1 is highly active in NK cells stimulated with IL-2/12 (Fig 3.19). mTORC1 is similarly active in NK cells stimulated with IL-2/12/18 as seen by the levels of phosphorylated S6 ribosomal protein when compared to the rapamycin treated negative control (Fig 5.5). The data shows that addition of IL-18 to IL-2/12 stimulated NK cells enhances NK cell blastogenesis and further increases lipid synthesis (Fig 4.1). IL-2/12/18 stimulates increases in glycolysis and OxPhos that are comparable to those measured in response to IL-2/12 (Fig 5.6). This increase similarly involves glycolytic reprogramming and increased expression of glucose transporters and glycolytic enzymes (Fig 5.7). While the expression of glycolytic enzymes was comparable in IL-2/12 and IL-2/12/18 stimulated NK cells IL-18 did induce an increase in Glut1 expression (Fig 5.7).

The data showed mTORC1 is critical to maintain SREBP activity and lipid synthesis in IL-2/12 stimulated NK cells but not in IL-2/12/18 stimulated NK cells. Given that we have demonstrated that SREBP activity is required for NK cell glycolysis, is glycolysis maintained in IL-2/12/18 stimulated NK cells in the absence of mTORC1 activity. Strikingly, inhibition of mTORC1 in NK cells stimulated with IL-2/12/18 has no effect on aerobic glycolysis (Fig 5.8a). mTORC1 activity is not required for OxPhos in IL-2/12/18 stimulated NK cells; as was observed in IL-2/12 stimulated NK cells (Fig 5.8b). Moreover, inhibition of mTORC1 did not decrease the glycolytic capacity of NK cells stimulated with IL-2/12/18 (Fig 5.9a). Glycolytic capacity is considered to be reflective of the glycolytic machinery available to the cell. However, mTORC1 inhibition significantly decreased the expression of hexokinase 2 (Fig 5.9b), Glut 1 (Fig 5.9c) and Ldha (Fig 5.9d). Nevertheless, mTORC1 is required for

other aspects of NK cell metabolism in IL-2/12/18 stimulated NK cells. Rapamycin treatment inhibits NK cell blastogenesis (Fig 5.10), as well as the expression of CD71 and CD98 in IL-2/12/18 stimulated NK cells (Fig 5.11).

Thus, it appears mTORC1 regulated expression of glycolytic genes and glucose transporter isn't the rate-limiting factor for elevated glycolysis in cytokine stimulated NK cells. These experiments show that levels of glycolysis in NK cells correlate with SREBP activity and argue that SREBP dependent maintenance of cytosolic NAD^+ is the rate-limiting factor for glycolytic flux in NK cells (Fig 4.14).

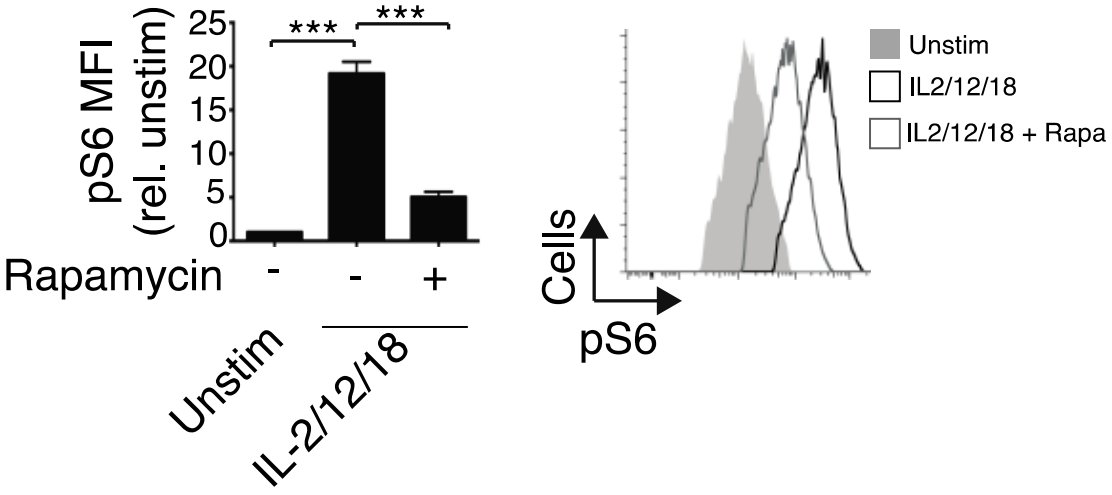

Figure 5.5. mTORC1 is active in IL-2/12/18 stimulated NK cells. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12/18 +/- Rapamycin overnight. Stimulated NK cells were subjected to flow cytometry analysis to determine the expression of pS6 in NK1.1+ NKp46+ CD3- NK cells. Data shown is mean +/- S.E.M of N=3 and representative histograms. (***p<0.001).

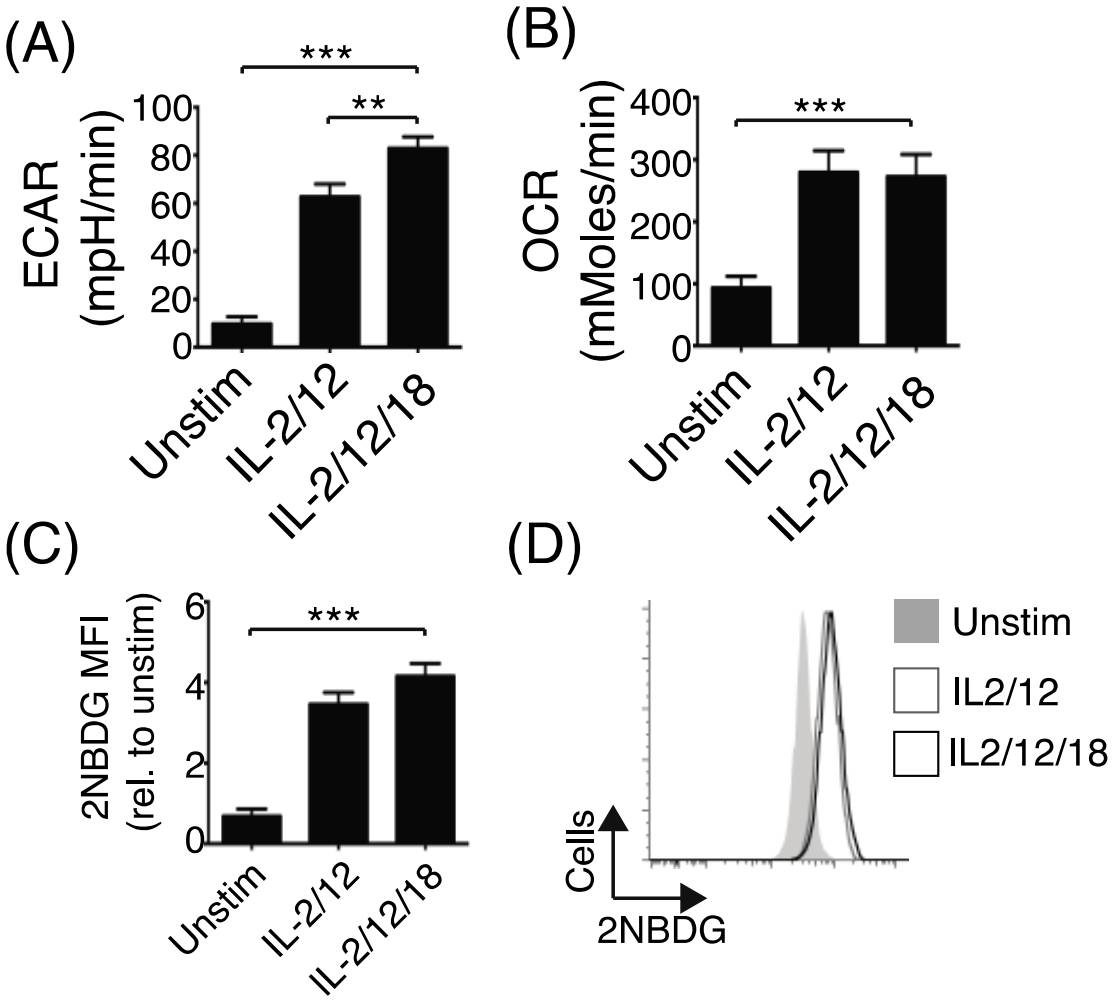

Figure 5.6. NK cells increase their rate of glycolysis and OxPhos when stimulated with IL-2/12/18. (A-D) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 overnight. Stimulated NK cells underwent detailed metabolic analysis using the Seahorse metabolic Flux analyzer that determined the glycolytic (a) and Oxidative Phosphorylation (b) levels of the cell. Glucose uptake based on 2NBDG was determined using flow cytometry (c-d). Data shown is mean +/- S.E.M of N=4 (a-c) or representative of N=4 (d) (** $p<0.01$, ***$p<0.001$).

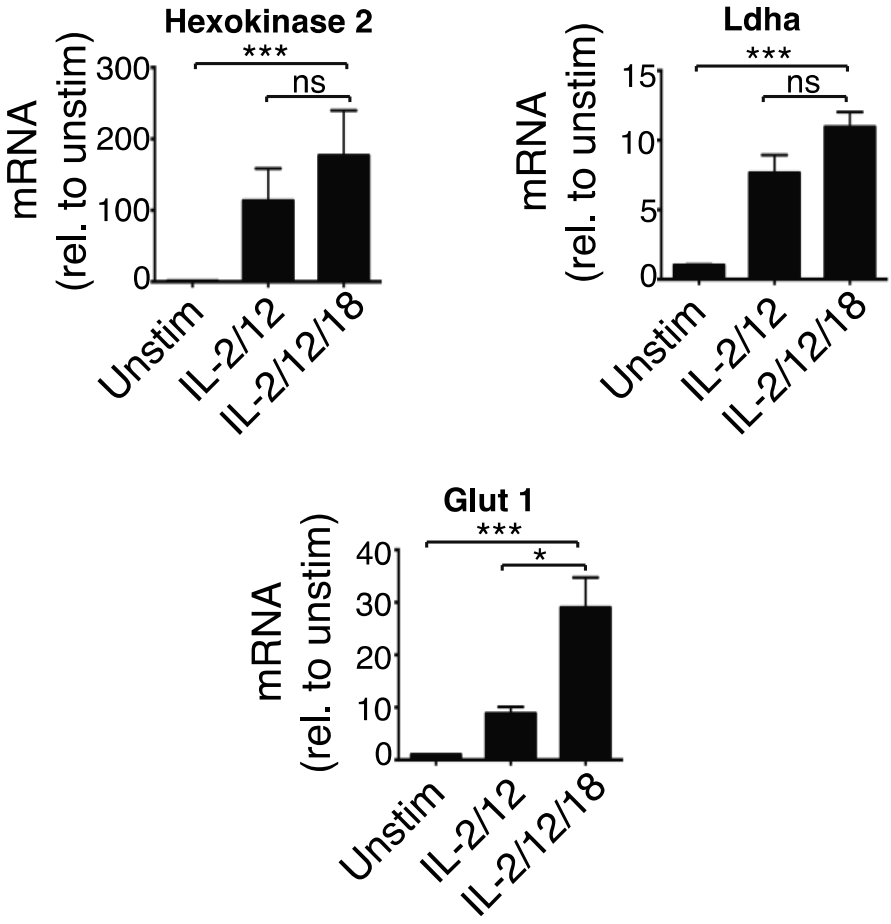

Figure 5.7. IL-2/12/18 cytokine stimulation induces metabolic reprogramming in NK cells. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12 or IL-2/12/18 overnight. RT-qPCR analysis of expression of Hexokinase (b), Glut1 (c) and Ldha (d) Data shown is mean +/- S.E.M of N=3. (ns=not significant, *p<0.05, ***p<0.001).

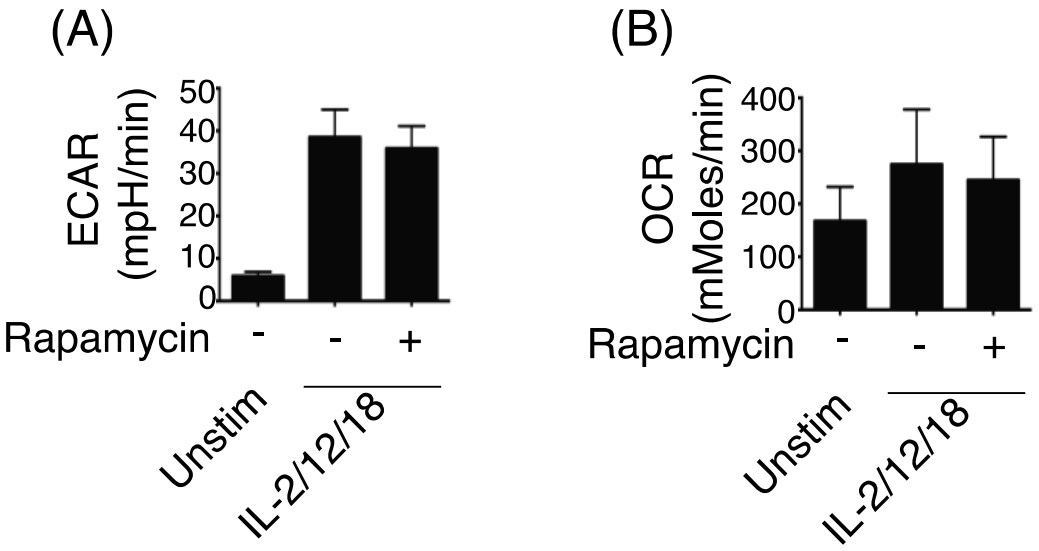

Figure 5.8. mTORC1 activity is not required for IL-2/12/18 induced increase in NK cell glycolysis or OxPhos. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12/18 +/- Rapamycin overnight. Stimulated NK cells underwent detailed metabolic analysis using the Seahorse metabolic Flux analyzer which determined the glycolytic (a) and Oxidative Phosphorylation (b) levels of the cell. Data shown is mean +/- S.E.M of N=3.

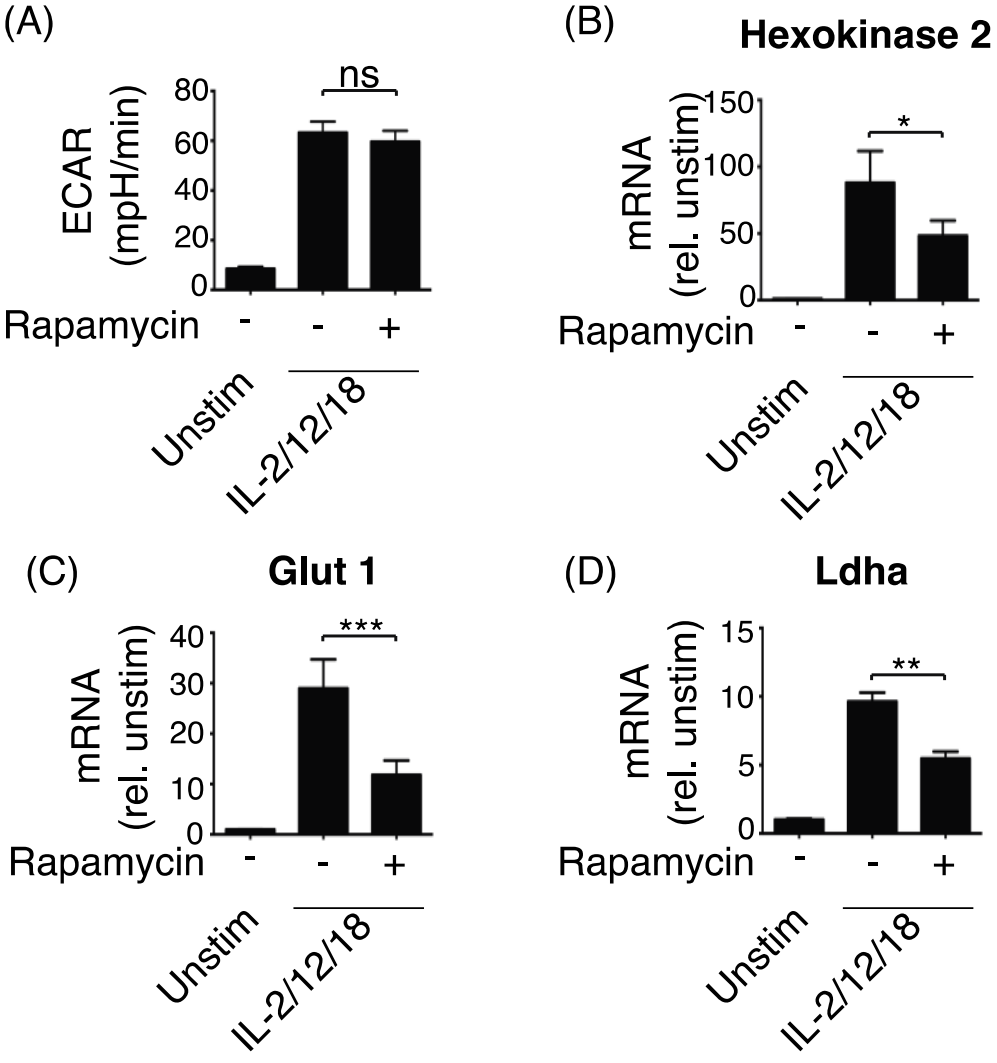

Figure 5.9. mTORC1 is required for IL-2/12/18 induced metabolic reprogramming. (A-D) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12/18 +/- Rapamycin overnight. Stimulated NK cells underwent detailed metabolic analysis using the Seahorse metabolic Flux analyzer which determined the glycolytic capacity (a) of the cell. RT-qPCR analysis of expression of Hexokinase 2 (b), Glut1 (c) and Ldha (d) Data shown is mean +/- S.E.M of N=3. (ns=not significant, * $p<0.05$, ** $p<0.01$, ***$p<0.001$).

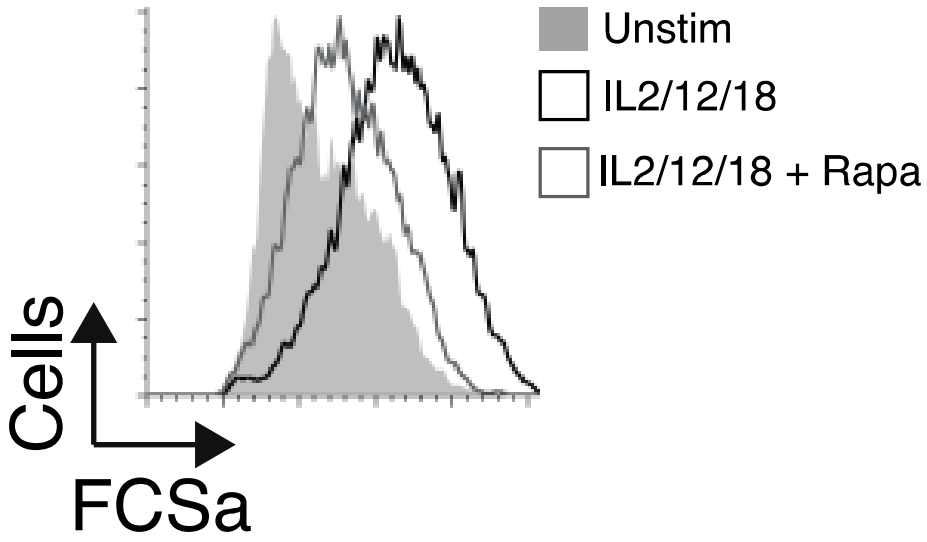

Figure 5.10. mTORC1 activity is required for IL-2/12/18 induced NK cell blastogenesis. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12/18 +/- Rapamycin overnight. Stimulated NK cells were subjected to flow cytometry analysis to determine cell size, based on FSCa, in NK1.1+ NKp46+ CD3- NK cell. Data shown is representative of N=10.

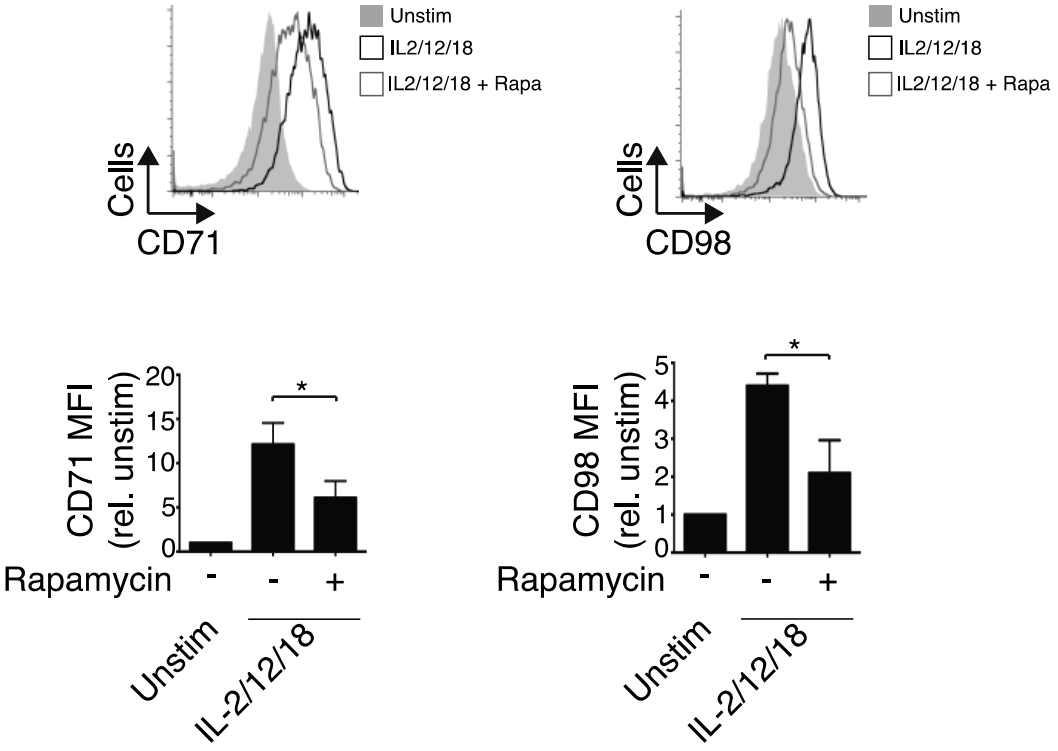

Figure 5.11. Inhibition of mTORC1 decreases the expression of CD71 and CD98. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12/18 +/- Rapamycin overnight. Stimulated NK cells were subjected to flow cytometry analysis to determine the levels of expression of CD71 and CD98 in NK1.1+ NKp46+ CD3- NK cells. Data shown is mean +/- S.E.M of N=3 and representative histograms. (* $p<0.05$).

5.4. mTORC1 independent glycoysis is required for IFNγ production in IL-2/12/18 stimulated NK cells.

We have previously described how mTORC1 dependent glycolysis is required for IFNγ production in IL-2/12 stimulated NK cells. However, the data show that elevated glycolysis in IL-2/12/18 stimulated NK cells is independent of mTORC1. Experiments were designed to investigate whether rapamycin treatment of IL-2/12/18 stimulated NK cells resulted in impaired IFNγ production. Inhibition of mTORC1 had no effect on the percentage of IFNγ$^+$ NK cells when stimulated with IL-2/12/18 (Fig 5.12). In fact, there was a subtle increase in IFNγ production in rapamycin treated NK cells (Fig 5.12). Therefore, mTORC1 is neither required for glycolysis or IFNγ production in IL-2/12/18 stimulated NK cells. Elevated glycolysis is required for IFNγ production in IL-2/12 stimulated NK cells. The question was asked whether elevated glycolysis was required for IFNγ production in IL-2/12/18 stimulated NK cells. We directly limited glycolysis with low dose of 2DG and also by culturing the NK cells in medium containing galactose instead of glucose as described in chapter 3. Similar to IL-2/12 stimulated NK cells, elevated glycolysis is required for IL-2/12/18 induced NK cell blastogenesis (Fig 5.13). In addition, limiting glycolysis also reduces the amount of IFNγ being produced by IFNγ$^+$ NK cells (experiment carried out by Ms Roisin Loftus) (Fig 5.14). Together this data describes a mechanism whereby glycolysis is independent of mTORC1 activity in IL-2/12/18 stimulated NK cells. However, the underlying principal of elevated glycolysis being required for IFNγ production remains.

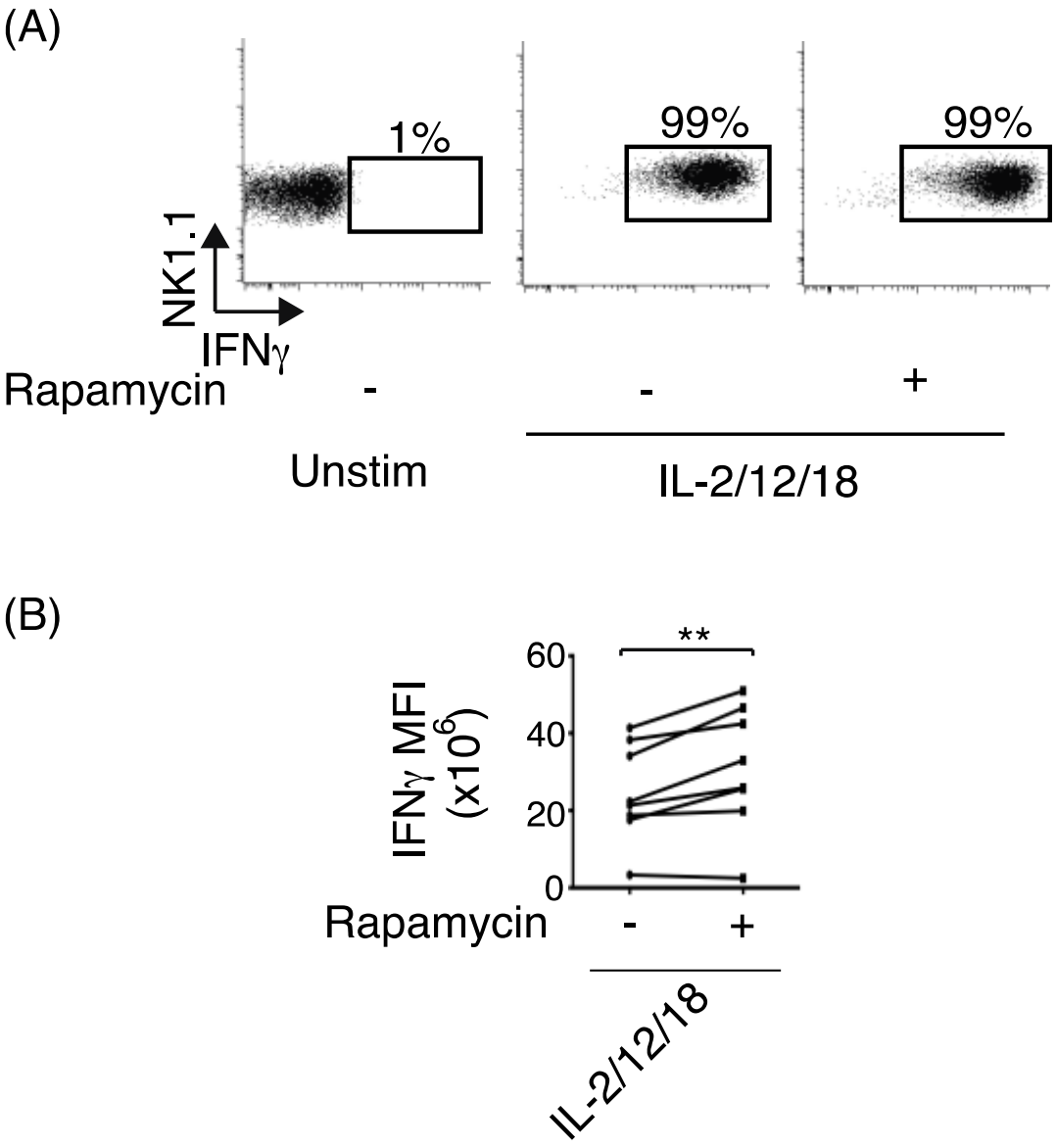

Figure 5.13. mTORC1 is not required for IFNγ production in IL-2/12/18 stimulated NK cells. (A-B) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12/18 +/- Rapamycin overnight. Stimulated NK cells were subjected to flow cytometry analysis to determine the % positive (a) and the levels of expression of IFNγ (b) in NK1.1+ NKp46+ CD3- NK cells. Data shown is mean +/- S.E.M of N=8 (b) and representative dot plot (a). (**p<0.01).

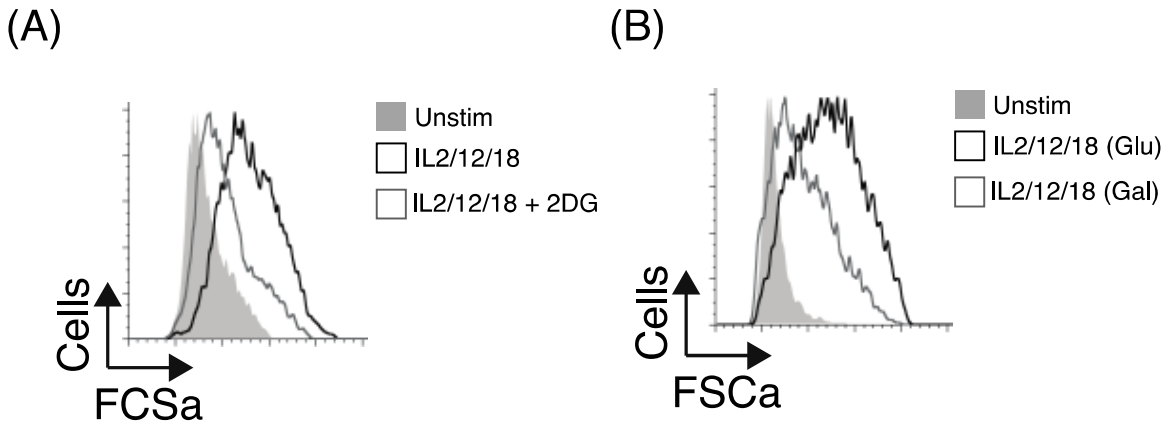

Figure 5.14. Elevated glycolysis is required for IL-2/12/18 induced NK cell blastogenesis. Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12/18 +/- 2DG (1mM) overnight. NK cells were also stimulated with IL-2/12/18 in galactose (10mM) instead of glucose (10mM). Stimulated NK cells were subjected to flow cytometry analysis to determine cell size, based on FSCa, in NK1.1+ NKp46+ CD3- NK cells. Data shown is and representative of N=4.

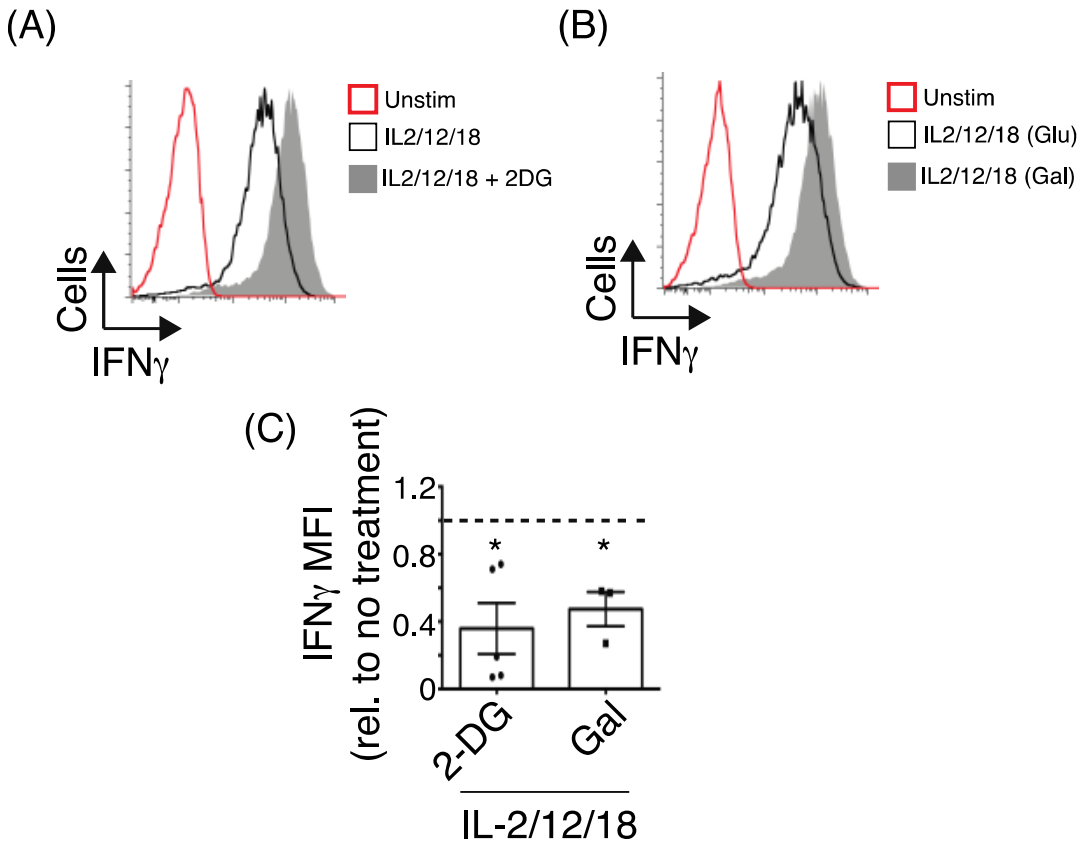

Figure 5.15. Elevated glycolysis is required for IFNγ production in IL-2/12/18 stimulated NK cells. (A-C) Expanded NK cells were maintained in IL-15 (5ng/ml) or stimulated with IL-2/12/18. Elevated glycolysis was inhibited in IL-2/12/18 stimulated NK cells with low dose 2DG (1mM) (a,c) or stimulated in medium containing galactose (10mM) instead of glucose (10mM) (b,c). Stimulated NK cells were subjected to flow cytometry analysis to determine IFNγ expression based on MFI of NK1.1+ NKp46+ CD3- NK cells. Data shown is representative histograms of N=4 (a,b) or relative MFI decreases between untreated and treated conditions. Data shown is mean +/- S.E.M of N=3 (a). (* $p<0.05$). Experiment carried out by Ms Roisin Loftus.

5.5. Discussion

In the two previous chapters we have shown how IL-2/12 stimulated NK cells increase glucose metabolism and *de novo* lipid synthesis. Moreover, we have demonstrated that SREBP activity is required to sustain glycolysis in cytokine stimulated NK cells. We have also demonstrated that mTORC1 activity is required to maintain glycolysis in IL-2/12 stimulated NK cells. Thus considering mTORC1 and SREBP independently control NK cell glycolysis and effector molecules when stimulated with IL-2/12, we hypothesised that mTORC1 activity is required to regulate SREBP in NK cells. Indeed, mTORC1 has been shown to regulate SREBP activity in various other cell types. Herein our data shows how mTORC1 is required for lipid synthesis in IL-2/12 stimulated NK cells in a SREBP dependent manner. Interestingly, similar observations were made in $CD8^+$ T cells (Kidani et al., 2013). Strikingly, we observed no significant decrease in SREBP activity when we treated IL-2/12/18 stimulated NK cells with rapamycin. mTORC1 is highly active in IL-2/12/18 stimulated NK cells, however data showed that it is not required for elevated levels of glycolysis or OxPhos. In addition, while mTORC1 has been known to regulate glycolysis in most lymphocyte populations, B cells engage in aerobic glycolysis independently of mTORC1 (Doughty et al., 2006). IL-2/12/18 stimulated NK cells undergo similar metabolic reprogramming to IL-2/12 stimulated NK cells. In fact, they have slightly elevated levels of glycolysis and glycolytic genes. This is potentially reflective of the increased biosynthetic burden arising with IL-2/12/18 stimulation. IL-2/12/18 stimulated NK cells undergo enhanced cellular growth compared to IL-2/12 stimulated NK cells. Moreover, our data showed that mTORC1 does not regulate the glycolytic capacity in IL-2/12/18 stimulated NK cells, despite the fact that rapamycin treatment results in significant reductions in glycolytic genes and glucose transporters. This data argues that factors, other than the genes analysed, are rate limiting for NK cell glycolytic capacity. Given that SREBP activity seems to correlate with the glycolytic capacity in IL-2/12/18 stimulated NK cells, we propose that

sustaining the pool of cytosolic NAD^+ may be the rate limiting factor for the maximal rate of glycolysis in these cells. Indeed, an interesting observation during this study was that targeting NAD^+ levels, by inhibiting SREBP, ACLY, CiC or NAMPT, resulted in NK cells operating at their max glycolytic capacity with no glycolytic reserve. Our data showed that mTORC1 regulated glycolysis was required for IFNγ production in IL-2/12 stimulated NK cells. However, IL-2/12/18 stimulated NK cells maintained high levels of IFNγ production in the presence of rapamycin. However, perturbations in elevated glycolysis resulted in decreased IFNγ production. Thus, while elevated glucose metabolism appears to be globally required for normal effector molecules in response to diverse cytokine stimuli, the mechanism controlling metabolic reprogramming appears to be dependent on the stimulation an NK cell receives. Our data shows that SREBP activity is not required for the expression of glycolytic genes or glucose transporter but is required for glycolytic capacity in cytokine stimulated NK cells.

Our data suggests that SREBP regulated cytosolic NAD+ levels is a rate limiting factor controlling elevated glycolysis in cytokine stimulated NK cells. mTORC1 signalling is essential for controlling SREBP activity in NK cells in IL-2/12 but not IL-2/12/18 stimulated NK cells. Therefore SREBP is regulated by a different mechanism in the presence of IL-2/12/18. There are multiple other kinases that are functionally similar with roles in regulating cellular metabolism (Hao and Rutter, 2008). Indeed they have similar substrate specificity as mTORC1 (Jastrzebski et al., 2011); Akt (Nagano et al., 2015, Kobayashi et al., 1999), PIM2 (Peng et al., 2007), MAPK and PASK (Kikani et al., 2010). Therefore, these kinases are potentially involved in regulating SREBP activity in IL-2/12/18 stimulated NK cells. Some of the potential candidates include:

1) Akt: Recent research has shown that Akt can promote SREBP expression independently of mTORC1. Yecies and colleagues showed

that Akt induces suppression of INSIG, an inhibitor of SREBP processing (Yecies et al., 2011). In addition, the ubiquitin ligase Fbw7 (F-box and WD repeat domain-containin 7) can interact with nuclear SREBP to promote its ubiquitination and degradation. Fbw7 activity is dependent on GSK3, which is negatively regulated by phosphorylation via Akt (Sundqvist et al., 2005). Akt can also upregulate ACLY mRNA levels via activation of SREBP which is essential for the citrate-malate shuttle to function (Sato et al., 2000). In lymphocytes PI3K/Akt signalling is frequently associated with being an upstream regulator of mTORC1 activity (Rao et al., 2010, Powell and Delgoffe, 2010). However, Finlay and colleagues showed that $CD8^+$ T cells undergo mTORC1-dependent metabolic reprogramming independently of PI3K/Akt signalling (Finlay et al., 2012). B cell glycolysis is independent of mTORC1 signalling and primarily relies on PI3K and Akt signalling. Indeed, IL-18 has been shown to be a strong inducer of PI3K/Atk signalling in other cell types (Yoo et al., 2005, Reddy et al., 2008, Hosotani et al., 2008). Therefore, Akt signalling may account for mTORC1 independent SREBP activity in IL-2/12/18 stimulated NK cells.

2) Proviral integrations of moloney virus 2 (PIM2): Studies have shown that IL-18 can also drive STAT3 signalling in NK cells (Kalina et al., 2000). PIM2 a serine/threonine kinase that has roles in cell growth, proliferation and the control of numerous signal transduction cascades (Alvarado et al., 2012) is activated downstream of JAK/STAT3 signalling (Beharry et al., 2011). Our data has shown that SREBP activity is required for IL-15 induced homeostatic proliferation. Studies have shown that PIM2 is required for cytokine-dependent proliferation and survival in T_H1 cells (Tahvanainen et al., 2013). Like mTORC1, PIM2 is implicated in multiple malignancies such as multiple myeloma (Lu et al., 2013). PIM2 and mTORC1 have been shown to independently regulate cell growth (Hammerman et

al., 2005). Furthermore studies have shown that Foxp3$^+$ T cells have a selective advantage to proliferate in the absence of mTORC1 activation (Zeiser et al., 2008, Strauss et al., 2009), with Treg cells more resistant to cell death (Strauss et al., 2009). This is likely due to the increased expression of PIM2 kinase (Basu et al., 2008). In this study we show that the addition of IL-18 to IL-2/12 stimulated NK cells results in rapamycin-resistant NK cell metabolism and IFNγ production. Interestingly, a study by Fox *et al* showed that T cells from PIM2 kinase knock out mice display an increased sensitivity to rapamycin. Furthermore, they showed that PIM2 kinase promotes rapamycin-resistant survival of T cells (Fox et al., 2005b). As previously mentioned CTLs treated with rapamycin are comparable in size to untreated cells (Finlay et al., 2012). On that note, Fox *et al* has shown PIM2 kinase is also responsible for inducing rapamycin-resistant T cell blastogenesis (Fox et al., 2005b). Therefore, PIM2 kinase is potentially implicated in the observed rapamycin insensitivity in IL-2/12/18 stimulated NK cells.

3) Mitogen-activated protein (MAP) kinase: MAPK are protein kinases that are strongly induced in NK cells upon IL-18 stimulation (Kalina et al., 2000). Interestingly, SREBP activity has been shown to be linked to the MAP-kinase cascade. Roth *et al* showed MAP kinase ERK1/2 phosphorylates SREBP1a at Ser 117 (Roth et al., 2000) to positively regulate its activity. Moreover, MAP kinase has also been shown to phosphorylate SREBP1c (Knebel et al., 2014) and SREBP2 (Kotzka et al., 2004) at multiple sites to induce SREBP activity.

4) Per-Arnt-Sim (PAS) Kinase: PASK activity is dramatically increased with elevated glucose concentrations (da Silva Xavier et al., 2004). PASK has recently been shown to induce SREBP activation by promoting the proteolytic maturation of SREBP in murine hepatocytes (Wu et al., 2014). Indeed, there are multiple ways being reported how

PASK positively regulates SREBP activity. Firstly, multiple studies have shown that PASK inhibits GSK3 activity by directly phosphorylating it on its inactivating phosphorylation sites Ser(9) (Semache et al., 2013) and Ser(640) (Skurat and Dietrich, 2004). Indeed, GSK3 can promote the ubiquitination of SREBP resulting in its degradation (Sundqvist et al., 2005). Moreover, PASK activity has been shown to negatively regulate AMPK (Hurtado-Carneiro et al., 2013, Shaw et al., 2004). AMPK kinase is known to inhibit SREBP activity (Li et al., 2014). Moreover, there is a dramatic reduction in lipid accumulation in mice with PASK deficiency, which was attributed to lower expression of several genes involved in lipid synthesis, including SCD1, a known SREBP target gene (Hao et al., 2007). The metabolic phenotypes of mice deficient in PASK are quite similar to those caused by AMPK activation or inhibition of mTORC1 signalling via S6K1 deletion (Zhou et al., 2001, Pende et al., 2000, Um et al., 2004). Indeed, PASK has multiple global similarities with mTORC1. Firstly, mice lacking PASK have similar metabolic phenotypes, including hypoinsulinemia (Pende et al., 2000) and resistance to diet-induced obesity (Um et al., 2004). However, importantly mTORC1 is not downstream of PASK as no decrease in mTORC1 activity was observed in PASK deficient mice (Leone et al., 2005). Instead, PASK and mTORC1 operate on different linear pathways with convergent roles. Inhibition of PI3K also has no effect on PASK activity (Cengel et al., 1998) and therefore PI3K/Akt signalling does not affect PASK activity. Therefore, PASK might play a role in inducing SREBP activity in IL-2/12/18 stimulated NK cells.

Chapter 6- Final Discussion

We have successfully characterised the changes in glycolysis and lipid synthesis that occur following cytokine stimulation. Furthermore, we have demonstrated that the molecular mechanisms required for elevated glycolysis is dependent on the cytokine stimulation an NK cell receives. IL-2/12 stimulated NK cells undergo mTORC1 dependent metabolic reprogramming, while IL-2/12/18 stimulated NK cells undergo metabolic reprogramming independently of mTORC1. Moreover, we have shown that SREBP activity is required to maintain elevated glycolysis independent of the cytokine stimulation an NK cells receives. The data argues that SREBP regulated citrate-malate shuttle is required to maintain cytosolic NAD^+ needed for the elevated glycolytic flux in cytokine stimulated NK cells. Furthermore, the data shows that elevated glycolysis is required for normal NK cell effector molecules. Previously the potential mechanisms regulating NK cell metabolism and function have been discussed. Herein I discuss how our work might be relevant in different physiological settings.

This study highlights a crucial role for signalling through the high-affinity IL-2R, CD25, in promoting elevated rates of NK cell glycolysis and concomitant enhanced NK cell effector molecules. It is becoming clear that NK cells act in parallel with T cells of the adaptive immune system during immune responses to diverse immunological challenges (Vivier et al., 2011, Lee et al., 2012, Bihl et al., 2010). Although it is apparent that T cells are not essential for NK cell responses (Kim et al., 2002b, Prlic et al., 2003, Sun and Lanier, 2011, Orange et al., 1995), our research suggests that the adaptive response may enhance NK cell effector function. This is achieved through the actions of the T cell–derived cytokine IL-2 in promoting elevated NK cell glycolysis. Additionally, given that a key mechanism through which regulatory T cells control NK cell responses is through sequestering IL-2 (Kerdiles et al., 2013, Sitrin et al., 2013), it seems likely that regulatory T cell–mediated suppression of NK cell effector molecules involves decreased IL-2–stimulated levels of NK cell glycolysis. Therefore, the data presented in this study promote the idea that T cell–controlled IL-2 availability may be a factor that

impacts upon the regulation of NK cell metabolism and function. We described a new regulatory axis that is required for the acquisition of normal NK cell effector molecules. This has widespread implications for our understanding of NK cell immune responses to viral infection, tumours, and other inflammatory situations.

The finding that mTORC1 is required to maintain glycolysis in activated NK cells is very important as it provides insight into the physiological setting in which NK cell function might be augmented by limitations on NK cell metabolism. mTORC1 is an acute sensor of the immune microenvironment whose activity reflects both the cytokine milieu, nutrient (glucose, amino acids) and oxygen availability (Heikamp and Powell, 2012, Colgan and Taylor, 2010). While temporal glucose deprivation will rapidly and directly limit glycolysis and thus NK cell effector molecules, sustained glucose, amino acid, oxygen or cytokine deprivation will inhibit mTORC1 activity in activated lymphocytes (Rolf et al., 2013, Sinclair et al., 2013). This results in reduced glucose transporter and glycolytic enzyme expression, thereby limiting glycolysis and disrupting NK cell functions. Competition for cytokines and nutrients between the large numbers of infiltrating immune cells at sites of inflammation can result in nutrient depletion (Colgan and Taylor, 2010, Nizet and Johnson, 2009). In addition, bacterial infections can also compete for nutrients with lymphocytes. Staphylococcus aureus can induce an infection that results in localised tissue hypoxia and an environment depleted of glucose (Vitko et al., 2015). Therefore, regulation of cell metabolism at inflammatory sites will be an important factor that dictates the pro-inflammatory function of NK cells.

In addition to the inflamed microenvironment, the solid tumour microenvironment can also experience substantial hypoxic and nutrient deprived conditions (Hirayama et al., 2009). Tumour cells have glucose uptake rates up to 10 fold greater than activated lymphocytes as they can efficiently sequester glucose from the tumour microenvironment away from infiltrating immune cells (Cham and Gajewski, 2005). In addition, several

tumour-derived molecules can further promote immune suppression by affecting tumour-infiltrating lymphocytes (Vesely et al., 2011). The tumour microenvironment is composed of various cell types, including tumour-associated macrophages with similar phenotypes and functions of alternatively activated or M2 macrophages i.e. expressing IL-10 and TGFβ (Mantovani and Sica, 2010). The end result being tumour initiation through the induction of immune suppression, and angiogenesis (Murdoch et al., 2008). Interestingly, unpublished data in collaboration with the Walzer group suggests that TGFβ can inhibit mTORC1 in NK cells. Similarly, it has been shown that the increased metabolism of l-arginine by myeloid cells can result in the impairment of lymphocyte responses to tumour cells (Bronte and Zanovello, 2005). There are some reports that suggest that arginine is essential for mTORC1 signalling though we have not tested this in NK cells yet (Kong et al., 2012, Bauchart-Thevret et al., 2010, Yao et al., 2008, Gonzalez et al., 2012).

Therefore, at the sites of tumours and infections infiltrating NK cells can encounter unfavourable conditions that may inhibit mTORC1 activity and/or elevated glycolysis. The data presented in this study supports a model which predicts that NK cell anti-tumour and anti-viral functions will be compromised by the metabolically inhospitable conditions of the tumour and inflammatory microenvironments. Indeed, NK cells infiltrating a range of human solid tumours have been shown to exhibit defective anti-tumour functions (Carrega et al., 2008, Mamessier et al., 2011, Platonova et al., 2011, Rocca et al., 2013). Our data suggests that disrupted NK cell glycolysis may be an important mechanism underpinning NK dysfunction in solid tumours.

The finding that SREBP activity and the citrate malate shuttle is required to sustain glycolysis is highly significant as it provides a deeper understanding into the settings where NK cell functions might be inhibited. Recent research has shown that tumour cells have the ability to secrete oxysterols such as 25-HC into their microenvironment (Raccosta et al., 2013). The release of these oxysterols has a significant impact on the immune response. DCs migration

towards the tumour microenvironment is inhibited by oxysterols (Raccosta et al., 2013). Interestingly, neutrophils have also shown to suppress the antitumour immune response (Fridlender et al., 2009, De Santo et al., 2010). Oxysterols released by tumours have the ability to recruit neutrophils within the tumour microenvironment, favouring neoangiogenesis and immunosuppression (Raccosta et al., 2013). Moreover, recent research has shown that macrophages and DCs can regulate the immune response via secretion of the oxysterol 25-HC. Macrophages and DCs express high levels of cholesterol 25-hydroxylase which converts cholesterol into the oxysterol 25-HC (Park and Scott, 2010). Based on our data it is highly possible that tumour cells as well as macrophages and DCs could also inhibit NK cell function through the release of 25-HC by inhibiting SREBP activity. Interestingly, oxysterols have previously been shown to inhibit NK cell cytotoxicity (Kucuk et al., 1992).

The amino acid tryptophan is used for *de novo* synthesis of NAD^+. Our data shows that de novo NAD+ synthesis is required for elevated glycolysis and maximal NK cell effector molecules following cytokine stimulation. Tryptophan concentrations and catabolism fluctuate between individuals and are highly influenced by environmental factors such as smoking and drinking (Deac et al., 2015). Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyzes the rate-limiting step in tryptophan catabolism. Studies have shown that IDO expression is induced by IFNγ, resulting in a decrease in pro-inflammatory immune responses (Yoshida et al., 1979). Originally this was thought to be a mechanism necessary for dampening down an immune response to restore homeostasis. However, evidence is now emerging those infectious agents themselves, such as influenza, can induce IDO expression resulting in decreased tryptophan levels during illness. Interestingly, studies have shown that most malignant tumours express high levels of IDO (Uyttenhove et al., 2003). Tumour cells can cause immunosuppression by release of IDO causing the breakdown of tryptophan in the tumour microenvironment and tumour-draining lymph nodes. This depletion of

tryptophan results in impaired T cell effector molecules (Uyttenhove et al., 2003). Subsequent removal of IDO from the same tumours can reverse the suppression of T cells (Uyttenhove et al., 2003). An interesting study by Okamoto *et al* showed that levels of IDO expression correlated with survival time in patients with ovarian cancer. Tumours expressing high levels of IDO resulted in a mean survival time of 11 months compared to 41 months for patients whose tumour expressed low levels of IDO (Okamoto et al., 2005). Moreover IDO expression is being used as a prognosis marker in endometrial and colon carcinomas (Ino et al., 2006, Brandacher et al., 2006). Macrophages and DCs are also able to regulate NK and T cell response by upregulating and releasing IDO (Frumento et al., 2002, Hwu et al., 2000). IDO is also implicated in immune suppression at the tumour-draining lymph node, where a population of IDO expressing DCs has been shown to inhibit T cell effector response (Munn et al., 2004). Furthermore, IDO levels are known to be elevated upon viral infection; HIV patients have been reported to have low levels of tryptophan (Fuchs et al., 1990). The exact molecular mechanisms by which IDO inhibit the immune response are not clear. Our data suggests that one mechanism may be due to a decrease in *de novo* NAD^+ synthesis, required to maintain glycolysis. Decreased NAD^+ would result in reductions in proinflammatory effector molecules as they are unable to increase their rates of aerobic glycolysis. In support of this hypothesis Peng *et al* demonstrated that pancreatic tumour cells inhibit NK cell function through the release of IDO. Moreover they saw reductions in IFNγ and granzyme B (Peng et al., 2014). In addition, metabolites of tryptophan including kynurenine, quinolinic acid and picolinic acid are directly toxic to T cells (Frumento et al., 2002). Additionally, IDO can also play an important role in promoting immune tolerance during infection, pregnancy, transplantation and autoimmunity (Uyttenhove et al., 2003, Mellor and Munn, 2004, Curti et al., 2007). Research in EAE mouse models, showed administration of FK866 after EAE onset successfully ameliorated the severity of the disease (Bruzzone et al., 2009) caused by T cells. NK cells have also been implicated in

autoimmune disorders (Schleinitz et al., 2010), and in such circumstances targeting NAD^+ might be of clinical interest.

Obesity is one of the leading risk factors for developing cancer and infections. Numerous studies have shown that NK cell function is impaired in obese patients (O'Shea et al., 2010, Laue et al., 2015). Obesity is normally associated with high levels of cholesterol. Moreover, the levels of the oxysterol 25-HC have been found to be elevated in hypercholesterolemic serum (Lappano et al., 2011). In addition, expression of SREBP is considerably lower in obese people (Kolehmainen et al., 2001). Strikingly, NAD^+ levels are considerably reduced in obese people, however the underlying mechanisms for this remain unclear (Choi et al., 2013). We hypothesize that obesity would result in decreased NAD^+ levels in NK cells due to cholesterol inhibition of SREBP activity and the citrate-malate shuttle. In support of this, recent research done in our lab in collaboration with Hogan and colleagues show IL-2/12 stimulated NK cells from obese people have reduced glycolysis and OxPhos levels (unpublished data). Moreover, recent research by Choi *et al* showed the microRNA-34a (miR-34a), is elevated in obesity. miR-34a directly targets and inhibits NAMPT, reducing NAD^+ levels and sirtuin activity (Choi et al., 2013).

One of the major findings from this study was that IL-18 in addition with IL-2/12 can promote mTORC1 independent glycolysis. IL-18 has been shown to have numerous roles in initiating immune cascades. It is involved in stimulating IFNγ production in T cells, assisting T_H1 cell development, enhancement of T_H1 cytokines and inducing NK cell cytotoxicity (Boraschi and Dinarello, 2006). It also has a role in enhancing IL-17 production in T_H17 cells in a TCR-independent manner, similar to its role in T_H1 cells (Harrington et al., 2006, Weaver et al., 2006). IL-18 has been shown to originate from numerous cell types from both immune and non-immune systems (Boraschi and Dinarello, 2006).

Rapamycin insensitive metabolism in IL-2/12/18 stimulated NK cells offers great therapeutic potential. As already stated one of the major problems of the tumour microenvironment and the inflammatory site is the competition for nutrients leading to decrease in mTORC1 activity. However, an NK cell with the ability to function in the presence of limited nutrient supplies through an mTORC1 independent mechanism would be able to persist in such an environment to clear infection and eradicate tumours. It would be very hard to influence the cytokine activation of NK cells *in vivo* as the immune system is very complex. However, there might be a potential strategy for preactivation of NK cells *in vitro* followed by adoptive transfer. Multiple studies have looked at *ex vivo* activation of NK cells followed by adoptive transfer as a potential cancer immunotherapy. One such study showed that NK cells preactivated with IL-2 were ineffectual against metastatic melanoma or renal carcinoma. However, there is evidence that IL-18 can promote a robust anti-tumour response (Parkhurst et al., 2011). Ni and colleagues show that IL-12/15/18 preactivated NK cells were shown to persist with sustained effector function *in vivo* leading to substantially reduced growth of established mouse tumours (Ni et al., 2012). IL-12/15/18 preactivated NK cells were shown to express high levels of IL-2Rα (CD25), and their rapid *in vivo* proliferation depended on IL-2 produced by $CD4^+$ T cells.

In conclusion this study has broadened the understanding of NK cell glucose and lipid metabolism. Interestingly, virally infected and transformed cells have adapted ways to evade the immune response through targeting lymphocyte metabolism. As outlined this can occur via targeting mTORC1 activity or disrupting SREBP signalling and NAD^+ supplies amongst others. With a greater understanding of NK cell metabolism we can strive towards improved therapeutics to restore immune homeostasis.

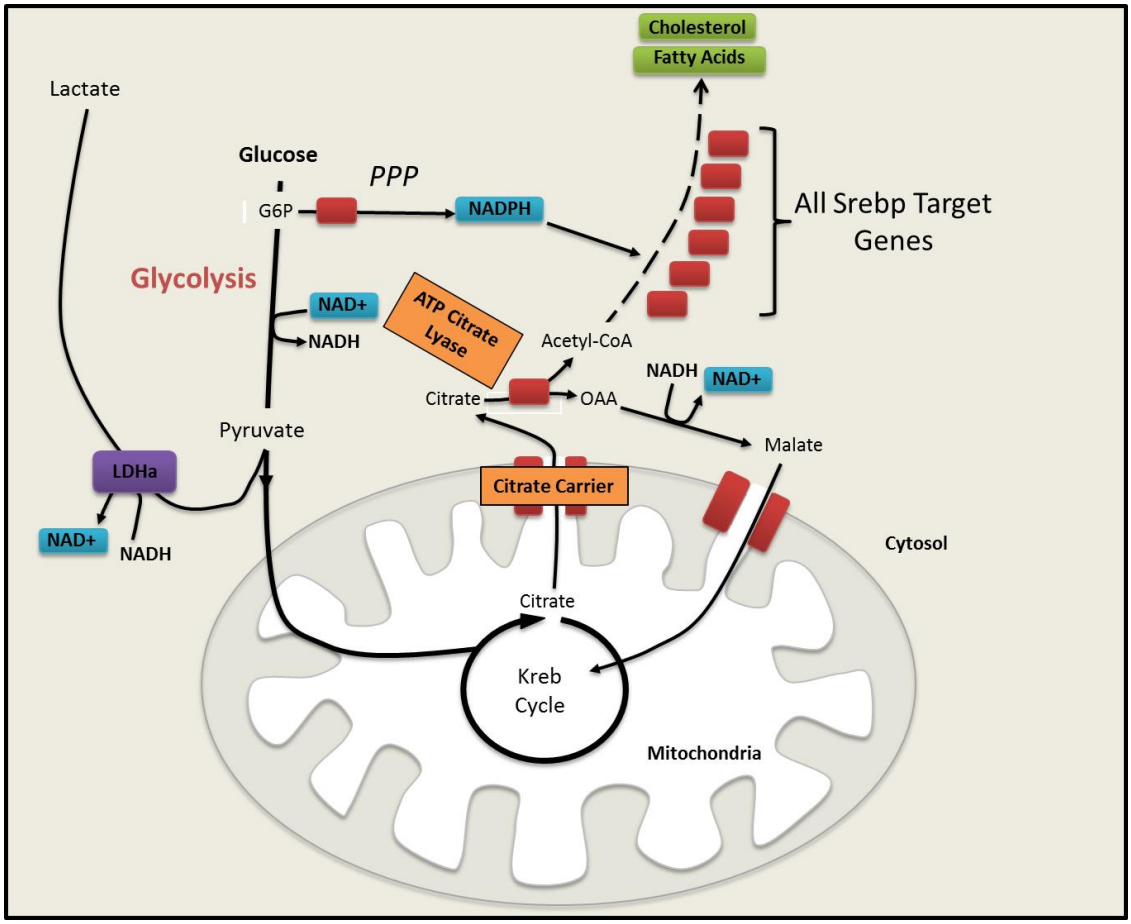

Figure 6.1. The citrate malate shuttle restores cytosolic NAD⁺ levels. Outlined above is a summary diagram demonstrating how cytosolic NAD⁺ can be restored through the citrate malate shuttle. Glucose derived pyruvate enters the mitochondria and enters the krebs cycle via acetyl-coA. The metabolite citrate is shunted out of the krebs cycle and the mitochondria via the mitochondrial citrate carrier, a SREBP target gene. Cytosolic citrate is subsequently cleaved into Acetyl-CoA and oxaloacetate by ATP citrate lyase, another SREBP target gene. Acetyl-CoA is furthered metabolized to produce lipids while oxaloacetate gets reduced to malate in a reaction that oxidizes NADH to NAD+ which is an essential cofactor needed for the glycolytic enzyme GAPDH.

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